Proceedings of the IUFRO Tree Biotechnology 2015 Conference: "Forests: the importance to the planet and society"

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4 authors, including:

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Italian National Research …

Tree Biotechnology 2015 Conference

8-12 June 2015
Florence, Italy

IUFRO Tree Biotechnology 2015 Conference

"Forests: the importance to the planet and society"

8-12 June 2015
Florence, Italy

Proceedings

Editors

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Presentation

The IUFRO Tree Biotechnology Conference is held every two years and is the official meeting of the IUFRO Working Group 2.04.06 (Molecular biology of forest trees). The year 2015 is the thirtieth anniversary of its initial gathering in 1985 in the U.S. (Avon Lake, Ohio), and for the first time organized in Italy, returning to Europe after 8 years. It is estimated that approximately 300 delegates from 50 countries will participate the Tree Biotechnology Conference being the major international forum to present and discuss new developments and ideas related to the current state and future of research in genetics, genomics and biotechnology in the forestry.

This conference brings together academics, scientists, public and private institutions of international, national and regional, governmental and non-governmental organizations, and other stakeholders to discuss all aspects of biotechnology and biosafety of forest trees.

Therefore, the "IUFRO Tree Biotechnology" offers a unique opportunity to share information and experiences, and to engage in an open and meaningful dialogue on the state of research in the field.

The main theme of the conference is "Forests: the importance to the planet and society" and how to preserve it in the light of global climate change to meet the growing demands of society for sustainable resources, renewable energy and biomass production.

There is a growing worldwide demand for wood and biomass in response to the needs of the society (paper, energy, etc.), and therefore we are witnessing an increase in forest plantations of high productivity (e.g. poplar and eucalyptus). The latest developments in biotechnology applications will contribute to meeting the global demands of the society by helping to preserve the natural forests and reducing deforestation of large forest areas important for the ecosystem preservation.

This conference is a unique and timely opportunity focusing on the current social and environmental issues and knowledge gaps related to the status of research, and to the development of new technologies. This implies a dynamic discussion on the intersection of science, regulatory and policy guidelines and society, which will be an innovative session of the Congress in which socio-economic studies related to biotechnology and regulatory issues are reported and discussed.

The Congress has the following thematics:

1) Tree Genomics and Climate Change.
2) Tree ecosystem, biodiversity, conservation and environment interactions.
3) Tree Biotechnology: molecular understanding of tree growth and development and applications for tree improvement.
4) Biosafety: environmental risk assessment, monitoring and management, and socio-economic implications.

IUFRO Tree Biotechnology Conference

The IUFRO Tree Biotechnology Conference is always the premier science for IUFRO Working Group 2.04.06 (Molecular biology of forest trees) on a bi-annual basis, a tradition started in 1985 in the U.S. (Avon Lake, Ohio).

Conference locations:

YEAR	LOCATION	ORGANIZERS
1985	Avon Lake, Ohio, USA	Howard Kriebel
1987	Petawawa, Ontario, Canada	Bill Cheliak
1989	Riksgransen, Lappland, Sweden	Petter Gustafsson
1990	Lake Tahoe, California, USA	Dave Neale & Claire Kinlaw
1992	Bordeaux, France	Antoine Kremer
1994	Scarborough, Maine, USA	Mike Greenwood
1995	Gent, Belgium	Wout Boerjan
1997	Quebec City, Canada	Pierre Charest
1999	Oxford, UK	Malcolm Campbell
2001	Skamania Lodge, Washington, USA	Steve Strauss and H.D. "Toby" Bradshaw
2003	Umeå, Sweden	Björn Sundberg and Göran Sandberg
2005	Pretoria, South Africa	Brenda Wingfield and Zander Myburg
2007	Ponta Delgada, Azores, Portgual	Maria Margarida Oliveira and Cristina Marques
2009	Whistler, British Columbia, Canada	Carl Douglas and Shawn Mansfield
2011	Arraial d'Ajuda, Bahia, Brazil	Dario Grattapaglia and Karina Zamprogno
2013	Asheville, North Carolina, USA	Matias Kirst and Jeffrey Dean
2015	Florence, Italy	Cristina Vettori, Giovanni G. Vendramin Donatella Paffetti, Davide Travaglini

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Giovanni Giuseppe Vendramin - Co-Chair IBBR-CNR
Donatella Paffetti - Co-Chair DISPAA-UNIFI
Davide Travaglini - Co-Chair GESAAF-UNIFI
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Fabio Migliacci - Webmaster ICCOM-CNR

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Janice Cooke - University of Alberta (Canada)
Jeffrey Dean - Mississippi State University (USA)
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Shawn Mansfield - University of British Columbia (Canada)
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Cristina Vettori - Institute of Bioscience and BioResources (Italy)

Organizing Secretariat

Scaramuzzi Team
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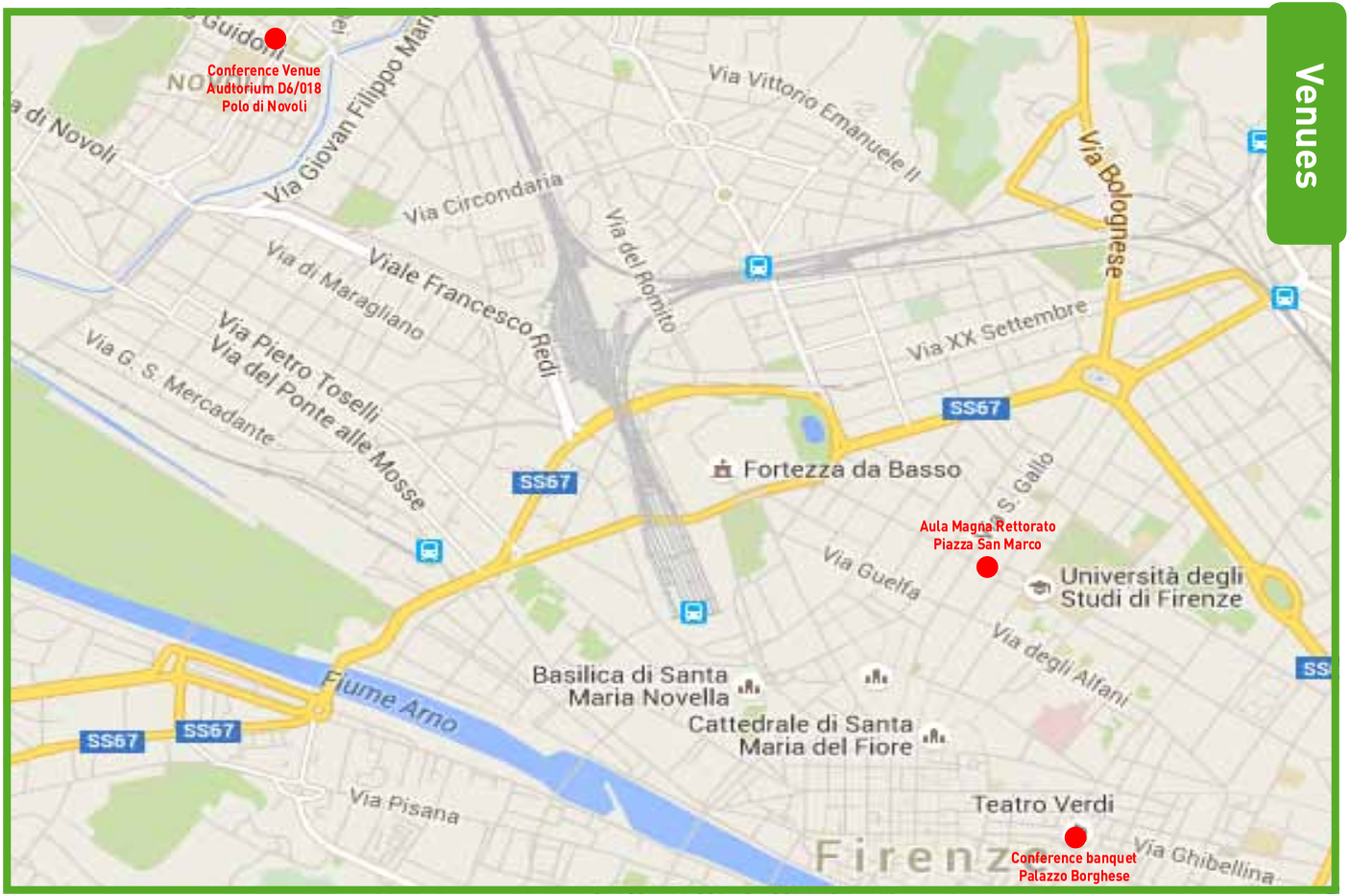
Program overview

Monday 8 June	Tuesday 9 June	Wednesday 10 June	Thursday 11 June	Friday 12 June
Morning	Morning	Morning	Morning	Morning
Aula Magna Rettorato Piazza San Marco	Auditorium D6/018 Polo di Novoli	Auditorium D6/018 Polo di Novoli	Auditorium D6/018 Polo di Novoli	Auditorium D6/018 Polo di Novoli
10:30 Registration	7:45 Registration Open	8:30-10:00 Session II	8:30-10:00 Session III	9:00-10:15 Session III
	8:00-8:30 Setting up posters			
	8:30-10:00 Session I			
	10:00-10:30 Coffee Break + Poster Viewing	10:00-10:30 Coffee Break + Poster Viewing	10:00-10:30 Coffee Break + Poster Viewing	10:15-10:45 Coffee Break + Poster Viewing
	10:30-12:00 Session I	10:30-12:00 Session II	10:30-12:00 Session III	10:45-12:15 Session IV
	Light lunch	Light lunch	Light lunch	Light lunch
Afternoon	Afternoon	Afternoon	Afternoon	Afternoon
15:45-17.55 Welcome and Opening Session	13:45-15:00 Session I	13:45-15:00 Session II	13:45-15:00 Session III	13:45-15:45 Session IV
	15:00-15:30 Coffee Break + Poster Viewing	15:00-15:30 Coffee Break + Poster Viewing	15:00-15:30 Coffee Break + Poster Viewing	15:45-16:15 Coffee Break + Poster tear-down
18:00 -19:00 Welcome Reception	15:30-16:30 Session I	15:30-16:30 Session II	15:30-17:00 Session III	16:15-17:30 Session IV
			17:00-17:30 Presentation of the next IUFRO Tree Biotech	17:30-17:45 Selection of next IUFRO Tree Biotech
	16:30-18:30 "Advancements in Conifer Genomics" Open Workshop	16:30-18:30 Evoltree Open Workshop	19:00-20:30 Florence Walking Guided Tour	17:45-18:00 Closing Session
			20:30-23:30 Conference Banquet	


Conference Venue
Auditorium D6/018
Polo di Novoli
Via Giovan Filippo Marr
Via Vittorio Emanuele II
Via Bolognese
Via Circondaria
Via del Romito
Viale Francesco Redi
Via di Maragliano
Via Pietro Toselli
Via del Ponte alle Mosse
Via G. S. Mercadante
Via XX Settembre
SS67
Fortezza da Basso
S. Gallo
Aula Magna Rettorato
Piazza San Marco
Università degli
Studi di Firenze
Via Guelfa
Via degli Alfani
Basilica di Santa
Maria Novella
Cattedrale di Santa
Maria del Fiore
Fiume Arno
Via Pisana
Teatro Verdi
Conference banquet
Palazzo Borghese
Via Ghibellina
Firenze

Concluding Remarks

Molecular Biology

The 2015 IUFRO Tree Biotechnology Conference – "Forests: The importance to the planet and society" showcased current and emerging applications in forest biotechnology aimed at addressing the growing need for sustainable, renewable sources of biomass, in the face of climate change. The conference was hosted in beautiful Florence, Italy by several Italian colleagues, lead by Drs. Cristina Vettori, Giovanni Giuseppe Vendramin, Donatella Paffetti and Davide Travaglini. The conference was split into fourth thematic areas, including: 1) Tree genomics and climate change, 2) Trees ecosystems, biodiversity, conservation and environmental interactions, 3) Tree biotechnology: molecular understandings of tree growth and development and applications for tree improvement, and 4) Biosafety: environmental risk assessment, monitoring and management, and socio-economic implications. The scientific presentations at the conference highlighted cutting-edge advancements in many facets of forest biotechnology research, including tree physiology, stress response, molecular breeding, wood development, and an update on the status of the releases of the conifer genomes. In addition, the social, economic, and societal impacts of genetically modified (GM) trees in were heavily discussed as 2015 marked the first deregulation of GM-Eucalyptus in Brazil. The conference attracted a diverse mix of students, postdoctoral fellows, and scientists from academia and industry. This dynamic group of international scientists shared their current results and visions for the future of tree biotechnology. In all, the conference was attended by ~300 registered participants, representing 25 countries. Support for the conference was drawn from a wide variety of Academia, Industry, and Government sources, and included financial support from several commercial genomic service providers.

The conference was a huge success, providing an exceptional mix of science, social activities and field excursions in a relaxed and collegial atmosphere in the backdrop of beautiful Florence Italy. The bi-annual conference will move to South America, to be hosted in the summer of 2017 by Dr. Sofia Valenzuela and her colleagues in Concepcion, Chile.

Shawn Mansfield, Dario Grattapaglia and Armand Séguin

Monday 8 June Aula Magna Rettorato (Piazza S. Marco)

10:30 Registration open

15:45-17:00 **WELCOME AND OPENING SESSION**
CHAIRS: *Cristina Vettori (IBBR-CNR), Giovanni Giuseppe Vendramin (IBBR-CNR), Donatella Paffetti (DISPAA-UNIFI), Davide Travaglini (GESAAF-UNIFI)*
HOST ORGANIZATIONS: *Università degli Studi di Firenze, Dipartimento di Scienze-BioAgroalimentari-CNR*
LOCAL AUTHORITIES: *Regione Toscana, Accademia Italiana di Scienze Forestali, Accademia dei Georgofili, Società Italiana di Selvicoltura ed Ecologia Forestale, Società Italiana Genetica Agraria*

17:00-17:10 **IUFRO Presentation**
Yousry A El-Kassaby (The University of British Columbia, Canada)

17:10-17:55 **OPENING LECTURE**
Sally Aitken (The University of British Columbia, Canada)

Millions of markers but what is the message? Understanding and managing local adaptation in a changing climate (ID: K.O1)

18:00-19:00 WELCOME RECEPTION

Tuesday 9 June Auditorium D6/018 (Polo Novoli)

07:45	Registration Open
08:00 - 08:30	Setting up posters
08:30 - 12:00	**SESSION I** Tree genomics and climate change CHAIRMANS: *Matias Kirst (University of Florida, USA) and Giovanni G. Vendramin (IBBR - CNR, IT)*
08:30 - 09:00	**KEYNOTE SPEAKERS** Genome structure and evolution in Angiosperms and Gymnosperms: when age matters! (No abstract) *Michele Morgante (University of Udine, Italy)*
09:00 - 09:30	Exploring the landscape of the conifer genome: from gene maps to visiting intergenic regions (ID: K.O3) *Jean Bousquet (Université Laval, Canada)*
09:30 - 09:45	**SELECTED TALKS** Assembly and comparative genomics of the quaking aspens *Populus tremula* and *P. tremuloides* (ID: S1.O1) *Nathaniel R. Street (VIB-Ghent University, Belgium)*
09:45 - 10:00	Searching for genes with various copy numbers in the white spruce genome using comparative genome hybridizations (ID: S1.O2) *Julien Prunier (Centre for Forest Research, Université Laval, Canada)*
10:00 - 10:30	COFFEE BREAK + POSTER VIEWING
10:30 - 10:45	**SELECTED TALKS** Gene expression and natural selection shape the evolution of protein-coding genes in *Picea* (ID: S1.O3) *Amanda R. De La Torre (Umeå University, Sweden)*
10:45 - 11:00	Epigenomic profiles of two antagonistic histone modifications, H3K4me3 and H3K27me3, in *Eucalyptus grandis* developing xylem (No abstract) *Steven G. Hussey (University of Pretoria, South Africa)*
11:00 - 11:15	Analysis of the role of chromatin remodeling in environmental control of annual growth cycle (ID: S1.O5) *Pal Miskolczi (Umeå Plant Science Center, Sweden)*
11:15 - 11:30	Transcriptomic responses to epitype inducing cultivation temperatures during somatic embryogenesis in Norway spruce leading to the formation of epigenetic memory (ID: S1.O6) *Igor Yakovlev (Norwegian Forest and Landscape Institute, Norway)*
11:30 - 11:45	Systems genetics dissection of lignocellulosic biomass and bioenergy-related traits in *Eucalyptus* (ID: S1.O7) *Zander Myburg (University of Pretoria, South Africa)*

Tuesday 9 June Auditorium D6/018 (Polo Novoli)

11:45 - 12:00 What MYB genes tell us about the specificities of woody plants? (ID: S1.O8)
Marçal Soler (Laboratoire de Recherche en Sciences Végétaux, France)

12:00 - 13:30 LUNCH

13:30 - 16:30 **SESSION I** Tree genomics and climate change (continue)
Chairmans: *Dario Grattapaglia (EMPRAPA, Brazil) and Zander Myburg (University of Pretoria, South Africa)*

SELECTED TALKS

13:45 - 14:00 Genome-wide association studies of adaptive traits in *Populus trichocarpa* (ID: S1.O9)
Man Zhang (Virginia Tech, USA)

14:00 - 14:15 Coupling metabolomics, mQTL and association genetics analyses to identify genes regulating the production of metabolites (ID: S1.O10)
Timothy Tschaplinski (Oak Ridge National Laboratory, USA)

14:15 - 14:30 A whole-genome association analysis of *Populus deltoides* implicates rare alleles in phenotypic variation for biomass growth and composition (No abstract)
Matias Kirst (University of Florida, USA)

14:30 - 14:45 Genomic selection for growth traits in *Eucalyptus benthamii* and E. pellita populations using a genome-wide *Eucalyptus* 60K SNPs chip (ID: S1.O12)
Barbara S. F. Muller (University of Brasília, Brazil)

14:45 - 15:00 Towards population genomics in Aleppo pine: the colonisation history of a keystone Mediterranean tree (ID: S1.O13)
Giovanni G. Vendramin (Institute of Biosciences and Bioresources, CNR, Italy)

15:00 - 15:30 COFFEE BREAK + POSTER VIEWING

SELECTED TALKS

15:30 - 15:45 Adaptation to drought in *Populus*: Is leaf growth a key trait? (No abstract)
Hazel Smith (University of Southampton, UK)

15:45 - 16:00 Geographic patterns of genetic variation observed in adaptive SNPs, growth chamber common gardens, and long-term field trials: implications for adaptation to climate change (ID: S1.O15)
Laura Gray (University of Alberta, Canada)

16:00 - 16:30 Common discussion of the session I: Chairmans, Invited speakers

16:30 - 18:30 "Advancements in Conifer Genomics" OPEN WORKSHOP
(No abstracts)

Wednesday 10 June Auditorium D6/018 (Polo Novoli)

08:30 - 12:00 **SESSION II** - Tree ecosystem, biodiversity, conservation and environment interactions
CHAIRMANS: *Peter Constabel (University of Victoria, Canada) and Antoine Kremer (INRA, France)*

KEYNOTE SPEAKER

08:30 - 09:00 Molecular signatures of adaptation in Mediterranean conifers at range wide and local spatial scale (ID: K.O4)
Santiago C. González Martínez (INIA, Spain)

SELECTED TALKS

09:00 - 09:15 Genomic diversity of a foundation tree species *(P. deltoides)* in relation to climate, soil, and geography: a northern perspective
(No abstract)
Julle Godbout (Laurentian Forestry Centre, Canada)

09:15 - 09:30 The relationship between genome, epigenome, and physiology in poplar in response to drought (ID: S2.O2)
Katharina Bräutigam (University of Toronto, Canada)

09:30 - 09:45 Inter- and intra-specific variation in drought response: insights from native and non-native conifers in Europe (ID: S2.O3)
Silvio Schueler (Federal Research and Training Centre for Forests, Austria)

09:45 - 10:00 Cell wall rearrangement in wood of poplars subjected to abiotic stresses (ID: S2.O4)
Mireille Cabané (Université de Lorraine, France)

10:00 - 10:30 COFFEE BREAK + POSTER VIEWING

SELECTED TALKS

10:30 - 10:45 Genomic and geographic analysis of the introgression between progenitor-derivative spruce species (*Picea mariana* × *P. rubens*) (ID: S2.O5)
Guillaume deLafontaine (Université Laval, Canada)

10:45 - 11:00 Functional genomics uncovers trait interrelations, pleiotropy, and potential tradeoffs in life-history evolution of forest trees (ID: S2.O6)
Ilga Porth (University of British Columbia, Canada)

11:00 - 11:15 How eco-evolutionary principles may guide tree breeding and tree biotechnology for enhanced productivity (ID: S2.O7)
Oskar Franklin (International Institute for Applied Systems Analysis, Austria)

11:15 - 11:30 Species-specific alleles at a beta-tubulin gene show significant associations with leaf morphological variation within *Quercus petraea* and *Q. robur* populations (ID: S2.O8)
Ilga Porth (University of British Columbia, Canada)

11:30 - 11:45 Discovery of Effector-like proteins in *Populus* during symbiosis formation (No abstract)
Xiaohan Yang (Oak Ridge National Laboratory, USA)

Wednesday 10 June Auditorium D6/018 (Polo Novoli)

11:45 - 12:00 Two sides of the coin: Investigating defence responses against *Phytophthora cinnamomi* in *Eucalyptus nitens* using dual RNA- sequencing (No abstract)
Sanushka Naidoo (University of Pretoria, South Africa)

12:00 - 13:30 LUNCH

13:30 - 16:30 **SESSION II** Tree ecosystem, biodiversity, conservation and environment interactions (continue)
CHAIRMANS: *Janice Cooke (University of Alberta, Canada) and Stephen Cavers (ETH, UK)*

SELECTED TALKS

13:45 - 14:00 *Eucalyptus grandis* defence responses against the myrtle rust pathogen, *Puccinia psidii* – insights from transcriptome profiling (ID: S2.O11)
Louise Shuey (University of Pretoria, South Africa)

14:00 - 14:15 A genomic view of lodgepole and jack pine responses to mountain pine beetle and their fungal associates in the face of mountain pine beetle range expansion (ID: S2.O12)
Janice Cooke (University of Alberta, Canada)

14:15 - 14:30 Primed host response in the conifer Norway spruce (ID: S2.O13)
Carl Gunnar Fossdal (NIBIO, Norway)

14:30 - 14:45 Molecular basis of resistance to myrtle rust (*Puccinia psidii*) in *Melaleuca quinquenervia* (No abstract)
Carsten Külheim (Australian National University, Australia)

14:45 - 15:00 The impact of North American root-rot disease fungus *Heterobasidion irregulare* on ecophysiological properties, volatile terpenoids and transcripts of *Pinus pinea* L. (ID: S2.O15)
Marco Michelozzi (Institute of Biosciences and Bioresources, CNR, Italy)

15:00 - 15:30 COFFEE BREAK + POSTER VIEWING

SELECTED TALKS

15:30 - 15:45 A comparative chemical and transcriptomic analysis of defence pathways activated in conifer trees by pathogenic fungi and herbivorous insect (ID: S2.O16)
Fred O. Asiegbu (University of Helsinki, Finland)

15:45 - 16:00 Biochar alters the soil microbiome: results next generation amplicon sequencing at three European field sites (No abstract)
Gail Taylor (University of Southampton, United Kingdom)

16:00 - 16:30 Common discussion of the session II: Chairmans, Invited speakers

16:30 - 18:30 EVOLTREE OPEN WORKSHOP on "Metagenomics of the tree microbiome"

Thursday 11 June Auditorium D6/018 (Polo Novoli)

08:30 - 12:00 **SESSION III** Tree Biotechnology: molecular understanding of tree growth and development and applications for tree improvement
CHAIRMANS: *Wout Boerjan (VIB/Ghent University, Belgium) and Shawn Mansfield (University of British Columbia, Canada)*

KEYNOTE SPEAKER

08:30 - 09:00 Transposon activation tagging in plants for gene function discovery (ID: K.O5)
Matthias Fladung (vTI, Germany)

SELECTED TALKS

09:00 - 09:15 Exploiting SNPs for CRISPR/Cas9-mediated biallelic mutations in *Populus* reveals 4-coumarate:CoA ligase specificity and redundancy (ID: S3.O1)
Chung-Jui Tsai (University of Georgia, USA)

09:15 - 09:30 Discovery of the biological role of PHENYLCOUMARAN BENZYLIC ETHER REDUCTASE (PCBER), one of the most abundant proteins in poplar xylem (ID: S3.O2)
Wout Boerjan (VIB/Ghent University, Belgium)

09:30 - 09:45 Specific tubulins involved in determining the cellulose microfibiril angle of fibres in eucalypt stems (No abstract)
Antanas Spokevicius (The University of Melbourne, Australia)

09:45 - 10:00 *Arabidopsis* proteins, MODIFYING WALL LIGNIN (MWL)-1 and 2 containing the domain of unknown function 1218 (DUF1218) function redundantly to alter secondary cell wall lignin content (No abstract)
Ritesh Mewalal (University of Pretoria, South Africa)

10:00 - 10:30 COFFEE BREAK + POSTER VIEWING

SELECTED TALKS

10:30 - 10:45 A waxy cover for *Populus trichocarpa* leaves (ID: S3.O5)
Eliana Gonzales-Vigil (University of British Columbia, Canada)

10:45 - 11:00 In planta deacetylation of glucuronoxylan – a way of enhancing sugar yields and deciphering cell wall nanostructure (ID: S3.O6)
Ewa J. Mellerowicz (Swedish University of Agricultural Sciences, Sweden)

11:00 - 11:15 EVE Regulation of Vessel Development (No abstract)
Matias Kirst (University of Florida, USA)

11:15 - 11:30 Quantitative proteomic and phosphoproteomic approaches for deciphering the signaling pathway for tension wood formation in poplar (No abstract)
Mélanie Mauriat (INRA, France)

11:30 - 11:45 Cold regulation of genes related to secondary cell wall biosynthesis affects wood structure and composition (ID: S3.O9)
Fabien Mounet (Laboratoire de Recherche en Sciences Végétaux, France)

Thursday 11 June Auditorium D6/018 (Polo Novoli)

11:45 - 12:00 Transcriptional networks of plastid targeted genes affecting carbon metabolism in *Eucalyptus* xylem (No abstract)
Desré Pinard (University of Pretoria, South Africa)

12:00 - 13:30 LUNCH

13:45 - 17:00 **SESSION III** Tree Biotechnology: molecular understanding of tree growth and development and applications for tree improvement (continue)
CHAIRMANS: *Jacqueline Grima-Pettenati (Université Toulouse II, France) and Matthias Fladung (vTI, Germany)*

SELECTED TALKS

13:45 - 14:00 Genome-wide patterns of recombination, nucleotide diversity and linkage disequilibrium in *Eucalyptus* from high density SNP genotyping and pooled whole-genome resequencing (ID: S3.O11)
Orzenil B. Silva-Junior (Empresa Brasileira de Pesquisa Agropecuária, Brazil)

14:00 - 14:15 Genome-wide prediction of individual tree ranking for growth, chemical and physical wood properties in *Eucalyptus* based on high-density SNP data (ID: S3.O12)
Dario Grattapaglia (EMBRAPA Genetic Resources and Biotechnology, Brazil)

14:15 - 14:30 A comparison of genomic selection models across time in interior spruce (*Picea engelmannii* × *glauca*) with use of unordered SNP imputation methods (ID: S3.O13)
Blaise Ratcliffe (The University of British Columbia, Canada)

14:30 - 14:45 From SNP discovery to operational testing of genomic selection in boreal black spruce (ID: S3.O14)
Patrick Lenz (Laurentian Forestry Centre, Canada)

14:45 - 15:00 Targeted repeat reduction in whole tree genomes prior to sequencing (ID: S3.O15)
David Kainer (The Australian National University)

15:00 - 15:30 COFFEE BREAK + POSTER VIEWING

SELECTED TALKS

15:30 - 15:45 Improving poplar for biofuel applications: elucidating the genetic basis of important traits in *Populus nigra* through an association genetics approach (No abstract)
Mike Allwright (University of Southampton, United Kingdom)

15:45 - 16:00 Genetic architecture of wood properties based on association analysis and co-expression networks in white spruce (ID: S3.O17)
Mebarek Lamara (Université Laval, Canada)

16:00 - 16:15 Towards a Corymbia reference genome: comparative efficiencies of Illumina, PacBio and hybrid de novo assemblies of a complex heterozygous genome (ID: S3.O18)
Orzenil B. Silva-Junior (Empresa Brasileira de Pesquisa Agropecuária, Brazil)

Thursday 11 June Auditorium D6/018 (Polo Novoli)

16:15 - 16:30 Characterization of the network of MYB transcription factors controlling condensed tannin synthesis in *Populus* (ID: S3.O19)
C. Peter Constabel (University of Victoria, Canada)

16:30 - 16:45 The *Eucalyptus* transcription factor EgMYB1 interacts with the histone linker EgH1 to modulate wood formation (ID: S3.O20)
Anna Plasencia (Laboratoire de Recherche en Sciences Végétaux, France)

16:45 - 17:00 Targeted regulation of woody biomass formation in poplars (No abstract)
Kyung-Hwan Han (Michigan State University, USA)

17:00 - 17:30 Presentations of the next IUFRO Tree Biotech Conference Venue

19:00 - 20:30 Florence Walking Guided Tour (only registered delegates)

20:30 - 23:30 CONFERENCE BANQUET (ticket required)

Friday 12 June Sala convegni D6/018 (Polo Novoli)

09:00 - 10:00 **SESSION III** Tree Biotechnology: molecular understanding of tree growth and development and applications for tree improvement (continue)
CHAIRMANS: *Jacqueline Grima-Pettenati (Université Toulouse II, France) and Matthias Fladung (vTI, Germany)*

SELECTED TALKS

09:00 - 09:15 Enhanced cytokinin signaling stimulates cambial activity and biomass production in tree trunk (No abstract)
Juha Immanen (University of Helsinki, Finland)

09:15 - 09:30 Investigating the molecular and physiological basis of drought tolerance in *Populus* (No abstract)
Henning Wildhagen (Georg-August-University Göttingen, Germany)

09:30 - 09:45 Polycomb and ABA mediate photoperiodic control of bud dormancy (ID: S3.O25)
Rishikesh P. Bhalerao (Bhalerao, Sweden)

09:45 - 10:00 Paternity recovery in maritime pine policross trial using SNPs: consequences for breeding (ID: S3.O26)
Marjorie Vidal (Institut Technologique FCBA, INRA, France)

10:00 - 10:30 Common discussion of the session III: Chairmans, Invited speakers

10:30 - 11:00 COFFEE BREAK + POSTER VIEWING

11:00 - 17:45 **SESSION IV** Biosafety: environmental risk assessment, monitoring and management, and socio-economic implications
CHAIRMANS: *Hely Hagman (University of Oulu, Finland), Elisabeth Waigmann (EFSA)*

KEYNOTE SPEAKERS

11:00 - 11:30 Forest Biotechnology and Sustainability (ID: KO6)
Stanley Hirsch (FuturaGene Group CEO)

11:30 - 12:00 Field trials with genetically engineered forest trees: past experiences and future prospects (ID: KO7)
Gilles Pilate (INRA, France)

12:00 - 12:30 RNAi based GM plants: EFSA activities in relation to environmental risk assessment (ID: KO8)
Elisabeth Waigmann (EFSA)

12:30 - 14:00 LUNCH

13:45-17:30 **SESSION IV** Biosafety: environmental risk assessment, monitoring and management, and socio-economic implications (continue)
CHAIRMANS: *Gilles Pilate (INRA, France), Stanley Hirsch (FuturaGene Group CEO)*

Friday 12 June Sala convegni D6/018 (Polo Novoli)

KEYNOTE SPEAKERS

14:15 - 14:45 Socio-economic implications in relation to the use of GMTs: concerns and acceptance by the public in South America (ID: KO9)
Sandra Sharry (Argentina)

INVITED TALKS

14:45 - 15:00 COST Action FP0905: final results (ID: S4.O1)
Hely Haggman (University of Oulu, Finland)

15:00 - 15:15 Biosafety of a genetically modified *Eucalyptus* with yield enhancement characteristics (ID: S4.O2)
Othon Abrahão (FuturaGene, Brazil)

SELECTED TALKS

15:15 - 15:30 EFSA GMO Panel Scientific Opinions on the safety assessment of plants obtained through cisgenesis, intragenesis or Site-Directed Nucleases (SDN-3) (ID: S4.O3)
Andrea Gennaro (EFSA, GMO Unit, Italy)

15:30 – 15:45 Containment technology for trees: a new frontier opens (ID: S4.O4)
Amy L. Klocko (Oregon State University, USA)

15:45 - 16:15 COFFEE BREAK + Poster tear-down

SELECTED TALKS

16:15 - 16:30 Assessing the risk of gene flow from plantation to native eucalypts in Australia (ID: S4.O5)
Brad Potts (University of Tasmania, Australia)

16:30 - 16:45 Model assessment of transgenic trees impact on nitrogen and carbon cycles in forest plantations (ID: S4.O6)
Konstantin Shestibratov (Institute of Bioorganic Chemistry RAS, Russia)

16:45 - 17:30 Common discussion of the session IV:
Chairmans, Invited speakers, Società Italiana Genetica Agraria

17:30 - 17:45 Selection of next IUFRO Tree Biotech Conference Venue

17:45 - 18:00 CLOSING SESSION

CHAIRS: *Cristina Vettori (IBBR-CNR), Giovanni Giuseppe Vendramin (IBBR-CNR), Donatella Paffetti (DISPAA-UNIFI), Davide Travaglini (GESAAF-UNIFI)*

Workshop on "Advancements in Conifer Genomics"

16:30 - 18:30 **Tuesday 9 June (Auditorium D6/018, Polo Novoli)**

The workshop is open to everyone interest in it!

Organized by representatives of the conifer genomics initiatives

Maria Teresa Cervera (Spain), Carmen Diaz-Sala (Spain), Par Ingvarsson (Sweden), John Mackay (UK-Canada), Jill Wegrzyn (USA)

Format: Open discussion workshop

Objective: The aim of this meeting is to briefly discuss the state of the art of the conifer genomics to foresee potential integrative initiatives

AGENDA

16:30- 17:10 Presentations of the conifer genomics initiatives

- Update of the most important scientific and technological results
- Current and future scientific activities
- Convergence among initiatives

17:10 - 18:10 Open discussion: Implementation of point-to-point research convergence

18:10 - 18:30 Financial programs to implement international research in plants including mobility

EVOLTREE WORKSHOP

"METAGENOMICS OF THE TREE MICROBIOME"

16:30 - 18:30 **Wednesday 10 June (Auditorium D6/018, Polo Novoli)**

The workshop is open to everyone interest in it!

CHAIRMAN: *Antoine Kremer (INRA, France)*

KEYNOTE SPEAKERS

16:30 - 17:30 Understanding the *Populus* Microbiome: Drivers of Community Variation and Potential Implications for Plant and Ecosystem Function (ID: S.EV.1)
Christopher W. Schadt (Oak Ridge National Laboratory, USA)

Endophytic bacterial microbiome of mountain birch recovered from moth herbivory (No abstract)
Anna-Maria Pirttilä (University of Oulu, Finland)

VOLUNTARY SPEAKERS

17:30 – 18:30 QTL mapping for phyllosphere microbial community descriptors in oak (*Quercus robur* L.) (ID: S.EV.3)
Boris Jakuschkin (Univ. Bordeaux, France)

Endophytic bacteria in poplars – Characterization and artificial inoculation to enhance growth parameter (ID: S.EV.4)
Heike Liesebach (Thünen Institute of Forest Genetics, Germany)

TreeType (ID: S.EV.5)
Stephen Cavers (ETH, UK)

POSTER SESSIONS

Poster Session I: Tree genomics and climate change

ID	ABSTRACT TITLE	AUTHORS
S1.P1	Exploring adaptive responses to change in environmental conditions in *Pinus halepensis* Mill.	Mariaceleste Labriola, Andrea Piotti, Ilaria Spanu, Tiziana Gentilesca, Cristina Leonarduzzi, Nicola Moretti, González-Martínez Santiago, Marco Borghetti, Giovanni Giuseppe Vendramin
S1.P2	Gene expression profiling in response to UV-B radiation in different *Populus alba* clones	Cristina Vettori, Kostlend Mara, Ilaria Spanu, Sabrina Raddi, Donatella Paffetti
S1.P3	Genetical genomics and physiological responses to drought stress in poplar (No abstract)	Francesco Fabbrini, Chiara Evangelistella, Simone Scalabrin, Sabine Schnabel, Riccardo Ludovisi, Simon Rüger, Maurizio Sabatti, Michele Morgante, Gail Taylor, Joost Keurentjes, Giuseppe Scarascia-Mugnozza, Antoine Harfouche
S1.P4	Molecular and physiological analysis of drought response in the Mediterranean conifer *Pinus pinaster* Ait.	Nuria de María, María Ángeles Guevara, José Antonio Cabezas, Enrique Sáez-Laguna, Marina de Miguel, Luis Manuel Díaz, Alberto Pizarro, Carmen Collada, David Sánchez-Gómez, Estrella Cadahía, Ismael Aranda, M. Teresa Cervera
S1.P5	Identification and analysis of candidate genes for drought stress tolerance and bud burst in European beech (*Fagus sylvatica* L.)	Markus Müller, Sarah Seifert, Reiner Finkeldey
S1.P6	Geographic variation in cork oak and its implications for expected impacts of climate change	Rodrigues Ana, Sampaio Teresa, Costa e Silva João, Patrício Maria Sameiro, Costa e Silva Filipe, Faria Carla, Correia Antonio, Varela Carolina, Pereira João Santos, Almeida Maria Helena
S1.P7	Gene discovery in white pine: a catalog of transcripts enriched for genes associated with freezing tolerance produced by Illumina mRNA-Seq. (No abstract)	Claire Rasheed-Depardieu and Ingo Ensminger
S1.P8	SuberStress - An integrated approach to identify regulatory genes in the crosstalk between abiotic and biotic stress response in cork oak (No abstract)	Pedro M Barros, Ana Leal, Natacha Vieira, Francisca Reis, Pablo R Hardoim, Andreas Bohn, A Cristina Coelho, Rui M Tavares, Teresa Lino-Neto, José C. Ramalho, Nelson Saibo, M Margarida Oliveira
S1.P9	Transcriptome responses to medium-term water deficit among *Eucalyptus* species of contrasting ecotype provide insight into key pathways responsible for adaptation to water limited environments	Antanas V Spokevicius, Josquin Tibbits, Philippe Rigault, Marc-Alexandre Nolin, Andrew Merchant
S1.P10	Ecotypic variation and global expression patterns in response to light spectra in Scots pine (*Pinus sylvestris*) (No abstract)	Sonali S. Ranade, M.Rosario García-Gil
S1.P11	Combining genetic, ecological and extinction risk to assess conservation management	Serra-Varela M.J., Ruiz-Daniels R., Alía R., González-Martínez S., Zimmermann N.E., Grivet D., Gonzalo-Jiménez J.
S1.P12	Comparative analysis reveals differential gene family expansions in stress genes in conifer species	Amanda R. De La Torre, Yao-Cheng Lin, Zhen Li, Yves Van de Peer, Pär K. Ingvarsson

Poster Session I: Tree genomics and climate change

ID	ABSTRACT TITLE	AUTHORS
S1.P13	Application of GBS and RADseq for discovering and genotyping of SNP polymorphisms in *Fagus sylvatica* and *Quercus robur*	Bartosz Ulaszewski, Jarosław Burczyk
S1.P14	The Genosuber project: initial sequencing and annotation of the cork oak (*Quercus suber* L.) genome	Inês Chaves, Genosuber Consortium
S1.P15	Insight into conifer karyotype evolution from high resolution comparative mapping between Pinaceae (n=12) and Cupressaceae (n=11)	Marina de Miguel, Jérôme Bartholomé, François Ehrenmann, Yoshinari Moriguchi, Kentaro Uchiyama, Saneyoshi Ueno, Yoshihiko Tsumura, Nuria de Maria, Maria-Teresa Cervera, Jean-Marc Gion, Jérôme Salse, Christophe Plomion
S1.P16	RuBisCo Evolution in Eucalypts	Hossein Valipour Kahrood, Philippe Rigault, Antanas Spokevicius, Gerd Bossinger Josquin Tibbits
S1.P17	Conservative microRNA studies and analyses in Scots pine (*Pinus sylvestris* L).	Baiba Krivmane, Ilze Šņepste, Vilnis Šķipars, Dainis Ruņģis
S1.P18	Development of chromosome- and organelle-specific SNP markers for different *Populus* genotypes	Matthias Fladung, Hilke Schroeder, Birgit Kersten
S1.P19	TOZ19 is a Y haplotype-specific gene in aspen	Birgit Kersten, Birte Pakull, Cristina Vettori, Matthias Fladung
S1.P20	Identification of genetic determinants of the rooting capacity and tolerance to *Mycosphaerella* sp. in *Eucalyptus globulus*	José Antonio Cabezas, María Dolores Vélez, Libertad Juez, Elena Carneros, Luis Manuel Díaz, Carmen Barbero, Claudia Escudero, Alicia del Amo, Nuria de María, Carmen Díaz-Sala, Carlos Tejedor, M. Teresa Cervera
S1.P21	The evolution and characterization of dehydrins in white spruce (No abstract)	Juliana Stival Sena, Isabelle Giguère, Philippe Rigault, Jean Bousquet and John Mackay
S1.P22	Analysis workflow for small RNA sequencing data of the conifer *Pinus pinaster* (No abstract)	Andreas Bohn, Andreia Rodrigues, Inês Chaves, Célia Miguel
S1.P23	Expanding the Genomes of Myrtaceae: Progress Towards the Guava Genome	Anita Severn-Ellis[1], Jonathan Featherston[2], D. Jasper G. Rees[3] and Charles A. Hefe
S1.P24	Effects of primed conifer defences on a tree-killing bark beetle-fungus complex	Carl Gunnar Fossdal, Hugh Cross, Adam Vivian-Smith and Paal Krokene
S1.P25	Using different molecular approaches to resolve the phylogeny and taxonomic complexity of the European black pine, *Pinus nigra* Arnold	Guia Giovannelli, Anne Roig, Ilaria Spanu, Giovanni Giuseppe Vendramin, Bruno Fady
S1.P26	The involvement of 5-methyl cytosine DNA Demethylases in the dormant-growth transition in poplar	Daniel Conde, Alicia Moreno-Cortés, Tamara Hernández-Verdeja, Jose M. Ramos-Sánchez, Mariano Perales, Pablo González-Melendi, Isabel Allona
S1.P27	Induction of poplar mutants by activation tagging with an inducible Ac/Ds transposon system	Matthias Fladung

Poster Session I: Tree genomics and climate change

ID	ABSTRACT TITLE	AUTHORS
S1.P28	Retrotransposon expression in response to in vitro inoculation with two fungal pathogens of Scots pine (*Pinus sylvestris* L.)	Angelika Voronova, Viktorija Belevich, Dainis Rungis
S1.P29	Phenotype dependent differences in the hypomethylome of *Norway spruce*	Eva M. Sehr, Maria Berenyi, Elisabeth Wischnitzki, Karin Hansel-Hohl, Kornel Burg, and Silvia Fluch
S1.P30	European Douglas-fir stands and seed sources: estimation of the varietal and geographic origin, and comparisons in morphology, bud burst and genetic diversity	Marcela van Loo, Tamara Eckhart, Wolfgang Josef Hintsteiner, Silvio Schüler, Hubert Hasenauer
S1.P31	Stucture of genetic diversity in *Abies alba* Mill. populations from the Czech Republic	Helena Cvrčková, Pavlína Máchová
S1.P32	Microsatellite markers using for clonal identification in the *Norway spruce* seed orchard	Pavlína Máchová, Helena Cvrčková

Poster session II: Tree ecosystem, biodiversity, conservation and environment interactions

ID	ABSTRACT TITLE	AUTHORS
S2.P1	Single-locus versus Multi-locus Effects Underlying the Genetic Architecture of Local Adaptation in Eastern White Pine (*Pinus strobus*)	Om P. Rajora, Andrew J. Eckert, and John W.R. Zinck
S2.P2	Does molecular diversity generally decrease from south to north? Contrasting results from a rare and scattered forest tree (*Sorbus domestica* L.)	George Jan-Peter, Konrad Heino, Collin Eric, Thevenet Jean, Ballian Dalibor, Idzojtic Marilena, Kamm Urs, Zhelev Peter, Geburek Thomas
S2.P3	Fine scale adaptation and adaptive potential in phenology of perennial species with contrasting life history traits (No abstract)	Albin Lobo, Erik Dahl Kjær, Lars Nørgaard Hansen and Jon Kehlet Hansen
S2.P4	Genetic structure of relict and isolated Scots pine (*Pinus sylvestris* L.) populations from Central-Eastern Europe: signals of recent population fragmentation?	Endre Gy. Tóth, Giovanni G. Vendramin, Francesca Bagnoli, Klára Cseke, Mária Höhn
S2.P5	*Inga ingoides* and *Inga edulis*: how close species are they for agroforestry purposes?	Maria Margarida Ribeiro, Alexandr Rollo, David Honys, Bohumil Mandák, Julio Chia Wong, Carmen Santos, Rita Costa, Bohdan Lojka
S2.P6	Genetic diversity of *Chukrasia* spp. as revealed by ISSR markers (No abstract)	Chong Wu, Chonglu Zhong, Yong Zhang, Qingbin Jiang, Yu Chen, Zhen Chen, Khongsak Pinyopusarerk, David Bush
S2.P7	How history and demography affected *Arbutus unedo* populations' genetic structure (No abstract)	Maria Margarida Ribeiro, Andrea Piotti, Alexandra Ricardo, Daniel Gaspar, Rita Costa, Giovanni Giuseppe Vendramin
S2.P8	Tickle me tree *"Lagerstroemia indica"*: the reason research	Jae In Park, Hyo Jin Han
S2.P9	Chloroplast DNA markers reveal the native locality of origin of *Eucalyptus globulus* at the Tre Fontane Abbey in Rome	Brad Potts, Sascha Wise, Jules Freeman and René Vaillancourt
S2.P10	Genetic diversity and structure of *Quercus trojana* populations in Italy	Claudia Mattioni, Maddalena Carabeo, Marco Cosimo Simeone, Carla Caruso, Laura Bertini, Marcello Cherubini, Francesca Chiocchini, Chiara Mattia, Fiorella Villani
S2.P11	Strawberry biophysical units' ecology to design provenance regions in Portugal using GIS tools	Luís Quinta-Nova, Natália Roque, Alexandra Ricardo, Maria Margarida Ribeiro
S2.P12	Relationship between internal morphology and germination of seeds of *Bauhinia longifolia* (Bong.) Steud. - Fabaceae, a Brazilian tree	Leticia Caravita Abbade, Silvio Moure Cicero
S2.P13	Genetic identification of maternal and progeny generation of Scots pine (*Pinus sylvestris* L.)	Agata Konecka, Justyna A. Nowakowska, Anna Tereba
S2.P14	Extensive clonal structure and moderate genetic diversity at peripheral populations of *Sorbus torminalis* (L.) Crantz, a scattered, self-incompatible tree species	Sandra Jankowska-Wróblewska, Jarosław Burczyk

Poster session II: Tree ecosystem, biodiversity, conservation and environment interactions

ID	ABSTRACT TITLE	AUTHORS
S2.P15	Genotypic characterization of cultivated and wild *Prunus cerasus* var. austera from Central Italy	Muriel Gaudet, Claudia Mattioni, Marcello Cherubini, Francesca Chiocchini, Isacco Beritognolo, Fiorella Villani
S2.P16	Nuclear and chloroplast SNP markers support successful poplar breeding	Hilke Schroeder, Tobias Bruegmann, Matthias Fladung
S2.P17	Cross-species transferability of EST-based SSRs and nuclear SSRs to Mexican white pines	Alma Rosa Villalobos-Arámbula, Dolores Barragán Reynaga, Judith Morales Saavedra, Luis Mario Valadez Sandoval, Jesús Aguirre-Gutiérrez
S2.P18	Protoplast isolation from leaf tissues of holm oak, a new tool for the study of DNA integrity	Elena Kuzminsky, Roberta Meschini, Cristian Silvestri, Serena Terzoli, liliana Pavani, Giuseppe Scarascia-Mugnozza
S2.P19	Genetic structure and diversity of natural black poplar (*Populus nigra* L.) populations along three rivers in Slovenia and Croatia	Gregor Bozic, Marjana Westergren, Davorin Kajba, Hojka Kraigher, An Vanden Broeck
S2.P20	Population structure of *Eucalyptus cladocalyx* based on SSR marker analysis	Freddy Mora, Osvin Arriagada, Carlos Maldonado, Paulina Ballesta, Nicol Vargas, Raúl Herrera, Eduardo Ruiz
S2.P21	Speciation within Betulaceae family – a look into organellar genomes (No abstract)	Omid Mohammadi, Jarkko Salojärvi, Sitaram Rajaraman, Olli-Pekka Smolander, Ali Amiryousefi, Pezhman Safdari, Airi Lamminmäki, Juha Immanen, Kaisa Nieminen, Lars Paulin, Petri Auvinen, Yrjö Helariutta and Jaakko Kangasjärvi
S2.P22	Range-wide analysis of genetic variation of *Pinus mugo* Turra using chloroplast microsatellites	Weronika B. Żukowska, Witold Wachowiak, Monika Litkowiec, Błażej Wójkiewicz
S2.P23	Sub-structuring of Scots pine across European distribution range based on cpDNA SSR markers	Błażej Wójkiewicz, Witold Wachowiak, Monika Litkowiec, Weronika B. Żukowska
S2.P24	Spatio-temporal gene flow patterns along an altitudinal transect in a marginal Norway spruce population from northern Apennines (No abstract)	Camilla Avanzi, Andrea Piotti, Stefano Leonardi, Cristina Leonarduzzi, Ilaria Spanu, Valeria Gallucci, Carlo Urbinati, Giovanni Giuseppe Vendramin
S2.P25	Contemporary seed and pollen immigration in an altitudinal transect of silver fir (*Abies alba* Mill.) in a fragmented area in Central Apennines (No abstract)	Cristina Leonarduzzi, Andrea Piotti, Ilaria Spanu, Giovanni Giuseppe Vendramin
S2.P26	Does human-induced selection influence the spatial genetic structure diversity and dynamics in beech forests	Donatella Paffetti, Cristina Vettori, Francesca Bottalico, Daniele Matteucci, Susanna Nocentini, Davide Travaglini
S2.P27	Conservation genetics of *Pinus heldreichii* var. leucodermis disjunct populations in the Pollino National Park (southern Italy) (No abstract)	Andrea Piotti, Sebastiano Pini, Francesca Bagnoli, Aldo Schettino, Giuseppe De Vivo, Ilaria Spanu, Dalibor Ballian, Giovanni Giuseppe Vendramin
S2.P28	Genotype by Environment Interaction in Radiata Pine for Growth and Biomass Traits in Chile	Sergio E Espinoza, Carlos R Magni, Rómulo E Santelices, Antonio M Cabrera

Poster session II: Tree ecosystem, biodiversity, conservation and environment interactions

ID	ABSTRACT TITLE	AUTHORS
S2.P29	Genetic diversity in candidate genes for drought stress. Photoperiod perception and cold tolerance along environmental gradients: evidence of climate-driven local adaptation in *Nothofagus* spp from Patagonia	Carolina Soliani, Maximiliano Estravis Barcalá, Susana Torales, Florencia Pomponio, Birgit Ziegenhagen, María Verónica Arana, Paula Marchelli
S2.P30	Phylogeography and Ecological Niche Modelling of Neotropical Myrtaceae *Eugenia uniflora* L. unveil distinct evolutionary scenario from Southern to Northern Atlantic Rain Forest Domain (No abstract)	Andreia C. Turchetto-Zolet, Fabiano Salgueiro, Caroline Turchetto, Fernanda Cruz, Nicole M. Veto, Michel J. F. Barros, Ana L. A. Segatto, Loreta B. Freitas, Rogério Margis
S2.P31	Role of RAV genes in tree seasonal dormancy	Tamara Hernández-Verdeja, Alicia Moreno-Cortes, Judith Lucia Gomez-Porras, Chris Dervinis, José Manuel Franco-Zorrilla, Matias Kirst, Isabel Allona
S2.P32	Local provenances of woody species in the face of climate change (No abstract)	Vander Mijnsbrugge Kristine
S2.P33	Evaluation of the adaptive potential of silver fir (*Abies alba*) along altitudinal gradients using reciprocal transplants	Anne Latreille, Christian Pichot, Bruno Fady
S2.P34	Molecular identification of *Fraxinus excelsior* L. population in terms of ash dieback	Anna Tereba, Justyna A. Nowakowska, Artur Pacia, Tomasz Oszako, Agata Konecka
S2.P35	Expression profiling of lodgepole and jack pine chitinase gene family in response to inoculation by mountain pine beetle fungal associate *Grosmania clavigera*	Elizabeth L. Mahon, Adriana Arango-Velez, Catherine I. Cullingham and Janice E.K. Cooke
S2.P36	Transcriptome profile of candidate genes associated with resistance to *Fusarium circinatum* in *Pinus radiata*	Angela Carrasco, Jill L. Wegrzyn, Andrea Donoso, Eugenio Sanfuentes, Álvaro Durán, David Neale, Sofía Valenzuela
S2.P37	Interspecific variation of a constitutive defense mechanism in conifers (No abstract)	Geneviève J. Parent, Isabelle Giguère, John J. MacKay
S2.P38	Natural variation in metabolites, growth and biotic interactions in aspen (*Populus tremula*) (No abstract)	Kathryn Robinson, Nathaniel Street, Benedicte Albrectsen, Stefan Jansson
S2.P39	Clonostachys rosea-mediated resistance against pitch canker disease in *Pinus radiata* (No abstract)	Priscila Moraga-Suazo, Eugenio Sanfuentes
S2.P40	Genetic diversity of Heterobasidion, the casual agent of conifer root and butt rot	Nicola Luchi, Donatella Paffetti, Paolo Capretti
S2.P41	Genetic and spatial structure of natural populations of *Ziziphus joazeiro* Mart. – strategy for seed collecting	Manoela M. Duarte, Antônio C. Nogueira, Elisa S. N. Vieira

POSTER SESSION III: Tree Biotechnology: molecular understanding of tree growth and development and applications for tree improvement

ID	ABSTRACT TITLE	AUTHORS
S3.P3	Linkage Disequilibrium and Genomic Selection in Maritime Pine (No abstract)	Fikret Isik, Jérôme Bartholomé, Alfredo Farjat, Emilie Chancerel, Annie Raffin, Leopoldo Sanchez, Christophe Plomion, Laurent Bouffier
S3.P4	SNP development and High-dense genetic maps construction of *Eucalyptus urophylla* and *E. tereticornis* (No abstract)	Fagen Li, Changpin Zhou, Qijie Weng, Xiaoli Yu, Mei Li, Siming Gan
S3.P5	Development of Candidate Gene-based KASP SNP Assays and their Application for Genetic and Association Studies in *Eucalyptus camaldulensis*	Prasad S. Hendre, Kamalakannan Rathinam, Anandh Kumar Chandran, Aparna L. Gurumurthy, Ramadevi Payyavula, Mohan Varghese, Rajkumar Rathinavelu
S3.P6	Dense genetic maps of *Eucalyptus urophylla* and *E. tereticornis* for genomic comparison and quantitative trait locus analysis (No abstract)	Fagen Li, Changpin Zhou, Xiaoli Yu, Qijie Weng, Yong Guo, Yu Wang, Xiaohong Zhang, Mei Li, Siming Gan
S3.P7	Development of biotechnological tools for genetic improvement of willow (No abstract)	Elena Palomo-Ríos, Huw Jones, Steven Hanley and Angela Karp
S3.P8	Biotechnologies and tree improvement for forest tree farming in highland areas in Italy	Fulvio Ducci, Anna De Rogatis, Maria Emilia Malvolti, Roberta Proietti, Piero Belletti
S3.P9	Breeding for browsing "resistance" in *Norway spruce*	Carlos Trujillo-Moya, Silvio Schueler, Heino Konrad
S3.P10	Annual Variation of Mating System in Seed Orchard of *Pinus thunbergii* Revealed by Microsatellite Markers	Young Mi Kim, Jae In Park,Yong Pyo Hong, Kyung Nak Hong , and Yu Jin Park
S3.P11	Top reasons to develop a quality control system for large-scale production of conifers: the example of white spruce (No abstract)	Julie Godbout, Patricia Lavigne, Laurence Tremblay, John MacKay, Jean Bousquet, Nathalie Isabel
S3.P12	Screening of 19.2 Mb of genomic sequence surrounding DArT markers associated to wood quality traits in *Eucalyptus globulus*	Pamela V. Villalba, Janet Higgins, Natalia C. Aguirre, Cintia V. Acuña, Martín Garcia, Eduardo P. Cappa, María C. Martinez, Pablo Pathauer, H. Esteban Hopp, Dario Grattapaglia, Norma Paniego, Susana Marcucci Poltri
S3.P13	Assessing patterns of genotype-by-environment interactions to aid planning for integrating genomic selection into tree breeding programs	Nicholas K. Ukrainetz, Alvin Yanchuk and Shawn D. Mansfield
S3.P14	Genomic selection in a multi-generation conifer breeding population: maritime pine as a study case	Jérôme Bartholomé, Christophe Boury, Joost van Heerwaarden, Fikret Isik, Christophe Plomion, Laurent Bouffier
S3.P15	Genomic Selection of *Eucalyptus elite* trees in northern Brazil by SNPs analysis (No abstract)	Yujiroh Fukuda, Tomotaka Shinya, Shinichi Onogi and Akiyoshi Kawaoka

POSTER SESSION III: Tree Biotechnology: molecular understanding of tree growth and development and applications for tree improvement

ID	ABSTRACT TITLE	AUTHORS
S3.P16	Efficient method to extract high quality DNA from dried *Eucalyptus* leaves	Durandeau Karine
S3.P17	Genetic variation between progenies of *Pinus caribaea* Morelet var. *caribaea* based on microsatellite markers	Janete Motta da Silva, Ananda Virginia de Aguiar, Edson Seizo Mori, Mario Luiz Teixeira de Moraes, Valderês Aparecida de Sousa
S3.P18	Measuring telomere length in forest trees and its relevance to tree breeding	Sacha Khoury, Paolo Latini, Francesco Fabbrini, Luca Proietti-De-Santis, Roberta Meschini, Giuseppe Scarascia-Mugnozza, Antoine Harfouche
S3.P19	Prediction Accuracy of Growth and Wood Attributes of Interior Spruce in Space Using Genotyping-by-Sequencing	Omnia Gamal El-Dien Ibrahim, Blaise Ratcliffe, Jaroslav Klápště, Charles Chen, Ilga Porth, Yousry A. El-Kassaby
S3.P20	Uncovering the embryo microRNA transcriptome of the conifer *Pinus pinaster* (No abstract)	Andreia S. Rodrigues, Inês Chaves, Andreas Bohn, Ana Milhinhos, Susana Lopes, André Rodrigues, Célia Miguel
S3.P21	Novel and known microRNAs identified in vascular tissues in Scots pine and Maritime pine	Ana Carvalho, Clara Graça, Victor Carocha, Susana Pêra, José Lima-Brito, Gregoire Le Provost, Jorge A. P. Paiva
S3.P22	Hairy root transformation of *Eucalyptus grandis*, a tool to investigate the function of genes involved in wood formation	Anna Plasencia, Marçal Soler, Annabelle Dupas, Guilherme Silva Martins, Nathalie Ladouce, Yves Martinez, Isabelle Truchet, Catherine Lapierre, Claudine Franche, Jacqueline Grima-Pettenati
S3.P23	Cultivable bacteria and fungi isolated from rhizosphere and phyllosphere of Guayacan trees (*Tabebuia chrysantha*) and evaluated as seed microbial inoculants for plantlet growth promotion (No abstract)	Luis X. Llacsa, Gorky V. Lajones, Luis Rodriguez, Liliana Solis, Ramón García, Eric Mialhe
S3.P24	Analysing the action of thermospermine during xylem development using the Arabidopsis root model (No abstract)	Andreia Matos, Ana Milhinhos, Osvaldo S. Ascenso, M. Rita Ventura, Célia Miguel
S3.P25	Molecular understanding of nitrogen-fixing nodule development in the tropical actinorhizal tree *Casuarina glauca*	Didier Bogusz, Jocelyne Bonneau, Claudine Franche
S3.P26	An integrative approach dissecting the control of wood formation (No abstract)	Juan Alonso Serra, Juha Immanen, Kaisa Nieminen, Ykä Helariutta
S3.P27	Tree selection including several QTLs related to growth and wood quality traits in *Eucalyptus grandis*	Martín García, Eduardo Cappa, Pamela Villalba, Cintia Acuña, María Martínez, Javier Oberschelp, Leonel Harrand, Juan López, Janet Higgins, Martín Marc, Norma Paniego, Susana Marcucci Poltri, Mauro Surenciski, Esteban Hopp
S3.P28	Novel winter-associated regulators of the circadian clock in poplar	José M. Ramos-Sánchez, Alicia Moreno-Cortés, Daniel Conde, Tamara Hernández-Verdeja, Mariano Perales, Isabel Allona

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S3.P30	Genetic structure and genome-wide association mapping for vegetative propagation traits in *Casuarina*	Kullan A.R.K, Suraj P.G, Ashok V. Kulkarni, Mohan V, Rajkumar R
S3.P31	Enrichment of a *Hevea brasiliensis* microsatellite-based genetic map and identification of candidate genes for growth-related traits (No abstract)	Da Silva Carla Cristina, Mantello Camila Campos, Souz Lívia Moura, Gazaff Rodrigo, Garcia Dominique, Le Guen Vincent, Garcia Antônio Augusto, Souz, Anete Pereira
S3.P32	Genomic prediction of growth traits in *Pinus taeda* using genome-wide sequence-based DArT-seq markers	Ananda Virginia de Aguiar, Dione Mendes Alves Teixeira-Freitas, Janeo Eustaquio de Almeida Filho, Valderês Aparecida de Sousa, Marcos Deon Villela Resende, Orzenil Bonfim Silva-Junior, Dario Grattapaglia
S3.P33	Genotyping and phenotyping of a *Eucalyptus grandis* training population towards the development of genomic selection models (No abstract)	M.M. Mphahlele, F. van Deventer, S.M. Reynolds, M.M. O'Neill, G.R. Hodge, F. Isik, A.A.Myburg
S3.P34	Leaf Development Characterisation in European Aspen (*Populus tremula*) (No abstract)	Barbara K. Terebieniec, Bastian Schiffthaler, Kathryn M. Robinson, Niklas Mähler, Nicolas Delhomme, Torgeir R. Hvidsten, Nathaniel R. Street
S3.P35	Genomic technologies to select for resistance to *Phytophthora* disease	Emily Telfer, Natalie Graham, Yongjun Li, Vincent Liu, Charlie Low, Lucy Macdonald, Grahame Stovold, Phillip Wilcox, Nari Williams, Rebecca Ganley, Heidi Dungey.
S3.P36	Defining Roles for SVP-like Genes in the Activity-Dormancy Transition of White Spruce Terminal Buds (No abstract)	Amanda S Gregoris, Jocelyn C Hall, Walid El Kayal, Janice EK Cooke
S3.P37	Sequencing of the *Pinus patula* shoot transcriptome and identification of antifungal defence genes (No abstract)	Erik Visser, Jill Wegrzyn, Emma Steenkamp, Alexander Myburg, Sanushka Naidoo
S3.P38	Exploring poplar GH5 enzymes	Erika Groth, Christine Ratke, Ewa Mellerowicz, Henrik Aspeborg
S3.P39	Characterization of the cork oak transcriptome dynamics during acorn development	Andreia Miguel, José de Vega-Bartol, Liliana Marum, Inês Chaves, Tatiana Santo, José Leitão, Maria Carolina Varela, Célia Maria Miguel
S3.P40	Isolation and characterization of four dehydrin genes from *Eucalyptus nitens*	Sofía Valenzuela, Paula Aguayo, Margaret Ochoa, Claudio Balocchi, Marta Fernández
S3.P41	Transcriptome analyses and gene expression profiling in different tissues of *Jatropha curcas* genotypes (No abstract)	Fatemeh Maghuly, Deak Tamas, Klemens Vierlinger, Stephan Pabinger, Margit Laimer
S3.P42	African Orphan Crops Consortium (AOCC): a Global Partnership to Address Food and Nutritional Requirements in Africa through Tree Genomics Applications	Prasad S. Hendre, Ramni Jamnadass, Alice Muchugi, Allen Van Deynze

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S3.P43	Identification of differentially expressed genes from Spanish *Ulmus minor* genotypes with contrasted tolerance to *Ophiostoma novo-ulmi*	Pedro Perdiguero, Martin Venturas, David Medel, Jorge Domínguez, Juan Antonio Martín, Luis Gil, Carmen Collada
S3.P44	Transcript level analysis of secondary cell wall formation in *Eucalyptus urophilla* x *E. grandis* (No abstract)	Tomotaka Shinya, Kenichiro Suyama, Yujiroh Fukuda, Eiji Iwata, Shinichi Onogi, Kazunori Hayashi, Antonio C. Rosa, Akiyoshi Kawaoka
S3.P45	Screening for candidate genes of stress response in pine	Dolores Abarca, Inmaculada Hernández, Alicia del Amo, Elena Carneros, Alberto Pizarro, Concepción Ávila, Francisco M. Cánovas, Marina de Miguel, Ángeles Guevara, Ismael Aranda, Maria Teresa Cervera, Carmen Díaz-Sala
S3.P46	De novo assembly and annotation of a transcriptome during xylogenesis in *Pinus canariensis* Chr. Sm. ex DC	Víctor Chano, Unai López de Heredia, Carmen Collada, Álvaro Soto
S3.P47	Quantitative proteomic and phosphoproteomic approaches to better understand the contrasting response to drought of three *Populus nigra* genotypes (No abstract)	Mélanie Mauriat, Jérôme Bartholomé, Marlène Daventure, Marie-Béatrice Bogeat-Triboulot, Hazel Katherine Smith, Henning Wildhagen, Andrea Polle, Michel Zivy, Gail Taylor, Christophe Plomion
S3.P48	Proteomic and transcriptomic analysis of carbohydrate metabolism involved in somatic embryogenesis of Brazilian Pine (*Araucaria angustifolia* Bertol. Kuntze)	Bruno Viana Navarro, André Luis Wendt dos Santos, Paula Elbl, Leandro Francisco de Oliveira, Amanda Ferreira Macedo, Amanda Pereira de Souza, Marcos Silveira Buckeridge, Eny Iochevet Segal Floh
S3.P49	Transcriptome sequencing and de novo assembly of the cold acclimated *E. nitens* using ion torrent	Catalina Lagos, José Luis Gaete, Sofía Valenzuela, Marta Fernández
S3.P50	HSFA2 transcription factor responds to cold acclimatation and might by related to dehydrin gene in *Eucalytus nitens*	María F Beltrán, Leonardo Gutiérrez, Marta Fernández
S3.P51	Identification of four dehydrin genes in *E. nitens*	Paula Aguayo, Margaret Ochoa, Darío Navarrete, Marta Fernández, Sofía Valenzuela
S3.P52	Towards elucidating the role of DREB1 and DREB2 transcription factors in *Eucalyptus* whole plants under abiotic stresses: gene regulation in leaves, stems or roots	Nguyen Chien, Cao Phi Bang, Marque Christiane, Ployet Raphaël, Mounet Fabien, Grima-Pettenati Jacqueline, Teulières Chantal
S3.P53	Understanding wood formation in *Eucalyptus* spp: anatomical and transcriptomics approach	Juan Pedro Elissetche, Isabel Carrillo, Camila Suazo, Sofía Valenzuela
S3.P54	Impact of potassium and sodium fertilization on the leaf transcriptome of *Eucalyptus grandis* submitted to water deficiency	Bénédicte Favreau, Hana Pereira da Silva, Livia Maria Franceschini, Marie Denis, Raphael Ployet, Fabien Mounet, Helaine Carrer, Jean-Paul Laclau, Carlos Lab
S3.P55	Assessing the role of a *Quercus suber* Omega-3 fatty acid desaturase in the response to biotic and abiotic stresses (No abstract)	Ana Leal, Natacha Vieira, Pedro M Barros, Tiago Lourenço, Nelson Saibo, M Margarida Oliveira

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S3.P56	Seasonal variations in the metabolic profiles and their relations to the fast-growth trait of *Pinus densiflora* (No abstract)	Eung-Jun Park, Mi-Na Cho
S3.P57	Using induced resistance in *Eucalyptus grandis* to identify broad spectrum defence mechanisms	Lizahn Zwart, Ronishree Mangwanda, Alexander A. Myburg, Sanushka Naidoo
S3.P58	Genomic resources in *Casuarina* sp L.	Ashok.V.Kulkarni, Kullan A.R.K, Kamalakannan R., Suresh Kumar R, Rajkumar Rathinavelu
S3.P59	Identification, characterization and gene expression analysis of three CBF transcription factors during cold acclimation of *Eucalyptus globulus*	Darío Navarrete-Campos, Claudio Balocchi, Sofía Valenzuela
S3.P60	De novo assembly and characterization of the leaf transcriptome from young rubber trees (*Hevea brasiliensis*)	Camila C Mantello, Carla Cristina da Silva, Erivaldo José Scaloppi Júnior, Paulo de Souza Gonçalves, Anete Pereira de Souza
S3.P61	Proteome analysis of proembryogenic masses and early somatic embryos of *Araucaria angustifolia* (Bert.) O. Kuntze (No abstract)	Neusa Steiner, Vanildo Silveira, Claudete Santa Catarina, Luciano F. Huergo, Emanuel Maltempi de Souza, Aurea Maria Randi, Eny Floh, Miguel Pedro Guerra
S3.P62	Root tip response to drought is altered in SUT4-RNAi poplar plants	Scott Harding, Liangjiao Xue, Christopher Frost, Chung-Jui Tsai
S3.P63	Molecular cloning and analysis of a gene encoding two-pore potassium uptake channel from *Cryptomeria japonica* (No abstract)	Yoshihiro Hosoo, Yukiya Kimura, Hirokazu Nishiwaki
S3.P64	Hyperthermophiles: A source of CAZymes for industrial lignocellulosic degradation (No abstract)	Jonathan Botha, Eshchar Mizrachi, Alexander A. Myburg, Don Cowan
S3.P65	The role of cork oak Myb1 transcription factor in phellogen development – an approach for the in vivo identification of the involved regulatory networks	Tiago Capote, Ricardo Ordás, Catarina Prista, Héctor Carrasco, Maria F. Duarte, Leonor Morais, Sónia Gonçalves
S3.P66	Functional characterization of a SHORT-ROOT (SHR)-like gene in *Populus* secondary growth (No abstract)	Andreia Miguel, Ana Milhinhos, Brian Jones, Célia M. Miguel
S3.P67	Inducible extreme expression of cellulases in poplar (No abstract)	Charleson Poovaiah, Yao Xiao, Heather Coleman
S3.P68	Towards establishing a morphological and ultrastructural characterization of proembryogenic masses and early somatic embryos of *Araucaria angustifolia* (Bert.) O. Kuntze (No abstract)	Neusa Steiner, Francine L. Farias-Soares, Éder C. Schmidt, Maria L. T. Pereira, Bruna Scheid, Gladys D. Rogge-Renner, Zenilda L. Bouzon, Daniela Schmidt, Sara Maldonado, Miguel Pedro Guerra
S3.P69	Development and use of embryo culture for micropropagation of threatened woody species	Mariam Gaidamashvili, Eka Khurtsidze

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S3.P70	Somatic embryo maturation using a high concentration of gellan gum promotes germination of somatic embryos of *Pinus armandii* Franch. Var. *amamiana* (Koidz.) Hatusima, an endemic and endangered species in Japan	Tsuyoshi E. Maruyama, Yoshihisa Hosoi
S3.P71	*In vitro* induction of tracheary elements in calli of *Eucalyptus* to study genes related to xylogenesis	Ana Paula Chiaverini Pinto, Carlos Alberto Labate
S3.P72	Cryopreservation of *Araucaria angustifolia* embryogenic cultures: cryotreatment optimization and cell viability evaluation	Hugo PF Fraga, Leila N Vieira, Jamily M da Silva, Catarina C Puttkammer, Miguel P Guerra
S3.P73	Fractioning and cell tracking of *Araucaria angustifolia* embryogenic cultures	Hugo PF Fraga, Leila N Vieira, Catarina C Puttkammer, Miguel P Guerra
S3.P74	Analysis of the root system architecture of trees (No abstract)	Federica Brunoni, Irene Perrone, Rubén Casanova-Sáez, Karin Ljung, Catherine Bellini
S3.P75	Simplified droplet vitrification protocol for eazy long term preservation of plant material for functional genomic research and genetic resources management	Harvengt Luc, Ranggeh Yanis, Reymond Isabelle
S3.P76	*In silico* identification and characterization of genes related to polyamines biosynthesis in embryogenic cultures of Brazilian pine	Leandro Francisco de Oliveira, Paula Maria Elbl, Bruno Viana Navarro, André Luiz Wendt dos Santos, Eny Iochevet Segal Floh
S3.P77	Improved lignocellulosic biomass yield of RAV1 engineered poplars in a SRC field trial	Alicia Moreno-Cortés, Tamara Hernández-Verdeja, Pablo González-Melendi, Jose M. Ramos-Sánchez, M. Mario Sánchez-Martín, Mercedes Guijarro, José Carlos Rodrigues, Hortensia Sixto, Isabel Cañellas, Isabel Allona
S3.P78	Development of Epigenomics based Agri-nutrigenomics: Study of Genome-wide Differential Methylation Patterns in *Eucalyptus camaldulensis* in Leaf and Secondary Xylem upon Fertilizer Application by *de novo* Genome Sequencing and Whole Genome Bi-sulfite Conversion	Prasad S. Hendre, Suresh Kumar Ramadoss, Roby Mathew, Anand Rajkumar Kullan, Rajkumar Rathinavelu
S3.P79	Strategies for improving the induction of fertile flowers in male and female early flowering poplar	Hoenicka H, Lehnhardt D., Ebbinghaus D., Fladung M
S3.P80	Organization of cis-acting regulatory elements in the promoters of four dehydrins genes of *E. nitens*	Paula Aguayo, Margaret Ochoa, Marta Fernández, Sofía Valenzuela
S3.P81	Genetic approaches to increase biomass yield in the woody perennial *Populus*	Bruegmann Tobias, Fladung Matthias
S3.P82	Dynamics of repressive and active epigenetic marks during cork development	Vera Inácio, Miguel Ramos, Yana Konoplych, José Graça, Leonor Morais Cecílio

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S3.P83	The epigenetic landscape of *Quercus suber* L. female flower development	Teresa Ribeiro, Carles Mir, Vera Inácio, Maria Manuela Ribeiro Costa, Leonor Morais Cecílio
S3.P84	Functional characterization of three *Eucalyptus* MYB genes controlling wood formation and evaluation of their impacts on saccharification	Anna Plasencia, Marçal Soler, Jorge Lepikson-Neto, Annabelle Dupas, Alicia Moreno-Cortés, Anna Alves, José Carlos Rodrigues, Isabel Allona, Isabelle Truchet, Fabien Mounet, Jacqueline Grima-Pettenati

POSTER SESSION IV: Biosafety: environmental risk assessment, monitoring and management, and socio-economic implications

S4.P1	Genome Engineering Improvement for Useful plants of a Sustainable agriculture	Nogué Fabien, Vergne Philippe, Chèvre Anne-Marie, Chauvin Jean-Eric, Bouchabké Oumaya, Déjardin Annabelle, Chevreau Elisabeth, Hibrand-Saint Oyant Laurence, Mazier Marianne, Barret Pierre, Guiderdoni Emmanuel, Mathis Luc, Sallaud Christophe, Matt Mireille, Pierron Jean-Philippe, Bonnel Eric, Foucrier Séverine, Toppan Alain, Trannoy Laure, Rogowsky Peter
S4.P2	Strategic biosafety communication: a new approach	Sandra Sharry, Andrea Sonnino
S4.P3	GIS-based models for environmental risk assessment of genetically modified plants: poplar case study in forest ecosystems in Mediterranean environment	Travaglini D., Paffetti D., Bottalico F., Buonamici A., Donnarumma F., Chelazzi L., Labriola M., Tomaselli V., Vettori C.

Abstract Keynote Communications

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Millions of markers but what is the message?
Population genomics of adaptation to climate in conifers

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Background

Most widespread tree species show moderate to strong local adaptation to climate. For centuries, foresters have understood the need to reforest with locally collected seed to ensure healthy forests that are adapted to planting environments. However, climate warming is rapidly creating a mismatch between trees and their local conditions. New policies are needed to guide *assisted gene flow* strategies to facilitate adaptation to new climates that are biologically appropriate (Aitken and Whitlock 2013) and broadly acceptable to stakeholders and end users (Hajjar et al. 2014).

While comprehensive provenance trials are available to inform assisted gene flow for some species, for many others they are inadequate. There is great interest in using genome scans to detect signatures of local adaptation to climate, either through genotype-environment association (GEA) or genome-wide association studies (GWAS) for climate-related phenotypes, but care must be taken in sampling design, control of neutral population structure, and statistical approaches to reduce false positives (Lotterhos and Whitlock 2014, 2015).

In the AdapTree project, we are using genome scans to inform and evaluate seed transfer policies for the most economically important tree species in Canada, lodgepole pine (*Pinus contorta*) and interior spruce (*Picea glauca, P. engelmannii,* and their hybrids). Here we use results from lodgepole pine to illustrate the potential and the pitfalls of using genomic data to understand local adaptation and develop assisted gene flow strategies.

Methods

We identified approximately 23,000 genes through transcriptome sequencing and assembly (Yeaman et al. 2014). These genes were re-sequenced in 600 trees from over 280 natural populations using targeted sequence capture, yielding ~11 million SNPs. After stringent filtering for SNP quality, genotyping success, and minor allele frequency over 0.05, ~1.1 million SNPs remained for genome scans of local adaptation. The GEA analysis used Bayenv2 (Günther and Coop 2013), and subset of ~250,000 SNPs with little missing data were also tested using LFMM (Frichot et al. 2013). The GWAS analysis used GCTA (Yang et al. 2011). SNPs in targeted non-coding regions with no evidence of outlier behavior were used to control for population structure using the covariance matrix from Bayenv2, and through multidimensional scaling for GWAS.

Seedlings from all populations were phenotyped for growth, biomass allocation, phenology, cold hardiness, drought hardiness and heat stress injury in a series of growth chamber experiments simulating seasonal temperature and moisture regimes for cold, warm, hot wet and hot dry climates. The phenotypes and genotypes of natural populations are also being compared with seed orchard-origin seedlings from local breeding programs.

Results and discussion

Many SNPs are associated with one or more of the 22 provenance climatic and geographic variables tested in the GEA analyses. At lower statistical thresholds, precipitation variables are associated with more SNPs than temperature variables, but at higher stringencies, temperature variables are associated with a large majority of outlier SNPs. Bayenv2 and LFMM produced similar results for genotype-climatic associations, but somewhat different results for associations with latitude and longitude.

Traits involved in local adaptation to climate such as growth phenology and cold hardiness are highly polygenic, with tens to hundreds of SNPs associated with individual traits ($p<0.0001$). While individual effect sizes are small, these SNPs collectively explain much of the trait variation. There were relatively few SNPs or genomic contigs in common between GEA and GWAS analyses.

These results need to be interpreted cautiously: in the absence of a linkage map, it is unclear how many of these SNPs are independent, and there will always be false positives when testing such a large number of markers. Individual adaptive loci mostly have small effect sizes and relatively weak

signatures of selection. Nonetheless, they collectively describe complex patterns of local adaptation in terms of both climate and trait variation.

Candidate markers for local adaptation as well as putatively neutral SNPs to control for population structure have been selected for a ~50K lodgepole pine SNP array. This is being used to validate GEA and GWAS results by genotyping ~6,000 additional trees from seedling and field common gardens. Results will also be used to empirically evaluate the efficiency of different sampling strategies and sample sizes for detecting local adaptation, and to assess the genome wide effects of selective breeding on adaptive SNP frequency and diversity. Results will be translated into recommendations for seed transfer policies and breeding strategies for managing adaptation to new climates.

Competing interests

The authors declare that they have no competing interests.

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Exploring the landscape of the conifer genome: from gene maps to visiting intergenic regions

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Key words: comparative genomics, gene mapping, intergenic regions, structural genomics

Conifers harbor among the largest plant genomes. Recently, spruce and pine genomes have been sequenced by relying mostly on shotgun sequencing, which represents a major achievement. But still, much work remains to be done in terms of improving the contiguity of the genome sequences obtained and comparative structural analysis. Concurrently, genetic mapping of conifer genomes has been pursued mainly by mapping genes, which facilitates comparative analysis of genome macrostructure based on gene orthology. However, given the physical size of conifer genomes, the link between fragmented genome sequence assemblies and current genetic maps is not obvious. We present an overview of the structural features of a white spruce (*Picea glauca*) composite genetic map containing nearly 9000 expressed genes. The comparative analysis with a loblolly pine (*Pinus taeda)* map reinforces the idea of the conserved nature of the genome macrostructure in the Pinaceae in spite of diversification dating back to more than 100 million years. Sequence scaffolds corresponding to intergenic regions matching segments of the white spruce genetic map were also identified. The length of these regions varies largely but common features were observed. The analysis of putative homologous intergenic regions between spruce and pine also revealed notable differences. The overall picture in the Pinaceae appears to be one of relative stability of the genome macrostructure, with more rapid evolution of microstructure.

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Molecular signatures of adaptation in Mediterranean conifers at range wide and local spatial scale

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(From July 1st, *UMR1202 BIOGECO, INRA, Bordeaux*)

Mediterranean conifers typically grow in fragmented habitats where small effective population sizes and isolation may have resulted in reduced adaptation. However, they also contain large genetic diversity due to a demographical history characterized by long-term survival in southern Europe glacial refugia. In this talk, we provide evidence on molecular adaptation to climate in two conifer species with highly contrasted population structure, the maritime pine (*Pinus pinaster* Aiton) and the English yew (*Taxus baccata* L.). In both cases, common gardens and quantitative genetic analyses of fitness-related traits were fundamental to either identify selection drivers prior to the molecular study (e.g. continentality in yew) or to validate genotype-environment associations (in maritime pine). Combination of genomic scans with fitness experiment is an attractive approach to provide must needed validation in reverse ecology studies. In this talk, we also present new insights on the relevance of polygenetic adaptation, in comparison with single-polymorphism effects, for climate adaptation in Mediterranean conifers, and on how studies that consider gene interactions may overcome previous limitations to identify relevant adaptive variation in forest trees. Finally, the need for deeper research on the spatial scale of genetic adaptation is presented, as illustrated by current studies at local spatial scales in maritime pine and comparison with rangewide adaptation patterns.

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Transposon activation tagging in plants for gene function discovery

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Key words: Gain of function; mutagenesis; transposition; jumping gene; functional genomics

Background

For black cottonwood (*Populus trichocarpa*) as the first tree, the sequence of the whole genome of was made available (http://www.phytozome.net/poplar) few years ago [1]. In general, sequencing and annotation of the genome of an organism yields a tremendous amount of data and suggestions for putative genes, however, often without any information on their function. Once the whole-genome information is available for an organism, the challenge turns from identifying the structural genomic parts to understanding the function of many genes as possible. The gained functional information can be used to understand how a plant cell genetically coordinates all the different life pathways and consequently, to improve the genetic architecture of a plant cell. A successful approach to unravel gene functions is to introduce a tag in the genome which integrates in or nearby genes. A tag disturbing gene function is useful in many plant species but not appropriate for dioeciously long-lived poplar. Stimulating of gene function by overexpression is known as activation tagging or gain-of-function mutagenesis, and transposons can be applied as tags to do this in a high-throughput, systematic manner. An introduction to transposons present in living organisms as well as to the Ac/Ds transposable element system of maize will be given.

Methods

Transposon tagging was very successful in maize leading to the isolation of different genes. It has been demonstrated that maize transposons could be transferred to and are active in other plant species. We have transferred the maize transposon *Ac* into poplar to check the usability of a transposon-based mutagenesis for the induction of “Knock-out” or “Knock-in” (“gain-of-function” or Activation tagging”) mutant [2], [3]. We clearly could demonstrate that the transposon *Ac* is active in the poplar genome and preferentially reintegrates near or in coding regions [4]. Further, the majority of the re-integrations were found scattered over many unlinked sites on other scaffolds than the one carrying the original integration locus, confirming that *Ac* does in fact cross chromosome boundaries in poplar [5]. Using the *rolC* gene as marker for transposon excision, indeed we could follow transposition of the “Activation Tagging Ds” system (ATDs; kindly provided by Y. Suzuki, University of Tokyo, Tokyo Japan [6], in combination with a transposase gene) on the phenotypic level but had no efficient screen to enrich putative tagged lines [7]. A new approach uses the negative selectable marker gene *tms2* from *Agrobacterium tumefaciens* to screen transposition of the ATD element.

Results and Conclusions

We could demonstrate the application of an efficient activation tagging system for poplar based on a transposon system (the non-autonomous “Activation Tagging Ds” [ATDs]) in a tree species for the first time [7]. Different independent transgenic lines could be obtained carrying a functional HSP::*TRANSPOSASE* construct. Two transgenic lines with highest transposase expression were selected and used for super-transformation with the ATDs-*rolC* construct based on the construct by [7]. In total, 22 double transgenic lines could be obtained with confirmed mobility of the ATDs element. Four rounds of activation-tagging

experiments were conducted yielding in total 12,083 individuals regenerated from putative ATDs transposed calli. From these, 18 different putatively tagged variants could be identified revealing phenotypic variations [7].
A fourth activation-tagging experiment unraveled that approximately only one third of the investigated individuals reveal transposition of the ATDs. Analyses of the new genomic positions of ATDs reveal a very high percentage of tagged genes. To increase the efficiency of the transposon-based activation tagging approach, the *rolC* gene was replaced with the negative selectable marker gene *tms2* from *A. tumefaciens* to obtain only lines with transposition of the ATDs. Different concentrations (10 µM, 25 µM, 50 µM, 100 µM) of naphthalenacetamid (NAM) were added to the regeneration medium. In theory, non-ATDs transposed poplar cells (still expressing the *tms2* gene) metabolize NAM to active IAA leading to callus formation instead of plant regeneration, thus only ATDs transposed poplar cells regenerate plantlets. First experiments indicate the usefulness of this novel activation tagging approach.

Competing interests
The author declares that he has no competing interests.

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Forest Biotechnology and Sustainability

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Key words: Governance, Innovation, Collaboration, Sustainable development

Introduction and Conclusions

Mechanisms to develop and ensure more sustainable forest management practices in an environment of constantly increasing and diversifying demand for forest biomass are an imperative. Scientific and technological innovations, including biotechnology, are core elements in this equation as is open dialogue with all stakeholders on appropriate governance frameworks for their uptake and deployment in approaches to raise forest productivity.

The development of a genetically modified eucalyptus variety with an approximately 20% increase in yield by FuturaGene in Brazil provides a lens through which to examine the commercial, environmental and social implications of producing more, whilst utilizing less resources. Productivity intensification has the potential to free land for other uses, such as food production and biodiversity, whilst further diminishing logging pressure on natural forests. Conditions that promote the diffusion and transfer of the technology could further integrate and benefit outgrowers who currently access proprietory genetic materials and clones to produce wood.

There is a window of opportunity for stakeholders to collaboratively develop a framework for the governance of forest biotechnology that would guide research towards productivity challenges and ensure that the deployment and diffusion of this technology is appropriately managed down to the local level.

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Field trials with genetically engineered forest trees: past experiences and future prospects

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It is a common agronomic practice to evaluate new varieties under natural field conditions. This applies to GM plants and for more than 25 years, numerous field trials were set up throughout the world to assess GM trees modified for an array of different traits. In this talk, I will stress some of the knowledge, we gained from these experiments.

While recently a few GM tree events have been authorized for commercial release, in Europe, GM tree field trials remain limited in numbers, mainly because it is becoming increasingly difficult to obtain authorization for a GM tree field trial. This is in sharp contrast with all the experimental results issued from GM tree field trial experiments:
1) phenotypic effects resulting from transgene expression in GM trees grown in the field appear to be stable, albeit variable
2) most field studies have validated earlier observations made under greenhouse conditions, although in some cases the modification of target traits was less obvious in fluctuating field environments, and in a few cases, GM trees had severe growth and developmental penalties
3) non-target effects were consistently within the range of natural variation.

Overall, the European GM tree field trials failed to identify any significant tangible risks. Based on this evidence, it seems appropriate that Europe should now move forward beyond small confined trials to larger scale experiments better fitted to a broader context of evaluation and environmental assessment.

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RNAi based GM plants: EFSA activities in relation to environmental risk assessment

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Genetically modified (GM) plants intended for market release can be designed to induce silencing of specific genes *in planta* or in target pests or pathogens through RNA interference (RNAi). As part of the pre-market risk assessment (RA), the European Food Safety Authority (EFSA) evaluates any risks that GM plants may pose to animal and human health and the environment. To discuss potential risks associated with the use of RNAi in GM plants, EFSA organised an international scientific workshop on 4-5 of June 2014 in Brussels, Belgium, bringing together experts from academia, RA bodies and the private sector. The workshop considered the biology underlying the RNAi mechanism, current and future applications of RNAi-based GM plants, and RA approaches. Risk assessment aspects were discussed in three separate break-out sessions, focusing on the main areas of GM plant risk assessment: molecular characterisation; food/feed safety assessment; and environmental risk assessment. The objective of the workshop was to solicit scientific expertise for the problem formulation phase of the risk assessment of RNAi-based GM plants. The outcome contributes to determine in which areas the existing RA approaches for GM plants are appropriate and if complementary or alternative RA strategies should be developed for RNAi-based GM plants. Selected key outcomes of the workshop will be presented.

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Socio-economic implications in relation to the use of GMTs: concerns and acceptance by the public in South America

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Key words: transgenic trees, biotechnology, public opinion.

The issue of genetic engineering and biosafety has captured unprecedented public interest and concern around the world. South America has not stayed out of this debate. This polarized debate about genetic engineering includes scientific discussion and many other concerns as ethical issues, possible impacts (environmental, economical and societal), health and technological dependence and sovereignty. Claims about the promise of GMTs are at times greeted with suspicion, doubt, ignorance, opposition and even indifference. Technology's acceptance is based not only on technological soundness but also on how it is perceived socially, politically and economically. Public acceptance in GM crops, including trees, is one of the factors that will largely influence the extent to which countries invest in and benefit from genetic engineering to modified quality of life. During the past years, biotechnology in South America (SA) has been growing at an accelerated pace. Different technologies have been developed or introduced (more GM trees ~~less~~ than GM crops). However, it currently faces constraints that limit its social acceptance. Viewpoints on GMTs differ within the countries of South America. Brazil and Argentina are the largest producers of GM crops, and the most active developers. Bolivia, Paraguay, Chile, and Uruguay allow production of GM crops but production is banned in Ecuador. These differences among countries are reflective of South America's cultural mosaic, where questions about the GMTs are based on each nation's needs and perception of their own interests. These interests, of course, vary from country to country. To this respect, the acceptance criteria changes based on whether it is a question about native forests or plantations. In mega diverse countries, the challenge is the biodiversity. In countries where GM are developing (Brazil, Argentina), the main point of discussion or obstacle for the acceptance is forest certification, technological dependence (technological sovereignty) and social impact. Usually, people acquire partial knowledge about GMOs from what they see in the media, from environmentalists NGO's campaigns and such information not always originates from reliable sources. Although numerous studies have been carried out on the public's attitude towards GMOs in South America, no research has been done about concerns and acceptance by the general population in relation to the use of GMTs. Empirically, we know that the public's reaction towards GMTs are influenced by different factors, such as information, social and cultural norms, beliefs, values and perceptions. Recently (5 March 2015), women from the MST (Movimiento de los Sin Tierra) took over operations of a genetically engineered tree company Futuragene in Brazil and occupied the CNTBio (Comisión Nacional Técnica de Bioseguridad) building. This action included the destruction of GE eucalyptus seedlings.
In the same way, the Asunción Declaration rejects all Genetically Engineered Trees (22 November 2014 in Asunción, Paraguay), including field trials. Most major international environmental NGOs, stressing the scientific uncertainties involved with GM trees, have tended to oppose them or at a minimum to urge extreme caution in their use. Local and Regional NGOs embrace policies developed by their organizations focusing on Global Level strategy, even though such policies might go against local needs, local culture and

local claims. In other words, international movements do not necessarily support innovation and technology developed at regional level to address true local needs. On the other hand, most people in Latin America do not connect GMTs to health related worries as they do with the genetically modified food. For the case forestry, the major concerns are possible ecological damage such as consequences from the release of a transgenic tree into the environment. For companies interested in GM, the situation is quite different, where marketing benefits is main their main motivation for becoming GM certified. However, one of the most prominent certification bodies, the Forest Stewardship Council, has barred the use of genetic modification (GM) in the forests that it certifies. Many leaders in the forestry industry in SA are examining the potential of GM, but recognise the anti-GM stance of the FSC and the NGOs, which support and help finance the FSC. As evident from the preceding discussion, the key arguments about GM trees are associated with competing views of ethical and moral imperatives and of the doubt about expected benefits of GM tree use. Concluding, the principal barriers for the plantation of GMTs in SA are: the process of certification, the environmental impact in native forests and the technological dependence. The acceptance of GMTs in South America shows different visions that depend on the country (culture), the type of social actor (NGOs, companies, people in general, students, others) and the degree of development that should have reached the technology GM in each country.

Abstract oral session I

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Assembly and comparative genomics of the quaking aspens *Populus tremula* and *P. tremuloides*

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Key words: Comparative genomics, genome assembly, divergence, natural variation

Background

The European and North American aspens *Populus tremula* and *Populus tremuloides* are keystone species while also being utilised as a model system for forest tree research. Currently, sequence based analyses have relied on use of the reference *P. trichocarpa* genome sequence, which often results in poor sequence alignment rates and that prohibits identification of loci that are aspen unique. Given these limitations, we undertook sequencing and assembly of these two aspen genomes in addition to re-sequencing 24 *P. tremula* and 22 *P. tremuloides* individuals.

Methods

We generated three paired end I(PE) llumina 2x100 bp sequencing libraries with insert sizes of 150, 300 and 650 bp to a genomic coverage of >80 in each of our target species. Asll individuals were re-sequenced at >20X using a single 650 bp PE library. To facilitate assembly scaffolding we produced Illumina mate-pair (MP) jumping libraries with target insert sizes of 3 kilo bp (Kbp) and 10 Kbp (median measured insert sizes were 2335 bp and 10011 bp respectively). After removing duplicated reads these libraries represented ~38X and ~724X span coverage respectively. We then performed genome assemblies using the *de Brjuin* graph assembly algorithms ABySS and CLCbio.

Given the levels of fragmentation present in the resultant Illumina short read *de Brjuin* graph assemblies, we produced ~10X coverage of 454 reads, comprising ~5X reads with mean length 450 bp and 5X with mean 700 bp. These data were assembled using the overlap-layout-consensus assembly tool gsAssembler (*a.k.a.* 'Newbler'). The assembly produced was again highly fragmented. Subsequently, as each assembly tool performs better for different regions of the genome, we employed an assembly merging approach whereby un-scaffolded, assembled contigs from each assembler were stepwise merged: Firstly the CLC and ABySS assemblies were merged (we refer to this as assembly GAM01); secondly

GAM01 was merged with the 454 assembly (GAM02). The GAM02 assembly was marginally more contiguous (LG50 4.9 Kbp). Finally, GAM02 was scaffolded using the two MP libraries, yielding an assembly (Potra01) with an LG50 of 42.8 Kbp. Although more contiguous than any of the individual assemblies, this assembly remained highly fragmented with clear evidence of haplotype spitting.

The assembled genome was then annotated using a combination of MAKER-P for *ab initio* gene prediction and PASA for support evidence based annotation refinement. The results from PASA were used to identify long intergenic non-coding RNAs (lincRNAs) in addition to novel protein coding genes. The resulting annotation was then compared to the reference *P. trichocarpa* v3 annotation using Kraken and comparative genome analyses were performed using Saguaro in combination with population-level genotyping results.

For population level analyses, re-sequencing data was quality control filtered and aligned to the genome using bwa-mem with SNPs calling performed using the GATK UnifiedGenotype pipeline.

Results and Conclusions

Using alignment-free *k*mer analyses and sequence alignments to the *P. trichocarpa* reference we first characterised the highly heterozygous nature of the aspen genomes in addition to their rapid divergence compared to the reference *P. trichocarpa* genome assembly. We subsequently used short read second generation sequencing technologies to produce *de novo* genome assemblies that we annotated and compared to the reference assembly. These efforts were complemented by extensive transcript sequencing efforts to support *ab inito* and *de novo* transcript assembly based gene annotation. A combination of whole genome alignment and population level genotyping data were used to identify regions of the aspen genome that have rapidly diverged from the reference *P. trichocarpa* genome as well as to identify novel, aspen specific features. Additionally differences between the two aspens were identified. Details of the genome annotations and comparative genomics analyses will be presented.

Characterisation of the assembled genomes revealed extensive evidence of haplotype splitting due to high rates of heterozygosity, driven by a combination of both SNPs and INDELs. The fragmented nature of the assembly limits many intended downstream uses of this resource. We are therefore assessing and undertaking new efforts utilising third generation sequencing technologies in combination with producing an intra-specific *P. tremula* genetic map to produce a substantially more contiguous genome assembly. We will present progress on these efforts including assessment of genomic shotgun and fosmid pool sequencing using the Pacific Bioscience (PAcBio) platform.

We will also briefly overview inclusion of the generated genomics resources, including community annotation tools, within the PlantGenIE.org web platform.

Competing interests

None

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Spatial Planning (Formas), the Swedish foundation for Strategic Research (SSF) in parts through the UPSC Berzelii Centre for Forest Biotechnology and the Trees and Crops for the Future (TC4F) project. The computations were performed on resources provided by SNIC through Uppsala Multidisciplinary Center for Advanced Computational Science (UPPMAX) under Project b2010014.

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Searching for genes with various copy numbers in the white spruce genome using comparative genome hybridizations

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Key words:
Copy Number Variations, Genome, White Spruce, Comparative Genome Hybridization

Background

DNA segments in Copy Number Variations (CNVs) and Presence/Absence Variations (PAVs) are increasingly investigated in human and model organism genomes since 2004 [1]. Although the presence of genes in various copy numbers and their impact on the phenotype have been shown a long time ago [2], the occurrence of CNVs in the genome is wider than previously thought [3, 4]. Among the various approaches aiming at detecting CNVs, comparative genome hybridizations on arrays (aCGH) were widely used and proven efficient to identify genomic regions in CNVs between a test and a reference genomes [5].
However, CNVs and their phenotypic impact have been rarely investigated in non-model organisms. In addition, the majority of CNV detection methods based on aCGH and currently available relies on probe mapping upon a relatively contiguous reference genome ([6], for instance). Such reference genome does not currently exist for white spruce, hence not allowing a similar approach. On the other hand, white spruce genome is already well characterized, its description including a large gene catalogue [7], a genetic map [8] and a genome capture assembly [9]. This knowledge allowed us to develop an original approach in order to detect genes in CNVs and PAVs and subsequently characterize pedigrees already used for genetic mapping of QTLs related to climate adaptation.

Methods

Hybridizing probes targeting 14,000 genes were designed from a genome capture assembly [9] and included in a series of CGH arrays to test dozen individuals from various pedigrees. White spruce pedigrees were sampled in East and West parts of the species distribution and each individual ('test' genome) was compare to one parent ('reference' genome) by aCGH (Fig.1 A). A pipeline of python programs and R scripts was developed to analyze intensity ratios and infer the genes in CNV or PAV in each pedigree. Ranges of detection criteria were tested, namely the intensity rate threshold to declare that a probe targeted a DNA sequence in CNV and the rate of significant probes per genes. The false discovery rate related to our approach was estimated using self/self hybridizations analyzed following the same pipeline of analyses. Genes in CNV/PAV were afterwards characterized regarding their annotation, their tissue expression in white spruce [10] and genome distribution [8].

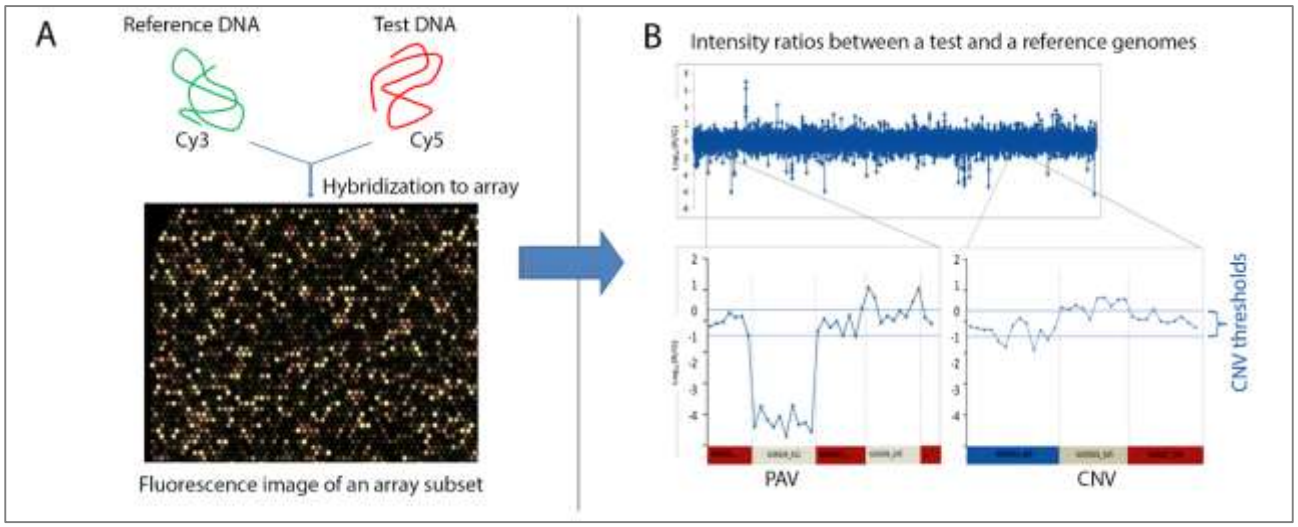

Figure 1: aCGH approach to detect genes in CNV/PAV in white spruce. A) Illustration of aCGH hybridization (inspired from [11]. B) Subset of intensity ratios (in $\log_2$) along the genes between one descendant (test genome) and the parent (reference genome); Gene IDs follow the white spruce gene catalogue [7].

Results

The reference genome was hybridized both as test and reference DNA three times and afterward simultaneously analyzed with all descendants from the pedigree. This procedure allowed to estimate the false discovery rate for each set of detection criteria and finally allowed to define an optimal set of criteria resulting in a FDR inferior to 1%, an intensity ratio threshold of |0.42| (=$\log_2$(4copies/3copies)) and a rate of 87% of significant probes per gene.
Thousands of genes in CNV/PAV were successfully identified over all individuals. Additional genes partially in CNV were identified, more likely showing the presence of "older" gene copies, such as derived pseudogenes for instance. The percentage of the gene space found in CNV/PAV was not different from those found in model species [12, 13]. According to their annotation, these genes represented a variety of molecular functions and biological processes, enriched in those related to adaptation.

Conclusions

This work represents a first step towards the investigation of gene in CNVs in relation to quantitative traits variations of economic interest or involved in climatic adaptation.

Competing interests

The author declares that they have no competing interests.

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Gene expression and natural selection shape the evolution of protein-coding genes in *Picea*

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Key words: gene expression, gene duplication, sequence divergence

Background
Differences in gene expression often result in functional evolutionary innovations leading to new phenotypes (Gallego-Romero et al. 2012; Jacquemin et al. 2014). Despite the importance of gene expression in phenotypic divergence and adaptation, the role of natural selection on the evolution of gene expression levels remains largely unknown in non-model species (Gilad et al. 2006; Bedford and Hartl, 2009). Gymnosperms are a major yet unstudied plant clade, in which genomic studies were limited by the lack of reference genomes (De La Torre et al. 2014). In this study, we use the first two fully sequenced representatives of the gymnosperm plant clade (*Picea abies* and *Picea glauca*) to test for the evidence of expression-mediated selection. We used whole-genome gene expression data to study the relationship between gene expression, codon bias, rates of sequence divergence, protein length, pathway position and gene duplication.

Methods
Whole-genome coding sequences of *Picea abies* (26,597 genes) and *Picea glauca* (27,721 genes) were retrieved from public databases. In addition, expression profiles for most of the genes in both *Picea* species were obtained from previous studies (Raherison et al. 2012; Nystedt et al. 2013). After open reading frames prediction, UTR removal, and frame correction, the resulting gene sequences were used for posterior analyses. An all-against-all BLASTP followed by a Markov Cluster algorithm was used to cluster orthologous protein sequences between the genomes of *P. abies* and *P. glauca* with the program Ortho-MCl (Li et al. 2003). Multiple alignments of orthologous gene families were generated using MUSCLE (Edgar et al. 2004).
We also estimated codon bias, measured as the frequency of optimal codons (Fop) with the program CodonW. Substitution rates (dN, dS and w) were calculated using the maximum likelihood method in Codeml from the PAML package (Yang et al. 2007). Functional over-representation of single-copy and large gene famiies were calculated using BINGO 2.44 (Maere et al. 2005). Pathway position of genes in the terpenoid pathway were calcualted following Ramsay et al. (2009). All correlations were tested using R ststistical package.

Results and Conclusions
By using recently available whole-genome resources in two *Picea* species, our study suggests gene expression and natural selection play an important role in the evolution of protein-coding genes in *Picea*. We found that gene expression is correlated with rates of sequence divergence and codon bias for translational efficiency. A strong correlation between gene expression and gene duplication was found, with genes in large multi-copy

gene families having, on average, a lower expression level and breadth, lower codon bias, and higher rates of sequence divergence than single-copy gene families. A correlation between pathway position and gene duplication was also found. Single-copy genes encoded ancient and essential biological functions and were under strong selective pressures to maintain their copy number in *Picea* species. In contrast, large paralogous gene families had great expression divergence and higher levels of tissue-specific genes. The recent expansion of some large families in *Picea* suggest these gene families are rapidly evolving in response to changes in their environment.

Our study highlights the complex relationships between gene expression, natural selection and gene duplication in shaping the evolution of protein-coding genes in *Picea* species, and sets the ground for further studies investigating the evolution of individual gene families in gymnosperms.

Competing interests
The authors declare that they have no competing interests.

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Analysis of the role of chromatin remodeling in environmental control of annual growth cycle

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Key words: Chromatin remodeling, dormancy, poplar, ChIP-seq,

Background

Perennial plants such as trees growing in boreal or temperate regions of the world display annual cycle of growth that alternates with dormancy. Transition from active growth to dormancy prior to winter is induced by the short day signal (1). Upon perception of short day signal, growth terminates in the shoot apical meristem which manifests in the formation of apical bud. Subsequently, after growth cessation, continued short day exposure leads to the establishment of dormancy that is characterized by inability of the meristem to respond to growth promotive signals such as long days. Exposure to low temperature results in breaking of dormancy and warm temperature subsequently reactivates growth.

Results and Conclusions

We previously performed microarray as well as metabolic profiling analyses to identify transcriptional programs associated with distinct stages of annual growth cycle in hybrid aspen tree (2,3). Recently, we extended these analyses generating genome-wide ChIP-seq data to identify chromatin modifications that underlie the massive changes in transcription during annual growth cycle. We focused on alterations in the repressive histone H3K27 tri-methylation mark in the apex during transition from active growth to growth cessation, dormancy and dormancy break. Our results show that massive genome-wide changes in the repressive mark accompany changes in gene expression during distinct stages of annual growth cycle. Based on the integrative analysis of the genome-wide changes in histone H3K27me3 levels on gene expression during distinct stages of annual growth cycle, the role of H3K27me3 in environmental control of annual growth cycle will be discussed.

Competing interests
The authors declare that they have no competing interests.

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Transcriptomic responses to epitype inducing cultivation temperatures during somatic embryogenesis in Norway spruce leading to the formation of epigenetic memory

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Key words: Picea abies, embryogenesis, epigenetic memory, RNA-Seq

Background

Trees as long-lived organisms with complex life cycles need to adjust their growth cycle to the environment to maximize growth and survival. Epigenetic variation is likely to contribute to the phenotypic plasticity and adaptive capacity of plant species (Bräutigam et al. 2013). The environmental conditions during embryo development lead to the formation of epigenetic memory that permanently affect vital adaptive traits, such as the timing of bud burst and bud set. Our goal is to determine the molecular changes underlying the formation of epigenetic memory caused by different temperature conditions during embryogenesis in Norway spruce. We focus our study on general transcriptional changes depending on temperature conditions and in particular on the predicted members of the epigenetic machinery.

Methods

We prepared nine whole-RNA libraries from somatic embryos of the same genotype at three different epitype inducing temperatures of 18°C, 23°C and 28°C and sequenced using Ion PGM® sequencing platform. RNA-seq analysis was done using RNA-Seq tool of the CLC genomics workbench software (v.8). As a reference, we used all the gene models defined from the Picea abies genome v1 and downloaded from the Norway spruce genome project - ConGenIE (http://beta.congenie.org/) site.

Results

From nine libraries we obtained 48 236 904 sequence reads with average length 107 – 138 bp. RNA-seq analysis gave more than 23 000 reproducibly expressed transcriptional units, of which ~10 000 were temperature-dependent differentially expressed genes (DEGs). Around 17 804 highly expressed gene models were annotated based on defined similarities with Pfam domains and/or known *Poplar/Arabidopsis* genes.

A comparison of DEGs between different temperatures allowed distinguishing of 15 clusters of genes, similar in transcriptional response. The amount of DEGs increased with rising of temperature difference between comparing pairs. There was a qualitative difference in abundant transcript families between the close to normal embryogenic temperature of 18°C compared to the epitype inducing temperatures of 23°C and 28°C, while the difference between 23°C and 28°C conditions was mostly quantitative, when most of shared gene families had higher transcript levels at 28°C. Defined clusters of genes with similar expression profiles were considering as canalization of response under epitype inducing conditions resulting in activation or silencing of genes and possibly whole pathways. Expression patterns of 12 unique transcripts differentially expressed relative to temperature conditions were confirmed by qRT-PCR as a positive control.

Major epigenetic modifications include DNA methylation, multiple histone modifications (methylation, acetylation, ubiquitination, phosphorylation and many more) and chromatin remodeling or incorporation of variant histones, and involve non-coding RNA based mechanisms (Boyko & Kovalchuk 2010; Heo & Sung 2011; Satake & Iwasa 2012; Saze 2008). All these modifications, referred as epigenetic marks, are reversible, have different stability and achieved with the aid of specific enzymes. Based on the classification and functional description epigenetic machinery used in biomedical research (Falkenberg & Johnstone 2014; Lu 2013; Tarakhovsky 2010), we defined extensive sets of genes, putatively involved in different aspects of epigenetic regulation, based on the assumption that the conifer genes are similar in function to their *Arabidopsis* or animal (human) counterparts. We decided to divide all epigenetic-related genes into three main groups: “writers”, “erasers” and “readers” of epigenetic marks, as well as several subgroups, based on protein domain structure and type of epigenetic modification role. This classification fits well to the molecular model of epigenetic memory, which involves several stages (Thellier & Lüttge 2013).

Epigenetic regulation in spruce could be realized through a substantial amount of genes. We found more than 700 highly expressed gene models encoded proteins involved in placing, removing, or binding to epigenetic marks. 262 of these genes were DEGs at different temperatures. Additionally we found 64 gene models involved in small RNA biogenesis, from which 38 were DEGs, and 19 genes, putatively involved in thermosensing, of which 8 were DEGs.

Conclusions

Epitype inducing conditions was accompanied by marked transcriptomic changes depending on culturing temperature. We suggest temperature dependent canalizing of gene expression during embryo formation, putatively based on chromatin modifications, leads to the formation of epigenetic memory. Remarkable similarities between the gymnosperm and angiosperm epigenetic machinery were identified. Based on homology we identified around 736 genes putatively involved in epigenetic regulation, 309 of which were DEGs responding to the epitype inducing temperature conditions. Our results contribute to the annotation of the Norway spruce genome, especially of genes related to the epigenetic regulation, and will provide support for future forward genetics approaches aiming to link genotypes with adaptive phenotypes.

Competing interests

The author declares that they have no competing interests.

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Systems genetics dissection of lignocellulosic biomass and bioenergy-related traits in *Eucalyptus*

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Background

What is the genetic architecture and unique biology of complex traits such as growth and development and cell wall properties of trees, and downstream processing traits like sugar release efficiency? In almost all cases growth and wood properties under selection by breeders are polygenic traits. Here, the genetic component is far from just additive, and is subject to the interaction of dozens to hundreds of loci and alleles, with many genes exhibiting pleiotropic effects due to common biological and biochemical pathways underlying these related traits [1]. To date we have seen previews of this biology through association studies and large, single gene perturbation effects, but these do not describe the complexity of interactions that ultimately lead to trait variation in field grown trees. Additionally, with increased accessibility of tree genomics research the transcriptomes of woody species can be explored to high resolution [2-4], although these still represent a single component of the biology, generally studied in isolation from other system components. We have collected and integrated genetic and phenotypic information in a structured *Eucalyptus* interspecific backcross population, in combination with data on hundreds of xylem transcriptomes and metabolomes from these same trees using eQTL prioritization techniques, with the aim of understanding the shared regulation and contribution of different molecular processes to trait variation. In addition to deepening our understanding of wood and tree biology, this work will inform molecular breeding approaches, as well as identify key regulatory and metabolic junctions for perturbation using genetic engineering.

Methods

We consider traits generally considered by all tree breeders (growth and wood density), as well as cell wall properties (broad composition of cellulose, xylan and lignin as well as S:G lignin monomer ratio) and bioenergy related traits (efficiency of 5- and 6-carbon sugar release). These traits, as well as xylem transcriptome (RNA-seq) and metabolome (GC-MS) variation were analyzed in F_2 progeny of an interspecific backcross of an F_1 hybrid of *E. grandis* and *E. urophylla* [5, 6]. We build on a novel systems analysis method based on network-based eQTL prioritization and multilevel data integration [7] to model and understand relationships of traits, genes, metabolites and biological processes, which are further resolved by looking at the common molecular pathways underlying different traits. We take into consideration metabolic and temporal flux, as driven by carbon availability and the molecular clock.

Results

We interrogated and report on the following relationships: molecular components explaining variation in traits, complexity of traits (reflected by the number and diversity of components such as genes and metabolites associated with them) and the biology and regulation shared among traits. Of all associated gene-trait relationships, between 1% and 88% are shared among different combinations of these traits, reflecting their shared (and in some cases antagonistic) biology. Amongst others, the important bioenergy trait C6 release efficiency was associated with a gene set enriched for secondary cell wall polysaccharide biosynthesis and modification. We further identify inverse relationships reflecting key drivers of differential carbon partitioning between polysaccharides and lignin.

Conclusion

This approach, integrating genetic correlations of segregating component traits with inherent metabolic coordination, has enabled us to begin to decipher biological programming controlling spatial and temporal partitioning of carbon in xylem cell walls, and importantly the interactions of component traits explaining complex growth, wood and processing traits in *Eucalyptus* trees.

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What MYB genes tell us about the specificities of woody plants?

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Keywords: Eucalyptus genome, wood formation, cambium, lignin

Background

Woody plants are characterized by their massive production of secondary xylem, which arises after the tangential divisions of the vascular cambium and become hardy lignified after cell differentiation. Secondary xylem gives support to the aerial parts of the plant and allows an efficient water transport thanks to their thick secondary cell walls made of cellulose, hemicelluloses and lignin. The main factors affecting wood formation are cambial activity and xylem differentiation, which require a sophisticated and precise regulation in time and space. Many studies have been carried out these last years to investigate how this two processes take place, and a hierarchical network of transcription factors have been proposed to control wood regulation through a model in which R2R3-MYB proteins have a prominent role (reviewed in Schuetz et al. [1]).

Objective

In order to get an insight into possible tree-specificities in the process of wood formation, we performed a genome-wide study of the R2R3-MYB family thanks to the recent publication of the *Eucalytpus grandis* genome [2] and compared this

family to those of other plant genomes already sequenced from woody and non-woody plants.

Methods

A total of 141 R2R3-MYB genes were identified in the *E. grandis* genome and we used them to construct phylogenetic trees including R2R3-MYB genes from other well-annotated woody (*P. trichocarpa* and *V. vinifera*) and non-woody (*A. thaliana* and *Oryza sativa*) species. Gene expression was assessed by high-throughput RT-qPCR using a wide list of different eucalyptus tissues and environmental conditions. Poplar and Arabidopsis transformed plants overexpressing *Egr*MYB88, either in its native form or as a dominant repressor fused to the EAR transcriptional repression domain, were generated. Phenotype was assessed at the histological, biochemical and transcriptomic levels.

Results

We detected three clades of genes that contained a higher number of sequences in woody species (Eucalyptus, poplar and grapevine) as compared to herbaceous species (Arabidopsis and rice), and we named them *woody-expanded subgroups*. Moreover, five clades of R2R3-MYB genes were only present in the analysed woody species, being completely absent in Arabidopsis and rice. Deeper analyses indicated that these clades are not specific of woody species but are preferentially found in woody plants; thus, we named them *woody-preferential* subgroups. Interestingly, both *woody-expanded* and *woody-preferential* subgroups were intensively affected by tandem duplication events, with 80-95% of genes arranged in tandem belonging to those groups in Eucalyptus, poplar and grapevine. By contrast, clades containing well-known regulators of secondary cell wall formation were well conserved across all of the species analysed and, generally, not affected by tandem duplication events. Transcriptomic analysis revealed that nearly all the Eucalyptus genes from the three *woody-preferential* subgroups and some genes from *woody-expanded* subgroups were preferentially expressed in the cambium enriched region. It is therefore tempting to speculate that these woody-related genes could regulate specific aspects related to cambium and secondary growth [3].

We selected one *woody-preferential* R2R3 MYB gene preferentially expressed in cambium (*Egr*MYB88) and generated both poplar and Arabidopsis plants overexpressing this gene either in its native form or as a dominant repressor by adding the EAR motif. No clear differences were observed in these transgenic lines when considering the cell wall structure of xylem cells or the total lignin content, but significantly higher amounts of some soluble phenolics were found in wood-forming tissues from overexpressing poplars, whereas they were reduced in dominant repression lines.

Conclusions

Our results suggest that xylem differentiation and secondary cell wall formation are processes highly conserved between woody and herbaceous plants, and key R2R2MYB genes involved in those processes are maintained across all analysed land plants. However, some groups of genes seem to be more abundant in woody

species thanks to a higher retention of tandem duplication events, suggesting that they may be related to environmental adaptation of trees. Taking into account that perennial woody plants have to overcome many biotic and abiotic challenges over their long lifetimes in order to be evolutionarily successful, they may need more sophisticated mechanisms to adapt secondary growth to a myriad of different environmental conditions [3]. In order to test this hypothesis, we started to functionally characterize one *woody-preferential* gene for which the most visible phenotype was the accumulation of soluble phenolic compounds in wood-forming tissues. We are currently investigating the role of these compounds in relation to wood development and stress responses.

Competing interests

The author declares that they have no competing interests.

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Genome-wide association studies of adaptive traits in *Populus trichocarpa*

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Background

Global climate change is re-shaping the distribution of terrestrial plants across the world with changes in temperature and precipitation regimes. In the absence of adaptation or migration, many plant species and populations will trend towards decline or even extinction [1]. In order to determine the potential for adaptation under climate change, we must first have a comprehensive understanding of the genomic basis for variation in climate-related phenotypic traits. With a widespread geographical distribution, populations of *Populus trichocarpa* exhibit latitudinal and altitudinal clines in fitness-related traits such as timing of bud set and flush, cold hardiness, height, and diameter [2]. In this study, we have three major objectives: 1) to detect loci underlying adaptive phenotypes using genome-wide association mapping; 2) to identify locally adaptive loci by assessing genotype-environment correlation; 3) to identify genomic targets of selection and relate these signals back to phenotypic variation;

Methods

We employed sequence capture followed by Illumina HiSeq sequencing to genotype 449 *Populus trichocarpa* individuals collected along both latitudinal and altitudinal gradients. Phenotype data from two common gardens (Virginia, USA and Vancouver Island, Canada) including height, diameter, cold hardiness and bud phenology were collected and clonal best linear unbiased predictors (BLUP) for adaptive traits were calculated. Association studies were performed with TASSEL. We then performed three analyses to identify loci under local adaptation. First we estimated genetic differentiation among 85 spatial subgroups as F_{st} in 1-kb nonoverlapping window throughout the genome with the *hierfstat* R package. Second, we estimated the correlation between allele frequency and key principal components of climate variables with *Bayenv2* software among the same spatial subgroups. We considered SNPs with Bayesian factor>10 and top 1% correlation ρ as candidate loci. The third selection scan estimated standardized iHS (integrated Haplotype Score) within allele frequency bins across 1 kb non-overlapping sliding window and 20 consecutive SNPs window. Genomic regions showing significant signals of selective sweeps were further studied by comparing with significant GWAS loci.

Results and Conclusion

Preliminary GWAS identified 220 SNPs associated with bud flush (percent variance explained (PVE) between 6.4-7.2%), 128 SNPs associated with bud set (PVE: 5.2 to 6.8%), and 40 SNPs associated with height (PVE: 7.0-8.5%). We also detected 21 SNPs (*PVE: 6.9- 8.3%*) associated with diameter in the Virginia

common garden. Several SNPs showed overlapping associations with different phenotypes, for example, a nonsynonymous SNP in 5' UTR of *Potri.005G045500.1,* which encodes cell division kinase, had significant associations with both height ($P = 3.48\times10^{-8}$) and diameter ($P = 2.24\times10^{-7}$). Moreover, we detected significant phenotypic clines along latitudinal and altitudinal gradients, suggesting local adaptation. Among the 14,815 SNPs associated with first 3 Bayenv climate PCs, 94 SNPs had significant association with adaptive traits. For example, a SNP in promoter region of *Potri.001G329800.1,* which is a ortholog of an Arabidopsis MADS-box gene (AGL47), was significantly correlated with Bayenv PC2 (*Bayes Factor 31.77, Spearman correlation coefficient -0.24074*) and Bud flush (P *value* 2.26×10^{-5}). We also found significant overlapping signals between the 2kb surrounding regions of associated SNPs and extreme iHS score (|iHS|>2) peaks, suggesting strong positive selection. However, complex evolutionary forces and different adaptation strategy may be responsible for most nonoverlapping selection outliers.

Competing interests

We declare that we have no competing interests.

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COUPLING METABOLOMICS, mQTL AND ASSOCIATION GENETICS ANALYSES TO IDENTIFY GENES REGULATING THE PRODUCTION OF METABOLITES

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Key words: metabolomics, mass spectrometry, metabolite, GWAS

Background: Coupling metabolite (m) profiling with identification of quantitative trait loci (QTL) using structured plant pedigrees can identify mQTL that are genomic intervals containing a gene(s) that regulates the production of metabolites. However, such intervals typically contain 10-300 genes that are potential candidate genes. In contrast, an association genetics approach pinpoints potential gene targets and can be used in concert with mQTL to confirm valid gene targets for subsequent gene function validation. High-throughput whole-genome resequencing and the ability to identify large numbers of informative single nucleotide polymorphisms (SNPs) and insertion-deletions (indels) in a large population of unrelated individual plants of a given species growing in a common environment, has resulted in the capability to identify and map large numbers of metabolite-gene associations.

Methods: We conducted a study targeting 56 metabolites in fine roots of 185 progeny of an interspecific backcross pedigree resulting from a *Populus trichocarpa* X *P. deltoides* (TD) hybrid backcrossed to a different *P. deltoides* (D') parent (TDxD' Family 13) at Thief River Falls, MN. Aqueous ethanol (80%) extracts of lyophilized and milled fine roots were analyzed for metabolites by gas chromatography-mass spectrometry following trimethylsilylation of metabolites. Additionally, we have conducted a collection of leaves from a Genome-wide Association Study (GWAS) consisting of a range-wide collection of >1050 clones of black cottonwood (*P. trichocarpa*) established at Clatskanie, OR. The 1051 samples (851 clones with 200 clones replicated) of fast-frozen leaves were similarly extracted and analyzed for >400 metabolites per plant with data derived following both manual and automated extraction. The resulting metabolite data were coupled with the genomic analysis to establish allelic variation for >8.2 million SNPs and indels.

Results and Conclusions: mQTL were identified for 48 metabolites (86%), with a total of 117 suggestive ($P<0.05$) and 38 definitive ($P<0.001$), with normally distributed mQTLs explaining 6-34% of the phenotypic variance in metabolite concentration. Similarly, statistically significant metabolite-gene associations were established for most metabolites analyzed in the GWAS study. Combining mQTL analysis from the pedigree study and the metabolite-gene associations from the GWAS population is confirming genes regulating the production of metabolites and is identifying putative functions for, heretofore, unknown genes. Furthermore, examples will be provided that indicate that metabolite synthesis pathways may be derived from the metabolite GWAS analyses.

Competing interests: There are no competing interests.

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Genomic Selection for growth traits in *Eucalyptus benthamii* and *E. pellita* populations using a genome-wide *Eucalyptus* 60K SNPs chip

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Key words: Genome-Wide Selection (GWS), high-throughput genotyping, tree breeding, eucalypt.

Background

Genomic Selection (GS) was proposed by Meuwissen *et al.* [1] and has been gaining interest for prospective application in forest tree breeding. This predictive methodology provides an alternative to Quantitative Trait Locus (QTL) mapping and association genetics, to increase genetic gain per unit of time through early selection. GS has good potential in tree species due to their long generation time and late expressing traits as was initially shown for *Eucalyptus* [2]. The application of GS typically requires thousands of markers covering the whole-genome depending on the organism and the extent of Linkage Disequilibrium (LD). Thus, it is expected that most alleles of interest will likely be in LD with at least one or more markers genotyped and, therefore, properly captured in the predictive models. High-throughput and low-cost genotyping platforms constitute an essential tool to apply GS in forest tree breeding. Recently, SNP genotyping has become more accessible and a high-throughput SNP chip was developed for *Eucalyptus* [3]. *Eucalyptus* are the most planted species of hardwood trees worldwide for energy, pulp, paper and solid wood. *E. benthamii* is a species of interest due to its cold tolerance and high quality wood for plantation as pure species or hybrid combinations in subtropical regions of southern Brazil. *E. pellita* on the other hand

is a fast growing tropical species used in hybrid breeding with *E. grandis* providing drought tolerance and resistance to some diseases and pests. In this study we report the development of genomic prediction models for growth traits in two breeding populations of *E. benthamii* and *E. pellita.* The overall objective is to accelerate breeding cycles of these two specialty germplasm resources to provide selected individuals to be used in advanced hybrid breeding programs of *E. grandis*.

Methods

This study was carried out using two progeny trials of *E. benthamii* and *E. pellita* as training populations. These trials were developed as part of the breeding program of EMBRAPA Forestry. For *E. benthamii* the trial involved 40 treatments, being 36 open-pollinated maternal families from wild Australian populations and 4 samples from mixed seed sources. The trial was composed by ~2,000 trees that were phenotyped at age 4 years and 8 months. The experimental design was a randomized complete block with 50 blocks in single-tree plots, with the 40 treatments and 50 repetitions per treatment, planted in May 2007 at Candói, PR, Brazil. The *E. pellita* trial was composed by 24 open-pollinated maternal families from a 1st generation seed orchard in Australia with 960 trees. The experimental design was a randomized complete block composed by the 24 progenies and 40 trees per progeny in single-tree plots planted in Rio Verde, GO, Brazil. In *E. benthamii* growth traits were measured on 506 trees: Diameter at Breast Height (DBH, cm), Total Height (HT, m) and Wood Volume (WV, m^3). For *E. pellita* only DBH was measured for 769 trees. In total 569 trees of *E. benthamii* and 772 trees of *E. pellita* were genotyped at GeneSeek (Neogen Corporation, Lincoln, NE) using the *Eucalyptus* Infinium EucHIP60k.br [3]. The genotypic data was filtered to remove SNPs with call rate ≤ 90% and a Minimum Allele Frequency (MAF) ≤ 0.05. These markers were used to construct a Genomic Relationship Matrix (GRM) following Powell's method [4]. Individual SNPs had their effects estimated adjusting all the allelic effects simultaneously using the genomic BLUP (GBLUP) approach. A 10-fold cross-validation approach was performed where 90% of the total population was used as a training population and 10% as the validation population. The selection gain of GS (GBLUP) was compared with traditional phenotypic selection (Pedigree BLUP, PBLUP) considering a reduced breeding cycle as a result of early selection.

Results and Conclusions

A total of 12,177 SNPs for *E. benthamii*, with a median of 10 individuals per family, were used to build prediction models for GS. The effective population size was estimated from SNP data as N_e ~51. Accuracies of Genomic Estimated Breeding Values (GEBVs) were 0.32 for DBH and 0.33 for WV. For *E. pellita* 18,605 SNPs were used and a median of 32 individuals per family. Accuracy of GEBV was 0.62 for DBH and the estimated N_e ~35. The selection responses of GS, assuming a conservative reduction of 50% in the length of the breeding cycle, were 237% and 239% for DBH and WV in *E. benthamii* respectively highlighting the increase in efficiency per unit of time. For *E. pellita* the traditional selection (PBLUP) could not be estimated due a detected inconsistency between the expected pedigrees based on family information and the realized pedigree based on the GRM built from SNP data. This result indicates that the original seeds allegedly from maternal families in fact were most likely mixtures from several families possibly due to tree labeling or seed collection errors in the original seed orchard. This observation revealed an additional advantage of using SNP data not only for genomic prediction but also to correct pedigree information for conventional breeding. This study demonstrated that GS could dramatically increase genetic gains per unit of time of these *Eucalyptus* breeding programs and aid in the correct estimation of quantitative genetic parameters.

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Towards population genomics in Aleppo pine: the colonisation history of a keystone Mediterranean tree

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Key words: ecological genomics, range expansion, adaptation, conifers

Background
Aleppo pine (*Pinus halepensis* Mill.) is a widespread circum-Mediterranean conifer. It grows in a highly vulnerable ecosystem in terms of climatic and land-use changes, and high wildfire risk. This species is known to have colonised the western Mediterranean from ancient populations in Greece and Turkey. This long-range colonisation is thought to have been accompanied by moderate bottlenecks and selection for colonisation traits (Grivet et al. 2009, 2011). However, until now, the scarce available molecular data limited our understanding on how this species had spread and adapted within the Mediterranean basin.

Methods
We present a new set of c. 200 candidate genes, the assembly of two Aleppo pine transcriptomes, and the development and application of a new SNP-genotyping assay based on these resources (Pinosio et al. 2014). Using these new resources, we combined a population genomics approach with association genetics between genotypes and phenotypes of important life history traits. Our comprehensive analyses allowed us to shed light on: i) the range-wide population genetic structure and levels of gene diversity in the different Aleppo pine gene pools, ii) the characterisation of the demographic history (i.e. the long-range expansion) of the species, and iii) the identification of candidate genes that may have been favoured the colonisation of new territories by the species.

Results and Conclusions
Aleppo pine showed a more complex population genetic structure than previously thought, with several gene pools identified in its western range. Reduction of effective population size during range expansion to the west was strong but it did not appear to prevent local adaptation. Finally, candidate genes potentially involved in Aleppo pine colonisation syndrome were identified. We conclude that the new genomic tools were successful in providing the means for understanding the adaptive evolution of this keystone Mediterranean forest tree species, characterised by long-range expansions and great differences in genetic diversity across its natural range.

Competing interests
The author declares that they have no competing interests.

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Geographic patterns of genetic variation observed in adaptive SNPs, growth chamber common gardens, and long-term field trials: implications for adaptation to climate change

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Key words: adaptation, climate change, white spruce, lodgepole pine

Background
For reforestation practices, commercial forestry program are often challenged to identifying patterns of genetic and adaptive variation over a tree species range from which to make seed selection for planting, often defaulting to the assumption that local seed is best which may no longer be valid given the warming trends and changes in precipitation patterns observed over the last several decades in western North America.

Methods
Here we pursue empirical, experimental and modeling approaches to guide assisted migration efforts for climate change in Alberta for natural populations and improved breeding stock, synthesizing data from 1) growth chamber common gardens for adaptive traits, 2) field trials for long-term growth traits, and 3) single nucleotide polymorphisms that are either linked to adaptive traits or climatic gradients. We ask how natural populations are adapted to climate, and incorporate the results into climate envelope model approach to map the patterns of adaptive variation across the landscape in an effort to aid modifications to the current provincial seed transfer guidelines.

Results and Conclusions
The analysis of growth chamber data revealed broadly defined groups of locally adapted populations that correspond well to Alberta's current species-specific breeding regions. Analyses of long-term growth traits found that genetic differentiaiton appears to be closely linked to temperature and precipitation. Seed originating in mild and wet Rocky Mountain Foothills ecosystems are poor performers in drier boreal ecosystems relative to local sources. Within the boreal region, lateral transfers west from drier and colder ecosystems outperform local sources in historically wetter environments. Anticipated future decrease in precipitation will likely have a negative effect on tree performance without human intervention through assisted migration of suitably adapted genotypes.

Competing interests
The author declares that they have no competing interests.

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Abstract oral session II

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The relationship between genome, epigenome, and physiology in poplar in response to drought

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Key words: drought response, genetics, epigenetics, phenotype

Background
For plants, it is of key importance to respond efficiently to environment perturbation. This is particularly important for tree species, that must be able to content with fluctuating environmental conditions throughout their long lifetimes, and in light of recent and predicted scenarios of climate change. To further our understanding of key stress responses, it is crucial to link phenotypic responses with underlying molecular patterns. Here we focus on a unique combination of physiological traits, hydraulic characteristics, genetic and epigenetic patterns in hybrid poplars, trees of economic importance in Canada and worldwide.

Methods
For five economically important genotypes, vegetatively propagated plants were grown in a common environment and studied under non-stress and stress conditions. Drought, one of the key factor determining tree growth and survival in *Populus*, was investigated to study genotype- and stress-specific effects. Genome responses through remodeling of the transcriptome were analyzed by next generation sequencing (RNA-seq), and plant growth and performance was studied by assessing biomass parameters and water relations.

Results and Conclusions (these last two either separate of together)
The analyses of resource allocation patterns (*e.g*. between above-ground and below-ground biomass) and physiological parameters (stomatal conductance, vulnerability to cavitation) uncovered different strategies employed by different genotypes to contend with drought, a key abiotic stress. Moreover, transcriptome analyses using deep RNA-sequencing (Illumina technology) revealed shared as well as distinct genotype-specific patterns. In addition to genetic factors, the importance of epigenetic patterns for plant performance has been reported recently. Differences in drought transcriptome responses in genetically identical genotypes were found to be paralleled by differences in DNA methylation (currently being investigated using whole-genome bisulfite sequencing) indicating a possible mechanism for adding an additional layer of plasticity in long-lived organisms. The work contributes to our understanding of plant-environment interactions, with applications related to genotype selection, plasticity of responses, and their impacts on future clone performance in plantations.

Competing interests
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Inter- and intra-specific variation in drought response: insights from native and non-native conifers in Europe

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Key words: conifers, drought response, genetic variation, wood properties

Background

Forest ecosystems have to cope with the consequences of climate change within the next centuries. In particular, long-lasting periods of drought are predicted to become more frequent and could severely affect the actual distribution of tree species. A trees' vulnerability to drought depends on numerous factors such as the anatomy of the water transport system, the ability to close stomata early or to alter the root to shoot ratio. However, only few studies investigated the variation in drought response within species, although knowledge of intra-specific variation of drought response is essential for reforestation and breeding schemes in future forest management. For the identification of drought resistant individuals and provenances, adaptive traits that are involved in mitigating drought stress need to be correlated to easy screenable tree and wood characters as selection criterion. The present study aimed at the following questions: (i) do species respectively provenances of economically important conifers show different response to drought stress?, (ii) Do commonly used wood properties represent suitable screening traits for identifying drought resistant trees?

Methods

We analyzed inter- and intra-specific variation of drought response within three of the most important coniferous tree species in Europe (Norway spruce [Picea abies], Silver fir [Abies alba] and Douglas-fir [Pseudotsuga menziesii). Additionally, our dataset encompassed five fir species with limited distribution around the Mediterranean, which are supposed to be potential substitute species for silver fir at its southern distribution limit (*Abies cephalonica*, *Abies cilicica*, *Abies borisii-regis*, *Abies bornmülleriana* and *Abies nordmanniana*). In total, data from 688 trees were taken from long-term provenance trials located at three different sites in the drought prone Eastern Austria. Past drought periods were identified using the standardized precipitation index (SPI) developed by McKee *et al*. (1993) focusing on two different time scales (three and six month, respectively). Drought response was assesssed

by four different response measures describing a trees' growth performance before, after and during a drought event (resistance, recovery, resilience and relative resilience) as suggested by Lloret *et al*. 2011. Moreover, x-ray densitometric measures of wood density and annual increment were used as potential screening traits. Climate data of seed origin were taken from the WorldClim database (Hijmans *et al*. 2005). Statistical analysis included analysis of variance with associated *posthoc* procedures to infer inter- and intra-specific variation in drought response. Correlation analyses were used for testing relationships between drought response and wood properties respectively climate at seed origin.

Results and Conclusions

Between three and five distinct drought periods per trial site were identified between 1970 and 2012. Drought periods differed significantly in their time of occurrence (spring vs. summer) as well as in their intensity and duration. We found significant differences in drought response and for wood properties among provenances of all three species as well as among the different conifers. For silver fir, variation in drought response among provenances was found to be less stable across successive drought events as compared to Norway spruce, Douglas-fir and to the variation among the *Abies* species.In Norway spruce, Douglas-fir and among *Abies* species, the ranks for the calculated drought response measures were stable across the different drought events, while *A. alba* provenances often changed their ranks even between two consecutive droughts. This suggests that genetic variation in drought response within *A. alba* is more limited than within the other species and among *Abies* species. We found a trade-off between the ability to withstand periods of drought (=resistance) and to recover from it. Ring density was found to be weakly correlated with drought response indices in *Abies spec*. and in Douglas-fir. In Norway spruce, significant correlations were found between ring density and drought resistance (negative), recovery (positive), and resilience (negative) in nearly all drought periods.
We conclude that genetic variation in drought response in silver fir is smaller than within Norway spruce and Douglas-fir. However, the drought response of silver fir was not significantly different from that of the Mediterranean Greek fir and thus silver fir is likely to withstand also future drought events. The observed genetic variation in drought response within Norway spruce and Douglas-fir suggests that assisted migration and breeding activities will help to improve drought resistance of future conifer forests.

Competinginterests

The author declares that they have no competing interests.

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Cell wall rearrangement in wood of poplars subjected to abiotic stresses

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Key words: cellulose, lignin, wood, abiotic stress

Background
Wood is an important economic resource valued by numerous industries for its varied uses, particularly as feedstock for bio-energy fuel. Biosynthesis of cellulose and lignin, both important components of wood, is highly controlled during the tree development and is affected by environmental stress factors. Since biomass production increased by elevated CO_2 levels and decreased by high ozone levels, it is particularly important to assess the impact these two gases have on the mechanisms of wood formation.
The purpose of this work was to understand the cellular mechanisms that control the biosynthesis of cellulose and lignin in poplar wood subjected to abiotic stresses.

Methods
Plants of hybrid poplar (*Populus tremula x alba* clone INRA 717-1-B4) have been exposed in controlled chambers to either filtered-air (control), elevated CO_2 (800µl l^{-1}), ozone (200nl l^{-1}) or a combination of the two gases. Trees were bent in order to induce tension wood formation on the upper side of the stem. Cellulose and lignin composition, capacity to incorporate new assimilates by using ^{13}C labelling and the genes involved in the cellulose and lignin biosynthesis pathways were analysed in tension wood and opposite wood. Any variations in wood anatomy were also investigated.

Results and Conclusions
Ozone treatment induced a reduction in carbon supply and effective lignin and cellulose synthesis as a result of ^{13}C labelling. The transcript abundance of CesA genes involved in the formation of the secondary cell wall, lignin synthesis and transcription factors (WND) were strongly reduced. On the other hand, the gene expression of CesA involved in cellulose synthesis of primary cell wall were strongly induced. The cellulose to lignin ratio as well as cell wall thickness were decreased suggesting a reorganization of the cell wall architecture. Under elevated CO_2, the ^{13}C incorporation into cellulose and lignin was increased, which suggested a stimulation of cellulose and lignin synthesis. However, the cellulose to lignin ratio was decreased probably due to a stronger stimulation of lignin synthesis compared to cellulose. The two environmental constraints in combination had an intermediate effect. Elevated CO_2 seemed to mitigate the ozone effect.
Environmental constraints (ozone and/or elevated CO_2) induced a rearrangement in the cell wall which probably involves a 'stress specific' control of cellulose and lignin biosynthesis.

Competing interests

The author declares that they have no competing interests.

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Genomic and geographic analysis of the introgression between progenitor-derivative spruce species (*Picea mariana* × *P. rubens*)

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Key words: asymmetric introgression, F_{ST} outliers, genomic clines, geographic clines

Background

According to the genic species concept [1], barriers to interspecific reproduction are porous to gene flow. This view implies that some genomic regions are hermetic to interspecific gene flow while other regions are exchanged freely between species through hybridization or introgression [2, 3]. As such, interspecific differences can be maintained despite ongoing gene exchange within contact zones [4, 5]. We assessed the heterogeneity of the genomes of a progenitor-derivative species pair (*Picea mariana* and *P. rubens*, black spruce and red spruce, respectively) [6-8] in the face of introgression. The two spruce species likely diverged in geographic isolation during the Pleistocene, and came into secondary contact during the Holocene [7].

Methods

A total of 303 single nucleotide polymorphisms (SNPs) distributed across the 12 linkage groups of black spruce [9, 10] were genotyped using the Sequenom iPLEX Gold genotyping assay for 388 individual trees from 33 populations distributed across the allopatric zone of each species (i.e., allopatric black spruce area, in the boreal forest of Northern Québec and allopatric red spruce area in the Appalachian Mountains of eastern US) as well as within the zone of contact (i.e., St. Lawrence Valley, Southern Québec). In order to assess the heterogeneity of the genomes in the face of introgression, we used an integrative approach based on three analytical procedures to scan the genomes: detection of F_{ST} outlier loci between populations of the two allopatric zones [11, 12], geographic clines [13-15], and genomic clines [16, 17].

Results and Conclusions

Taken together, the three analytical approaches cumulated a total of 48 loci exhibiting low introgression, 56 strongly introgressed loci, and 199 loci displaying neutral patterns of introgression. Of these, congruence among at least two approaches jointly identified 20 loci recalcitrant to introgression and 19 loci readily introgressed. Preliminary results from the present study reveal the semipermeable nature of the barriers to interspecific reproduction of these progenitor-derivative spruce species, in line with the general trend that spruces evolve reproductive barriers very slowly [18,19]. We suggest that loci showing limited introgression are constituents of the interspecific barrier to gene flow perhaps under divergent selection (or tightly linked to such regions), that neutral loci can introgress freely between the two spruce species, whereas strongly introgressed loci might either reflect

shared ancestral polymorphism or balancing selection (or tightly linked to such regions). Our results indicate that the morphological and genetic delimitation of these progenitor-derivative spruce species was maintained since their secondary contact despite significant gene exchanges affecting most of their genomes in the zone of contact. Additional analyses suggest that introgression is asymmetric between the two species with red spruce genome more readily introgressed by black spruce than the opposite.

Competing interests

The author declares that they have no competing interests.

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Functional genomics uncovers trait interrelations, pleiotropy, and potential tradeoffs in life-history evolution of forest trees

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Key words: QTL mapping, genetic correlations, GWAS, evolutionary quantitative genomics

Background

Understanding the molecular basis of forest tree growth and development is crucial for the selection of stress resilient trees with enhanced biomass productivity. In particular, knowledge about trait interrelations can help to foresee the expected response to selection for one trait relative to another (or others). Gene-based master regulons that underlie massive extent of phenotypic trait variation and that involve multiple traits are termed pleiotropic. When such genes are knocked out, this can have detrimental effects on plant development. Therefore, exclusively forward genetics approaches such as Quantitative Trait Locus (QTL) mapping or genome-wide association studies (GWAS) are employed to discover such genes. Furthermore, the uncovered genetic pleiotropy in trait interrelation may even be related to evolutionary tradeoffs in adaptation and in life-history evolution (resistance to insect herbivory, growth and reproductive development).

Methods

QTL mapping for phenotypic traits (insect attack; oviposition; pre-attack height growth; pre-attack leader length: pQTLs) involving gene expression variation (spruce shoot tissue; transcriptomics: eQTLs) as well as phenotypic and genetic correlation estimations (total and partial correlations, respectively) on the same phenotypic traits (including transcriptome variation in 14k spruce genes that yielded informative Blast results) were conducted in four *P. glauca*P. engelmannii* (188 Interior spruce individuals) progenies segregating for spruce weevil resistance and (pre-attack) height growth.
433 natural and unrelated *P. trichocarpa* (black cottonwood) accessions throughout most of the species' range were investigated for signals of genome-wide associations of allelic variants with phenotypic variation in field traits related to phenology, wood quality, and pathogen resistance. Genetic differentiation among the four distinct climatic zones following the western North American coastline inwards was assessed in these phenotypic traits as well (Q_{ST} estimates). The genetic loci associated with adaptive traits in poplar (among 29k SNPs tested through GWAS) were also tested for signals of diversifying selection by employing different outlier detection methods (F_{ST}; spatial ancestral analysis SPA).

Results and Conclusions

We could show that forward genetic approaches, QTL mapping (here the employed genetical genomics) and GWAS, are successful in uncovering the presence of functional pleiotropy in forest trees. In spruce, we identified significant interrelation between the tree's inherent growth rate and constitutive resistance to herbivory. Points of evidence included: (a) overall positive phenotypic correlation between growth and attack, (b) overall positive correlations between the pQTLs for both traits, (c) all allelic effects derived from coinciding pQTLs underlying the phenotypic variation in growth and attack positioned at eQTL hotspots were positively correlated, (d) the identified master regulons have proposed functions in general developmental processes but also in biotic defense responses in plants. We further studied the directionality in the genetic correlations between quantitative traits (resistance

to herbivory, pre-attack growth) using large-scale gene expression data. Here, for the first time, intricate gene-gene interaction networks determining the different (the positive and the negative, respectively) gene-based relationships between growth, development and pest resistance in perennials were uncovered. Our results also highlighted the importance of integrating inherent growth characteristics of the host in functional genomics studies related to defense mechanisms in its interactions with the herbivore, research that thus far has not been given much attention in forest trees.

In poplar, we uncovered functional pleiotropy on a gene-by-gene basis by employing GWAS. Genes that associated with different phenotypic traits that themselves are functionally unrelated were considered pleiotropic sensu lato. This is because trait characteristics that are genetically correlated are constraint in their ability to evolve independently. We present the poplar Class III HD ZIP REVOLUTA (PtREV) gene as an example for functional pleiotropy. The three identified functional variants within PtREV that associated with fungal resistance, leaf drop and cellulose content, respectively, evolved at different times, the leaf rust related genetic variant being more ancient. This confirms PtREV's primary function in auxin signaling, further reflects the functions of auxin as a growth hormone and in the dynamics of plant immunity against pathogens. Both rust resistance and leaf drop are also adaptive to local climate (non-neutral Q_{ST}). Moreover, F_{ST} and SPA analyses revealed the pathogen and the phenology related SNPs under diversifying selection. We identified a recessive haplotype related to rust susceptibility in the northerly located populations that are under weaker selection pressure by the pest. Furthermore our results demonstrated the functional evolution of PtREV in supporting fundamental processes in vascular plant development (secondary growth) and leaf life span. Such pleiotropic gene functions might be facilitated by alternative gene splicing processes giving rise to different protein isoforms and/or gene expression levels. Furthermore, we uncovered several other genes through GWAS, including those implicated in nitrogen uptake/nitrate distribution, which were pleiotropic for adaptive traits related to autumn phenology, biomass acquisition and rust resistance. In conclusion, different types of functional genomics approaches that apply forward genetics and that are conducted in genetically untransformed forest trees help to uncover trait interrelations as the basis of tree growth and development. Furthermore, studying naturally occurring mutations in the natural environment enhances our understanding regarding genetic pleiotropy in normal gene functions (related to master regulons).

Competing interests

The authors declare that they have no competing interests.

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How eco-evolutionary principles may guide tree breeding and tree biotechnology for enhanced productivity

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Key words: theory, risk management, aridity, mortality

Background

Tree breeding and biotechnology can enhance forest productivity and help alleviate the rising pressure on forests from climate change and human exploitation (Fenning et al. 2008). While many physiological processes and genes are targeted in search of genetically improved tree productivity, an overarching principle to guide this search is missing (Nelson and Johnsen 2008). Traditional reductionist, bottom-up approches to genetically improve particular physilogical processes and traits at the cell or organ scale do not necessarily lead to increased forest productivity at the stand scale, where interactions among processes and with the environment regulates growth. Here we propose a method to identify which traits can be modified to enhance productivity at the stand scale, based on the differences between trees shaped by natural selection and 'improved' trees with traits optimized for productivity

Methods

We developed a tractable model of plant growth and survival to explore potential trait modifications to increase productivity under a range of environmental conditions, from non-water limited to severely drought-limited sites. The traits we consider are related to water use (conductance regulation), resistance to drought (wood density), resistance to herbivors and pathogens (investment in defenses), and competitive behavior. The natural values of these traits were determined based on the eco-evolutionary principle that traits have been adapted to maximize fitness (approximated by lifetime reproductive production (Franklin et al. 2012)) in their natural environment. Using the natural traits as a baseline, we explored the potential to modify the traits to maximize stem wood productivity in managed forests rather than fitness in nature.

Results and Conclusions

We show how key traits are controlled by a trade-off between productivity and survival, and that productivity can be increased at the expense of long-term survival by reducing isohydric behavior (stomatal regulation of leaf water potential) and allocation to defense against pests compared to native trees. In contrast, at dry sites occupied by naturally drought-resistant trees, the model suggests a better strategy may be to select trees with slightly lower wood density than the native trees and augment isohydric behavior and allocation to defense. Thus, which traits to modify, and in which direction, depend on the original tree species or genotype, the growth environment, and wood-quality versus volume production preferences. In contrast to this need for customization of drought and pest resistances, consistent large

gains in productivity for all genotypes can be obtained if root traits can be altered to reduce competition for water and nutrients. Our approach illustrates the potential of using eco-evolutionary theory and modeling to guide plant breeding and genetic technology in selecting target traits in the quest for higher forest productivity (Franklin et al. 2014) (Näsholm et al. 2014).

Competing interests

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Species-specific alleles at a *beta-tubulin* gene show significant associations with leaf morphological variation within *Quercus petraea* and *Q. robur* populations

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Key words: Quercus spp, diversity, gene-flow, species integrity

Background

European oak species belong to different taxonomic sections within the subgenus *Lepidobalanus* Endl. For many of its species, the genus *Quercus* is characterised by a high variability in morphological and ecological traits, the occurrence in mixed stands, and the presence in large population sizes. A large body of results show high levels of interspecific gene flow and frequent natural hybridizations. Karyotypic similarities among *Quercus* spp. allows for a high permeability of oak genomes to gene-flow among different species. A previous study by Scotti-Saintagne et al. (2004) on *Q. petraea* /*Q. robur* indicated substantial heterogeneity including large variation of F_{ST} values along the genome. In the present study, various natural oak populations of interfertile *Quercus spp*. were investigated for their variability and differentiation patterns at a *beta-tubulin* gene in a Europe-wide survey. This gene was chosen as a possible candidate among loci subjected to selection and maintaining integrity between species.

Methods

Detailed molecular characterisation of the first intron in *tubulin* was performed following identification of its substantial sequence variation. PCR fragment-length variation was employed enabling high-throughput genotyping. We performed a) estimates of differentiation between four oak species and more detailed between *Q. petraea* and *Q. robur* involving tests for departure from neutral expected patterns, b) detailed spatial analysis, and finally c) comparison of genotypic and allelic variance (at the most discriminating alleles, i.e. showing highest F_{ST}) and leaf morphology. Linkage mapping was performed using an intraspecific *Q. robur* progeny and a hybrid *Q. petraea*/*Q. robur* progeny (Porth et al., 2005) to locate the *tubulin* locus on the *Quercus* genome (linkage group, LG).

Results and Conclusions

Two frequent alleles showed particularly high genetic differentiation between *Q. robur* and *Q. petraea* (ranging from 12% to 30% in 7 out of 10 mixed stands for one allele, from 5% to 18% in 4 stands for the other allele), each allele being specific to one particular species. Among all allelic sequences related to the first intron of *beta-tubulin*, the shortest, and specific to *Q. petraea*, showed the ability to form specific stem/loop structures. The critical

role of introns in pre-mRNA stabilisation or processing has been already recognized. This phenomenon of increased secondary structure potential in introns relative to exons usually occurs in genes under extreme selection pressure for function (i.e. were the coding region is extremely conserved; evidenced in the case of the CDS for *beta-tubulin* that is under strong purifying selection (P. Garnier-Géré, pers. obs.)) and has an important functional role at the translational level. For three mixed *Q. petraea* /*Q. robur* stands the studied intronic *tubulin* gene fragment significantly departs from expected neutral differentiation patterns and thus could be considered to be under selection pressure. Significant associations were found between genotypic and allelic variation and variation in five morphological traits, after having accounted for both species and stands effects. *Beta-tubulin* maps to a chromosomal region on oak LG 7 for which colocations with QTLs for adaptation have already been shown. While *beta-tubulin* showed normal Mendelian segregation in the offspring of the pure *Q. robur* cross, significant segregation distortion was observed for the interspecific cross derived progeny. *Q. petraea*/*Q. robur* known for being under intensive genetic exchange, show limited gene-flow for *beta-tubulin* based on the two common alleles, which might be due to selection acting either directly on the locus or due to hitchhiking effects from closely linked sites under selection. In stands with a substantial number of trees with recorded intermediate morphology and no high F_{ST}, as expected, no significant phenotype-genotype associations were uncovered.

Competing interests
The authors declare that they have no competing interests.

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***Eucalyptus grandis* defence responses against the myrtle rust pathogen, *Puccinia psidii* – insights from transcriptome profiling**

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Key words: Plant-pathogen interactions, RNA-seq, Forest pathology, Myrtle rust

Background

The Myrtle rust pathogen, *Puccinia psidii* Winter, was initially described on native guava in Brazil (*Psidium guajava*), and has since spread to many areas of the world. The host range of *P. psidii* is growing and is recorded for more than 400 species from 70 genera (Giblin and Carnegie, 2014). It has caused much damage to native forests and industries relying on species of *Myrtaceae* since its introduction to Australia in 2010 (Pegg et al., 2014). In 2013, an ornamental tree infected by *P. psidii* was discovered in South Africa (Roux et al., 2013). It has since been found in two other provinces but has to date not been identified from eucalypt plantations in South Africa. It is imperative for the survival of eucalypt (*Eucalyptus* and *Corymbia*) forestry industries worldwide that breeding programs, incorporating resistance to this pathogen, commence.

RNA sequencing has proven to be a valuable tool in deciphering disease resistance genes and mechanisms in forest trees (Hayden et al., 2014, Liu et al., 2013). However, to gain a true understanding of the responses, factors influencing both resistance and susceptibility of the host must be understood. This study highlights possible mechanisms employed by *E. grandis* in response to *P. psidii* in both susceptible and resistant systems.

Methods

Eucalyptus grandis seedlings of mixed provenances from eastern Australia were identified as resistant and susceptible to *P. psidii* (Southerton and Pegg, unpublished) based on rating methods developed by Junghans et al. (2003). The inoculated tissue was removed and the seedlings were grown in glasshouse conditions for eight weeks. Desiccated *P. psidii* urediniospores (BRIP# 57793) stored at -80°C were brought to room temperature and resuspended for one hour in distilled water to a concentration of 1×10^5. Tween® 20 was added to the suspension at a rate of 0.02%. Distilled water with Tween® 20 was used as the control.

The seedlings were sprayed with either the spore or the control mix with a pressurised spray unit until both the upper and lower leaf surfaces were uniformly coated with a fine mist. The seedlings were wrapped in black plastic bags to maintain a humid environment for 24 hours to facilitate infection. Young leaves were harvested at 12, 24, 48 hour (hr) and 5 day (120 hr) time points for both resistant and susceptible groups. Fifteen leaves were harvested per repetition, from a selection of 60 plants, and snap frozen in liquid nitrogen. Four repetitions per time point were harvested. The plants were observed after two weeks for signs of a positive infection.

The harvested tissue was sent to BGI (Hong Kong) for RNA extraction and sequencing. RNA-seq was performed on an Illumina HiSeq2000 and generated 30 million 50 bp paired end reads. Reads were mapped to the *E. grandis* genome version 1.1 and differentially expressed genes were determined using programs from the Tuxedo suite (Trapnell et al., 2012)

Results and Conclusions

Within the Australian *E. grandis* provenances, a range of responses were apparent to *P. psidii* challenge. Development of uredinia were absent in resistant genotypes 14 days after inoculation, but apparent in highly susceptible genotypes.

The comparative transcriptome analysis of both the resistant and susceptible host responses of *E. grandis* to *P. psidii* showed significant differences in the expression profile. There were 21 genes up-regulated in the resistant interaction that were down-regulated in the susceptible interaction. There were 16 genes up-regulated in the susceptible interaction that were also down-regulated in the resistant interaction (Figure 1). This mirrored differential expression could be an indication that these genes are associated with resistance and susceptibility to *P.psidii*. Some of the genes identified were key plant-pathogen hormone-response markers, but several were unique to *E. grandis* with no known function. This work identified novel genes for plant host-pathogen interactions, and provides potential candidates for future investigation into resistance against this devastating pathogen.

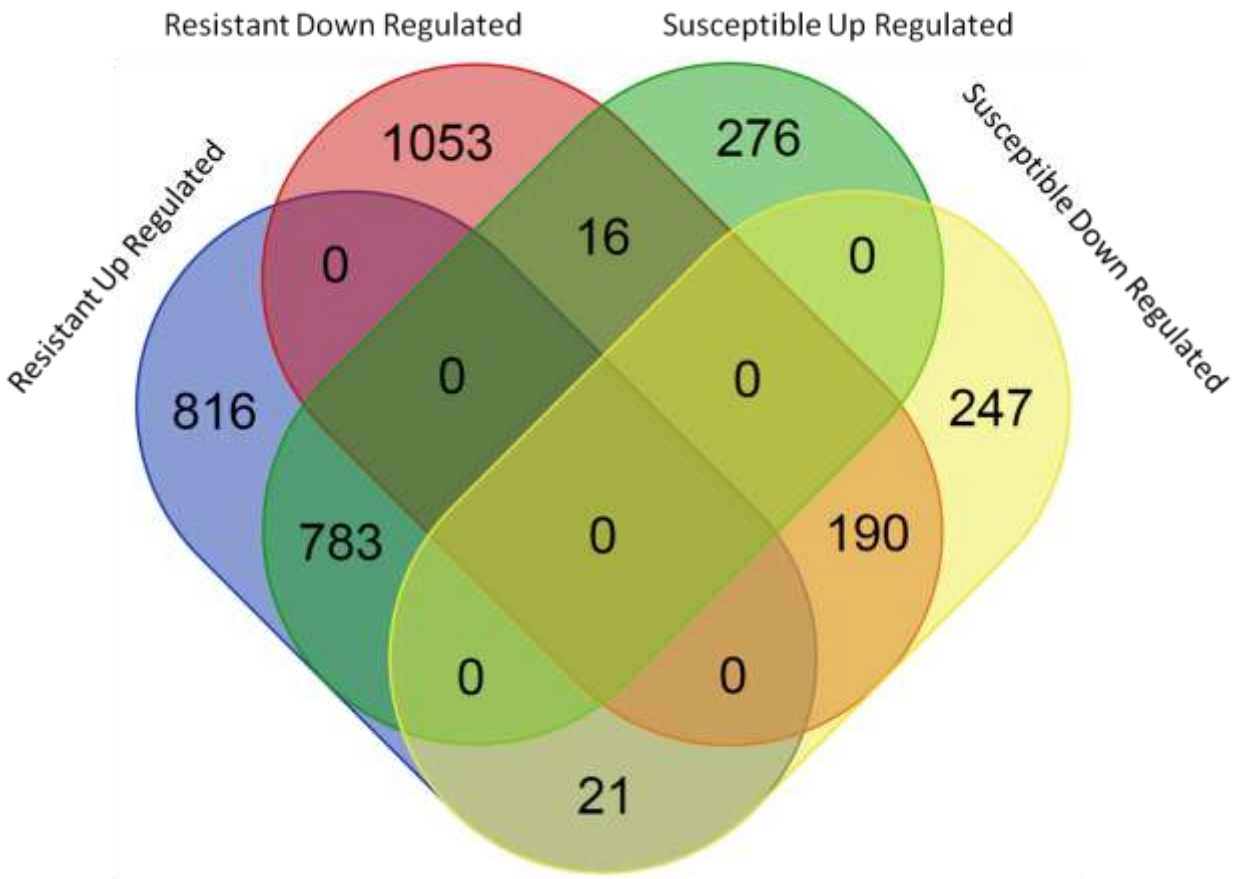

Figure 1: The differential expression of *Eucalyptus grandis* genes in response to *Puccinia psidii* at 24 hours post inoculation. A CuffDiff analysis found 1869 unique differentially regulated genes in the resistant interaction, and 523 in the susceptible. Additionally, 21 genes that were up-regulated in the resistant intereaction were down-regulated in the susceptible, and 16 that were up-regulated in the susceptible interaction were down-regulated in the resistant.

Competing interests

The author declares that they have no competing interests.

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A genomic view of lodgepole and jack pine responses to mountain pine beetle and their fungal associates in the face of mountain pine beetle range expansion

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Background

The current mountain pine beetle (MPB; *Dendroctonus ponderosae*) epidemic has impacted more than 28 million hectares of pine forests in western North America [1-3]. Lodgepole pine (*Pinus contorta var. latifolia*), with a range overlapping that of MPB, is the main species of pine affected by the present outbreak. From its historic range in central British Columbia, MPB has spread into novel habitats in northern Alberta where lodgepole pine naturally hybridizes with jack pine (*Pinus banksiana*), a boreal species spanning eastward to the Maritime Provinces. We used species-distinguishing markers to redefine this hybrid zone, and demonstrated that MPB has undergone host range expansion to jack pine, a novel host [4]. Whereas lodgepole pine shares a co-evolutionary history with MPB, jack pine is considered an evolutionarily naïve host. We are testing the hypotheses that molecular aspects of host suitability differ between lodgepole and jack pine, and that abiotic stresses such as water limitation affects molecular features of host suitability of these species.

Methods

Sanger, Roche (454) and Illumina platforms were used to generate lodgepole and jack pine transcriptome assemblies [5; 6]. SNP discovery was performed using CLC Genomics; genotyping was performed using Illumina Golden Gate and/or Sequenom; and population genetics analyses were carried out according to [7-11]. Experiments were conducted with two year old seedlings subjected to well watered or water deficit conditions. MPB attack was simulated by inoculation with the MPB fungal associate *Grosmannia clavigera*. Hydraulic conductivity was determined as in [12]. Hormones were quantified using methods modified from [13, 14]. Microarray analyses and quantitative RT-PCR were as described [13].

Results

SNP analyses revealed that the lodgepole pine - jack pine hybrid zone is larger and patchier than previously thought, and that there is an even larger zone of introgression. Loci exhibiting signatures of selection were identified using Fst-based and environmental correlation methods.

Water limitation treatments were sufficient to increase ABA levels and decrease stomatal conductance and photosynthesis in both lodgepole and jack pine seedlings, but were not sufficiently severe to alter stem hydraulic conductivity. While both lodgepole and jack pine are conservative water users, jack pine exhibited attributes that suggest greater avoidance of water deficit-induced stress. The ability of jack pine to better maintain function under water deficit was consistent with distribution modeling of lodgepole and jack pine within western Canada, in which we found lower precipitation to be a significant predictor for jack pine ancestry.

G. clavigera-induced lesion development was slower in jack pine than lodgepole pine, and further delayed by water deficit. G. clavigera inoculation significantly increased levels of jasmonic acid in both species. Microarray analyses revealed that thousands of genes are invoked in the response of pines to *G. clavigera* infection, that there are substantial differences in responses of lodgepole and jack pine, and that water limitation alters this transcriptional programme in part by attenuating expression levels of defense-associated genes. Some differentially expressed genes also showed signatures of selection in the population genomic analyses. Combining these transcriptomic analyses with microscopy and histochemistry provided additional clues as to how lodgepole and jack pine defenses differ.

Conclusions

Molecular signatures for host suitability for MPB of the evolutionarily co-evolved lodgepole pine differs from that of the naïve host jack pine, and environmental stresses such as water deficit affect these molecular signatures. Ongoing collaborative investigations will determine the degree to which molecular signatures affect pine interactions with MPB, and thus influence MPB population dynamics and the potential for further spread into novel habitats.

Competing interests

The authors declare that they have no competing interests.

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Primed host response in the conifer *Norway spruce*

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Key words: Conifer, Resistance, Priming, Wounding

Background

Plant defense to necrotizing fungi, wounding and methyl jasmonate (MJ) leading to induced host responses may induce priming of the host defence, making them more responsive or resistant to a later challenge. Priming and systemic defense responses are present in gymnosperms such as Norway spruce (1, 3, 4). When the passive boundary of bark periderm and rhytidome on the outer surface of trees is breached, constitutive and inducible defenses in the secondary phloem serve to protect the vascular cambium and sapwood against pathogens (2). Increased production of lignans, terpenes and PR-proteins are induced upon wounding and infection to protect the tissues against invading pathogens. Phenolics and terpenes are important components of the constitutive and inducible defense to pathogenic fungi and bark beetles in Norway spruce. Induced responses to MJ, wounding and the bark beetle-associated necrotizing fungus *Ceratocystis polonica,* as well as the devastating root-rot fungus *Heterobasidion annsoum s.l.,* have been observed and the priming persist for weeks or longer after treatment (1, 3, 4), but the repertoire of Norway spruce genes involved in the priming response is still scanty. The protective defenses of living conifer bark (phloem) are associated with increased lignification and phenolic compounds (1,2). We have examined the primed defence responses in Norway spruce to wounding, and re-wounding 12 months later, and how priming impact on ethanol-extractable and hydrolysable cell wall-bound phenolics, lignin levels and on the monolignol biosynthesis-pathway gene transcripts in healthy and wounded bark tissue.

Methods

Plants: Two ramets of Norway spruce for the resistant and less resistant clone were used under the same conditions. Each ramet were wounded and stem-inoculated with *H. annosum* s.l. and re-wounded 1 year later (See reference 1).

qRT-PCR: qRT-PCR was run using SYBR Green PCR from Applied Biosystems for C4H, 4CL, CCR, CAD, HCT-like, C3H, COMT, CCoAOMT, F5H transcripts using a-Tubulin as reference (1).

Free and bound phenolics: Six compounds were analyzed from methanol extracts: taxifolin glycoside, astringin, tetrahydroxy-stilbenoids I and II, piceid and isorhapontin. Cell wall-bound phenolics were six compounds characterized as catechin, coniferin, isorhapontin, tetrahydroxy-stilbenoid, dihydroflavonol and piceid (1).

Lignin quantification: Measured using the LTGA assay.

Results and Conclusions

The constitutive levels of most monolignol biosynthesis pathway transcripts differed in healthy tissue of treated trees, compared with levels in healthy tissues 12 months earlier, suggesting a primed or at least heightened basal defense state due to inoculation and wounding (12 months after the challenge). Furthermore, the PR protein PaChi4, a marker for effectively induced host defense responses, is more highly expressed in healthy tissue of trees that have fully recovered from inoculations and wounding made 12 months earlier.

Transcriptional changes were observed in the bark due to priming after re-wounding. Prior exposure that heightens or increases sensitivity and response rate of the host defense induction upon a later challenge to the same or similar stress are hallmarks of primed reactions. The transcriptional responses to re-wounding 12 months after the initial challenge tended to follow a similar but not identical pattern of induced defence reactions. For C3H, CCR, COMT, CCoAOMT and 4CL the levels tended to reach maximum after the re-wounding, whereas the opposite trend occurred with F5H and CAD. The fact that we see both indications of a heightened basal level of transcription and differential expression beyond that observed in the initial year is consistent with a priming effect.

The levels of lignin in healthy tissue were higher after 12 months for both clones (and both clones recovered from the treatments) but this increase was distinct only in the more resistant clone. Lignin content tended to increase in both clones after wounding in both years, but reached maxima after the second challenge and, in these terms, the increase in the second year was most clear for the least resistant clone. The higher constitutive levels of lignin found in the more resistant clone, as well as the induced lignification following wounding and inoculation, suggest that defense processes related to lignification, may be of great importance in explaining the level of resistance to *Heterobasidion* in these Norway spruce clones.

There were clear changes in the methanol-extractable and hydrolyzable cell wall-bound phenolics in healthy samples 12 months after the initial challenge as compared with healthy samples from the initial year, suggesting a heightened or primed increase in the basal level of these compounds. There were also clear differences in the levels of these compounds after wounding between the 2 years, but the additional change in phenolics compared with the ‘primed’ healthy control were generally less.

The transcriptional changes indicate that both clones show primed or heightened levels of phenolics and lignin pathway-related transcripts 12 months after the initial treatments (1). The resistant clone had higher quantities of lignin before treatment and it was able to more effectively reinforce the bark tissues with further lignin deposition following treatments. However, these results also suggest that both the more- and the less-resistant clone have efficient defense signaling pathways affecting the level of monolignol-related expression and both recovered from the treatments. Thus Norway spruce show primed or altered levels of phenolics and lignin pathway-related expression 12 months year after the initial treatments, but it still remains to be verified whether such primed defense responses may last for several growth seasons or even longer. Furthermore, primed defense responses and other types of long lasting induced resistance likely include lasting changes in gene expression trough molecular mechanism similar to those employed in epigenetic phenomena such as chromatin remodeling and noncodingRNAs; further studies will focus on these aspects of the primed response.

Competing interests
No competing interests.

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The impact of North American root-rot disease fungus *Heterobasidion irregulare* on ecophysiological properties, volatile terpenoids and transcripts of *Pinus pinea* L.

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Keywords: *Heterobasidion irregulare, Heterobasidion annosum, Pinus pinea*, transcripts, volatile terpenoids.

The North American root and butt-rot disease fungus *Heterobasidion irregulare* (Underw.) *Garbel. & Otrosina* was introduced in Italy during the World War II from North America (Gonthier et al. 2004; Garbelotto et al. 2013). The fungus has become invasive and is currently distributed on Italian Stone Pine (*Pinus pinea* L.) forests along about 100 Km of Italian coastline west of Rome (D'Amico et al. 2007; Gonthier et al. 2007, 2014). In the same forest is present the European vicariant native *Heterobasidion annosum* (Fr.) Bref. sensu stricto (s.s.), a serious root and butt-rot fungal pathogen of conifers.

Conifers are prolific producers of oleoresin, which is a mixture of different classes of terpenoids and phenolics. Numerous secondary terpenoids act as primary defensive chemicals against herbivorous and microbial attacks; however the production of these compounds have many other functions which are indispensable for the survival of the species. Numerous terpenes are involved in mediation of vital interactions viz., plant-plant, plant-pathogens, plant-mammal herbivores, insect-insect and plant-insects (Langenheim 1994). Individual terpenes show broad-spectrum effects but the function of these compounds is dosage dependent and is enhanced by interaction with other terpenoids since these substances may act additively or synergistically (Langenheim 1994). Thus, mixtures of compounds (either among terpenoids or with other classes of chemicals) appear to be more effective in chemical defences than individual constituents because evolution of resistance in herbivores is difficult with regard to a combination of different compounds (Cates 1996).

Objective of this study was to investigate the impact of *H. irregulare* on ecophysiology, volatile organic compounds and transcripts of stone pine. An inoculation experiment was carried by using 3-year-old Italian seedling.

Material and Methods

Twelve seedlings were used in each of the following treatments: i) *H. irregulare*; ii) *H. annosum*; iii) mock-inoculation; iv) Unwounded, as control. For each seedling, a cylinder strip of bark containing phloem and cambium, was removed 3, 14 and 35 days after inoculation from two locations: a) at the inoculation site (IS), and b) in the distal portion (DP), approx. 12 cm above the inoculation site. Gas exchange properties were measured using a portable infra-red gas analysis system (LI-COR, Lincoln, NE, USA) equipped with a conifer cuvette, while terpenoid were analysed by GC-FID and GC-MS.

Trascripts analysis on bark samples was carried out by using six different genes involved in plant-fungus interaction (Chitinase, Peroxidase, Antimicrobial peptide, Cinnamoyl alcohol dehydrogenase, Xyloglucan endotransglycosylase, Phenylalanine ammonia-lyase). Real-time PCR reactions were performed using StepOne™ Real-Time PCR System (Applied Biosystem, Forest City, CA). All qRT-PCR data were normalized to the reference gene Actin using the 2^{-DDCt} method (Pfaffl 2001), comparing the data for each gene in the inoculated samples with the data for non-inoculated samples (un-wounded samples).

Results and Conclusion

Variation in photosynthesis and stomatal conductance were detected under the attack by the North American and European *Heterobasidion* species. Fungal infections strongly affected the production of volatile terpenoids: differences in the relative proportions (percentages) of constitutive and induced terpenes with *Heterobasidion* spp. were observed. Stem base inoculation with *Heterobasidion* also induced changes in total terpenoids contents.

In both site bark collected, the expression of all six gene tested did not reveal significant differences between two pathogens, and between pathogens and wounding samples.

Analysing the genes transcripts levels in the distal portion (DP), we found that almost all gene were more up-regulated in comparison with the transcripts levels in IS.

Analysis of transcripts showed differences in gene expression revealing also a systemic effect of the pathogens toward the host plant.

These findings on modulation of response to fungal attack by Italian stone pine are discussed in the context of an integrated disease management program to minimize the spread of the introduced forest pathogen *H. irregulare* in Italy.

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A comparative chemical and transcriptomic analysis of defence pathways activated in conifer trees by pathogenic fungi and herbivorous insect

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Key words: Fungi-Insects, Scots pine, Terpenes, Transcriptomics

Background

During their lifetime, forest trees are exposed to numerous pests including phytopathogenic fungi and herbivorous insects (1, 2). To protect themselves against pests, trees have developed a broad repertoire of protective mechanisms (3, 4). Many of the plant's defence reactions are activated upon fungi or insect attack, and the underlying regulatory mechanisms are not entirely understood yet, in particular in conifer trees. Here, we present the results of our studies on the transcriptional response and chemical compounds production of Scots pine upon attack by the root and butt rot conifer pathogen (*Heterobasidion annosum*). The results were comparatively analysed with similar studies done on Scots pine following challenge with the large pine weevil (*Hylobius abietis*) feeding.

Methods

Based on stem phloem lesion size, we selected highly susceptible and least susceptible trees for detailed terpene and transcript profiling analysis . Terpenes were analysed with the aid of GC-MS while micro-array was used to analyse Pine transcriptome (36.4K cDNA elements).

Results and Conclusions

The highly susceptible tree accumulated ten times more terpenes compared to the least susceptible tree. The absolute concentrations of 3-carene was higher in the least susceptible tree. The concentration of delta-3-carene was negatively correlated with lesion length. The microarray analysis indicated that transcipts involved in terpene pre-cursor synthesis through the methyl-erythritol-phosphate (MEP) pathway were up-regulated. Alpha-pinene synthases were up-regulated in the most susceptible tree. Weevil feeding also caused large-scale changes in pine transcriptome. In total, 774 genes were significantly up-regulated more than 4-fold ($p \leq 0.05$), whereas 64 genes were significantly downregulated more than 4-fold. Additionally, weevil feeding on stem bark of pine significantly increased the total emission of volatile organic compounds from undamaged stem bark area. Emission levels of monoterpenes and sesquiterpenes were also

increased. Interestingly and comparatively, we could not observe any correlation between the increased production of terpenoid compounds and expression levels of terpene synthase-encoding genes upon weevil feeding. The potential relevance of the results in identifying chemical markers for screening of Scots Pine trees for resistance to pests will be discussed.

Competing interests
The author declares that they have no competing interests.

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Abstract oral session III

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Exploiting SNPs for CRISPR/Cas9-mediated biallelic mutations in *Populus* reveals 4-coumarate:CoA ligase specificity and redundancy

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Background

CRISPR (clustered regularly interspaced short palindromic repeats), a naturally occurring immune system of bacteria and archaea, has been repurposed as a genome editing tool in eukaryotes. Co-expression of the *Streptococcus pyogenes* CRISPR-associated endonuclease (Cas9) with a chimeric guide RNA (gRNA) cleaves the targeted gene [1]. Subsequent repair by the error-prone non-homologous end-joining mechanism often leads to small indels that, when located within a coding sequence, can generate frame-shift mutations. The CRISPR/Cas9 system has been successfully applied in several herbaceous plant systems, including *Arabidopsis*, rice, wheat, tobacco, tomato and soybean [2, 3]. Here, we report its application in the woody perennial *Populus*, by targeting the 4-coumarate:CoA ligase (*4CL*) gene family as a case study. We achieved 100% mutational efficiency for two *4CL* genes tested—every single primary transformant we examined contains biallelic DNA modifications. For outcrossing species with a highly heterozygous genome, gRNA design must take into account the frequent occurrence of SNPs to achieve efficient genome editing.

Methods

CRISPR binary vectors were constructed as described [4], with gRNAs designed based on the GN_{19}-NGG motif. *Populus tremula* x *alba* (717-1B4) transformation, amplicon sequencing and plant characterization for phenylpropanoid-derivatives, wood properties and recalcitrance were performed using established procedures.

Results and Conclusions

CRISPR mutation of the Class I, lignin-associated *4CL1* gene resulted in strikingly uniform phenotypes among independent transgenic events. Lignin levels were reduced by ~20%, with a concomitant decrease of S/G lignin ratio by ~30%. A common side effect associated with lignin modification is wood discoloration, and indeed, all *4CL1* mutant lines exhibit a uniform reddish-brown color in the wood. The uniform pattern was unlike the patchy discoloration associated with uneven *4CL* suppression by other gene manipulation methods (*e.g.*, antisense, sense co-suppression or RNAi silencing) in previous studies. Amplicon-sequencing confirmed biallelic mutations in all transgenic

events examined, consistent with a null *4CL1*. The high levels of residual lignin in these mutants thus suggest functional redundancy of the *4CL* family.

Class II *4CL* has been associated with flavonoid biosynthesis based on its expression pattern, but this function has not been confirmed by reverse genetics. CRISPR mutation of *Populus 4CL2* resulted in significantly reduced levels of condensed tannins, by 50-90%, in roots without affecting stem lignin. Interestingly, several *4CL1* mutants also showed reduced CT accrual in roots, suggesting a minor role of *4CL1* in flavonoid biosynthesis. Chlorogenic acids (caffeoylquinate isomers) are another abundant class of phenylpropanoids in *Populus*. Their synthesis also depends on 4CL, but it remains unclear which isoform is involved. We showed that foliar chlorogenic acid levels were reduced significantly, by ~30%, in *4CL2*, but not *4CL1*, mutants. This is the first transgenic characterization of *4CL* involvement in chlorogenic acid biosynthesis.

For highly heterozygous species such as *Populus*, it is crucial to consider sequence polymorphisms in gRNA design for the success of CRISPR-mediated mutagenesis. This was demonstrated with a “negative” experiment, where mismatches between gRNA and its target sequences abolished cleavage for a third (*4CL5*) gene. To assess the impact of SNPs on gRNA specificity, we identified gRNA candidates for 41,264 *Populus* gene models using publicly available design algorithms. Of the ~390,000 exonic gRNAs we examined, ~50% harbor indels of SNPs in the target sequences. This suggests that standard gRNA designs may suffer from a high probability of failure (inefficient CRISPR editing) due to the frequent occurrence of SNPs in outcrossers. Given that 717 is among the most widely used clones for *Populus* transformation, a database was developed to facilitate screening of custom gRNAs for the presence of SNPs and/or indels based on ~20X resequencing data of 717. The resources should also be useful when allele-specific CRISPR editing is desired.

The ability to generate “instant-nulls” by the CRISPR/Cas9 system is highly desirable for forest trees with long generation cycles. When used in conjunction with early flowering strategies, transgene-free progenies can be obtained in one breeding generation. Elite clones carrying targeted gene mutation(s) without foreign DNA may ultimately help increase public acceptance of bioengineered agricultural products.

Competing interests

The author declares that they have no competing interests.

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Discovery of the biological role of PHENYLCOUMARAN BENZYLIC ETHER REDUCTASE (PCBER), one of the most abundant proteins in poplar xylem

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Background

Wood is the most abundant renewable natural material on earth and a prime source for timber, pulp and paper. In addition to these traditional sectors, wood is increasingly considered as an alternative for the production of second generation biofuels such as bioethanol. Given the worldwide importance of wood, it is striking that the biological role of one of the most prominent proteins in poplar xylem, Phenylcoumaran Benzylic Ether Reductase (PCBER), has remained obscure. PCBER was discovered 15 years ago as one of the most abundant proteins in 2-dimensional gels made from poplar xylem (Vander Mijnsbrugge 2000a; 2000b). PCBER belongs to a large class of NADPH dependent oxido-reductases. Davin et al (1999) showed that both the poplar and the pine PCBER can reduce dehydrodiconiferyl alchol (DDC) *in vitro*. DDC is a dimer of coniferyl alcohol, one of the main lignin monomers. Given that PCBER is a cytoplasmic protein and that monolignol coupling and further polymerization to lignin are believed to occur in the cell wall, the question raised what the *in vivo* substrate of PCBER could be, why PCBER is so abundant, and what its biological role is.

Material and methods

Transgenic poplars (*Populus tremula x P. tremuloides*) were made that were downregulated for PCBER by an RNAi approach. Young developing xylem was scraped (~0.5 cm deep) with a scalpel while frozen, from 40 cm long debarked stems harvested from the greenhouse grown poplars. The material was then ground to a fine powder in a mortar cooled with liquid nitrogen, and immediately processed or stored at -70 °C. Immunodetection was according to standard procedures using a polyclonal antibody raised against recombinant poplar PCBER (Vander Mijnsbrugge et al., 2000b). Phenolic profiling was done according to Niculaes et al. (2014). PCBER enzymes used for enzyme activity tests were heterologous produced by PSF (http://www.vib.be/en/research/services/Protein-production-purification-and-analysis/Pages/default.aspx).

Results and conclusion

Transgenic poplars downregulated for PCBER by an RNAi approach were first analyzed by Western blotting, which revealed the strong downregulation of PCBER protein abundance. Lignin amount and composition, as analyzed by acetylbromide, thioacidolysis and NMR, and cellulose amount, were similar between transgenic and wild type trees, indicating that PCBER had no major role in cell wall biosynthesis. Comparative metabolite profiling of the transgenic poplars downregulated for *PCBER* revealed the *in vivo* substrate and product of PCBER. Based on mass spectrometry and NMR data, the substrate was identified as a hexosylated 8–5-coupling product between sinapyl alcohol and guaiacylglycerol and the product as its benzyl-reduced form (Figure 1). This activity was confirmed *in vitro* using a purified recombinant PCBER expressed in *Escherichia coli*. Assays performed on 20 synthetic substrate analogs revealed the enzyme specificity. In addition, the xylem of *PCBER*-downregulated trees accumulated over 2000-fold higher levels of cysteine adducts of monolignol dimers. These

compounds could be generated *in vitro* by simple oxidative coupling assays involving monolignols and cysteine. From these data we deduce that apparently, some of the monolignols that are biosynthesized in the cytoplasm are oxidized to radicals in the cytoplasm, upon which they spontaneously couple to form dimers (and higher order oligolignols). The coumaran type of dimers are substrates for PCBER and are reduced to molecules that become themselves radical scavengers. In the absence of PCBER, the cell makes less of the reduced dimers (thus less scavengers) and the cytoplasm accumulates products derived from the increased oxidative conditions in the cytoplasm. Thus, the data suggest that the function of PCBER is to reduce phenylpropanoid dimers *in planta* to form antioxidants that protect the plant against the oxidative damage associated with lignifying cells. In addition to unveiling the catalytic activity of one of the most abundant enzymes in wood, our data provide the first experimental evidence for an *in planta* antioxidant role of a monolignol coupling product.

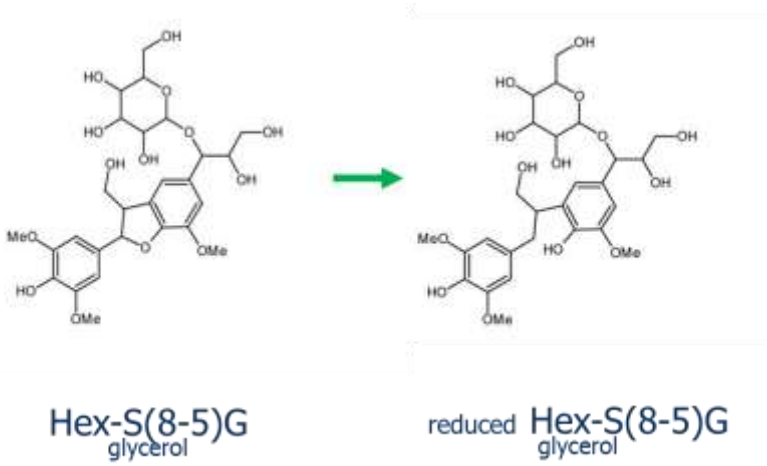

Figure 1. *In vivo* substrate and product of PCBER

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A waxy cover for *Populus trichocarpa* leaves

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Key words (max 4): poplar, cuticular waxes, natural diversity

Background

The cuticle is the extracellular lipid-rich film that covers all the aerial surfaces of land plant organs. The outermost layer of the cuticle is embedded in cuticular waxes consisting of a mixture of lipids including hydrocarbons, alcohols, free fatty acids, aldehydes and ketones. As the interface between the plant and the environment, the cuticle is responsible for protection from abiotic and biotic stresses [1].

We are using the genetic and genomic resources available through the POPCAN project to identify the enzymes responsible for the biosynthesis of wax components in *Populus trichocarpa*. As a first step towards this goal, the chemical diversity in cuticular wax was assessed in a collection of genotypes. This experiment has produced a large metabolite dataset that is being mined in combination with genome and transcriptome data to uncover the genes involved in the biosynthesis and regulation of cuticular wax components of *Populus* leaves.

Methods

Leaf samples were taken from 37 *P. trichocharpa* genotypes growing in a common garden at Totem Field in the University of British Columbia. From each genotype, leaves spanning plastochron index two through ten were collected to explore the changes in composition with leaf development. Discs from each leaf were dipped in chloroform to release cuticular waxes, and *n*-tetracosane was used as internal standard. The wax composition and quantity was measured by gas chromatography (GC) of the trimethylsilyl derivatives, and expressed per unit leaf area. Peak identification was based on comparison of retention time and mass spectra with authentic standards and with mass spectra provided by the NIST library.

The position of the wax alkene double bond was determined by oxidizing alkenes to α-diols with OsO_4, followed by GC-MS of the trimethylsilyl derivatives [2].

Results and Conclusions

The results clearly show that wax composition varied quantitatively and qualitatively between genotypes, even when grown under the same environmental conditions. A two-fold difference in total wax load between trees was observed. Moreover, categorization into compound classes showed that alkanes and alkenes are the two predominant classes, whereas primary alcohols and free fatty acids are minor constituents. Nevertheless there are some differences between genotypes, for example alkanes are the major wax class in LAFY30-2 genotype, whereas alkenes are the main components in SKNP10-2 genotype (Figure 1).

Along with the differences between genotypes, we observed large changes in wax load and composition within genotype during leaf development (Figure 2). The epicuticular wax components of individual leaves diverged such that they are more strongly related to developmentally equivalent leaves than they are to genotype of origin. Notably, the accumulation of alkenes is under strong developmental control, with younger leaves containing trace amounts and older leaves containing up to 62%.

Alkenes are major components of the cuticular waxes of mature *P. trichocarpa* leaves. However, a few genotypes were identified that lack these compounds. Additionally, preliminary evidence shows that the presence of alkenes is correlated with resistance to diseases such as *Septoria musiva* leaf spot. Alkenes are linear hydrocarbons with one or more double bonds, and despite their potential as biofuels their biosynthetic origin is unknown. By comparing the leaf transcriptome of alkene-accumulating and alkene-lacking genotypes, we identified a 3-ketoacyl-CoA synthase (KCS) as the primary candidate gene for the biosynthesis of alkenes. KCS is part of the Fatty Acid Elongation complex, a membrane-bound enzymatic complex composed of four enzymes that sequentially add two carbon units to a growing acyl chain. KCS utilize an acyl-CoA primer and malonyl-CoA to produce a β-ketoacyl-CoA. KCS enzymes differ in the carbon length of the acyl-CoA primer that they can use as substrate, as well as in the number of cycles to which they can participate. We hypothesized that the KCS identified in this study (herein called *PtKCS1*) has a preference for monounsaturated-CoAs, recruiting oleic acid ($18:1^{\Delta 9}$) for the synthesis of cuticular waxes. Supporting this hypothesis, the unsaturated bond in the alkenes recovered from leaves of *P. trichocarpa* was found at position 9. Moreover, the expression level of *PtKCS1* predicts alkene accumulation in an independent set of over 100 genotypes with an accuracy of greater than 95%.

PtKCS1 is part of a large gene family that has undergone recent expansion in *P. trichocarpa*, which has 39 putative KCS encoding genes, eight of these are found in a cluster on chromosome 10. The high similarity within copies in the cluster and the fact that *Arabidopsis* does not have a close ortholog, indicate that this cluster has recently expanded. The expansion of this *KCS* gene family may have allowed the evolution of the alkene biosynthesis in *P. trichocarpa*.

In conclusion, *P. trichocarpa provides* a unique biological system and new tools to study the biosynthesis of elusive alkenes in plants. Secondly, our observation of an expanded *KCS* gene cluster in *P. trichocarpa* that may contribute to alkene biosynthesis provides an interesting example of gene duplication leading to the evolution of a potentially adaptive trait. Thirdly, the knowledge generated could be used to breed and/or engineer plants more resistant to diseases.

Figure 1. Cuticular wax composition of leaves from two *P. trichoarpa* genotypes, LAFY30-2 and SKNP10-2. Per cent composition of the four major compound classes is shown on the pie-chart. an: alkane, en: alkene, ic: fatty acid, ol: primary alcohol.

Figure 2. Cuticular wax composition of leaves during development. an: alkane, en: alkene, ic: fatty acid, ol: primary alcohol.

Competing interests

The authors declare that they have no competing interests.

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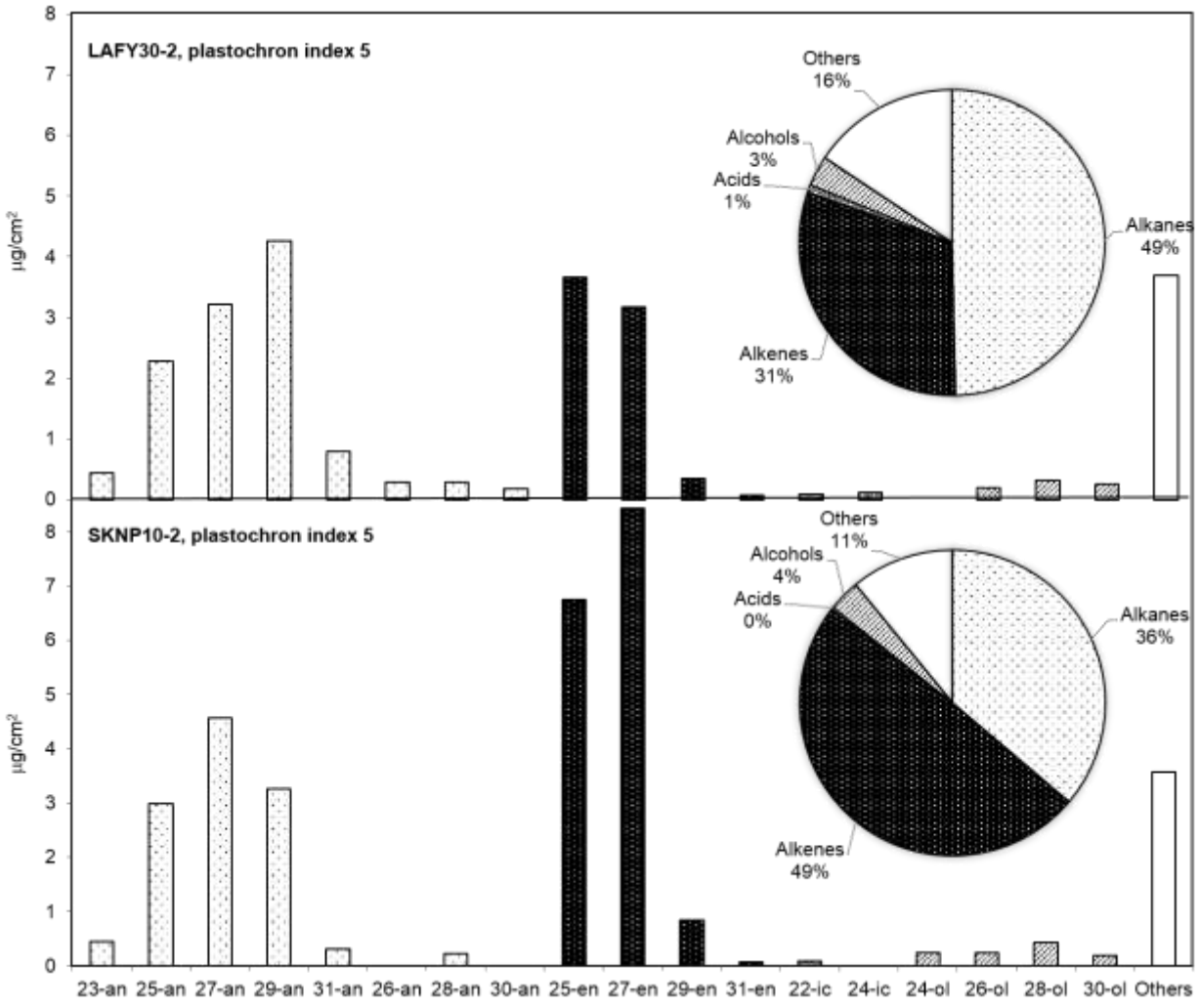
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Alcohols 3%
Acids 1%
Alkanes 49%
Alkenes 31%
SKNP10-2, plastochron index 5
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Alcohols 4%
Acids 0%
Alkanes 36%
Alkenes 49%
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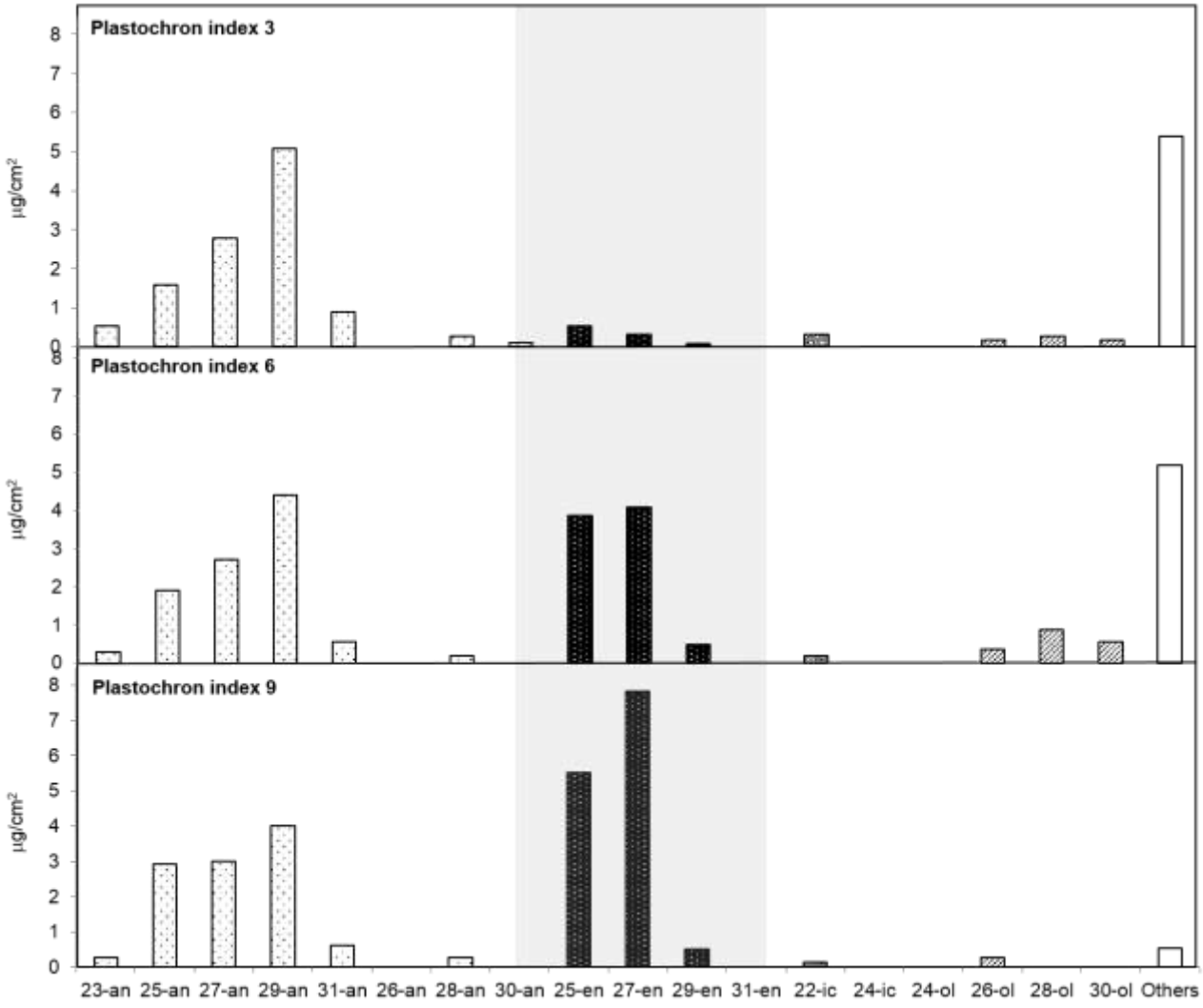
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28-an
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Others

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In planta deacetylation of glucuronoxylan – a way of enhancing sugar yields and deciphering cell wall nanostructure

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Key words: Populus, secondary cell wall, wood, xylan acetyl estarase

Background

Xylan is one of the most abundant biopolymers on earth. 60-70% of xylopyranosyl residues in hardwood xylan backbone are acetylated at *O*-2 and/or *O*-3 position. Enzymatic and chemical deacetylation of xylan can potentially increase the susceptibility of lignocellulosic biomass to enzymatic saccharification (Pawar et al., 2013). Hence, our aim was to study the feasibility of *in planta* xylan deacetylation for improved saccharification properties of wood.

Methods

Xylan acetyl esterase AXE1 from *Aspergillus niger* (Kormelink et al., 1993; Koutaniemi et al., 2013) was expressed in hybrid aspen and targeted to cell walls. Plant growth was followed in the greenhouse, and cell wall composition and properties were studied by wet chemistry and spectroscopy approaches (Biswal et al., 2014; Gandla et al., 2014). Saccharification of lignocellulose without and with acid pretreatment was carried using commercial enzyme mixtures Celluclast 1.5 L and Novozyme 188 (Gandla et al., 2014).

Results and Conclusions

The independent transgenic lines showed decreased acetylation of xylan. Their growth was not affected. The overall composition of the cell wall was unchanged, but the crystallinity of the cellulose increased. Interestingly, the S/G ratio of the lignin of the transgenic lines decreased. Enzymatic saccharification of transgenic lines yielded more glucose without pretreatment and after acid pretreatment as compared to wild-type plants.
To understand the source of these differences, we isolated different fractions of lignin carbohydrate complexes (LCC) for detailed analysis. The composition of LCCs fractions showed clear differences between transgenic and wild type plants indicating that xylan deacetylation affected the LCC formation and/or lignin extractability.

The results indicate that post-synthetic modification of *O*-acetylation of xylan is a promising approach to increase sugar yields and to study complex interactions between different cell-wall polymers.

Competing interests

The authors declare that they have no competing interests.

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Cold regulation of genes related to secondary cell wall biosynthesis affects wood structure and composition

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Key words: Eucalyptus, cold stress, Transcription Factors, Secondary Cell Wall

Background

The fast growing Eucalyptus is by far the most widely planted broad-leaved tree around the world and is emerging as one of the best source of lignocellulosic biomass for pulping and fuel production in Europe [1]. Very recently, this species was officially approved for forest plantation in France, but frost still represents the main limitation for the expansion of this non-dormant tree.

The availability of successful strategies aiming to select cold resistant clones adapted to french forest conditions is an important issue. However, it also appears crucial to evaluate the effect of low temperature on wood composition and quality which directly impact its energetic potential. Scarce data from literature suggest that low temperature regulate the expression of secondary cell wall (SCW) related genes and influence lignin or polysaccharide deposition as well as xylem cell patterning [3, 4]. However these results are highly heterogeneous among different species and focuse, for most of them, on non-woody tissues.

The plant signaling pathways triggered by cold temperature are now very well documented in model plants for which the *DREB1/CBF* transcription factors have been shown as key regulators of plant tolerance [2]. In Eucalyptus, our recent genome-wide

analysis highlighted a surprising high number of *DREB1/CBF* (17) compared to other dicotyledons species, due to specific intense duplications [3]. Transgenic *Eucalyptus* (*urophylla x grandis*) overexpressing the cold responsive factors *DREB1/CBF* were used to mimic a permanent cold stress exposure.

To evaluate the impact of a long term cold exposure on xylem development and highlight the regulators involved in these modifications, we have analyzed composition, structure and transcriptome of xylem from both cold-acclimated Eucalyptus and CBF overexpressors. Several high potential candidate genes were targeted for functional characterization.

Methods

We performed a long term cold acclimation (4°C during 7 weeks) in controlled condition using commercial *Eucalyptus gundal* clones (provided by FCBA, France). Secondary stem tissues were harvested at different time points (before cold and 2 days, 15 days and 49 days after cold exposure).The *Eucalyptus* (*urophylla x grandis*) lines constitutively overexpressing *DREB1/CBF1A* or *DREB1/CBF1B* [6] were grown in standard conditions. Secondary stem tissues were harvested in overexpressors and transgenic control line (empty vector) PK7. All samples were frozen and kept at -80°C. Klason, thioacidolysis and analytical pyrolysis were performed on samples to analyze lignin content and composition. Histochemistry analyses combined with automatic measurements of xylem cell parameters (ImageJ software ©) were performed on LR White (SIGMA) resin embedded xylem sections. Expression profiles of more than 100 genes related to lignin biosynthesis and regulation of SCW deposition were assessed in each sample using high throughput Real Time QPCR (Fluidigm Biomark®).

Results and discussion

The first part of this work relies on a long term cold acclimation in controlled conditions of 6 months-old eucalyptus. The aim was to correlate transcriptomic data with biochemical and histochemical modification of SCW deposition in xylem cells. Thanks to this integrative approach, as well as the long period of acclimation, strong effects induced by low temperature were detected in xylem cells. SCW thickness was significantly increased in fibers, parenchyma cells and vessels. By comparing transverse stem sections of eucalyptus grown at 25°c and 4°C, we showed that the lignification pattern of differentiating xylem cells was altered. Low temperatures triggered a deposition of lignin compounds immediately below the cambium and a significant increase of lignin content. Expression profiles clearly showed an induction of genes related to lignin, cellulose and hemicellulose synthesis [7].

The analyses of SCW structure and composition in eucalyptus CBF transgenic lines confirmed that the induction of cold signaling led to an increase in lignification. Genes related to SCW deposition, including lignification, were overexpressed in the transgenics. It confirmed that CBF pathway is involved for at least a part of the response observed in eucalyptus submitted to cold.

This work pointed out several transcription factors of unknown function that could be involved in xylem SCW remodeling in cold conditions. The most promising genes were selected based on the knowledge of our team, recently involved in the publication of eucalyptus genome and the fine annotation of gene families related to wood formation [8, 9, 10, 11, and 12]. Their functional characterization is underway in transgenic poplars and eucalyptus roots.

Competing interests

The author declares that they have no competing interests.

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Genome-wide patterns of recombination, nucleotide diversity and linkage disequilibrium in *Eucalyptus* from high density SNP genotyping and pooled whole-genome resequencing

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Key words: recombination rate, linkage disequilibrium, nucleotide diversity, eucalyptus.

Background

Meiotic recombination is a key process driving evolution and selective breeding. While mutation generates new allelic variants, recombination amplifies the existing genetic variation. Understanding the genome-wide patterns of recombination and nucleotide diversity provides important insights into the evolutionary processes that have shaped the species' genetic history and has, together with demographic factors, direct impact on the patterns of linkage disequilibrium (LD). The extent of LD in turn determines our ability to dissect quantitative traits and predict complex phenotypes. Powerful statistical methods allow extracting information about the patterns of recombination from genome-wide SNP data in natural populations and estimating the "population-scaled recombination rate" ($\rho = 4N_e c$, where c is the probability of crossover per base pair per generation and N_e is the effective population size) [1, 2]. Genome-wide studies in model species have corroborated the ample variation in recombination rates along genomes, which in turn reflects a wide variation in LD [3, 4]. Furthermore, by looking at how the nucleotide diversity (θ) varies along chromosomes, such genome-wide surveys also allow estimating the ratio of ρ/θ, i.e. the importance of mutation relative to recombination in shaping genetic variation in the population.

A rapid decay of LD (<1kb) has been reported for a number of forest trees based on limited sampling of SNP data in short sequence stretches, providing a rationale for undertaking association genetics [5]. Recent genome-wide studies analyzing thousands of markers at variable distances, however, have started to challenge this view, revealing longer range and more variable LD, highlighting the need to better investigate recombination at multiple genomic scales [6-8].

Studies in humans, *Drosophila* and *Arabidopsis* [9-11], have reported significant LD over distances longer than those predicted by standard population models, while others have shown less LD than would be expected in short genomic segments. Features such as

demographic fluctuations, local variation in recombination rates and the impact of gene conversion interrupting LD at short distances, contribute to this apparent discrepancy [12]. Such a nonlinear relationship between recombination and physical distances between sites indicates that more important than looking at the average extent of LD understands its variance as a function of distance between all heterozygous sites over moderate to large genomic regions. In this study we looked at this issue by describing genome-wide LD and population recombination rates across a set of variable distances between SNPs using data from two different sources, Infinium genotyping and pooled whole-genome resequencing. These estimates in turn allowed us to infer patterns of the demographic fluctuations in the history of *E. grandis* and the relative importance of recombination versus mutation in shaping its genetic diversity.

Methods

Plant material, SNP genotyping and whole-genome pooled resequencing. A set of 48 *E. grandis* trees from wild populations in Australia were genotyped with the EUChip60K [13]. Whole genome resequencing data for 36 of the 48 *E. grandis* trees were obtained in pools without sample barcoding, with estimated ≈3.5X coverage of each haploid genome by shotgun sequencing (paired-end 2x100; ~500 bp insert size).

LD and population-scaled recombination rate from genome-wide EUChip60K SNP data. Genome-wide LD measured by r^2, was estimated using *LDcorSV* [14] that corrects for population structure and relatedness. We used *PHASE* 2.1.1 [15] to obtain sequence haplotypes within windows with fixed SNP numbers. Phased data was used to obtain estimates of the population-scaled recombination rate, $\rho = 4N_e c$, for each window along the chromosomes. Two coalescent-based approaches differing in how LD is modeled between sites were used: (a) the composite likelihood approximation for all pairs of SNPs assuming that loci are independent implemented in *LDhat* 2.2 [16] and (b) the "products of approximate conditionals" (PAC) model implemented in *Hotspotter* [17] that besides considering all loci simultaneously also relates patterns of LD directly to the underlying recombination process.

Nucleotide diversity and population recombination rate from whole-genome pooled sequencing data. Short read sequence data of the pooled samples were aligned to *E.grandis* reference genome using *Novoalign*. Alignment information was extracted per nucleotide-coordinate as pileup files assuming a single sample. *Popoolation* [18] was used to identify SNP sites and calculate the genome-wide nucleotide diversity ($\theta_w = 4N_e\mu$) considering that

the sample represents a population sample with 36 diploid individuals. In a second approach we used *Snape-pooled* [19] to initially detect heterozygous sites in the pooled sample and used *mlRho* [20] to estimate the population-scaled recombination rates for SNPs sets at variable pairwise distances from a single bp up to 50 Kb.

Results and discussion

A total of 36,020 SNPs, an average of 3,275 SNPs/chromosome, with distances varying from 30 bp up to several Mb, provided >5 million pairwise estimates of r^2 per chromosome. At such a genome-wide scale LD decayed to $r^2<0.2$ within 5-7 kb (half-decay of r^2 at ≈3 kb), a considerably slower decay than previous reports based on the analysis of a few SNPs in short genic stretches and consistent with recent estimates in Populus [7]. LD was however quite variable across the genome and pairwise estimates of r^2 spanned the entire range of values, from absence to complete LD even up to 50-kb distances, likely a reflection of variable effective population size histories and uneven rates of recombination across the genome.

Genome-wide population recombination rates were estimated at ρ=0.0005 with *LDhat* and ρ=0.0011 with *Hotspotter*, 10 to 100 times lower than estimates obtained in candidate gene studies, and therefore consistent with the slower than expected decay of LD observed at the genome-wide scale. A more informative picture of the overall pattern of population recombination was obtained however, when ρ was estimated at different pairwise SNP distances using the whole-genome sequence data. Recombination rate estimates over all pairs of SNPs at very short distances (1-100 bp) was remarkably high, at ρ=0.142, consistent with estimates from candidate gene studies. When ρ was estimated over all pairs of SNPs at distances >100 bp up to 1Kb, it was 10 times lower, ρ=0.011, and when all pairs of SNPs at distances >1Kb were considered, ρ=0.001 was 100-times lower, converging to the estimates obtained from the genome-wide Infinium data, where SNPs are spaced at ~10kb on average. These results indicate that only with very large numbers of LD estimates from independent pairs of sites one can provide meaningful inferences on the population recombination rate. Furthermore, the observed variability in recombination rate likely reflects a large impact of gene conversion-like-processes unaccompanied by crossovers at short distance scale, overestimating recombination and disrupting LD, while leaving LD largely unaffected at long distances, as observed in previous studies in plant, humans and animal genomes [21-24].

These variable estimates of ρ in turn allowed inferences on the demographic history of *E. grandis* by estimating effective population sizes, both recent and in the more distant past. A

likely overestimated estimate of N_e= 1.1 million was obtained from the estimate of ρ obtained at short distances as a result of the inflated estimates possibly caused by the undistinguishable impact of gene conversion. A more reasonable estimate of N_e= 87,724 was obtained when ρ from SNPs at intermediate distances was considered, and a smaller N_e= 7,909 was calculated when recombination was considered for SNPs at distances >1Kb. These results indicate considerable fluctuations in the effective population size of *E. grandis* over different time scales, consistent with short range LD reflecting more ancestral N_e, while longer range LD expected to inform about more recent N_e [11, 25]. These estimates of N_e in turn provided estimates of the Time to the Most Recent Common Ancestor (TMRCA) from ancestral recombination graphs (ARG) showing divergence of *E. grandis* at 2-5 MYA, and population bottlenecks in the Pleistocene-Holocene 16 to 50 KYA, consistent with evidences from fossil-calibrated data [26]. Whole-genome data confirmed the high genome-wide nucleotide diversity of *Eucalyptus* (θ_w = 0.0168) observed in previous reports [27], revealing that mutation is more important than recombination in shaping the diversity in *Eucalyptus* (ρ/θ_w = 0.664).

In conclusion, our results show that the estimate of population recombination rate and consequently LD varies by orders of magnitude depending on the scale of observation adopted. At closely spaced SNPs recombination rates are likely overestimated and LD underestimated by the confounding impact of gene conversion, such that estimates of recombination and LD at intermediate to longer pairwise SNP distances better reflect the true pattern of genome-wide LD. At such a scale, LD in *E. grandis* shows a considerably slower decay, when compared to previous reports based on the analysis of limited sequence stretches in a few candidate genes. While a slower decay of LD facilitates the detection of associations it complicates the precise pinpointing of causative polymorphisms in GWAS. On the other hand it indicates bright prospects for the adoption of genome-wide approaches that work on the principle that LD alone provided by genome-wide marker genotyping can capture all relevant QTL effects into accurate phenotype prediction models.

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Genome-wide prediction of individual tree ranking for growth, chemical and physical wood properties in *Eucalyptus* based on high-density SNP data

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Keywords: genomic selection, growth, wood properties, *Eucalyptus*

Background

Notwithstanding their rapid growth rates, tropical *Eucalyptus* breeding cycles generally take 12-18 years to deliver clonally tested elite genotypes. While growth traits are measured in all individuals of a progeny trial, the assessment of wood chemical and physical properties is usually carried out in a considerably smaller number of trees in the late stages of the selection process. This procedure precludes taking advantage of the full range of existing genetic variation in wood properties. Despite the discovery of numerous QTLs and a few associations between genes and wood properties in *Eucalyptus* [1] the information has not been used in tree breeding for a number of reasons that have been discussed [2]. Genomic Selection (GS) has been proposed to predict phenotypic performance based on a genome-wide panel of markers whose effects on the phenotype are estimated simultaneously in a large and representative 'training' population of individuals without applying rigorous significance tests [3]. This approach might shorten tree breeding cycles and allow indirect selection while increasing selection intensity for all traits simultaneously, consequently enhancing genetic gains per unit time [4].

Methods

*Population, growth data and SNP genotype***s.** A full-sib progeny trial involving 58 hybrid families between *Eucalyptus grandis* and *E. urophylla*, was used as a 'training' population for genomic prediction models. Diameter at breast height (DBH) and height were measured at age 4. Mean annual increment in volume (MAI) was calculated using an equation that takes into account a tree form factor. A randomly stratified sample of 999 trees across 45 full-sibs families was selected for the study.

*Wood chemical and physical phenotyping***.** Sampling of wood flour for chemical analyses and increment cores for physical assessment was performed at age 5. Near-infrared spectra of wood flour were obtained for all samples. To optimize wet chemistry lab procedures, a diverse and representative subset of 350 samples was selected based on NIR spectra. Several wood chemical and

physical properties traits were measured including: cellulose content (%), hemicellulose (%), lignin monomer composition (Syringyl:Guaiacyl, S:G ratio), soluble lignin (%), insoluble lignin (%) and total lignin (%, a composite trait by the sum of soluble and insoluble lignin), wood density ($kg.m^{-3}$), microfibril angle (°; MFA), fiber length (mm), fiber width (µm) and coarseness ($g.100m^{-1}$). NIR calibration models were built and used to predict traits in the remaining 649 samples.

*Genomic predictions, validation and ranking***.** A total of 29,090 SNPs were obtained with the Infinium EuCHIP60K [5]. Genomic predictions were developed using RR-BLUP and a BLASSO approach. A 10-fold cross-validation scheme was carried out by random subsampling replication. Predictive abilities and accuracies were estimated for all traits. To assess the effective accuracy of GS in identifying top trees, an analysis was carried out by comparing the coincidence of individual tree ranking between the two genomic methods (RR-BLUP and BLASSO) and BLUP phenotypic selection for volume growth, density and S:G ratio.

Results

Predictive abilities reached similar estimates with the two analytical approaches, varying from a low of 0.10, for microfibril angle, to 0.42 for volume growth, and up to 0.83, for lignin traits. Correlations between genomic and phenotypic predictions ranged between 0.771 and 0.929 and were best for wood chemical traits, density and growth. Both genomic prediction models yielded a coincidence of 77% for the top 30 trees ranked by phenotypic selection for volume growth, wood density and S:G ratio, and 60% in the top 10 trees. When tandem multi-trait selection was applied to these traits simultaneously, 15 out of the top 25 trees selected based on phenotypes were also selected by genomic selection (GS).

Conclusions

Accurate genomic-enabled predictions were obtained for wood chemical traits, particularly related to lignin content and composition, wood density and growth. Good accuracies were also obtained for some physical fiber properties such as fiber length and coarseness. Cellulose content, MFA and fiber width still require improvements of phenotyping methods to allow amassing large numbers of phenotypes with high precision. No difference was seen between the two modeling approaches to genomic prediction, indicating that a model involving a large number of QTLs of small and equal effect fits the data well for all traits examined. Ranking of individual tree GEBVs largely overlapped with ranking derived from standard phenotypic selection both at the individual trait level and by tandem multi-trait selection. Our results corroborate earlier results in different eucalypt populations [6] by which GS could significantly reduce the length of a breeding cycle in *Eucalyptus* by applying ultra-early selection of genomically multi-trait ranked seedlings, potentially precluding the progeny trial stage.

Competing interests

The authors declare that they have no competing interests.

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A comparison of genomic selection models across time in interior spruce (*Picea engelmannii* × *glauca*) with use of unordered SNP imputation methods

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Key words (max 4): genomic selection, tree improvement, marker imputation, genotyping-by-sequencing

Background

The principal limitation in most tree improvement programs is the time required for the completion of one cycle of breeding, testing and selection. In some programs, it can take up to 30 years to complete a single cycle of breeding, specifically for late expression traits. Strategies to maximize genetic gain per unit time should then be the primary focus to rationalize the enormous spatial and economic requirements associated with forest tree improvement practices (White et al, 2007). The concept of genomic selection (Meuwissen et al, 2001) has promised to reduce the time associated with breeding cycles, and has since established itself as a paradigm in animal (Hayes et al, 2009) and plant (Heffner et al, 2009) breeding systems. Though, this movement has yet to occur within a forest tree species context. Genome complexity, lengthy life-spans, and exposure to wide ranging environmental conditions necessitates extensive testing of current methodologies before genomic selection can be adopted into forest tree breeding programs.

The novel genomic selection approach combines phenotypes and genotypes of a training population to develop a prediction model that estimates genomic breeding values (GEBV) for selection candidates, requiring only their genotypic information (Meuwissen et al, 2001). This method may circumvent the need for the long testing phase that forest trees require to attain accurate phenotypic data for traditional pedigree based estimation of breeding values, and offers a unique opportunity to substantially increase the response to selection through increasing the number of selection candidates. Genomic selection has been enabled by current generation DNA sequencing technologies. Additionally, cost efficient genotyping platforms such as genotyping by sequencing (Elshire et al, 2011), has opened the doors to applying genomic selection in non-model species.

Methods

Two Interior spruce (*Picea engelmannii* × *glauca*) progeny test sites near Prince George, British Columbia, Canada with 29,475 phenotyped trees from 175 open-pollinated families were used to estimate breeding values as a proxy of true breeding value (TBV) using a pedigree based method. We explored the implementation of genomic selection using a genotyped subset of 769 trees belonging to 25 families. A series of six repeated tree height measurements through ages 3 – 40 years permitted a temporal analysis with estimates of age-age genetic correlations. Genotyping by sequencing was used for single nucleotide polymorphism (SNP) discovery. SNP tables with 60% missing information were imputed using three methods, k-nearest neighbour, singular value decomposition, and mean imputation, resulting in a maximum number of 50,803 SNPs. The imputed SNP tables were utilized for GEBV prediction using three genomic selection models: Ridge-regression (Whittaker et al, 2000; Endelman 2011), generalized ridge regression (Shen et al, 2013), and BayesCπ (Hayes et al, 2009).

Combinations of genomic selection models and imputation methods were evaluated based on predictive accuracy and temporal predictive accuracy obtained from 10-replication 10-fold random cross validation, and their associated marker effects. Under this scenario, 90% of available data is selected randomly as the training population, while the remaining 10% is designated as the validation population. Prediction accuracy of genomic selection is defined as the mean Pearson product-moment correlation between TBV from the pedigree based model and GEBV from the genomic selection models for the validation population from the 10 replications, i.e. r(GEBV,TBV). The temporal prediction accuracy is then, $r(GEBV_j,TBV_k)$, where k is mature tree height at age 40 and j is tree height at an age less than 40.

Results and conclusions

Overall, moderate levels of predictive accuracy (0.31 - 0.55) were observed for tree height at different ages. Additionally, the predictive accuracy varied substantially through time seemingly with spatial competition among trees. As expected, temporal predictive accuracies were strongly correlated with age-age genetic correlations (r = 0.99), and decreased substantially with increasing difference in age between the training and validation populations (0.04 - 0.47). Our imputation comparisons indicate that k-nearest neighbour and singular value decomposition yielded higher predictive accuracies on average than imputing with the mean. Furthermore, on average, the ridge regression and BayesCπ models both yielded equal, and better predictive accuracy than generalized ridge regression. The efficiency of genomic selection was found to be greater than that of pedigree based selection under an early age selection scenario. Likewise, in a mature age selection scenario that assumes a 25% decrease in breeding cycle length attributed genomic selection, it was found that genomic selection was more efficient than the pedigree based method.

Competing interests

The author declares that they have no competing interests.

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From SNP discovery to operational testing of genomic selection in boreal black spruce

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Key words: genomic selection, breeding, wood traits, black spruce

Background: Black spruce (*Picea mariana* (Mill.) B.S.P.) is a highly planted conifer in the North American boreal forest with more than 100 million seedlings every year. Due to its economic and ecological relevance, the species has been the subject of several breeding initiatives aiming to improve growth and more recently wood properties [1]. Genomic selection appears as a valuable approach to overcome the time-consuming selection for mature traits such as wood quality, and it should be particularly appropriate in breeding populations with small effective size [2]. However, to test such applications in black spruce, genomic resources needed to be developed. Hence, the first objective of this study was to develop a large-scale SNP registry and a genotyping chip. Based on these resources, the second objective was to test genomic selection in an advanced breeding population of the Quebec Ministry of Forests.

Development of genotyping resources: A SNP registry representative of the black spruce transcriptome was constructed by compiling SNPs from previous studies [3,4,5,6] and by

discovering new SNPs using exome capture. Solid-phase capture with 454 GS-FLX-Titanium sequencing and liquid-phase capture with Illumina HiSeq sequencing were used with *Picea glauca* designed probes, generating more than 680M sequences. An in-house bioinformatics pipeline allowed identifying more than 97K high-confidence SNPs from 21K sequence contigs, reaching 96% success rate in genotyping and exceeding the 92% rate obtained previously for white spruce high-confidence SNPs [7]. About 4993 SNPs covering as many gene contigs were selected to construct an Illumina Infinium iSelect array used for genotyping progeny for genomic selection experiments.

Phenotyping of the genomic selection population: The 734 trees of this study belong to 34 full-sib families from a partial diallel mating scheme and were established on two ecologically contrasted sites in eastern Quebec. Four growth and wood traits were analysed: height and diameter at breast height (DBH), wood density and cellulose microfibril angle (MFA). Both wood traits are related to mechanical wood quality and were obtained from wood cores sampled at DBH.

Precision of genomic prediction models: Model quality was evaluated through the correlation between cross-validated breeding values and known breeding values for each individual and trait. When all marker loci were considered, results indicate an overall high accuracy of selection models, with values around 0.8 for wood density and height growth, and somewhat lower values for the two remaining traits. Accuracies were generally congruent with those obtained for *Picea glauca* biparental families for the same traits [8]. The relative efficiency of genomic selection models versus conventional ones was high, with values between 95% and 105%. Only for DBH, a slightly higher absolute gain was estimated for the pedigree-based approach. Similar patterns of model precision and estimated gains were observed when genomic selection models were trained and validated on different sites. This suggests low G-by-E interaction in this species, which should however be confirmed on a wider range of sites. Ongoing analyses deal with investigating the optimal number of markers and individuals that may be used for the construction of genomic selection models in advanced-breeding populations in order to balance model precision and costs in view of application in larger breeding populations. Preliminary findings show that the number of markers may be reduced without a significant loss of model accuracy. This further indicates that much of accuracy is due to both long-range linkage disequilibrium and relatedness existing between the training and testing datasets.

Conclusions: The present results are encouraging steps towards operational application of genomic selection in slow-growing boreal conifer species. Wood quality traits are expensive to evaluate and cannot be assessed until maturity is reached. Our results indicate that genomic selection for these traits is possible at the seedling stage, with genetic gain per year being two to three times higher than that obtained with the conventional selection approach.

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Competing interests
The author declares that they have no competing interests.

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Targeted repeat reduction in whole tree genomes prior to sequencing

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Keywords: repeat regions, genome complexity reduction, whole genome sequencing

Background

Whole-genome re-sequencing is becoming an increasingly common approach in large scale population studies that require the analysis of dense markers across the genome. In many plants including important trees, repetitive regions, such as transposable elements (TEs), make up a very large part of the genome (Table 1). For example, the *Eucalyptus grandis* genome is 49% repetitive, the very large *Pinus taeda* (Loblolly Pine) genome is 82% repetitive and *Zea mays* (Maize) is up to 85% repetitive. When sequenced as short reads via whole-genome shotgun sequencing, these repeats are notoriously difficult to assemble or map to a reference, which in turn makes them impractical for variant calling with any degree of confidence. Consequently many analysis pipelines discard or ignore these regions post-sequencing, which essentially means much sequencing is performed but the data unused. We have developed a cost-effective approach for the removal of a significant proportion of a genome's repetitive elements prior to sequencing (i.e. during library preparation) via 'in-solution capture', thus avoiding such wastage.

The value of removing repeat regions has been well appreciated for some time. Almost the entire repetitive proportion (85%) of the 2.3 Gb Maize genome was captured in-solution using a customized array of 720,000 probes designed to tile across all annotated repeats [1]. Our approach differs by recognising that due to the inherent nature of repeats, certain specific sub-sequences (e.g. 40-mers) are present in great frequency across the genome [2]. A relatively small set of short probes, designed to hybridize to the most repetitious sub-sequences, can therefore capture a large number

of shotgun fragments from repeat regions. For a given genome and number of probes, our method can determine a near-optimal set of short probes that will map to the largest proportion of the repeat regions as possible. These probes can then hybridize to common repeat fragments in-solution, leaving the remaining genomic fragments of the genome enriched prior to sequencing.

Table 1 Genome size and repetitiveness in selected reference trees and plants

Species	Common Name	Genome Size (Gb)	% Repetitive
Picea abies	Norway Spruce	19.6	70
Pinus taeda	Loblolly Pine	20.1	82
Eucalyptus grandis	Flooded gum	0.64	49
Populus trichocarpa	Black Cottonwood	0.42	40
Zea mays	Maize	2.3	85

Methods

We focused on published reference genomes and split them into repeat regions (G-repeat) and non-repeat regions (G-normal) based on available annotations. We then identified every k-mer of a specific size (e.g. 40-mers) in G-repeat and dismissed those with low frequency, resulting in a candidate set of short sequences. A greedy algorithm was implemented that selects specific k-mers from the candidate set based on their ability to cover the maximal amount of G-repeat bases without covering many G-normal bases. The output was a final set of N k-mers to use as probes. Whilst the greedy algorithm is unlikely to find the true optimal set of N k-mers that provides the maximal coverage of G-repeat for that N, it will typically approach the optimal.

Results and Conclusions

We applied the method to the annotated reference genomes of *E. grandis* and *Z. mays* obtained from Phytozome [3]. The algorithm was able to continue to add k-mers to the probe set for as long as there were further candidate k-mers and an un-mapped portion

of G-repeat. However, adding more probes resulted in diminishing returns with regards to increasing the total repeat regions covered by the set (Fig 1). The percentage of repeat region bases covered for N=1000 reached as high as 64.2% in *Z. mays* and 33.2% in *E. grandis*.

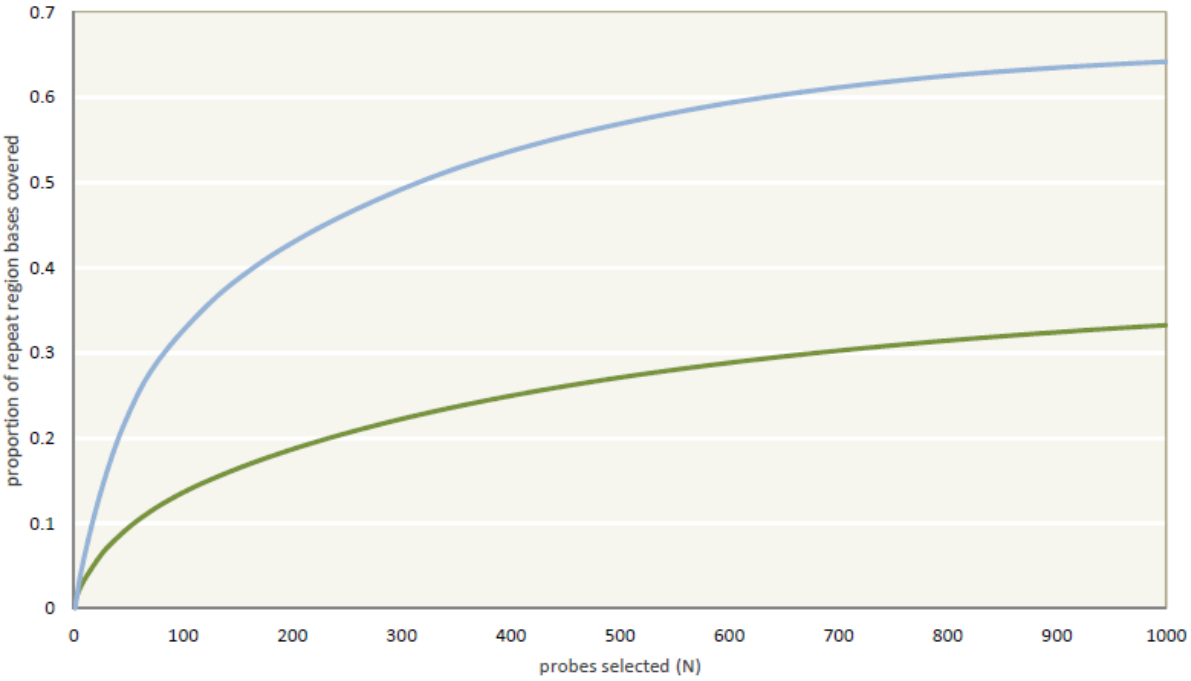

Figure 1 Proportion of repeat bases covered as the size of the set of probes (N) increases. Green and blue lines show output curves for *E. grandis* and *Z mays* respectively.

As shown in Figure 1, the method produces a curve that allows a user to select a set of probes of size N that provides a good coverage of the repeat regions for a relatively small cost. Further filtering of the probe set would be required for practical use in order to minimize coverage of genomic regions and to avoid issues such as cross-hybridization of probes in-solution. For large populations of highly repetitive genomes, this method could result in either a reduction in overall sequencing costs, an increase in coverage per sample for the same sequencing cost, or the ability to sequence more samples for the same sequencing cost. As genome size in trees appears to scale with the proliferation of TEs, whole genome studies in trees with larger genomes probably have the most to gain for relatively little cost.

Competing interests

The author declares that they have no competing interests.

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Genetic architecture of wood properties based on association analysis and co-expression networks in white spruce

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Keywords: white spruce, association genetics, co-expression network, quantitative genetics

Background

A growing number of studies show that combining association studies, molecular function and expression data could ultimately help increase understanding of the genomic architecture of complex traits and the genetic basis of variations in trait expression[1]. Developing insights into the genomic architecture of complex traits in forest trees will also require the testing of more SNPs and genes than reported in most studies to date[2]. This will enable the development of global and less biased understanding and the evaluation of pathways and gene networks.

The objectives of this study were: 1) to identify and compare the number, nature, and functional annotations of genes identified by association studies of different wood traits in white spruce; 2) to study the relationship between the genetic architecture and quantitative genetic parameters between different wood traits; 3) to determine what are the main expression profiles represented among genes associated with wood traits; 4) to conduct co-expression network analysis.

Methods

We obtained phenotypes and genotypes for 1694 trees from a population of open-pollinated families (consisting of half-sibs). In total, 6385 high quality SNPs in 2652 genes were available for analyses and were used in an association study of four wood traits determined from wood cores: wood density (WD), microfibril angle (MFA), modulus of elasticity (MOE) and ring width (RW) as described in [3]

The association analysis between SNPs and traits were performed in TASSEL. In this study, we focused on genes harboring nominally significant SNP associations with wood traits at $P < 0.05$ to maximize discovery and to gain insight into the genomic architecture

and biological processes underlying quantitative traits. The expression patterns of these genes are characterized using two transcriptome profiling experiments[4]. A BlastX search for the 2652 candidate genes sequences was performed with the Blast2GO. Enrichment analyses were carried out for both GO terms and Pfam domains. A gene co-expression network was developed based on pairwise gene expression correlations.

RESULTS

The narrow-sense heritability of traits varied between the EW and LW stages and between the different wood traits. In EW, a relatively high level of heritability was observed for WD (h_i^2 = 0.65), suggesting that this trait is under strong genetic control. Moderate heritability was observed for MFA (h_i^2 = 0.36) and MOE (h_i^2 = 0.29). RW was less heritable (h_i^2 = 0.18) than physical wood traits. The heritability estimates indicated that all of the traits were clearly under genetic control, both in EW and LW.

Association study and functional enrichment

The number of significantly associated genes varied from 229 to 292 depending on the wood traits. Pairwise comparisons of EW and LW sets of significant genes showed that many genes were shared between the two stages of wood formation for MFA and MOE, i.e. 205 (54%) and 170 (41%) respectively, and fewer genes were shared between EW and LW for WD and RW. This variation between EW and LW indicated that different stages of wood formation and growth are possibly controlled by different sets of genes for some traits but may be more conserved for others. We observed also that the number of associated genes that were shared between traits (Figure 1) or between growth stages was directly proportional to the magnitude of their genetic and phenotypic correlations.

An enrichment analyses identified 17 GO terms and 40 protein families. Overall, both the enriched GO terms and the enriched Pfam domains were very different between traits. Together, these observations indicated that the genes significantly associated with the different traits are functionally diverse and that the different traits may vary in their levels of functional specialization.

Gene expression and network reconstruction

The genes significantly associated with all of the traits except for WD in LW were overrepresented in co-expression group M2a, which is characterized by strongly preferential expression in secondary xylem and moderate expression levels in all other tissues (Figure 2). The gene expression profiles indicated that genes significantly associated with wood traits are overrepresented among co-expression groups with expression that is uniquely preferential to secondary xylem tissues compared to the overall set of genes tested.

The gene with the highest connectivity score was *PgNAC-7*, which was connected to 50 other genes in M2a. The other genes among the 20 top-ranking hub genes include MYB transcription factors and several biosynthesis enzymes of secondary cell wall polysaccharides (e.g., cellulose) and lignin. The present study identified *PgNAC-8* as part of an expression network of genes significantly associated with wood traits.

Conclusion

Our results reveal important links between genetic architecture and co-expression networks underlying wood properties. They argue in favor of the development of expression and network approaches that complement association genetics methods in forest trees.

Competing interests

The author declares that no competing interests exist

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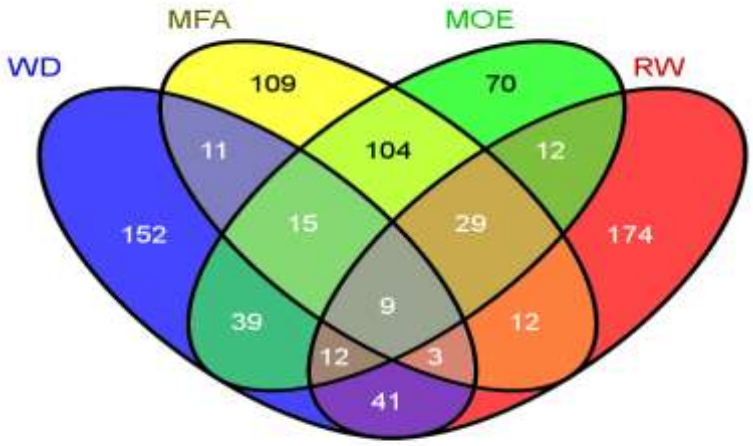

Figure 1. Overlap among sets of significantly associated genes after association testing ($P < 0.05$) between the different traits as determined for earlywood in white spruce. Venn diagram showing the full extent of overlaps of associated genes between traits. WD, wood density; MFA, microfibril angle; MOE, module of elasticity; RW, ring width.

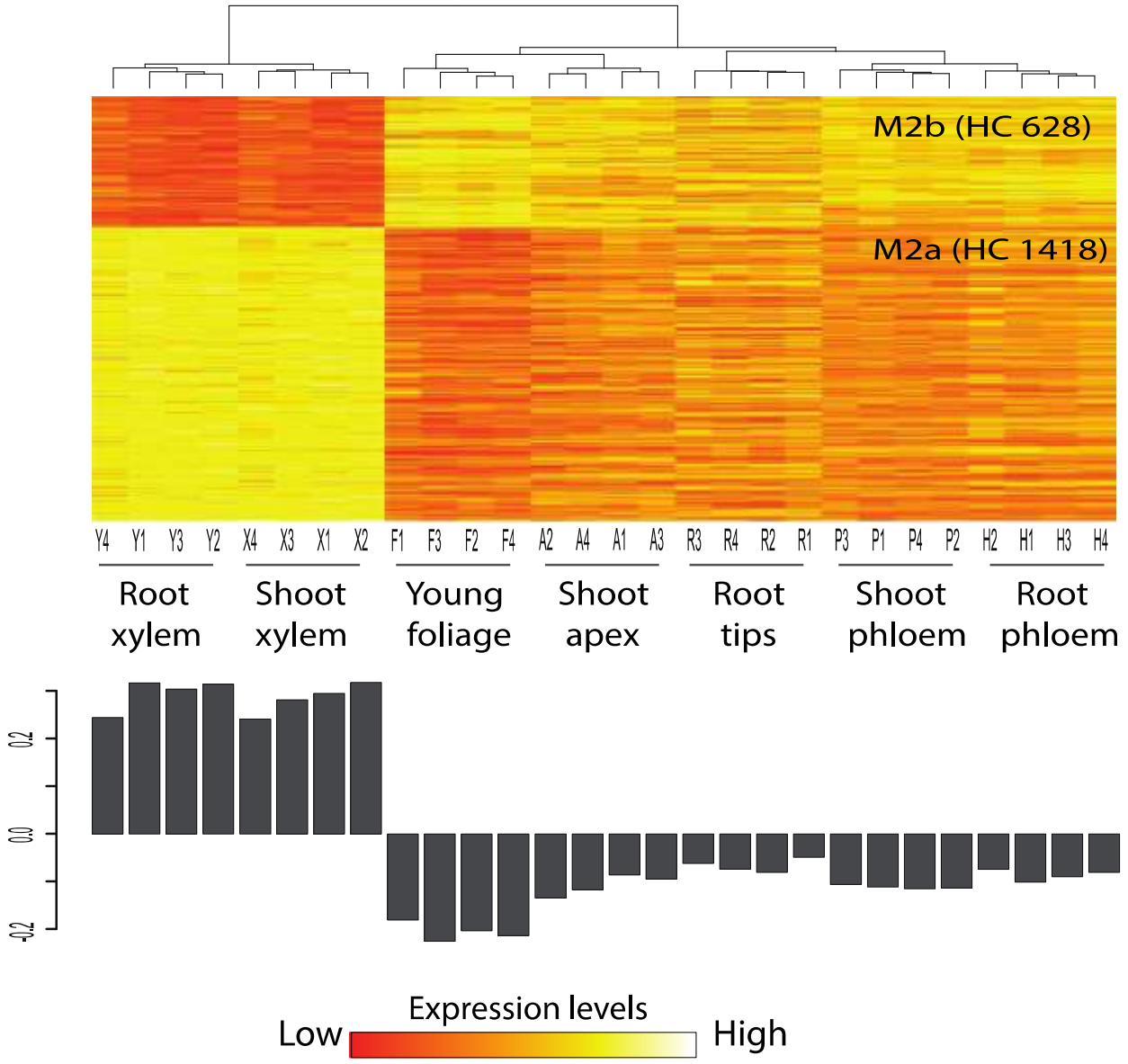

Figure 2. Heatmap of gene co-expression groups M2a and M2b in white spruce (*Picea glauca*) according to Raherison et al. (2015). Out of the 22 such groups, M2a was the group with expression uniquely preferential to secondary xylem tissues. Number in parentheses, number of high-confidence (HC) variable genes in the co-expression group. Rows (y-axis), genes which are grouped in M2 expression module; columns (x-axis), each of the four replicates of tissues (P1–4, shoot secondary phelloderm; Y1–4, root secondary xylem; R1–4, root tip; X1–4, shoot secondary xylem; F1–4, young foliage; A1–4, shoot apex; H1–4, shoot secondary phelloderm). Bar plots display the eigengene modules (or the first principal components).

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Towards a *Corymbia* reference genome: comparative efficiencies of Illumina, PacBio and hybrid *de novo* assemblies of a complex heterozygous genome

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Key words: Myrtaceae; Pacbio, gap filling, scaffolding

Background

Corymbia, a sister genus to *Eucalyptus* includes 88 species amongst which *C. citriodora* (spotted gum) stands out with a prominent role in forestry. It has risen in importance over the past few decades because of its use for timber, essential oil and energy production in drier subtropical regions of Australia, China, India and Brazil. The aim of the *Corymbia* genome consortium is to generate a high quality genome sequence anchored to a dense genetic map to underpin evolutionary studies, germplasm management and accelerated breeding by genomic technologies. Availability of a *Corymbia* genome and comparative studies with the reference *Eucalyptus grandis* genome will facilitate transfer of genomic resources between the taxa, and broaden our understanding of the diversification and evolutionary history of the eucalypt group. *Corymbia* reportedly has a small genome size of 370 Mb [1], ~40% smaller relative to *E. grandis*. Here we update our progress towards a draft genome assembly. More specifically, we present statistics of *de novo* assembly efforts using Illumina short reads, PacBio long reads and hybrid approaches using data of the two sequencing technologies. We show that hybrid assembly strategies even with low coverage PacBio data have already taken us to a draft assembly considerably better than several plant genome sequences published in the last two years. The *Corymbia* draft genome assembly will be released as an open-source platform and we are currently seeking additional partners.

Methods

The *Corymbia citriodora* subsp. *variegata* tree 1CCV2-054 was selected for sequencing based on a number of attributes, including its vigor, ease of propagation, disease resistance and wide use as a pollen parent in breeding programs. A 1CCV2-054 high-density linkage

map is under construction using DArT-seq [2]. Small-insert (180bp, 300bp and 600bp) and large-insert mate-pair libraries (2Kb, 5Kb and 10Kb) were HiSeq sequenced 2x150bp. ALLPATHS-LG error correction was applied on the raw reads to produce 616,939,334 and 288,011,326 error-corrected reads, respectively. Initial *de novo* short-read contig assembly and scaffolding was undertaken using ALLPATHS-LG. A total of 14.7Gb of long read sequence data was obtained from 20 PacBio P6C4 chemistry SMRT cells yielding 2,184,405 filtered continuous long reads (CLRs) with a mean read length of 6.7Kb, and a theoretical raw sequence coverage of ~40x. Constraints in getting higher molecular weight DNA for the PacBio runs resulted in only ~12x coverage of sequences >15Kb and <5x coverage of sequences >20Kb. Error correction routines of CLRs were based on two different approaches: (a) the multi-alignment of the CLRs themselves using SPRAI [3] and (b) taking as input the unitigs generated from the short read assembly step and using ECTOOLS [4]. After trimming and correction using SPRAI we had 7.4Gb of long read data, accounting for 940,149 reads >3Kb (~15x), 229,692 reads >10Kb (7x). The ECTOOLS reads correction is still running as we write. Following SPRAI error correction, draft assemblies were built using three different approaches: (a) *de novo* long read contig assembly using a Celera Assembler 8.2 with error-corrected reads from SPRAI; (b) a hybrid approach in which the error-corrected CLRs were aligned with PBJelly [5] to the pre-assembled contigs from short read data, and used to compute the contig linkage; and (c) a hybrid approach in which the mate paired-end reads were aligned to the pre-assembled contigs and used to compute the contig linkage from the alignment, with further gap filling and scaffolding performed using CLR data.

Results and discussion

Statistics for each assembly strategy are presented to allow for a comparison of their relative efficiencies (Table 1). Both the Illumina and PacBio assemblies have converged to an estimated genome size of ~490Mb, based on the kmer multiplicity and sequence coverage, considerably greater than the cytometry-based estimate of 370Mb [1]. This most likely reflects possible errors still in the reads, an expected high repetitive content and genome heterozygosity. Nevertheless, our reported contig N50 of ~120Kb is considerably better than those recently reported for published genome assemblies based on Illumina reads alone such as *Sesamum indium* (52.2Kb)[6] and *Cicer arietinum* (23.54Kb)[7], and well in the same range of more modern sequencing projects based on Illumina+PacBio reads such *Thlaspi arvense* (130Kb)[8]. Although we could consider having a publishable draft genome for *C. citriodora* at this point, by adding the linkage map information we are aiming at a considerably higher quality, in line with the current trend toward the “resurgence of high quality plant genomes” as recently discussed at PAG 2015 [9].

Currently, the best assemblies produced using Illumina and Illumina+PacBio data converged to draft sequences size of 395.2Mb and 412.2Mb (including gaps) respectively (Table 1), only ~7% to ~11% greater than cytometry-based estimate of the *Corymbia citriodora* genome size. The PacBio *de novo* assembly produced 1,663 contigs with a maximum contig size of 334,607bp. This result was quite remarkable considering that no scaffolding was carried out after contig assembly. However the still low coverage of PacBio sequence data (~20x) prevented us from generating a more complete draft, and only ~35% of the expected genome size assembled in contigs >50Kb. The first hybrid approach only

showed marginal improvement of the short read assembly metrics. However, a notable improvement was achieved with the second, when mate paired-end reads and CLRs were used. Despite the low coverage of PacBio long read data due to suboptimal HMW DNA, PacBio still contributed to gap filling and scaffolding. Error-corrected CLRs promoted additional gap filling and reduced the number of contigs/scaffolds in the final assembly, although the improvement of the N50 was not noticeable.

Besides the ongoing linkage mapping effort using a large (n=370) progeny with an expected map of >5,000 markers, RNA-seq by short and long read data is currently under production for annotation purposes. Pooled genomic resequencing of *C. citriodora* trees has also generated an initial SNP discovery carried out in the context of developing an Infinium chip [10]. Besides the more breeding and conservation oriented objectives of this project, by having a high quality genome for *Corymbia* we expect to shed light on the reasons underlying the genome size difference between *Corymbia* (370Mb) and *Eucalyptus* (600-640Mb). Based on the divergence of *Corymbia* and *Eucalyptus* dated at 55-85 Mya [11, 12], this size difference is not likely to be explained by the absence of the whole genome duplication that occurred in eucalypts around 109.9 Mya [13].

Table 1. Statistics of the current *Corymbia citriodora* sequencing data and *de novo* assembly.

Parameter	Illumina		PacBio	
Average read length (bp)	109		6,700	
Amount of sequence (Gb)	137		14.7	
Coverage of error-corrected sequence	Short fragment: 150 x Mate Paired-End: 70x		20x	
Assembly Method	**Allpaths-LG**	**Allpaths-LG + SSPACE**	**Celera Assembler**	**Allpaths-LG + SSPACE + PBJelly**
Assembly Size without gaps (bp):	483,144,404	453,061,813	516,987,126	499,616,141
Gap Size (bp)	114,014,948	41,286,783	NA	10,264,722
# of scaffolds	28,285	13,987	24,969	9,431
Largest scaffold size (bp)	1,242,073	698,263	334,607	919,614
N50/L50	95,410/1,576	122,063/1,193	28,484/5,324	123,067/1,212
Scaffolds > 10 Kb				
Size without gaps (bp)	430,753,213	434,094,424	466,015,797	486,538,024
Gap Size (bp)	111,841,864	39,882,701	NA	10,100,205
# of scaffolds	8,415	6,213	17,061	6,471
Scaffolds > 50 Kb				
Size without gaps (bp)	330,486,174	**365,427,914**	124,027,141	**404,431,917**
Gap Size (bp)	84,308,416	**29,783,928**	NA	**7,761,002**
# of scaffolds	3,265	3,055	1,663	3,173

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Characterization of the network of MYB transcription factors controlling condensed tannin synthesis in *Populus*

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Key words: R2R3 MYB transcription factor, proanthocyanidin, polyphenol

Background

The condensed tannins (CTs) are the most widespread secondary plant metabolites. They are especially prevalent in woody plants, and constititute a significant carbon sink in many forest trees. Condensed tannins can accumulate throughout the plant including in leaves, bark and roots; ultimately, CTs accumulate in the soil and can influence ecosystem processes such as nutrient cycling. In poplars and aspens (*Populus* spp.) CTs are typically the most abundant phenolics with concentrations reaching up to 25% leaf DW. They are also highly abundant in roots. The CTs have been associated with a variety of ecological functions including defense against herbivores and pathogens. They are also hypothesized to function in protection against abiotic stresses such as UV and aluminum toxicity.

In *Populus* leaves, CT accumulation induced by stresses such as wounding, UV light, and nitrogen deficiency. The CTs are polymeric flavan-3-ols and products of the flavonoid pathway. With the exception of the polymerization step, their biosynthesis is generally established, but the genes that control the pathway are only partly known. In *Populus*, we have identified several R2R3 MYB transcription factors that regulate CT accumulation. The poplar MYB134 gene is a positive regulator of CTs, and its overexpression in transgenic poplar trees causes a dramatically enhanced accumulation of CTs throughout the plant (Mellway et al., 2009). The characterization of this gene led to the identification of other positive and negative regulators of the flavonoid and CT pathway that are now being investigated further (Yoshida et al., 2015). We are now characterizing a suite of MYB repressor-like genes which are implicated in regulating the poplar CT pathway.

Methods

To study the function and mechanism of MYB regulators of CT biosynthesis, multiple approaches were used. Transgenic overexpression and RNAi poplars and hairy root cultures have been generated. The interaction of MYB repressors and activators, as well as of other co-factors, is investigated using the yeast-two-hybrid system, and the yeast one-hybrid is used to test for direct activation of promoters. Transient expression activation assays in biolistically transformed poplar suspension cells are being used to examine promoter activation with combinations of transcription factors.

Results

Our results demonstrate that all the MYBs tested are functional and act on the flavonoid and CT pathway. The two positive MYB regulators promote CT accumulation and can activate relevant flavonoid promoters in transiently transformed poplar cells. Four negative regulators (repressor-like MYBs) were also identified, which all repress the activation of the flavonoid pathway genes by the positive regulators. The promoters of the MYB genes are

themselves targets of the regulators in the transient expression assays, suggesting negative feedback loops. Furthermore, these experiments demonstrate the requirement for basic helix-loop-helix (bHLH) co-factor proteins for MYB function. Overall, our results to date indicate a complex network of transcriptional regulators.

To distinguish the specificities of these repressor-like MYBs, transgenic plants that overexpress and repress these MYBs have been generated. Several repressor MYB overexpressors show a low CT phenotype, confirming that these are negative flavonoid and CT regulators. Recent results have determined that at least one of the repressor MYBs, when overexpressed in transgenic plants, leads to strong low CT phenotype in roots. Transcriptomic analysis of these plants have identified a suite of potential target genes, which will ultimately delineate specific functions for the set of repressor and activator MYBs.

Conclusions

The gene regulatory network that controls CT accumulation in poplar is a complex web of MYB activators and repressors that provide for positive and negative feedback loops. Repressors appear to have broader specificity than activators, and have the potential to control the CT as well as other flavonoid branch pathways. The responsiveness of the CT pathway to a variety of environmental conditions and stresses may contribute to the evolution of this highly complex regulatory pathway.

Competing interests

The authors declare that they have no competing interests.

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The *Eucalyptus* transcription factor *Eg*MYB1 interacts with the histone linker *Eg*H1 to modulate wood formation

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Keywords: Wood formation, secondary cell wall, protein-protein interactions, lignin

Background

Wood, which is also known as secondary xylem, is the most abundant raw material on Earth, and it is widely used for many industrial applications. Specialized mature xylem cells, typically fibers and tracheary elements, are characterized by thick secondary cell walls composed mainly of cellulose, hemicelluloses and lignin. The structure and composition of these secondary cell walls are the key determinants of wood properties, making them more or less suitable depending on the industrial end-uses.

Wood formation requires a fine tight spatial and temporal regulation, mainly orchestrated at the transcriptional level. Recent studies performed in the model

plant Arabidopsis led to propose a regulatory model involving a multilevel hierarchical network of transcription factors (reviewed by Schuetz et al. [1]). However, although protein-protein interactions are known to be crucial to regulate the activity of transcription factors, their roles in the network controlling wood formation has been largely underexplored. The few studies addressing this post-translational regulation mechanism have clearly shown that protein-protein interactions add an important layer of protein activity modulation for a fine control of xylem development (reviewed in Grima-Pettenati et al. [2]).

Our team focus is the transcriptional regulation of wood formation in Eucalyptus, one of the most planted hardwood worldwide mainly for pulp and paper production. We have previously characterized one major regulator of wood formation, the MYB transcription factor *Eg*MYB1, a repressor of the secondary cell wall biosynthetic pathway [3]. Several lines of evidence suggested that this transcription factor is part of transcriptional complexes involving protein - protein interactions.

Objective

In order to get a deeper understanding of the complex regulation of wood formation in Eucalyptus, we analysed the post-translational regulation of EgMYB1 activity by identifying and characterizing its protein interactors and investigating the role of these interactions.

Methods

(i) A yeast-two-hybrid library was constructed from Eucalyptus xylem cDNAs and screened using *Eg*MYB1 as a bait.

(ii) Suitability of identified interacting candidates was first assessed by analysing their subcellular co-localization with *Eg*MYB1, and then, physical interaction was validated *in planta* using Förster Resonance Energy Transfer – Fluorescence Lifetime Imaging Microscopy (FRET-FLIM).

(iii) Interactions were functionally characterized by constructing Arabidopsis transgenic lines overexpressing *Eg*MYB1 and the identified protein partners, simultaneously and individually.

(iv) Transgenic lines were characterized at several levels. Structural analyses were performed using histochemical methods and scanning electron microcopy (SEM); chemical analyses by pyrolysis-GC, thioacidolysis and UPLC coupled to mass spectrometry; transcriptomic analysis using CATMA microarrays and RT-qPCR.

Results

We constructed and screened a Eucalyptus xylem yeast-two-hybrid library using *Eg*MBY1 as a bait. Several candidates arose from this screening but the most abundant was a Histone linker (*Eg*H1) protein. Since MYB proteins belong to a big multigenic family with a highly conserved R2R3 domain, we verified the specificity of the interaction by targeted yeast-two-hybrid assays including other

MYB proteins. We then showed that *Eg*H1 co-localizes with *Eg*MYB1 in the nucleus of transiently transformed cells. We further validated this interaction *in planta* using the FRET-FLIM technology and we also confirmed the specificity of the interaction by testing if other MYB proteins were able to interact with *Eg*H1 *in planta*.

To better understand the biological function of this interaction, we generated Arabidopsis plants overexpressing either *Eg*MYB1 or *Eg*H1 alone, and also overexpressing both genes together. As previously described by Legay et al. [3], transgenic lines overexpressing *Eg*MYB1 showed xylem cells from inflorescence stem with thinner cell walls and reduced lignin levels. In plants overexpressing *Eg*H1 no clear differences could be observed regarding cell wall thickness and lignin content. In contrast, the double transformants (35S::*Eg*MYB1::*Eg*H1) showed xylem cells with substantially thinner walls and with strong reduction of lignin content. Concerning lignin monomeric composition, an important increase of the percentage of H (p-hydroxycinnamyl) units occurred in 35S::*Eg*MYB1::*Eg*H1 plants, which was twice higher than in wild type plants.

Transcriptomic analysis revealed that secondary cell wall biosynthetic genes were repressed in 35S::*Eg*MYB1::*Eg*H1 plants, and to a lesser extent also in 35S::*Eg*MYB1 plants. Moreover, and specifically for 35S::*Eg*MYB1::*Eg*H1, there was a strong induction of defence genes, further supported by a strong accumulation of glucosinolates and, to a lesser extent, flavonoids.

Conclusions

Our results allowed the identification and characterization of a new interaction between proteins regulating wood formation and, considering the severity of the phenotypes observed, they could be an important regulatory step overlooked in previous studies. Moreover, the induction of *Eg*H1 expression after stress treatments and the induction of defence genes in 35S::*Eg*MYB1::*Eg*H1 plants suggests that *Eg*MYB1 may play a key role in the crosstalk between wood formation and defence mechanisms.

Competing interests

The author declares that they have no competing interests.

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Polycomb and ABA mediate photoperiodic control of bud dormancy

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Key words: Chromatin, ABA, dormancy

Main text

Trees growing in temperate and boreal regions undergo growth cessation and establish dormancy prior to the advent of winter. The establishment of dormancy is controlled by photoperiod since exposure to short days is essential for the establishment of bud dormancy. The molecular basis of bud dormancy establishment is poorly understood. We have identified evolutionarily conserved components of Polycomb and plant hormone ABA as key components of SD mediated bud dormancy establishment. We show that downregulation of FIE, a key component of polycomb repression complex or suppression of ABA response leads to a failure to establish bud dormancy in hybrid aspen. Moreover we show that photoperiodic signal acts via polycomb to increase ABA response in establishment of bud dormancy. I will outline our recent results that describe the molecular targets of polycomb and ABA in photoperiodic control of bud dormancy establishment and provide insight into the regulation of bud dormancy in model tree hybrid aspen.

Competing interests
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Paternity recovery in a maritime pine polycross trial using SNPs: consequences for breeding

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Key words: Pedigree reconstruction, polymix breeding, genetic parameters.

Background

Maritime pine (*Pinus pinaster* Ait.) is the main plantation species in France with nearly one million hectare of planted forests in the South West. A breeding program has been implemented since the early 1960s using a recurrent selection scheme. The breeding strategy combines two successive experimental designs: polymix crosses to evaluate parental breeding values and full-sib crosses from which selections are made for the next generation. This strategy was efficient both to increase genetic gains (maritime pine varieties reach 30% expected gain in volume and in stem straightness) and to maintain genetic variability in the breeding populations (1).

Future improved varieties must be adapted to major biotic and abiotic cues and to wood industry diversification. In such a rapidly changing context, forest tree breeding programs must integrate new selection criteria and propose a panel of varieties adapted to new threats and different end-uses. Accelerating breeding cycles to renew variety composition is thus becoming mandatory. Recent advances in the development of molecular marker and current trend in cost drop-off provide opportunities to deal with these challenges. Shortening selection cycle could be reached by polymix (PMX) breeding with parental analysis of progeny as proposed by Lambeth *et al* (2) instead of the current full-sib breeding and testing strategy. In

this context, our main objective was to study the feasibility of PMX breeding. Specific objectives were to: i/ assess pollen contamination rate, *i.e.* fathers not belonging to the PMX composition; ii/ study the deviation from equal paternal contribution; iii/ investigate the violation of assumption of true half-sibs in each PMX scheme; iv/ study the bias in breeding values and heritabilities due to unknown paternity identity.

Methods

A total of 1000 progenies (100 families, 10 progenies/ family) from two different polycrosses (PMX) were phenotyped for diameter at breast height, height and stem straightness, and were genotyped with 80 SNPs using the mass array technology of Sequenom. 66 SNPs were selected for paternity analysis.

Results and Conclusions

Pollen contamination rate (the proportion of offspring which could not be assigned to a known pollen parent from the PMX composition) was 11%, but it varied according to the families. We found that almost all genotyped pollen donors were present in the progeny pool), although their contribution was variable. Despite this unequal paternal contribution, the resulting violation of the assumption of true half-sibs in the progeny of both polycross trials was weak. Finally genetic parameters were estimated with the partial and reconstructed pedigree, and the results suggest that heritability values based on partial pedigree were either overestimated or underestimated depending on the trait considered. The maternal estimated breeding values (EBV) were not biased, and the correlation between parental EBV estimated with partial and complete pedigree information were high (0.88 to 0.95 according the trait considered). Although paternity recovery did not offer significant advantages in backward selection (but a better accuracy), it should permit forward selection of progeny with greater genetic gain and complete control of coancestry for future breeding decisions.

Competing interests

The author declares that they have no competing interests.

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COST Action FP0905: final results

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Background

The European Cooperation in Science and Technology (COST) Action FP0905 is aimed at evaluating the scientific knowledge of genetically modified (GM) trees related to biosafety protocols and coordinating existing and new information from various European countries [1]. This will help to provide a basis for future EU policy and regulation recommendations regarding the use of GM forest trees. A large, but diverse, body of knowledge on the environmental effects and biosafety issues of transgenic trees and other transgenic organisms has been acquired in many countries over approximately the past 25 years. Because of the potential unification of European states, there is now an urgent need to compile, collate, and analyze this scattered knowledge in order to create a unique platform of knowledge particular to the European environment [1]. The Action started the 12th of April 2010 and ended the 11th of April 2014. In this action, 27 COST countries (Austria, Belgium, Bosnia and Herzegovina, Bulgaria, Croatia, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Israel, Italy, Latvia, Netherlands, Norway, Poland, Portugal, Romania, Serbia, Slovak Republic, Slovenia, Spain, Sweden, The Former Yugoslav Republic of Macedonia, and United Kingdom) have signed the Memorandum of Understanding (MoU). Seven NON-COST countries (Albania, Australia, Canada, China, New Zealand, South Africa, USA) were participating to the Action.

The main objective of the COST Action was to evaluate and substantiate the scientific knowledge relevant for GMT biosafety protocols by putting together already existing information generated in various European and Non-EU countries as basis for future EU policy and regulation for the environmental impact assessment and the safe development and practical use of GMTs.

Methods

The Action work plan was organized in 4 Working Groups (WGs) focussed on: (i) the biological characterization of GMTs aiming to evaluate existing knowledge including the experience from expert scientists in the field of forest GMTs (WG1), (ii) the assessment of possible environmental impacts and monitoring of GMTs in the whole production chain from plantation to final products (WG2); (iii) the socio-economic implications and public acceptance and concerns of potential use of GM forest trees and R&D investments in the framework of Cost-Benefit Analysis (WG3), and (iv) increasing public awareness and understanding of GM forest plantations by providing science-based information through management of the www.cost-action-fp0905.eu dynamic website (WG4).

For each WG, the main methodology used was desk research through the use of PC to collect information from internet in specific web-site and specialized scientific journal. Each participant was contributing existing data from own research carried out with own national funds. In addition, questionnaires (surveys) have been worked out and sent to institutions to collect data on which GMT are “in the pipe”.

Each Working Groups (WGs) was led by a WG Leader and Vice Leader to facilitate the coordination and the exchange between and within each WG. The leader of each WG was supported by a Task Group (TG), identified within the participants to the WGs, to organise the collection the data.

Results and conclusions

WG1 launched the building of a database that gather the current knowledge on field trials and greenhouse experiments with transgenic trees. The knowledge gained was ultimately be used to guide the safe use and management of GMTs in forest tree plantations and to protect forest ecosystems. To support this goal, WG2 was launching and moving through expert-based surveys to: (1) evaluate the environmental impacts of the GMTs already developed and (2) assess the efficiency of existing transgene containment strategies, and to (3) investigate effective pre- and post-market monitoring techniques. The outcome from WG1 was used to develop a clear factual overview of the status of GMTs in European and non-European countries in order to provide sound scientific data for risk assessment to be further evaluated and monitored in WG2. In addition, it was very important to understand the kind of policies needed to meet the concerns of the society in relation to the possible use of GM trees which are widely spread in many of the Europe countries.

Therefore, two types of surveys were conducted worldwide by WG3: one to define a set of environmental and socio-economic indicators to be included in Cost-Benefit Analyses, and a Knowledge Attitude Perception (KAP) survey to explore public attitude towards adoption of transgenic forest trees. The cross- country results of the KAP surveys are expected to provide policy support to the European Commission with regards to public acceptance of transgenic forest trees and their potential conflicts of values.

On the other hand, through WG4, accurate and science-based information were communicated through our website to educate the general public on technical, socio-economic and environmental aspects of GM forest trees. In addition, the website provided an open discussion forum on transgenic forest biotechnology and biosafety, as well as on the potential impact of transgenic tree plantations on the current established forestry practices.

Figure 1. GMT database homepage.

WG1 provided a list of the principal biological characters of existing and potential GMTs in EU and non-EU countries and the WG4 established the GMT database with the main information on forest GMTs (Figure 1). Since the end of the

COST Action, the GMT database is free available to the scientific community and Europe organisations.
The EU COST Action FP0905 was expected to generate important benefits as it also has foreseen a strong collaboration among R&D bodies and legislative directives. This was fundamental to address policy-making efforts and to allow the scientific community to discuss to public concerns in a responsible way, particularly concerning socio-economic implications and biosafety issues of transgenic tree plantations.

Competing interests
The author declares that they have no competing interests.

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Biosafety of a genetically modified *Eucalyptus* with yield enhancement characteristics

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Keywords: Eucalyptus, yield, biosafety, transgenic

Background

Eucalyptus (*Eucalyptus* spp L.) comprises the largest planted forestry crop in Brazil. In 2013, the country had approximately 5.5 million hectares of *Eucalyptus* plantations. In the last ten years, the cost of wood for short fiber pulp production from *Eucalyptus* in Brazil has increased more than 90%. Thus any medium term improvements in the production chain, resulting in a lower cost of raw material, would be welcome. FuturaGene has conducted a biotechnology program since 2001, focussed on improving the performance of planted forests. In 2003, it produced the genetically modified H421 *Eucalyptus* event, designed to increase the accumulation of biomass. The increased growth of event H421 is due to the expression of 1,4-β-endoglucanase (named Cel1), a plant enzyme isolated from *Arabidopsis thaliana*. The yield enhanced *Eucalyptus* H421 has been under evaluation in containment for more than 10 years and in the field since 2006, and biosafety studies have been conducted in laboratory and field conditions.

Methods and Results

The H421 *Eucalyptus* was produced by genetic transformation mediated by *Agrobacterium tumefaciens*. In the transformation process, a T-DNA vector containing the expression cassette of the *cel1* gene was transferred to the *Eucalyptus* genome. The expression of the Cel1 protein in *Eucalyptus* plants results in greater cellular expansion and increases the volume of wood produced per area. H421 also expresses the neomycin phosphotransferase type II (NPT II) protein, which functions as a selection marker by conferring resistance to kanamycin.

Molecular characterization shows that the DNA inserted in H421 is present in a single locus and contains only one functional copy of the expression cassettes.The stability of the integrated DNA was observed by PCR analysis, in which both the construct inserted in the *Eucalyptus* genome and the regions of insertion were preserved in its progenies. The insertion stability was confirmed by molecular analysis, showing that the inserted sequences are inherited in a Mendelian fashion, as expected.

Expression of the Cel1 and NPT II proteins was quantified in tissue samples of H421 obtained from laboratory and field experiments. Results showed that the *cel1* and *npt II* genes are transcribed in low levels in all sampled tissues. Cel1 concentration does not represent more than 0.35% of the total protein, which means less than 0.05% relative to the tissue composition of the genetically modified event, whereas NPTII concentration do not exceed 0.003% of the total protein. Both proteins do not share any similarities with amino acid sequences of known allergens or toxic proteins. This was shown in the results of extensive evaluations using bioinformatics tools. In Brazil, other genetically modified events presenting the *npt II* gene have been approved by CTNBio and are commercially planted.

Proximate analysis of leaf samples collected from four experimental areas, at different plant ages, shows that the composition of H421 is equivalent to its conventional counterpart (CC).

The results showed no significant difference between H421 and CC. In addition, a comparative biomass degradation study showed no difference in degradation rate and residue quality of H421 compared to CC. Therefore H421 and conventional *Eucalyptus* are equivalent in composition and in their capacity for cycling in the natural environment.
Phenotypic and ecological interactions analysis indicated that H421 does not present risk of becoming invasive or dominant in a natural environment. The assessments were based on a combination of laboratory and field experiments. In each of these studies, the H421 *Eucalyptus* was compared with the conventional hybrid which was the basis for the genetic transformation, thus presenting the same genetic background of H421. These assessments included seed germination, pollen grain characteristics, plant growth and development data and observations of plant-insect and plant-disease interactions and response to abiotic stress factors.
The seed germination study results demonstrated no differences in the tests conducted in the laboratory and in field trials. Statistical differences were also not detected in the dimensions or average viability of pollen from H421 compared to CC. Phenotypic trait evaluation in material collected from experimental fields, at different developmental stages, showed no significant differences between *Eucalyptus* H421 and CC. Data from communities of arthropods as well as from the evaluation of biotic stresses (insects and diseases) and from abiotic stresses (drought, wind and shortage of nutrients) were collected in field trials to examine the ecological interactions of *Eucalyptus* H421 compared with CC. Comparative observations made in field trials planted since 2006 showed no significant or consistent repetitive differences between the H421 *Eucalyptus* and CC in relation to attraction or repulsion of species or populations, or any changes in the susceptibility or tolerance to the ecological stresses. Environmental analysis of H421 indicates that it represents no greater risk to indicator organisms. The assessment took into consideration toxicity tests of tissue, pollen and honey from H421 in representative indicator organisms. These included two aquatic species (fish and Daphnia), a soil decomposer (earth worm), microorganisms and species of beneficial insects (such as honey bees and stingless native bees). Analysis of composition of honey produced in an experimental area of genetically modified *Eucalyptus* and in an area of conventional *Eucalyptus* did not show any significant difference in the composition of the product collected from the hives. Honey and pollen clumps collected from the same hives were also used in controlled laboratory exposure studies against honey bees and stingless native bees.
A gene flow study was conducted, analyzing the ability of pollen from an experimental area of genetically modified yield enhanced *Eucalyptus* to cross with isolated conventional *Eucalyptus* individuals at different distances from the experimental area. Pollination tended to zero at distances greater than 600 m.

Conclusions

In conclusion, the results of the regulatory studies demonstrate that the H421 *Eucalyptus* does not present negative environmental impacts when compared with conventional *Eucalyptus* and has no ability to become invasive. A substantive dossier containing the entire evaluation has been submitted to the Brazilian National Biosafety Commission (CTNBio) for commercial approval. A summary of these studies will be presented during the Conference.

Competing interests

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EFSA GMO Panel Scientific Opinions on the safety assessment of plants obtained through cisgenesis, intragenesis or Site-Directed Nucleases (SDN-3)

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Key words: New breeding techniques, Zinc finger, TALEN, risk assessment

Background

Genetically modified (GM) plants and derived food and feed products are subject to a risk assessment and regulatory approval before entering the market in the EU. In this process, the European Food Safety Authority (EFSA) evaluates any risks that GM plants may pose to human and animal health and the environment. The safety assessment is performed based on risk assessment guidelines developed by the EFSA GMO Panel (EFSA, 2010a, b, 2011a, b) and following the data requirements for the risk assessment of GMOs laid down in the Implementing Regulation (EU) No 503/2013 (EU, 2013).
The deployment of new biotechnology-based plant breeding techniques (NPBT) offers new opportunities for plant breeding, including trees (Lusser et al., 2012). They enable the efficient alteration of the genetic and epigenetic make-up of the plant, and accelerate the breeding process (Yamagishi et al., 2014; Rinaldo & Ayliffe, 2015). Yet, the use of NPBTs also raises several risk assessment and regulatory challenges (Podevin et al., 2012; Pauwels et al., 2014). Since plants and derived products obtained through NPBTs approach commercialisation (e.g., cisgenic scab-resistant apple) and more novel products are in the development pipeline, the European Commission requested EFSA and its GMO Panel to provide scientific advice on the safety assessment of novel plants and derived products developed through NPBTs. EFSA's assessment focused on cisgenesis, intragenesis (EFSA, 2012a) and Zinc Finger Nuclease 3 and other Site-Directed Nucleases (SDN-3) with similar function (EFSA, 2012b). Cisgenesis and intragenesis both rely on the same transformation methods as used to develop transgenic plants, but in contrast to transgenesis, only transfer genes or genetic elements from the breeders' gene pool between crossable plant species. The SDN-3 technique is used for the targeted insertion of DNA. With respect to the genes introduced, SDN-3 does not differ from the other genetic modification techniques currently used, and can be used to introduce transgenes, intragenes or cisgenes. The main difference between the SDN-3 technique and the transformation methods currently used in transgenesis is that the insertion of DNA is targeted to a predefined genomic region.

Methods

As requested by the European Commission, EFSA assessed the following two questions in its Scientific Opinions (EFSA, 2010a, 2011a): (1) what are the risks that plants and derived products developed through cisgenesis, intragenesis or SDN-3 could pose to humans, animals and the environment, and (2) is there a need to develop new risk assessment guidelines for these three NPBTs or do existing guidelines remain applicable.
To address the abovementioned questions, EFSA established a dedicated working group of relevant experts with expertise in risk assessment, plant biology, molecular biology, conventional and molecular breeding and genome organisation which considered all

relevant information available in the scientific literature during the development of the Scientific Opinions.

Results and Conclusions

In its Scientific Opinions, the EFSA GMO Panel compared the hazards associated with plants obtained through cisgenesis, intragenesis and SDN-3 with those from plants obtained, either by conventional breeding techniques or transgenesis. The Panel concluded that similar hazards can be associated with cisgenic and conventionally-bred plants, while novel hazards can be associated with intragenic and transgenic plants. In case of genes introduced via SDN-3, the Panel indicated that SDN-3-related hazards are similar to those associated with transgenesis obtained with currently used genetic modification techniques. Whether identified hazards translate into risks to human and animal health and the environment is dependent on exposure factors such as the extent to which the plant is cultivated or its derived products consumed.

The Panel considered that its existing risk assessment guidelines for plants and derived products developed through transgenesis are applicable to those developed through cisgenesis, intragenesis and SDN-3, but that less event-specific data may be required, depending on the case under consideration.

Competing interests

The author declares that they have no competing interests.

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Containment technology for trees: A new frontier opens

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Background

Genetic engineering can be used to obtain trees with a wide variety of desired traits, however, concerns and regulatory restrictions on gene flow into wild and feral populations present a significant barrier to field studies and commercial use. We are therefore developing means for highly effective, stable genetic containment under field conditions. In contrast to established methods for tree sterility—which provide only male-sterility—we are seeking both male and female sterility to provide high levels of social and ecological risk reduction. We are testing both the classical technique of RNA-interference (RNAi), and the newly-developed direct-gene targeting method CRISPR-Cas mutagenesis, to interfere with the functions of essential floral genes in *Populus*. We will describe results from analysis of a four hectare field trial of three different poplar clones that were transformed with 23 different constructs that target

ten floral pathway regulatory genes, both singly and in various combinations. This is likely to be the largest field experiment of this kind in the world. We focus on phenotypes and gene expression of trees where the poplar homolog of the floral meristem determinacy gene *LEAFY* (*LFY*) was suppressed; it appears to show strong and stable sterility, and normal vegetative growth.

Methods

We created 23 different RNAi, overexpression, and dominant negative constructs targeting ten floral pathway genes. The plantation experiment included 3,414 total trees and 10 to 25 insertion events per construct (mean of 15). These constructs were transformed into three poplar clones; female clone 6K10 (*Populus alba*), female clone 717 (*P. tremula x alba*), and male clone 353 (*P. tremula x tremuloides*). Shoots were propagated to obtain four ramets per independent event. Rooted trees were planted in 2011 in a 4 hectare field trial, and trees began flowering in 2014. Floral phenotypes were initially assessed through indoor flushing of dormant floral buds followed by observation of field-opened buds. Tree size was measured each dormant season. Additional vegetative traits, (leaf area, total chlorophyll, leaf dry weight) were measured for all control and Pt-LFY:RNAi trees from clone 6K10 in summer 2014. We created three CRISPR-Cas9 constructs targeting *LFY*, two targeting individual regions of *LFY* and one targeting both, which should result in formation of large deletions. These constructs were transformed into female clone 717.

Results and Conclusions

Trees in our field trial started flowering in 2014, with 21% of the 6K10 trees flowering this first season, representing all but one construct. A screen for phenotypes of interest revealed that four constructs targeting the *LFY* and/or *AGAMOUS* (*AG*) genes led to mutant phenotypes. The Pt-LFY:RNAi trees were of particular interest, as two events had extremely tiny flowers with underdeveloped carpels that lacked stigmas, but showed robust vegetative growth (Figure 1). These phenotypes were stable; the same traits were observed for these events in 2015. In addition, we identified two more Pt-LFY:RNAi events with small flowers, and two events with intermediate floral phenotypes, indicative of partial gene-suppression. Analysis of gene expression showed that events with small flowers had less expression of *LFY* in floral buds than control trees (Figure 2). However, analysis of expression in open catkins showed higher expression of *LFY* in small flowers. We hypothesize that this difference reflects a lack of floral meristem development occurring in the RNAi trees, with individual floral meristems remaining undifferentiated

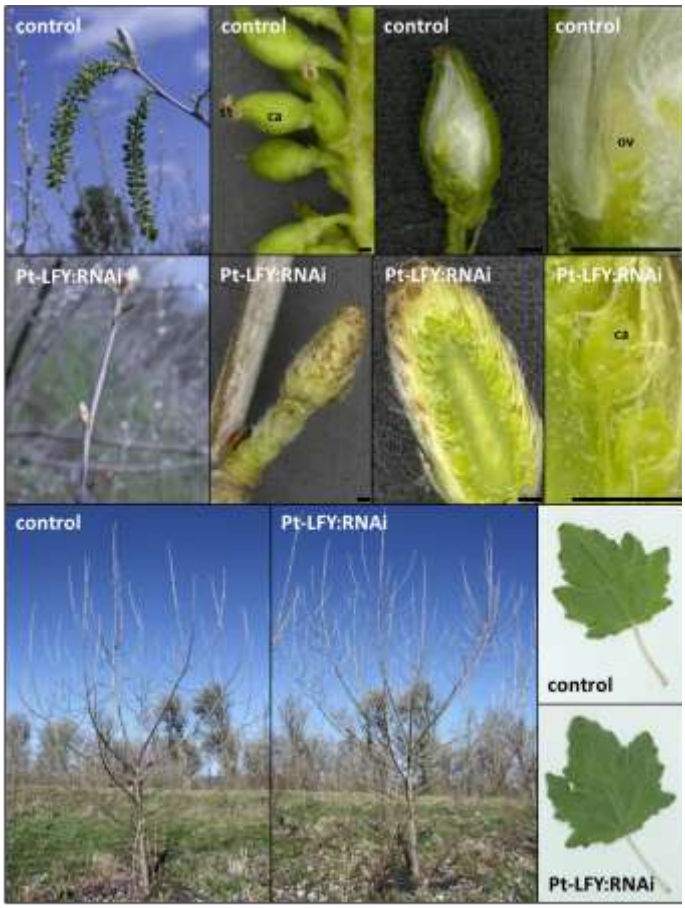

Figure 1: *Pt-LFY:RNAi* trees with small flowers had robust vegetative growth
While control trees developed large catkins with carpels (ca), stigmas (st) and ovules (ov), *Pt-LFY:RNAi* trees had tiny catkins that remained small and contained undeveloped carpels lacking stigmas. These carpels were smaller than ovules. Bar = 500 µm. A comparison of vegetative traits including tree growth, form, leaf size, chlorophyll content, and leaf mass did not detect any significant differences between control and Pt-LFY:RNAi trees.

instead of developing into mature carpels. *LFY* is expected to be expressed at a high level in developing meristems and decrease with organ differentiation. The high level of *LFY* in the RNAi trees likely corresponds with the undeveloped state of its carpels.

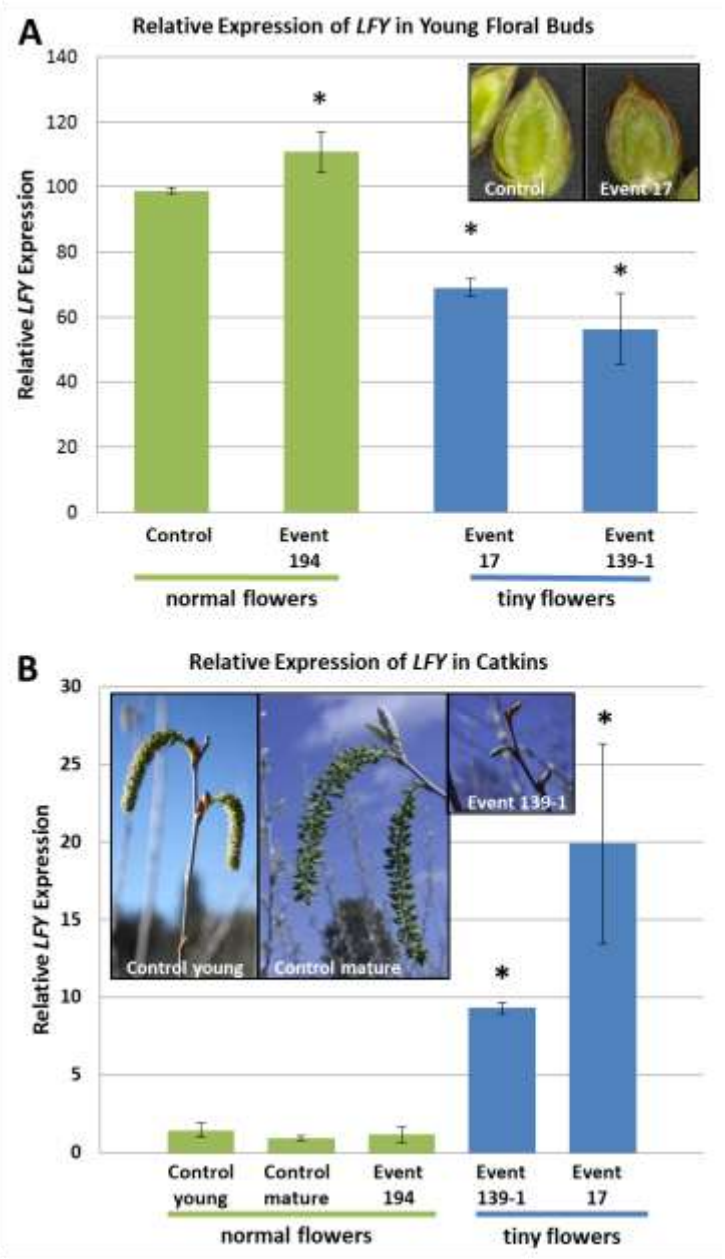

Figure 2: *Pt-LFY:RNAi* trees have altered expression of *LFY*. (A) *Pt-LFY:RNAi* events with small catkins have reduced expression of *LFY* in floral buds, but have (B) increased expression of *LFY* in open catkins. Bars show standard error, significant differences ($P < .05$) are shown by asterisks.

Our initial set of results for the CRISPR-Cas mutagenesis of *LFY* shows that we are obtaining a high rate of potentially bi-allelic mutations. We have recovered several hundred independent transformation events; a first set of shoots has undergone sequence analysis. Of the first 27 events tested for one of the single CRISPR constructs, 20 events had alterations (insertions or deletions) in the *LFY* gene sequence, several of which are complex and thus appear to be biallelic; they will be cloned and sequenced for verification shortly. Many of these mutations should lead to transcripts with early stop codons, which would encode a non-functional LFY protein. The abundant regeneration of mutants suggests

that although *LFY* is known to be expressed in vegetative tissues, it is not essential for vegetative meristem and shoot development. Trees with confirmed biallelic mutations will be induced to flower by retransformation with the *FT* gene to determine their effects on floral development.

Overall, our data suggests that it is possible to obtain trees with highly modified, sterile flowers while maintaining healthy vegetative growth. RNAi of poplar *LFY* demonstrated that this gene is an excellent target for suppression, while the high rate of CRISPR-based mutagenesis shows great potential for the identification of true, and putatively non-revertible and biallelic loss of function mutations. We believe that we are nearing the time when it will be possible to produce predictable, stable, and completely sterile trees that might facilitate field research and wider social acceptance of GE technology for biofuels and intensive forestry.

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Assessing the risk of gene flow from plantation to native eucalypts in Australia

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Key words: hybridisation, crossability, genome-wide scans, genetic distance

The large-scale translocation of species for agricultural, forestry or fisheries purposes has created numerous environmental issues world-wide which require management for industry sustainability and certification purposes (Laikre *et al.* 2010). One such issue is 'exotic gene flow', which in Australia may occur from plantation to native eucalypts. The last 25 years has seen a rapid expansion of eucalypt plantations in Australia (Gavran 2014). The estate is nearing 1 million ha, is mainly in temperate regions, and is dominated by *Eucalyptus globulus* and *E. nitens* (subgenus *Symphyomyrtus*), which are now mainly grown outside their natural range. While hybrids between eucalypt species within subgenera are often reported, including with these plantation species, hybridisation does not occur across subgenera (Potts *et al.* 2003).

Concerns were raised in the late 1990's that pollen flow from plantation eucalypts may threaten the genetic integrity of adjacent native eucalypt forests (Potts *et al.* 2003). It thus became important to implement research to enable the development of risk assessment and management strategies to minimise the offsite genetic effects of plantations. We have provided this research for 16 years and backed its application to management. Our initial focus was the island of Tasmania where *E. nitens* was introduced in the late 1980's and by 2013 there was 208,000 ha of plantations, often close to native eucalypt forests of conservation value. We have assessed barriers to hybridisation between *E. nitens* and all of the Tasmanian *Symphyomyrtus* species, and developed a risk assessment framework which is operational (Roberts *et al.* 2009). We have now expanded this research to *E. globulus* plantations on mainland

Australia (Barbour *et al.* 2010), assessing gene flow from both pollen (Larcombe *et al.* 2014a) and seed (Larcombe *et al.* 2013) dispersal.

Biotechnology has played a small, but significant, role in the development of risk assessment strategies and monitoring. This is because eucalypt plantation establishment in Australia is recent and pollen-mediated gene flow involves F_1 hybrids which are easily identifiable from their distinctive juvenile morphology (Barbour *et al.* 2005). Nevertheless, genetic markers have been important in validating F_1 hybridity. Initial validation of pollen-mediated gene flow from plantations used species diagnostic allozymes (Barbour *et al.* 2002; Barbour *et al.* 2003), but these markers were rapidly replaced by nuclear microsatellite loci which have been used to validate natural (Barbour *et al.* 2010; Larcombe *et al.* 2014a) and artificial (Larcombe *et al.* 2014b) hybridisation.

More recent advances have exploited high throughput DArT marker systems. These systems, coupled with the availability of a reference genome (Myburg *et al.* 2014), have allowed genome-wide scans with 1000's of sequence-tagged markers. These scans have allowed the identification and positioning of species differentiating markers on the reference genome (Hudson *et al.* in press) and the first genome-wide assessments of the relationships at species-level (Steane *et al.* 2011). Combined with a large-scale artificial hybridisation program, they have also allowed modelling of the relationship between genetic distance and reproductive compatibility amongst species (Larcombe *et al.* 2015). Understanding the relationship between crossability and genetic distance allowed us to substantially reduce the number of species considered to be at genetic risk from exotic gene flow.

A key future application of these molecular technologies will be the development of a robust system for species and hybrid identification to allow land managers to better monitor and manage exotic hybridisation for certification purposes.

Competing interests

The author declares that they have no competing interests.

Acknowledgements

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Model assessment of transgenic trees impact on nitrogen and carbon cycles in forest plantations

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Background

Growing global demand for wood is resulting in deforestation of natural forest areas. Plantation forestry based on fast-growing tree species seems to be promising in restoration of natural forests. Currently such plantations, occupying only 5% of forest land, give approximately 25% of all the timber production. Plantation forestry requires new genotypes created by different methods, including genetic engineering techniques. However, the use of transgenic trees needs the environmental risk assessment. Unlike studying the leakage of alien genes by pollen and impact on non-target organisms, possible impact of transgenic plants on global processes such as C and nutrients cycling in ecosystem has received less attention. The aim of our study was to evaluate decomposition rates of transgenic trees plant residues and to develop a mathematical model simulating the impact of transgenic plantations on soil organic matter dynamics and environmental conditions of Northern Eurasia.

Methods

In this work, we used a two-year plants of aspen and birch with pine glutamine synthase gene *GS1* with enhanced productivity and aspen plants with genes to modify the properties of wood (gene of xyloglucanase Xeg and RNAi construct harboring inverted fragments of 4-coumarate:CoA ligase *4CL*), and non-transgenic control [1]. Decomposition rates of plant residues were estimated by the mass loss at 22-24ºC and CO_2 emissions at temperature 22, 12, 2 ºC and at moisture 80, 50 or 20% water holding capacity (WHC). The mass loss was determined after 0.5 (for leaves only), 1, 2, 3.5, 5, 7, 9 and 12 months (for leaves, stems and roots) The CO2 emission dynamics was measured by gas chromatography (totally 21 measurements within 365 days). To simulate the effects of transgenic trees on soil nitrogen and carbon cycles, the model of organic matter dynamics in forest soils ROMUL was used [2]. Model scenarios assumed aspen plantations with *Xeg* gene and *4CL* RNAi construct growing with 30 - and 60-year rotations on sod podzolic soils of Northern Eurasia for use in the pulp and paper industry.

Results and conclusions

Only few studies on the decomposition of plant residues of transgenic trees are known [3, 4], with only one decomposing plant part, roots or leaves, used in the experiments with short-term incubation periods (100 – 130 days). Our research was conducted with three vegetative organs (leaves, stems and roots) exposed to the decomposition under various temperature and moisture conditions, with the incubation period as long as 1 year.

It is shown that the decomposition rate of leaves, stems and roots of transgenic aspen Xeg and 4CL plants expectedly decreased by near 2 times with decreasing temperature from 22 to 2 ºC. The moisture effect on the decomposition of different plant organs was

statistically insignificant, while the differences between transgenic and control plants were found. The stems of aspen clones with *4CL* RNAi construct decomposed faster, whereas Xeg samples decomposed slower than those of the control. The CO_2 emission by root decomposition of Xeg and control plants was similar, while that by the decomposition of the Pt4CL2c clone was significantly higher than for the Pt4CL4a clone and control (Figure 1). The birch stems with *GS1* gene demonstrated an increased decomposition rate due to low C:N ratio in stem tissues. Decay of plant tissues consisted of fast (50-70 days of the experiment) and slow (70-365 days) stages of decomposition. The data on decomposition rates were used to calibrate the mathematical model.

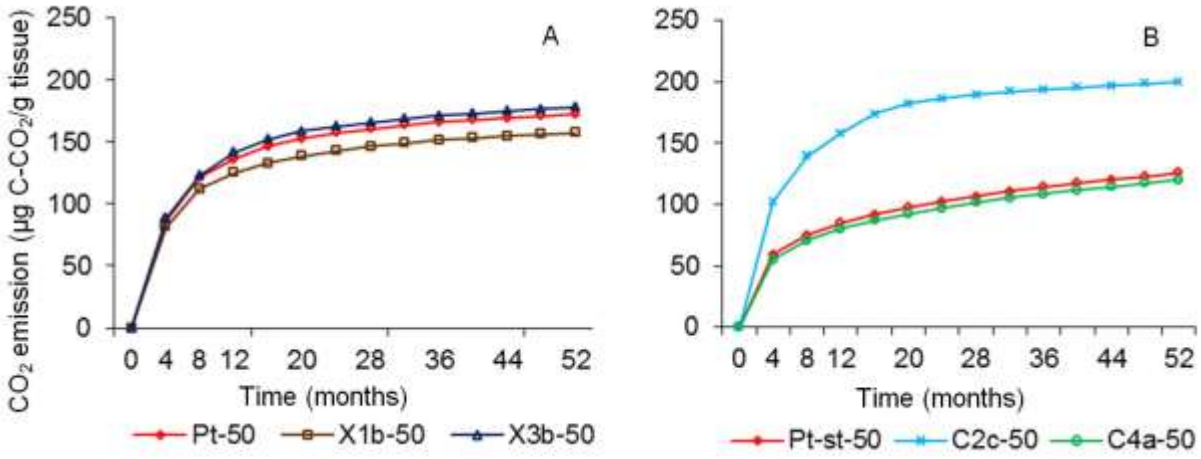

Figure 1 Decomposition of root material from transgenic Xeg (A), 4CL (B) and wild type plants estimated by CO_2 emission. Lines Pt-50, X1b-50, X3b-50 are emission of wild type, PtXeg1b, PtXeg3b plants decomposed at moisture 50% WHC, respectively. Lines Pt-st-50, C2c-50, C4a-50 are emission of wild type, Pt4CL2c, Pt4CL4a plants decomposed at moisture 50% WHC, respectively.

Mathematical models have previously been applied for the analysis of the forest plantations and their impact on the carbon cycle in the ecosystem [5], but the analysis of the nitrogen and carbon cycles for transgenic trees we performed for the first time. The rate constants of mineralization and humification of plant litter differed for transgenic and control plants, and these differences were consistent with the changes in their chemical composition. The simulation results showed that the litter storage in the control and 4CL plants was almost identical, as for the Xeg plants it was about 1.5 times higher (Figure 2). In mineral soil, on the contrary, the control was close to the stock of organic matter in the Xeg variant, whereas in 4CL variant it was somewhat lower. However, changes in the pools of carbon and nitrogen were in the range of 5-7%, which does not exceed the actual impact of standard silvicultural operations, particularly clear cut.

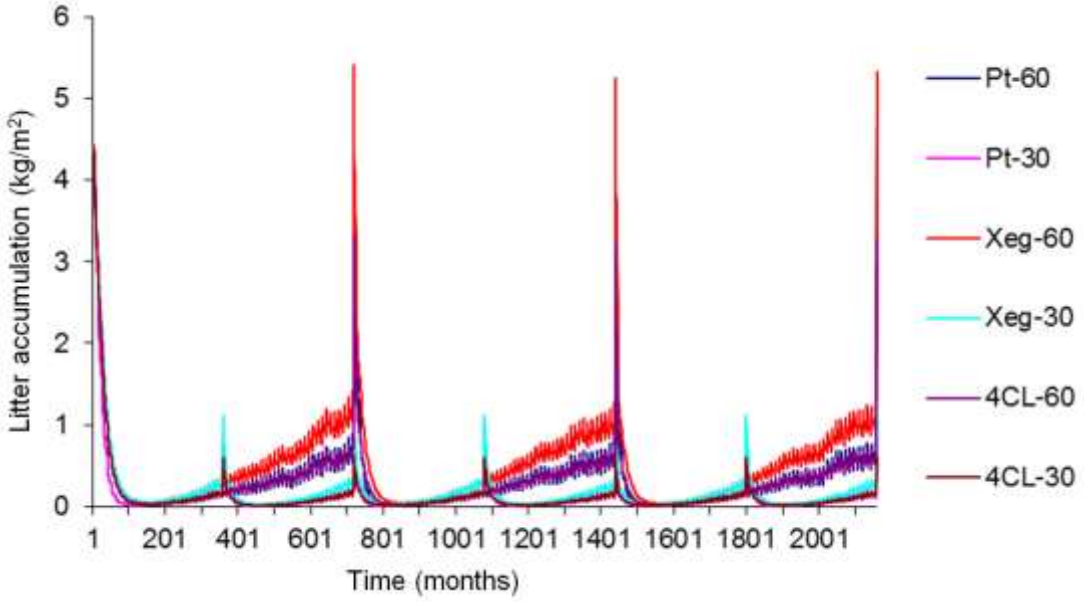

Figure 2 Mathematical model of the litter accumulation in the forest plantations from transgenic (with 4CL or Xeg modification) or wild type aspen. Lines Pt-60, Xeg-60, 4Cl-60 are the litter accumulation in wild type, PtXeg1b, Pt4CL2c aspen plantations at 60-year rotation, respectively. Lines Pt-30, Xeg-30, 4Cl-30 are the litter accumulation in wild type, PtXeg1b, Pt4CL2c aspen plantations at 30-year rotation, respectively.

Based on these data, we assume that the introduction of transgenic trees in the plantation forestry does not result in irreversible changes in C and N biological cycling in the relevant forest soils, and will not lead to possible degradation of forest ecosystems. Full-system models of forest ecosystems simulating both tree growth and soil organic matter dynamics should be used, along with conducted field trials, for a more detailed analysis of the impact of transgenic forest plantations on the environment.

Acknowledgements
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Abstract EVOLTREE Workshop

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Understanding the *Populus* Microbiome: Drivers of Community Variation and Potential Implications for Plant and Ecosystem Function

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Populus spp. are a genetically diverse genus of tree species that are broadly distributed across temperate environments of North America and have become an important model tree species. Additionally, fast growth rates, clonal propagation, and the ease with which *Populus* clones and hybrids can be grown on land otherwise unsuitable for food production, have made them good candidate feedstocks for pulp and bioenergy. For these reasons, it is important to understand the role of the root microbiome as it relates to the health and productivity of *Populus*. As part of the Plant Microbe Interfaces project (PMI – http://PMI.ornl.gov), we have been examining the root, rhizosphere and soil communities of *Populus deltoides* in natural riparian habitats in the Eastern US, as well as controlled common garden populations of *P. trichocarpa* in the Western US. Microbiome data for both bacteria and fungi are analyzed against the corresponding soil properties, geographic factors, tree phenotype, and tree genotype data in order to understand how these properties influence microbiome structure. We have shown that the rhizosphere and endosphere environments feature highly developed, diverse and to a large degree exclusive communities of bacteria and fungi. Endophytic bacterial diversity is highly variable, but on average tenfold lower than the rhizosphere, suggesting root tissues provide a distinct environment supporting relatively few species. Fungal endophytic species are more numerous than bacterial endophytes, but also less than rhizosphere spp. Both fungal and bacterial rhizosphere samples show distinct phylogenetic composition patterns compared to the more variable endophyte samples. Contrary to initial expectations, both *Populus* spp. have low natural levels of colonization by ectomycorrhizal fungi (EMF) and arbuscular mycorrhizal fungi (AMF), but high levels of presumed fungal endophytic taxa. Finally, the effects of *Populus* genotype on the composition of its rhizosphere appears to be limited when compared to the effects of local soil environment while endophyte influences are less clear. Our current research is, 1) expanding both the number and geographic range of species of *Populus* examined, 2) moving beyond the rooting zone to total microbiome studies of *Populus,* as well as, 3) adding additional functional examinations enabled by developments in metagenomics.

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QTL mapping for phyllosphere microbial community descriptors in oak (*Quercus robur* L.)

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Key words: Quercus robur, phyllosphere microbiota, community genetics, QTL

Background

A wide range of bacteria and fungi survive and proliferate on the plant leaves – the phyllosphere [1,2]. Phyllosphere microorganisms influence plant functional traits, plant fitness, and ecosystem properties and services [3]. The phyllosphere as microbial habitat is influenced by the environment[2] and by the plant traits[1]. The latter are ultimately coded in the plant genome. Thus we expected to find quantitative trait loci (QTL) underlying microbial associations on plant leaves.

Methods

We collected leaves from 180 oaks (*Quercus robur* L.) from a full-sib mapping population[4] in the south-western France. We analyzed the fungal and bacterial communities of these trees using the DNA barcoding technique[5] and next-generation-sequencing methods. Following microbial community descriptors were used as traits for the QTL-mapping: inverse Simpson index for fungal and bacterial diversity, axis projections of principal coordinate analysis (PCoA) of the microbial community using the Bray-Curtis dissimilarity index. We used composite interval mapping to identify putative QTLs.

Results and Conclusions

We identified one QTL for fungal diversity on the linkage group (LG) 6 and three QTLs for the axis projections of PCoA on the LGs 6, 9 and 10. Thus we report here four oak genomic regions which effect extends from population to the community level, influencing the fungal diversity and overall microbial composition of the oak phyllosphere.

Competing interests

The author declares that they have no competing interests.

Acknowledgements

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Endophytic bacteria in poplars – Characterisation and artificial inoculation to enhance growth parameters

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Key words: Populus sp., endophytic bacteria, plant growth-promotion, Paenibacillus sp.

Background

The community of endophytic bacteria may influence many phenotypical traits of plants, their adaptability to environmental factors or resistance characteristics. Therefore, microorganisms become more and more important as a factor to be managed in plant cultivation. Endophytic bacteria may promote plant growth with their metabolic capabilities, e. g., to assimilate nitrogen or to produce phytohormones.
In several studies, we analysed the community structure of endophytic bacteria in poplars, characterised a plant growth-promoting endophytic *Paenibacillus* strain in detail and applied several isolated strains to study the effects of inoculation. The investigation of plant growth-promoting effects of selected endophytic bacteria strains was carried out *in vitro* and under greenhouse and field conditions.

Methods

First, community structure of the endophytic bacteria obtained from different poplar clones was analysed by T-RFLP and clones libraries of the 16S rRNA gene [1]. Further, culturable endophytic bacteria were isolated from productive poplar clones *in vitro* and under field conditions. The isolates were phylogenetically classified by sequencing of the 16S rRNA gene as well. To test the ability to fix molecular nitrogen, the isolates were transferred onto N-free growth medium. The influence of one N_2-fixing *Paenibacillus* strain on the metabolism of poplars was characterised by GC-MS (MPI-MP Golm).
Meristem plants free from culturable bacteria were used to test the effect of single bacterial strains on plant growth *in vitro*. For greenhouse tests and field trials, cuttings were incubated in overnight broth cultures of the respective strains before planting.

Results and Conclusions

Previous studies with field grown poplar clones showed a large spectrum of endophytic bacteria studied with different approaches using the 16S rRNA gene [1]. The community structure displayed clear differences regarding the presence and relative proportions of bacterial taxa among the studied poplar clones. Large percentages of α-*Proteobacteria* and β-*Proteobacteria* and smaller amounts of γ-*Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes* were observed in the clone libraries. In contrast, the culturable bacteria are dominated by *Actinobacteria* and γ-*Proteobacteria*.

In tissue culture material from poplar, certain bacterial strains seemed to accumulate under long-term *in vitro* conditions without visible negative influences on the plant development [2]. One of these isolates (P22), identified as *Paenibacillus* sp. based on the sequencing of the 16S rRNA gene, was able to promote the rooting of cuttings.
The influence of this strain on the metabolism of poplar shoot explants was compared to control plants free from culturable endophytic bacteria. Approximately 70 metabolic compounds were detected and quantified by GC-MS [3]. The tested strain significantly influenced numerous compounds, mainly associated to the N-metabolism. The findings were in accordance with the ability of *Paenibacillus* sp. P22 to fix molecular nitrogen. Recently, the genome of this strain was sequenced [4] which revealed a gene encoding nitrogenase, and genes of the auxine-pathway were also found.

Current studies focused on the effects of selected endophytic bacterial strains on different poplar clones under laboratory, greenhouse and field conditions [5]. Inoculation with single strains of the genera *Stenotrophomonas* and *Paenibacillus* induced an enhanced root and shoot growth of *in vitro* plants [2, 5]. Under greenhouse and field conditions, inoculation of poplar cuttings with *Stenotrophomonas* and *Paenibacillus* strains resulted in an increased plant growth and biomass (Fig. 1). Here, the results of inoculation were found to be influenced by the poplar clone used and the growth conditions during the experiment.
Besides the screening for new plant growth-promoting isolates and the characterisation of genes involved in increasing plant productivity, practical nursery aspects are emphasised in planned research projects.

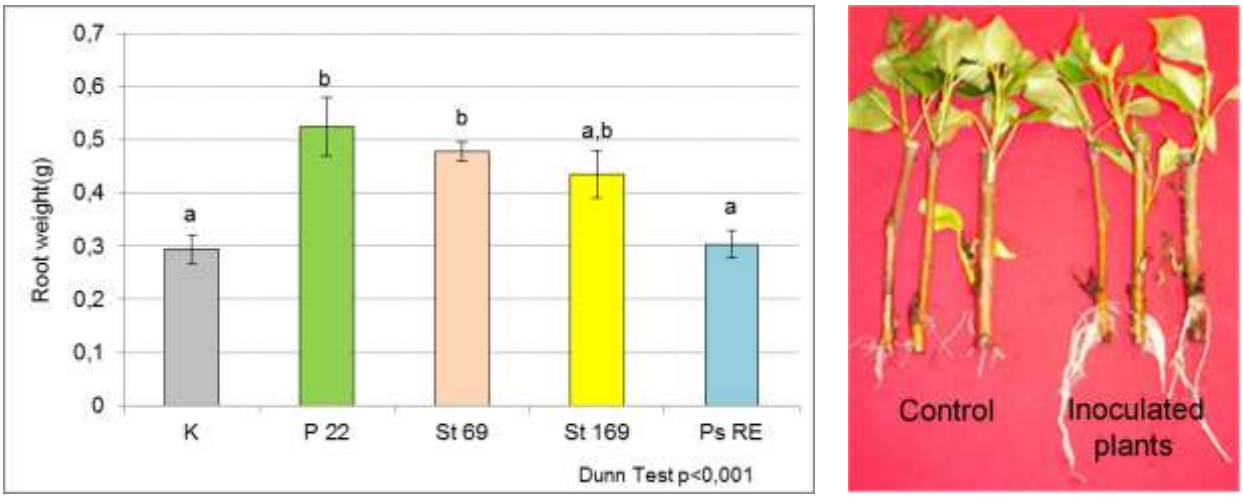

Figure 1:
Left: Root weight of poplar cuttings (clone Max2) inoculated with different bacteria in comparison to the non-inoculated control (K), analysed under greenhouse conditions. P22-

Paenibacillus sp. P22, St 69- *Stenotrophomonas* sp. 69 (strain collection Uni Graz), St 169- *Stenotrophomonas* sp. 169, Ps RE- *Pseudomonas* sp. RE (strain collection Uni Graz). Different letters indicate significant differences.
Right: Effect of *Paenibacillus* sp. P22 on the rooting of cuttings under greenhouse conditions.

Competing interests

The author declares that they have no competing interests.

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TreeType

Stephen Cavers, ETH (UK)

Collecting tree phenotypes in the field - a new, open-source project from EVOLTREE

In the age of easy next-generation sequencing, a lack of good phenotypic data is a major bottleneck in tree genetic research. Although a lot of data is collected from experimental trials, much less is routinely collected from the very different conditions of forest populations in the field. Yet such data can provide important insights into the balance between local adaptation and phenotypic plasticity in tree populations.

A new initiative from EVOLTREE will attempt to kick-start widespread collection of data on some simple phenotypic traits for European trees. The project will be open to participation by anyone with the enthusiasm and skills to record the data for the trees of their choice and data will be made openly available.

We will create an online recording website, providing standard protocols and guidance, and making it easy to upload new records to a common database and track the progress of data collection for your species. A simple voting tool will allow the proposal of new species to record, with data recording initiated once a critical mass of recorders has been reached.

The project will run for an initial trial period of two years, with a critical review at the end of that time - if the idea is taken up enthusiastically by the community, the project may continue and a valuable long term resource could be created!

Abstract poster session I

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Exploring adaptive responses to change in environmental conditions in *Pinus halepensis* Mill.

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Key words (max 4): Pinus halepensis, adaptive responses, gene flow, phenotype-genotype associations

Background

Mediterranean forests are biodiversity hotspots, currently threatened by several natural and anthropic factors (Fady *et al.* 2003, Vendramin *et al.* 2008). *Pinus halepensis* Mill. is widely distributed in the Mediterranean basin (Barbéro *et al.* 1998), and it is considered a key species for its ecological and economic importance, besides its recreational and aesthetic values (Fady *et al.* 2003). *P. halepensis* is considered as a pioneer, drought and fire resistant species (He *et al.* 2012, Hernández-Serrano *et al.* 2013).

As a consequence of climate change, a significant increase in extreme climatic events and aridity is foreseen for the Mediterranean region (Petit *et al.* 2005) and negative consequences on genetic diversity and adaptive potential of forest trees are expected (Grivet *et al.* 2009). Therefore, *P. halepensis* was chosen as a model species for studying adaptive responses to changing environmental conditions, identifying an *ad hoc* study site to:

- Assess the extant genetic diversity at SSRs and SNPs
- Analyse gene flow patterns
- Study phenotype-genotype associations

Methods

The study site is located in the Gargano peninsula (southern Italy), in a valley with a rather continuous distribution of *P. halpensis* from 0 to 550 m a.s.l. Sampling was carried out in two large stands, hereafter called H (~500 m a.s.l.), and L (~150 m a.s.l.), located along a steep precipitation gradient (Fig.1). Mean annual precipitation is ~750 and ~450 close to H and L, respectively. Long term series for temperature and precipitation were reconstructed from nearby climatic stations.

Since polymorphic nSSRs for *P. halepensis* were lacking, new markers were developed from the *Pinus halepensis* transcriptome (Pinosio *et al.* 2014), to assess the extant genetic diversity and to perform gene flow studies.

A SNP array (Pinosio *et al.* 2014) was used to genotype selected adult trees along the altitudinal gradient.

In order to study the adaptive plant responses to drought, tree cores were extracted from the same individuals used for SNP genotyping for dendrochronological and ecophysiological analyses. The patterns of growth were evaluated and five time-spans were identified within each chronology. Plant physiological trait variations (*i.e.* intrinsic water use efficiency, iWUE, as the ratio between assimilation and stomatal conductance) were assessed by $\delta^{13}C$ and $\delta^{18}O$ isotope analyses for each group of dated rings.

A reciprocal transplant experiment was set up nearby the H and L stands, where 80.000 seeds were collected.
The experimental design was a complete randomized and balanced split-plot, with 4 plots (~6×5 m) at each altitude (H and L) composed by 10 sub-plots/split-plots (~1×2.5 m). In order to test for differences between local light conditions within each experimental site, plots were placed under light and shade conditions. Four replicates/plots/blocks for each 'site × light conditions' combination were set. Overall, 160 lots were prepared (80 samples for each population). Each lot grouped 500 seeds from 25 families (20 seeds per family).
The experiment was approximately visited every month. During each visit, all newly emerged seedlings were labelled, and the date of emergence and death was recorded.

Results and Conclusions

For population genetics analyses, 100 adult trees and 100 juveniles in each plot were sampled. In both plots, several cones were collected from 25 mother trees to obtain enough seeds for paternity analysis and the reciprocal transplant experiment (Fig.1).
DNA extractions are completed. Additional samples to genotype will be available once reciprocal transplant experiment will finish.

SSRs genotyping is currently ongoing with previously available (8) and newly developed (14) SSRs, selected for their polymorphism and profile quality from a larger set.

For SNP genotyping, 50 adult individuals, 25 from each plot, were sampled. The genotyping of 384 SNP array was completed and an exon capture and genotyping by sequencing experiment is in progress. Measurements for the phenotypic characterization of these adult trees is completed.

Data collection and analysis for the reciprocal transplant experiment are in progress. Since the sown had been carried out (March 2014) until February 2015, 364 seeds germinated and only 155 are still alive.
Preliminary data analysis showed that the altitude but not the origin of seeds seems to influence the emergence of seedlings. At high altitude, emergence has been higher and negatively affected by light conditions. At low altitude, local conditions determined an extremely low global emergence rate regardless light availability.

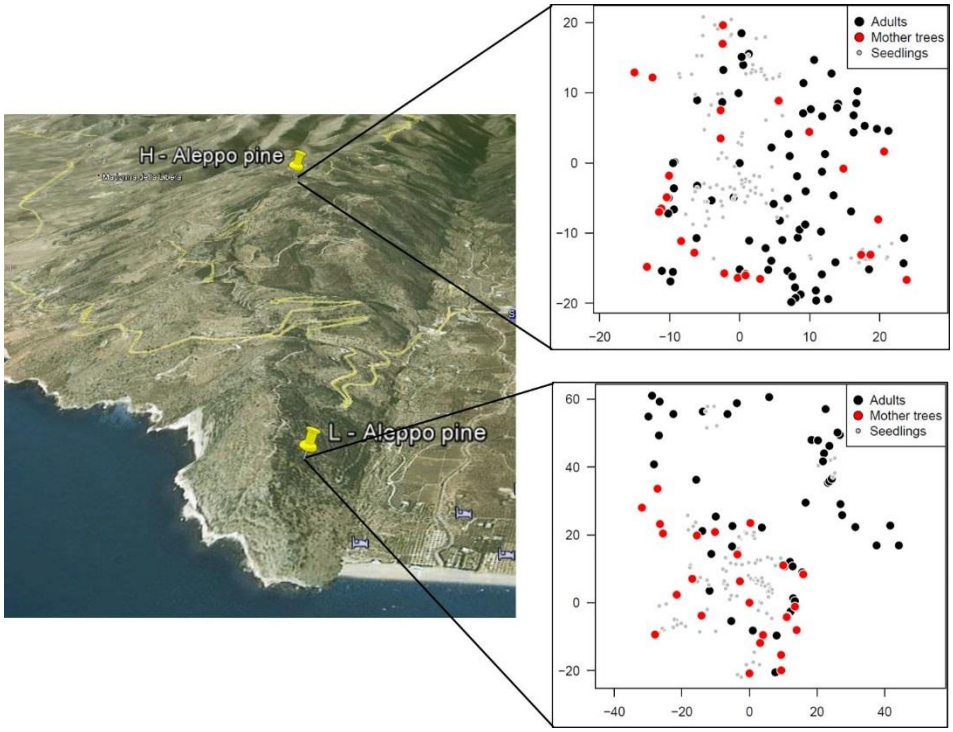

Fig. 1 Plot H and L, and relative maps of sampled individuals for gene flow study. Black dots represents adult individuals, grey dots are juveniles and red dots represent trees from which seeds were sampled.

Competing interests

The author declares that they have no competing interests.

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Gene expression profiling in response to UV-B radiation in different *Populus alba* clones

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Keywords: UV-B stress; Gene expression; Ecophysiology; Poplar

Background

Long-term depletion of the stratospheric ozone layer contributes to the increase in terrestrial solar ultraviolet-B radiation (UV-B), an environmental change with potentially deleterious consequences for plants. When exposed to elevated UV-B (280-315 nm), plants display a wide variety of physiological and morphological responses. Consequently, determining the molecular bases for acclimation to normal fluence and tolerance of high UV-B are important factors in sustaining plant yield.
To better understand the processes of UV-B acclimation, which result in altered plant morphology and physiology, we investigated gene expression in leaves of 5 different white poplar genotypes-clones at high UV-B fluence rate and exposure times.

Methods

Three years old plants were acclimated in two chambers identical for temperature and photoperiod but differing for the UV light. An UV-B lamp array and an ozone breakdown system is present in the UV chamber. The following parameters can be controlled inside the chambers: temperature, relative humidity, CO_2 concentration, O_3 concentration, visible (photosynthetic active radiation, PAR) and UV (UV-A and UV-B) radiation. The environmental light is developed to guarantee a good PAR-UVA-UVB ratio; in each chamber eighteen 36W fluorescent lamps are placed: 9 Osram Biolux 965 (day light), 9 Osram Fluora 77 (red blue light). Furthermore, 4 halogen Osram Power Star HQI-TS 400W/D ensure the presence of blue and UV-A radiation. Seven Sankyo Denki G40T10 (UVB radiation) lamps were used for UV-B treatment. UV-B lamps were wrapped in cellulose acetate film to cut radiation with wavelengths below 280 nm (UV-C).). Plants of 5 different clones of white poplar were grown in a nursery for 1 year and then acclimated in the chambers for 2 weeks before the start of the UV-B treatment. In order to simulate an environmental condition, plants were treated for 12 hours with an $UV\text{-}B_{be}$ (UVB biologically effective radiation) supplementary dose of 6 KJ/m^2/day (0.15 W/m^2 at 100 cm from the lamps) and allowed to recover during the night (12 hours). Growing conditions were as follow: i) 25°C temperature during the day and 20°C during the night, ii) 60% of relative humidity, iii) CO_2 concentration (450 ppm), iv) photoperiod of 12 hours of light and 12 hours of dark with a simulation of sunrise and sunset, v) 6 KJ/m^2/day supplementary dose of $UV\text{-}B_{be}$ during the daylight photoperiod. Four genes were selected for transcription analyses on the basis of the results of eco-physiological observation. Transcription analyses were used to analyze gene expression of plants treated for 3, 6, 12 and 36 hours with elevated levels of UV-B. These

genes resulted significantly down- or up-regulated (P value < 0.05 and fold change < 0.5 or >2) during the treatment. We analyzed the response of each genotype comparing (unpaired t test) the transcription levels between the treatment within and between the genotypes, to find out when the transcription levels were high, in the first case and which genotype responded to the treatment differently, in the second case. Before the start of UV-B treatment (0 hours), all genes transcription levels variation between control and treated samples, was not statistically significant for all the genotypes. Pooled leaf samples of the 5 clones were sampled from control and UV-B treated plants (3, 6, 12 and 36 hours); chlorophyll fluorescence of PSII was measured and RNA was extracted for qRT-PCR analysis of genes putatively related to UV-B response such as Chalcone Synthase (CHS), Caffeic Acid 3-O-Methyl Transferase (COMT2), Ribulose-1.5-Bisphosphate Carboxylase/Oxygenase large subunit (RuBisCo), and CPD-photolyase class II

Results and Conclusions

The eco-physiological observations were focused on the photosynthetic efficiency, as estimated by chlorophyll fluorescence measurements. The photosynthetic efficiency values decreased for all the genotypes already after the first day, ranging from values around 0.83, up to values around 0.7/0.6 after 72 hours of analyses (the end of the treatment). This decrease in chlorophyll efficiency is not significant to suppose damage or photoinhibition to PSII, but rather a decrease of efficiency in the treated samples due to the presence of an elevated UV-B radiation. Therefore, analyses regarding this parameter did not show a permanent damage to the leaves for all the different genotypes.

Eco-physiological data indicated that the different genotypes were not able to recover in the same way after 36 hours of UV-Bb radiation. For this reason, the transcriptional levels of genes (*chs*, *comt*, *rubisco* and *cpd photolyase*) putatively related to UV-B response, were investigated through a time-course qRT-PCR analyses. We analyzed the response of each clones comparing (unpaired t test) the transcription levels between the treatment within and between the clones, to find out when the transcription levels were high, in the first case and which clone responded to the treatment differently, in the second case. Regarding to the *chs* gene, Policoro showed high levels of transcription already in the early hours and increased its levels during the treatment. Val Bormida as well, increased its transcription levels after 12 and 36 hours of treatment. The same two clones that present high transcriptional levels of *chs* gene showed a higher recovery during the fluorescence analysis, indicating an early activation of defense mechanisms with constitutive production of protection pigments. Similar trend was observed for *comt* gene that showed a different pattern of expression during the hours of treatment for all the clones. Differences were noticed in particular after 12 and 36 hours, where Val Bormida and Policoro genotypes showed higher levels of transcription compared to the others. This is explained with the fact that at the beginning we have first the increase of the *chs* levels, the first gene in the phenylpropanoids pathway, related with the production of anthocyanins, and then arrive to the synthesis of lignin.

We therefore hypothesize that Policoro and Val Bormida are the 2 clones that better tolerated to UV-B radiation, Policoro having the highest transcription of *chs* and *comt* gene leading to the hypothetical production of both anthocyanins and lignin, and Val Bormida having higher transcriptional levels than the other clones for *comt* with the production of lignin. From our analyses emerged that the transcription of CPD-photolyase gene is not influenced by the treatment. Regarding RuBisCo large subunit (*rbcl*) the trend of transcription levels for the different clones was similar.

In the first hours, the transcription levels did not seem to be influenced by the treatment. Instead in the following hours, after 12 and 36 hours of treatment, the transcriptional levels resulted down-regulated. This result, together with the results obtained from the

physiological measures which did not include damage to the leaves, photoinhibition damage to PSII, confirm the hypothesis that is RuBisCo, and not PSII, the primary target involved in inhibiting photosynthesis after exposure to UV-B radiation. The decrease of the photosynthetic efficiency found in the eco-physiological experiments is probably due to a decrease of the transcriptional levels of RuBisCo for all the clones.
In conclusion, the results evidenced that white poplar clones respond to high UV-B fluence with a differential transcription of analyzed genes. An important next step is understanding correlation between the individual response of clone and UV-B tolerance.

Competing interests
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Molecular and physiological analysis of drought response in the Mediterranean conifer *Pinus pinaster* Ait.

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Key words: drought response, molecular dissection, functional characterization, Pinus pinaster

Background

Drought is a major environmental stress factor that determines the growth, development and survival of plants living in water scarce environments. Climate change predictions point at increasing dryness over the Mediterranean region. *Pinus pinaster* Ait. is an important Mediterranean conifer subjected to recurrent drought periods. Notwithstanding its relatively small geographical range this species, which is found along a rainfall cline, is characterized by a significant genetic and adaptive diversity. Different morphological and physiological responses appear to play an important role in drought adaptation of this conifer [1]. These complex responses to drought, from perception to transcriptional, metabolic and physiological changes, need to be considered at a global systems biology level to study the multiple interactive components [2]. Integration and analysis of multidisciplinary datasets will likely increase our understanding of molecular mechanisms controlling *Pinus pinaster* response to drought [1].

Methods

Different approaches were designed to analyze the processes involved in *Pinus pinaster* response to drought, all of them based on a progeny from an *ad-hoc* designed full-sib cross (Gal1056xOria6), that segregates for the response to this environmental factor:

Transcriptome and miRNA characterization: cDNA and miRNA libraries were constructed using RNAs extracted from different tissues from Gal1056xOria6 individuals vegetatively propagated and subjected to different treatments. cDNA and miRNAs libraries were sequenced using GS FLX Titanium and Illumina, respectively (3, Sáez-Laguna & Cervera, unpublished results, de María et al, unpublished results). Assembled reads were annotated and differentially expressed genes selected as potential candidates for qRT-PCR studies. SNPs were identified in the transcriptome.

Construction of genetic maps: Genetic maps of different INIA's progenies were developed mainly based on SNP segregation using Illumina SNP arrays (i.e. Infinium, Golden Gate; 3, 4). Additionally, in the frame of the ProCoGen project, an exome capture system is being developed to study segregation on a set of thousands sequences in different conifers and perform conifer comparative mapping.

QTL analysis: Identification of genome regions involved in the genetic control of *Pinus pinaster* response to water stress was carried out searching for associations between morpho-functional and molecular variants in the corresponding linkage maps [3]. This analysis allowed identification of positional candidate genes.

Study of cytosine methylation: Cytosine methylation during drought response was study at different scales: total cytosine methylation (HPLC analysis), genome-wide methylation changes at anonymous CCGG motives (MSAPs; 5) as well as detailed methylation pattern of a collection of candidate genes in response to the stress (collaboration with Dr. Díaz-Sala, UAH).

Global metabolomic analysis: A set of samples were also used to conduct global metabolomic profiling in order to analyze changes associated with drought stress

in different tissues as well as to analyze metabolite composition associated to the response to combined stresses.

Phenotypic analysis: Growth and different morpho-functional traits related to water and carbon plant economy were analyzed for contrasted genotypes in response to the interaction of water stress and different concentrations of atmospheric CO_2. (6, Sanchez-Gómez & Aranda unpublished results).

Results and Conclusions

More than 20 cDNA libraries were constructed representing different tissues and growing conditions. Differential expression analysis is ongoing to identify potential candidate genes that are further subjected to qRT-PCR. Additionally, a 1,536 SNP array has been developed based on SNPs associated to the reference mapping population and used to construct, together with additional SNPs, dense genetic maps [3]. These maps will be further saturated using an exome capture system designed by ProCoGen consortia in collaboration with Dr. Kirst (UFL, USA) for conifer comparative mapping.

Clones from Gal1056xOria6 showed high variability in their response to drought [6], i.e osmotic adjustment capacity differing between clones. Heritability values for stomatal conductance and intrinsic water use efficiency were moderate.

Genetic maps have also been used for the dissection of leaf gas exchange (photosynthesis and stomatal conductance to water vapour), chlorophyll fluorescence parameters and water use efficiency in response to drought, detecting QTLs that explained 10-20% of the observed phenotypic variability for each trait. Untargeted analysis of metabolic profiles allowed the discrimination of genotypes with contrasting drought response. Different metabolites, such as glutamate family amino acids, polyols and lipids, were correlated with some of the ecophysiological traits responding to drought.

Competing interests

The author declares that they have no competing interests.

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Identification and analysis of candidate genes for drought stress tolerance and bud burst in European beech (*Fagus sylvatica* L.)

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Key words: Fagus sylvatica, candidate genes, RNA-seq

Background

European beech (*Fagus sylvatica* L.) is one of the most important deciduous forest tree species in Central Europe. Several studies revealed a high neutral genetic diversity for this species which is regarded as a good basis for adaptation processes. Nevertheless, little is known about the genetic variation within genes relevant for adaptation. An increasing atmospheric CO_2 concentration, higher annual mean temperatures as well as changes in precipitation may affect the vegetation period, growth, health and distribution of trees (European Environment Agency 2012). Therefore, adaptive traits relevant for an adaptation to climate change like bud burst and/or drought stress tolerance might gain in importance in future. Thus, candidate genes for bud burst and drought stress tolerance were identified and analyzed in the present study.

Methods

A translocation experiment was established with progenies of beech populations growing under different environmental conditions in Northern Germany. Repeated observations of bud burst revealed significant differences among populations. Comparative sequencing of parts of ten different bud burst-related candidate genes revealed 116 SNPs (single nucleotide polymorphisms). In total, 46 SNPs were successfully used for genotyping of more than 1,400 individuals of the translocation experiment, which were selected based on their bud burst timing. Association analyses for bud burst data of three consecutive years were conducted to identify potentially adaptive SNP markers.
To identify candidate genes for drought stress tolerance in European beech, a controlled drought stress experiment was conducted. Samples of five stress and five control plants were taken at five different time points during drought treatment. Thus, in total, 50 samples were used for RNA sequencing using an Illumina HiSeq 2000 platform. *De novo* transcriptome assembly and analysis of differentially expressed genes between the stress and control group was conducted using the CLC Genomics Workbench 7.04 (http://www.clcbio.com). The software BLAST2GO (Conesa et al. 2005) was used to annotate the contigs.

Results and Conclusions

Association analyses were conducted using pooled individuals from the different populations to increase sample size. In total, 24 significantly associated SNPs with bud burst were identified. The phenotypic variation explained by the significantly associated SNPs with bud burst was low, but in accordance with other studies in forest tree species. In addition to the

association analyses, FST outlier analyses were conducted revealing five different SNPs, which are potentially under directional selection. In total, three potentially adaptive SNPs were simultaneously revealed by both outlier and association analyses. These might have the highest probability of being involved in the manifestation of bud burst. Currently, the SNP set is tested in further populations in Germany to confirm associations.
The *de novo* assembly of sequencing reads revealed 44,826 contigs with an average length of 764 bp. In total, 608 genes were significantly differentially expressed between the stress and control group ($p<0.05$; $FDR<0.05$). These genes are new candidate genes for drought stress tolerance.

Competing interests

The authors declare that they have no competing interests.

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Geographic variation in cork oak and its implications for expected impacts of climate change

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Cork oak (Quercus suber L.) is a protected tree species in Portugal, being also the source of raw material for the cork industry, a major player in Portuguese economy (representing 3% of GDP in 2010). The future climatic scenarios for Portugal point to an increase in average summer temperatures from 0.3 to 0.7ºC between 2016 and 2035, and up to 4.6ºC until 2100. In addition, precipitation estimates suggest a reduction of annual rainfall from 20 to 40%, especially in southern Portugal. Water stress will, therefore, be a leading constraint to primary production. The combined effects of drought and high temperatures will lead to decreases in carbon assimilation and increases in tree mortality, and consequently current reforestation efforts will need to account for these expected adverse outcomes through the sustainable use of suitable genetic material.

There are several reasons that can be highlighted to emphasize the need for an efficient management of cork oak genetic resources in Portugal, namely: i) to avoid cork import, and thus to increase cork production to meet the industry demands; ii) to overcome a generally poor area of natural regeneration, which does not help to ensure an *in situ* conservation of genetic resources; iii) to deploy adapted genetic material for afforestation/reforestation; and iv) to develop a gene resources conservation program, as cork oak is a vital component of agro-silvopastoral systems in the Mediterranean region.

Given the broad native range of the species, involving significant environmental and geographic gradients, a high level of genetic variation can be expected. It is possible that disruptive selection has caused a large differentiation in adaptive traits among populations, namely in the ability to tolerate different environmental stress events (e.g. drought and frost) and to cope with pests and diseases. Between 1998 and 2011, we have collected data involving survival, growth, phenology and water-use efficiency traits from five common-garden provenance trials (including family structure in two of the trials), that were established in Portugal under a concerted action launched by the EUFORGEN's network. These multi-site field experiments are based on up to 35 tested provenances covering the entire natural distribution of cork oak, and results obtained from the genetic evaluation of the trials have indicated significant differences among populations for all the measured traits at all observed ages. Four of the tested provenances (Alpujarras – Haza de Lino, Puglia – Lucci - S. Teresa, Landes - Soustons, Rif Occidental – Ain Rami) were then chosen according to their contrasting field performance for growth, phenology and water-use efficiency (WUE), and were further evaluated under controlled-environment conditions where drought stress was induced. In this context, the main drivers of drought adaptation appeared to be early stomatal closure and root investment, which also showed significant differences among the selected provenances. The responses to drought over time also varied among these studied populations, and seemed to be related to their differences in growth rhythm.

The Ain Rami population seemed to be most prone population to endure drought conditions. Facing a water deficit scenario this population, with highest growth, showed a higher investment on roots compared to the Haza de Lino population, that even under optimal hydration status, had lower biomass values, more reduced transpiration area (smallest size, with lowest Specific Leaf Area), leading to a lower water consumption. This population showed a delay in onset of stress when compared to other populations, only revealed no stomatal limitations with high stress levels. Furthermore, Ain Rami showed higher WUE under drought conditions both in the field trials and under controlled conditions, but average WUE in wet conditions.

The results from the field and controlled-environment experiments were consistent in that geographic origin had an important influence on the performance of fitness surrogates and functional traits, and thus providing a strong indication that seed origin must be considered in cork oak reforestation programs.

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Transcriptome responses to medium-term water deficit among *Eucalyptus* species of contrasting ecotype provide insight into key pathways responsible for adaptation to water limited environments

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Key words (max 4): transcriptomics, water deficit, eucalyptus, ecotypic comparative

Background

Climate change predictions suggest an aridification within the native range of many *Eucalyptus* (L. Hér.) species as well as in a most plantation growing regions. Climatic and edaphic conditions over geological timescales have generated enormous diversity of adaptive traits and a high degree of speciation within the genus. Eucalypt species occur from sea level to the alpine tree line, from high rainfall to semi-arid zones and from the tropics to latitudes as high as 43° S (Gillison, 1994). Despite several morphological and metabolomic characterizations, little is known regarding gene expression differences that underpin differences in ecotype.

Using species with contrasting ecotype; *E. globulus* a more 'mesic' or 'wet' climate eucalypt and *E. cladocalyx* a more 'xeric' or 'dry' one (Merchant *et al*, 2006), this study combines physiological characterization using RNAseq sequencing to identify genes involved in eucalypt responses to medium term water limitation and hence key pathways responsible for adaptation to water limited environments.

Methods

Eucalyputs cladocalyx and *E. globulus* seedlings of similar height and leaf area were selected and allocated to two treatments: well watered (WW) and severe water deficit (SS). SS plants were given 45% of water used by WW to exhaust the reservoir of water in the pot by week eight of the treatment period. Patterns in leaf gas exchange across the light period were determined using a LICOR 6400 infra-red gas analyser where net CO_2 assimilation rate (A, µmol m^{-2} s^{-1}) as well as stomatal conductance to water vapour (g_s, mmol m^{-2} s^{-1}) were recorded and used to compute the ratio of intercellular to atmospheric CO_2 concentration c_i/c_a.

Tissue for RNAseq analysis was collected at the end of week eight with five tissue types collected across both treatments in *E. globulus* (Apical Tip (AT), Fully Expanded Leaf (FL), Primary Stem (S1), Secondary Xylem (XY), Secondary Phloem (PH)) and four in *E. cladocalyx* (AT, FL, S1, Secondary Stem (S2)). Post RNA extraction of only two of the tissue

types in *E. cladocalyx* had sufficeint RNA for library creation. RNAseq libraries where created and sequenced on a GAIIX Illumina sequencer.

Fastq sequence files were post run filtered to retain high-quality reads using Nuclear software (Gydle Inc. http://www.gydle.com/) and were mapped to the E. grandis v1.0 genome annotation v1 (Myburg *et al*., 2014). Differential expression was analysed using R v3.0.1 (http://www.r-project.org) using the edgeR package v3.2.4 (Robinson *et al*., 2010) modelling species, tissue and treatment effects and interactions. Likelihood ratio tests identified genes with statistically significant effects and resulting p-values were adjusted for multiple testing using Benjamini-Hochberg FDR adjustment approach. Information from the plant metabolic network database Plantcyc (http://www.plantcyc.org/) and the pathway/genome database Biocyc (http://websvc.biocyc.org/) as well as the Gydle mapping as part of this study were used to visualise gene pathway networks using Cytoscape (V3.1.1, http://www.cytoscape.org/).

Results and Conclusions

Leaf gas exchange in control (WW) plants was consistent for both species throughout the study compared to severe stress (SS) treated plants, with average net carbon assimilation values higher for *E. globulus* than for *E. cladocalyx*. Both net carbon assimilation, stomatal conductance and sub stomatal cabon concentrations reduced with treatment intensity indicating that water availability was the main limitation for the SS treatment.

Overall 119,335,560 reads were aligned to 23,623 of the 33,916 annotated genes and used in the differential expression analysis. In total 460 genes showed a significant change in expression due to the water deficit treatment (FDR < 0.01) of which 80 showed a significant species specific response. Thirty three *E. cladocalyx* genes showed significantly higher expression in the SS treatment, while 41 had a significantly higher expression in the WW control, and six a significantly higher expression in *E. globulus* in the WW control. No genes showed significantly higher expression in *E. globulus* due to the SS treatment. Twenty of these responsive genes displayed a significant tissue specific response with 80% in both *E. cladocalyx* and *E. globulus* having a stem tissue response to treatment. Key pathways and genes that differentiated ecotypes were linked to photoprotection/redox balance, phytohormone/signalling molecules, primary photosynthesis/cellular metabolism and secondary metabolism pathways.

These results highlight; a more definitive response to water deficit by a ‘dry’ climate eucalypt, the significant role played by stem tissue in this response, and, key pathways and genes that are responsible for the ecotypic differences between ‘wet’ and ‘dry’ climate eucalypts. Identifcation of these pathways and genes provides the opportunity to further investigate the mechanisms and genetic variation linked to this important environmental response as targets for genomics efforts to assist with managing native populations and/or tree breeding/improvement programs under future climate scenarios.

Competing interests

The author declares that they have no competing interests.

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Combining genetic, ecological and extinction risk to assess conservation management

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Key words: Species Distribution Models, Climate Change

In regards to climate change, the conservation of biodiversity constitutes a major task to integrate into ecosystem management. Within this framework, genetic diversity has been highlighted to be a key component of biodiversity as it increases the species´ capacity to adapt to changing environments. Both neutral and adaptive patterns must be considered as they reflect different evolutionary processes. Still, conservation plans often do not integrate this information because of the lack of reliable data, and of a conventional procedure to follow. To get around these limitations, ecological variation within a species range can be a good proxy of genetic differentiation and it has been widely used to define conservation and management units (e.g. Koskela *et al.*, 2013).

Within this context, Species Distribution Models (SDMs) become a very useful tool as they enable to quantify, compare and describe the ecologic niche of different taxa. Furthermore, SDMs also allow predicting whether one species will be able to cope with climate change, even though they do not take into account adaptation or migration processes, both having a major impact on species distribution patterns. Another aspect that limits our capacity to predict precisely the future distribution of a species is the wide range of future climate models available. Until today, there is still no way of determining which of these models is more reliable than other, and given the large differences between them, we may obtain radically different conclusions depending on which model we consider.

In the present work, we provide a robust methodology to determine Conservation Management Units (CMUs) across the whole distribution range of any species. We base

CMUs on a combination of genetic and ecological information and also on risk extinction assessment for each population. We propose a novel approach to quantify the risk that each population is facing in regards of climate change based on the integration of all the different future climate models available.

We provide an example with two Mediterranean pines: the maritime pine (*Pinus pinaster* Ait) and the Aleppo pine (*Pinus halepensis* Mill). First, we genetically define groups across the entire distribution area of these two species. We then further account for adaptive differentiation within genetic groups considering contrasting ecotypes based on temperature and precipitation gradients, two relevant bioclimatic proxies for these pines' distribution. We use SDMs to predict future clade distribution under 42 different future climatic models and associate a risk value to each population, based on the number of models that predict extinction/survival in the future.

Finally, we propose conservation priorities for each of the genetic groups and ecotypes, based on the risk they face in regards to future climate change.

Competing interests

The author declares that they have no competing interests.

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Comparative analysis reveals differential gene family expansions in stress genes in conifer species

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Key words: gene family, LEA, stress genes, dehydrins

Background

Conifers have an outstanding capacity to withstand dry and cold environments (Neale et al. 2014). Late embryogenesis abundant (LEA) are a group of hydrophilic proteins that accumulate during the last stage of seed maturation and water deficit in plant vegetative organs (Battaglia et al., 2009). Recent studies in conifer species suggest some LEA families have diverse patterns of expression profiles suggesting great diversification and functional specialization leading to increased freezing tolerance (Joonsen et al., 2006; Raherison et al., 2012). Differential levels of nucleotide polymorphisms between differentially expressed genes suggest an adaptive role of some Dehydrins, a group of LEA genes, in *P. sylvestris* (Wachowiak et al., 2009).

In this study, we used whole-genome sequences of 15 species to understand how LEA genes have evolved in time. We were interested in knowing whether different patterns of gene family expansions can be found between angiosperms and gymnosperms; and whether the diversified gene expression patterns previously observed in conifers could be related to recent gene families expansions.

Methods

Whole-genome coding sequences of *Picea abies*, *Picea glauca* and *Pinus taeda* were retrieved from public databases (Nystedt *et al*., 2013; Birol *et al*., 2013; Wegrzyn *et al*., 2014). *Pinus pinaster* and *Pinus sylvestris* whole-transcriptomes were assembled using Newbler (v2.8.1). For *Picea sitchensis*, FLcDNAs and PUTs were retrieved from PlantGDB and integrated. In addition, the genomes of nine plant species (*Physcomitrella patens, Selaginella moellendorffii*, *Ostreococcus lucimarinus*, *Amborella trichopoda*, *Arabidopsis thaliana, Populus trichocarpa, Vitis vinifera, Oryza sativa, and Zea mays*) were retrieved from the PLAZA 2.5 database (Van Bel *et al*., 2012). Following open reading frames prediction, UTR removal, and frame correction, the resulting gene sequences were used for posterior analyses. We clustered orthologous protein sequences between the 15 plant genomes by using an all-against-all BLASTP followed by a Markov Cluster algorithm with the program Tribe-MCL (Enright *et al*., 2002). MUSCLE (Edgar et al. 2004) was used to generate multiple sequence alignments of orthologous proteins. Phylogenetic trees were generated by PhyML (Guindon & Gascuel 2003). Genes and gene families showing Pfam domains related to the LEA super family were selected for further analysis.

Results and Conclusions

After orthologous protein clustering, we obtained 608k proteins sequences clustered in 82k gene families in 15 species. From these, we selected 1719 genes from 101 Tribe MCl families contained in 9 functional LEA families (one functional family may contain more than one TribeMCl families) across all species according to LEA's classification in Battaglia *et al*., (2008). Our results indicated significant differences in gene family evolution of LEA genes in angiosperms than in gymnosperms. Recent clade or lineage-specific expansions were present in gymnosperms species in all LEA families with the exception of LEA3 (PF0298.11) and LEA 5C (PF03168.8). In addition, some LEA families such as LEA2 (Dehydrins), LEA7 (ABA/WDS induced protein), and LEA 5B (PF03242.8) were significantly larger in gymnosperm than in angiosperms suggesting recent gene family expansions. Other LEA families such as LEA5C were largely expanded in both angiosperms and gymnosperms, showing a large number of genes in *Zea mays*, *Oryza sativa* and *Pinus taeda*. Our studies support recent studies in conifers suggesting LEA genes have a high degree of gene expression diversification (Raherison et al., 2012).

By using large-scale comparative genomics analyses, our study suggests conifers have more diversified LEA gene families than angiosperms, which may be in relation to a greater capacity to withstand cold and dry environments.

Competing interests

The author declares that they have no competing interests.

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Application of GBS and RADseq for discovering and genotyping of SNP polymorphisms in *Fagus sylvatica* and *Quercus robur*

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Key words: genotyping by sequencing, restriction site associated sequencing, SNP, Fagaceae

Background

Restriction site associated DNA-sequencing (RADseq; Baird et al. 2008) and genotyping-by-sequencing (GBS; Elshire et al. 2011) are reduced genome representation sequencing methods, which are increasingly used in population genomics. They are attractive for species without known reference genomes, and are expected to produce large amounts of genomic data in a cost effective way. The methods were found to be useful in discovery and genotyping of SNP polymorphisms, particularly in species or populations exhibiting low to moderate levels of genome-wide polymorphism and complexity. However, most forest trees are known for their high genetic diversity and heterozygosity, genome complexity, which potentially may complicate the usefulness of these methods in population genomics of forest tree species.

We have applied RADseq and GBS methods to study genetic diversity of English oak (*Quercus robur* L.) and Common beech (*Fagus sylvatica* L.), which are important broad-leaved trees species in Europe. At the time of the study both species had no reference genomes established. Our main interest was to investigate the utility of GBS and RADseq methods for SNP discovery and SNP calling.

Methods

Individuals chosen for this study were selected from various, widely distributed populations. For each species 91 individuals were selected, with 4 of them replicated (95 samples in total). The same sets of samples (isolated DNA), were send out to two leading genomic facilities (Floragenex and Cornell University) which prepared the libraries and sequenced the samples. RADseq libraries were prepared based on the *PstI* restriction site for beech and oak, however GBS libraries employed *ApeKI* and *EcoT22I* restriction sites for beech and oak, respectively. Received raw data was processed by STACKS (Catchen et al. 2013) bioinformatical pipeline for SNP detection.

Results and conclusions

Neither of the two methods performed perfectly. Differences between them were revealed at several steps of analyses. GBS method gave two-times more the raw data than RADseq, after the quality filtering 76% in *F.sylvatica* and 92% in *Q.robur* of reads remained for further processing. On the other hand RADseq method gave less data but better quality reads, for both species 96% of reads passed the quality filtering step. Initial parameter optimization for STACKS pipeline was done based on replicated 4 individuals within each species. We were interested to find STACKS parameters which minimize locus and allele errors, and proportion of missing loci, while still keeping the number of reads (loci) as large as possible. In general, the optimization step has shown large differences between the two platforms and the studied species. Mean number of samples per polymorphic loci depended on STACKS parameters and varied for beech from 3.99 – 4.95 for RADseq and from 3.35 –

4.35 for GBS data. On the other hand for *Quercus robur*, GBS method provided better results in terms of the mean number of samples per polymorphic loci (4.58 – 6.40) than RADseq method (3.64 – 4.94). The change in STACKS parameters had pronounced effects on error levels, proportion of missing loci and the overall number of reads (loci) detected.

Results based on 95 samples per species were comparable. In general, *F. sylvatica* exhibited greater number of loci than *Q. robur* for RADseq data, which might reflect differences in sampling scenarios between the two species. However, GBS method provided much larger numbers of reads for *Q. robur* than for *F. sylvatica,* probably due to different restrictions sites employed. Nevertheless, analyses revealed serious problems with the extent of missing data and inconsistency of genotyping between replicates. In our opinion, broader application of RADseq or GBS methods in highly diverse and heterozygous species such as forest trees might be limited, unless specific genomic library protocols will be specifically optimized and/or software pipelines will be designed to account for errors and missing data. Recently available software pipelines (PyRAD, Eaton 2014; AftrRAD, Sovic et al. 2015), which account for indels and apply rigorous quality filtering hold promise but still need to be tested for forest trees.

Competing interests

The authors declare that they have no competing interests.

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The Genosuber project: initial sequencing and annotation of the cork oak (*Quercus suber L.*) genome

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Background

Cork oak is the main forest species in Portugal, playing a significant social, economic and ecological role. Cork is the main product obtained, for which Portugal accounts for around one third of the total worldwide production. Despite its clear importance, cork oaks are presently threatened by several (a)biotic stresses. Moreover, advances are also needed for traits of great economic importance for the producers and the industry. In order to achieve these goals and to advance the scientific knowledge on cork oak, the availability of a fully sequenced and annotated genome is essential.

Methods

The project is employing sequence data generated in different platforms, including Illumina, 454 and Pacific Biosciences, to generate a *de novo* assembly of the cork oak genome. Transcriptomic data, collected by sequencing a large variety of cork oak tissues derived from several biological conditions, will be used to annotate the genome. In order to organize the genome into chromosomes an F1 cork oak population, comprising 320 individuals and produced by a controlled pollination procedure, will be used. Currently, genotyping-by-sequencing is being applied to generate the marker data needed to build the first ever cork oak linkage map.

Additionally, the mechanisms involved in the formation of cork are also being investigated, in order to elucidate the regulation, at the genetic and physiological levels, of the cork formation processes. For this purpose, phellogen, a tissue deeply involved in the formation of cork, and the associated phloem and xylem were selected, as well as the leaf, which will be considered for comparison, as it is not directly involved in cork formation. All tissues will be subjected to a comprehensive characterization, which will include determination of the transcriptome, methylome, epitranscriptome and non-coding transcriptome, as well as to proteomic and metabolomic profiling. This approach will enable a systems biology approach to identify the main genes and pathways that regulate cork formation.

Results

A total of 15 paired-end and mate-pair libraries were sequenced in the Illumina platform, including 3 libraries each for the 170 bp, 500 bp and 800 bp paired-end libraries, and 6 and 3 libraries for the 2 kb and 5 kb mate-pair libraries, respectively. The read lengths used were 100 and 50 bp, for the paired-end and mate-pair libraries, respectively. In total, approximately 4,451 million reads were available to be used in the *de novo* assembly process. The genome coverage per library type varied from 31x (mate-pair 5 kb) to 123x (paired-end 170 bp), for a total estimated genome coverage of 436x, assuming a cork oak genome size of 800 Mb.

Pre-processing of the Illumina reads was performed, in order to remove from the dataset reads that displayed average low quality scores and/or other common problems, like the presence of undetermined nucleotides or adapter sequences. We used as thresholds to filter the reads a minimum average quality score of 20, and minimum read lengths of 80 and 40 bp for the paired-end and mate-pair libraries, respectively, a strategy that resulted in keeping for analysis 84% of the initial number of reads.

Several software tools that perform de novo assembly of genomes were evaluated, in order to identify the one with the best performance for this cork oak dataset. The best results were obtained using the Platanus assembler (Kajitani et al., 2014) with sequence data that was error-corrected using SGA (Simpson and Durbin, 2012). The number of scaffolds longer than 1kb, 10kb and 100kb was 17,601, 9,978 and 1,890, respectively. The percentage of Ns detected in these scaffolds was 15%. The maximum scaffold size was 690.4 kb and the size of the cork oak genome was estimated at 820 Mb.

These results represent a promising start for the effort of sequencing the cork oak genome. Even though the maximum insert size for all libraries used was only 5 kb, we were able to assemble a significant number of scaffolds longer than 100 kb. Since the results are at this stage still preliminary, the total number of scaffolds is large. However, the project is currently generating a considerable number of additional sequencing data and other resources expected to improve the assembly metrics of the cork oak genome.

Conclusions

The results and information generated in the project will be made available to the scientific community, industry, producers and other partners. This knowledge will allow new studies and applications in cork oak research, including the implementation of breeding programs, the mapping of QTL and development of genetic markers.

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Insight into conifer karyotype evolution from high resolution comparative mapping between Pinaceae (n=12) and Cupressaceae(n=11)

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Key words: conifer, evolution, genome structure, orthology.

Background

Conifers are dominant species in different ecosystems worldwide with a significant ecological and economic importance. The huge genome size of conifers, formed by a large proportion of repetitive elements and pseudogenes, hinders the analysis of genome structure and evolution, especially at the physical scale. On the other hand, at the genetic scale, conifers have shown highly conserved chromosomal structures between them despite the ancient divergence between taxa, at least within the Pinaceae. The aim of this work is to benefit from the high degree of sequence conservation between conifer species to develop high density comparative genetic mapping to shed new lights on genome structure evolution between representatives of two important Families: Pinaceae (with n=12) and Cupressaceae (with n=11).

Methods

A total of 18 genetic maps from 6 different species were used in this study to analyse the synteny and collinearity between three conifer genus. Firstly, a composite linkage map for *P. pinaster* was constructed using previously published maps [1–4]. This new composite map for *P. pinaster* was utilized, together with already published maps for *P. taeda* [5], *Picea abies* [6], *Picea glauca* and *Picea mariana* [7], in the construction of a composite linkage map for the *Pinaceae* family. Finally, this new composite map for the *Pinaceae* family was compared with a previously published genetic map of *Cryptomeria japonica* [8], as representative of the *Cupressaceae* family. This approach of merging different linkage maps allowed to maximize the number of mapped markers for comparative mapping. *P. pinaster* unigene sequences [9] were used as reference for comparative mapping. *LPmerge* software [10] was used to create all composite maps.

Results and Conclusions

The *Pinaceae* composite map was produced from the aggregation of 4 base maps (one per species) merged into 2 component maps (one per genus). The total length of the this map was 2,138.5 cM with a total of 6,912 mapped markers (5,971 identified in the *P.pinaster* unigene [9]) in 12 chromosomes, 514 of them being shared between *Pinus* sp. and *Picea* sp. Overall, the synteny between the two genus was remarkable (94%). When this unified *Pinaceae* linkage map was compared to that of one representative of the Cupressaceae, *C. japonica* (n=11), using a set of 215 orthologous markers, the most striking result was that each *Pinaceae* chromosomes corresponded to two different pieces of *Cupressacea* chromosomes and vice versa, suggesting differential suffling of at least 22 ancestral chromosomal blocks that have shapped the 12 and 11 modern chromosomes of the studied Pinaceae and Cupressaceae species. A model of karyotype evolution is under construction and will contribute to the understandig of genome structure and evolution between distant taxa.

Competing interests

The author declares that they have no competing interests.

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RuBisCo Evolution in Eucalypts

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Key words: RbcL, Eucalyptus, Natural Selection

Background: RuBisCo, the key enzyme in fixation of carbon dioxide in photosynthetic organisms, is particularly interesting from an evolutionary point of view as both nuclear and organellar genomes co-operate to encode the holoenzyme. Because of its crucial role in Calvin Cycle of photosynthesis, the protein has been hypothesized to undergo fine-tuning of its structure to adapt its activity to different climates through selection. Accordingly, *rbc*L, the large cp-DNA encoded subunit, has been the focus of exploring adaptive evolution in plant taxa. *Eucalyptus* is the dominant tree species within Australia, readily hybridising between closely related species and occurring across a large range of contrasting climate, making it an excellent model to explore naturally-occurring genetic variation that is under selection. This study therefore aims to find regions of *rbc*L under natural selection in eucalypts through a combination of *in silico* and function studies at both inter- and intra-species levels.

Methods: For the *in silico* studies, *Rbc*L sequences of 39 species from five genera of the Myrtle family (mainly *Eucalyptus*) were sourced from whole-genome Illumina reads and aligned using Nuclear (GYDLE Inc.), as used in Bayly et al. [1]. To measure the rate of evolution, the Phylogenetic Analysis by Maximum Likelihood package (PAML) [2] was applied to undertake a number of likelihood ratio tests (LRTs) which was followed by Bayes Empirical Bayes (BEB) calculations [3] to find sites under positive selection. Based on the *in silico* analysis, the *rbc*L gene in populations of *Eucalyptus globulus* sub. *globulus* was further sequenced to determine whether promising sites identified as being under natural selection are also segregating intra-specifically. This information was then used to conduct a glasshouse study involving a heat treatment and measurement of *in vivo* temperature response in this species to gain insights into any functional links to the *rbc*L selected sites. Photosynthetic responses of the seedlings with selected and non-selected amino acid residues were measured at between 20 – 40 °C.

Results: *Rbc*L was identified as a strongly positively-selected gene in LRT of all site-models. BEB analysis located four sites -namely T142P, I251M, M309I, and S328A with high probability (>%99) of being positively selected, all having *d*N/*d*S ratios of over 9. LRT in the Branch-site model also showed that the *Eucalyptus* branch has different omega ratios compared to its sister lineage, *corymbia+Angophora*. At intra-specific level, interestingly, two of these substitutions were also shown to be segregating among trees of Tasmanian blue gum, and seedlings with selected amino acid residues (P & M) are shown to have higher photosynthetic response in comparison to those with non-selected residues (T & I) either across all temperatures or specifically at highest temperature. Linear mixed model analysis showed that the selected haplotype (PM) has significantly higher rate of net assimilation rate at 40 °C compared to other haplotypes (PI or TM).

Conclusions: *Rbc*L is under natural selection in eucalypts and the branch leading to *Eucalyptus* but not *Corymbia+Angophora* lineage is characterised by an accelerated

substitution rate. The four missense mutations were also reported in both C_3 [4-6] and C_4 plants [7, 8] and were shown to have important functional consequences on RuBisCo protein. In this study too, it is uniquely shown seedlings with selected *rbc*L haplotype have desired rates of photosynthesis. This is presumably because Proline being a unique and rigid amino acid can accept only few conformations and lower conformational entropy of the unfolded state of the protein, and Methionine being one of the two sulfur-containing amino acids can facilitate forming strong bonds called 'sulfur-aromatic motif', a more vigorous interaction than 'salt bridges' [9]. Indeed, the contribution of the two selected residue would enhance the protein thermostability at higher temperature. Likewise, it is hypothesised in this study that periodical high temperature in the past, particularly at the time of seed germination or seedling establishment, would have acted as selective pressure for trees of supposedly temperate forests (*Eucalyptus* lineage). Such adaptive mechanisms for *Eucalyptus* species to withstand heat stress may also exist for other tree species, hence making RuBisCo a pivotal enzyme for selecting trees in (possible future) climates of prolonged heat wave and/or drought.

Competing interests

The authors declare that they have no competing interests.

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Conservative microRNA studies and analyses in Scots pine (*Pinus sylvestris* L).

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Key words (max 4): microRNA, Scots pine, IonTorrentPGM

Background

MicroRNAs (miRNAs) are non- protein coding RNAs ~20-24 bp in length that play an important role in many biological and metabolic processes, including regulation of gene expression, influencing plant growth and developmental processes, as well as responses to stress and pathogens (Bartel, 2004). miRNA studies in plants have been mainly concentrated in angiosperms, with few reports in conifers. There is no published data about Scots pine microRNAs.

Scots Pine is a long-lived organism, with a long generation time, which requires the tree to adapt to a wide range of growing and environmental conditions (Bräutigam et al., 2013). Scots pine covers ~40% of Latvia's territory, and is an economically important natural resource due to its productivity and high wood quality. Stress conditions (e.g. pathogen infection), influence genome stability, induce genomic rearrangements, and influence the recombination rate (Rohde & Juntilla, 2008), which result in new genotypes which can be selected for.

Methyl jasmonate (MJ) is plant hormone synthesized from linolenc acid and plays a role in plant responses to abiotic and biotic stresses, including wound response and plant defence (Westernack, 2007).

The aims of this study are to identify and characterise microRNAs in Scots pine, to elucidate the role of miRNAs in defence responses, and to detect miRNA expression levels under stress conditions and to clarify potential target genes.

Methods

To investigate Scots pine miRNAs, we utilised the IonTorrent PGM platform in collaboration with the Norwegian Forestry and Landscape Institute. Six Scots pine ramets from 1 clone were used in the experiments. 3 ramets were treated with 10 mM methyl jasmonate and 3 untreated ramets were used as controls. Ramets were grown in growth chambers, at 17-22^{0}C under short day conditions (16 h light + 8 h dark). After 2 weeks growth, needles were collected and total RNA isolated from 100 mg of needles, using liquid nitrogen and phenol:chloroform:isoamyl alcohol extraction. Total RNA and miRNA quality, quality and integrity number (for total RNA) was determined using the Agilent Technologies 2100 Bioanalyzer**.** Total RNA samples were enriched for small RNA and 6 small RNA non-barcoded libraries were prepared. miRNA and cDNA library quality and quantity were analysed with the Agilent Technologies 2100 Bioanalyzer. Then template-positive Ion Sphere™ Particles (containing clonally amplified DNA were prepared with the Ion OneTouch™ 2 Instrument. Ion Sphere particle quality and quantity were assessed using the Qubit® 2.0 Fluorometer and Ion Sphere™ Quality Control Kit, and then enriched and sequenced using the IonTorrent PGM.

Using the IonTorrent PGM, we identified potential mature miRNAs in Scots pine, which need to be further characterised and validated. We are currently analysing these data using the CLC Genomics Workbench software. We identified conserved miRNAs using previously reported miRNA sequences from various tree species as well as other plant species from the miRNA Registry Database (Release 20, http://www.mirbase.org). The potential precursor miRNAs were identified using NCBI Blast EST, Congenie and Compbio databases. Predicted miRNA hairpin stem loom structures were calculated using the Mfold web server.

Results and Conclusions

Sequencing of the six small RNA libraries yielded approximately 4.5 million reads with an average length of 21 nt.A total of approximately 1 million potential small RNA sequences were identified from all the small RNA libraries, of which 4975 were annotated and 957 were ambiguously annotated.

For miRNA annotation analysis we utilised sequences from 11 species in miRBase, of which 6 are woody species, including 3 conifers. Comparing our data with miRBase *Pinus taeda* sequences, we found that 91.7% of annotated *Pinus taeda* miRNA sequences were also present in the Scots pine sequences (Table 1).

Table 1. Comparison with miRNA databases

Resource	Sequences in resource	Sequences found	Percentage found
miRBase (*Acacia auriculiformis*)	7	4	57.1%
miRBase (*Arabidopsis thaliana*)	298	90	30.2%
miRBase *(Oryza sativa*)	592	82	13.9%
miRBase (*Picea abies*)	40	30	75.0%
miRBase (*Pinus taeda*)	36	33	91.7%
miRBase (*Pinus densata*)	30	21	70.0%
miRBase (*Populus euphratica*)	4	1	25.0%
miRBase (*Populus trichocarpa*)	352	73	20.7%
miRBase (*Nicotiana tabacum*)	162	30	18.5%
miRBase (*Vitis vinifera*)	163	27	16.6%
miRBase (*Zea mays*)	172	37	21.5%

Of the 4975 annotated sequences identified, 33.7% were identified from *Picea abies*, 29.3% from *Pinus taeda* and 11.8% from *Pinus densata* (Table 2).

Table 2. Small RNAs

Annotation	Count	Percentage
Annotated	4,975	0.5%
with miRBase (*Acacia auriculiformis*)	41	0.8%
with miRBase (*Arabidopsis thaliana*)	457	9.2%
with miRBase (*Oryza sativa*)	307	6.2%
with miRBase (*Picea abies*)	1,676	33.7%
with miRBase (*Pinus taeda*)	1,459	29.3%
with miRBase (*Pinus densata*)	586	11.8%
with miRBase (*Populus euphratica*)	2	0.0%
with miRBase (*Populus trichocarpa*)	162	3.3%
with miRBase (*Nicotiana tabacum*)	82	1.6%
with miRBase (*Vitis vinifera*)	132	2.7%
with miRBase (*Zea mays*)	71	1.4%
Unannotated	1,016,721	99.5%
Total	1,021,696	100.0%

Potential precursor miRNA sequences were identified by sequence alignment of the obtained mature miRNA sequences with the previously mentioned databases. These potential precursor miRNA sequences were analysed for the potential to form the required stem-loop structure, and the minimal folding free energy indexes were calculated.
In addition to the annotation and identification of novel and conserved Scots pine miRNAs, patterns of differential expression before and after methyl jasmonate treatment will also be analysed. In addition to the miRNA sequence data, we also have Ion Torrent PGM gene expression data which were obtained from the same samples in parallel. This data is yet to be analysed and will be able to be compared with the miRNA results.

Competing interests

The authors declare no competing interests.

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Development of chromosome- and organelle-specific SNP markers for different *Populus* genotypes

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Key words: Chloroplast; mitochondrion; protoplast fusion; somatic hybridization; hybrid; cybrid

Background

In the genus *Populus*, several genome-wide duplications have occurred during evolution with a most recent one about 8 to 13 Million years ago [1]. The recent duplication affected nearly 92% of the Populus genome including about 8000 pairs of genes and intergenic regions mostly duplicated as blocks and forming chimeric chromosomes. Hence, in general, the development of SNP-markers in *Populus*, especially of chromosome-specific SNP-markers, is hindered by the possible presence of paralogous genes and intergenic regions of similar evolutionary age and, therefore, similar sequence. Besides the nuclear genome, also chloroplast and mitochondrial DNA contribute to the genomic constitution of a plant cell. The application of molecular SNP-markers developed for the different genomes of the poplar cell is manifold. Thus, they can be used to differentiate between the 19 chromosomes in respect to different *Populus* species, barcode the species of the genus *Populus*, determine the direction of the cross, and reclassify putative pure lines as hybrid lines.

Methods

The genomic sequences (nuclear and chloroplast) and scaffolds of the mitochondrial genome of *P. trichocarpa* were used to select genes from each chromosome, or inter-genic spacer in the chloroplast and mitochondrion to search for nucleotide polymorphisms differentiating the *Populus* species. Homologous sequences from other *Populus* species available in public databases were compared with the *P. trichocarpa* sequences *in silico*. Putative SNPs were first validated in three genotypes of each *Populus* species by Sanger sequencing of PCR amplicons. Second, as many genotypes as possible from the same *Populus* species representing the natural distribution area were included to validate putative species-specific SNPs [2]. A species-specific consensus sequence for each gene/gene fragment/intergenic region was designed. A database containing all species- and chromosome-specific sequences is being developed.

Results and Conclusions

In frame of the project “ZUEND” funded by the Federal Ministry of Food, Agriculture and Consumer Protection (BMELV) on behalf of the Agency for Renewable Resources (FNR), chromosome- and organelle-specific SNP-markers have been developed in genotypes belonging to different *Populus* species. These SNP markers have successfully been used for the analysis of somatic fusion lines and sexual hybrids.
Somatic hybridization by e.g. protoplast fusion approaches allows combination of chromosomes and organelles from rarely or not cross-compatible *Populus* species. Beside tetraploid fusion hybrids, the occurrence of fusion hybrids identified as aneuploid and ones with aberrant chromosomes are well known. Chromosome-specific SNP-markers can be applied in aneuploid somatic hybrids to determine the origin of each chromosome from the respective fusion partner but also chromosome breakages. To further deepen our understanding on the somatic combination of the cytoplasm of two different *Populus* species

during somatic hybridization, organelle-specific SNP-markers could be applied to analyze the destiny of chloroplasts and mitochondria during the regeneration process [3, 4].
In one fusion line, we were able to identify a chromosome break in chromosome 15, while the second was still intact. Fine mapping of this chromosome revealed that the position of the chromosome break was between Potri.015G095500 and Potri.015G106900 (terminal end of chromosome 15). Further, we were able to identify a somatic hybridization between two “parental” lines: the somatic fusion line revealed to be heterozygous in all five investigated gene fragments belonging to five different chromosomes.
In future, the development of chromosome- and organelle-specific SNP-markers could accelerate poplar breeding, *i.e.* interspecies- and intersectional hybrids could be identified very easily in respect to their hybridization genealogy, and possibly, also crossing-over events can be detected physically.

Competing interests
The authors declare that they have no competing interests.

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TOZ19 is a Y haplotype-specific gene in aspen

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Key words:
Populus tremula, Populus tremuloides, sex, molecular marker

Background

The genus *Populus* features a genetically controlled sex determination system, located on chromosome 19. However, different *Populus* species vary in the position of the sex-linked region on the respective chromosome, and the precise mechanism of sex determination in *Populus* is still unknown. Depending on the *Populus* species, XY- or ZW-systems were reported [1], although evidence for male heterogamety is on the increase [2].
In aspen, the sex-determining region was shown to be located on a central position of linkage group XIX with male heterogamety (XY-system) [3]. In the male *P. tremuloides* clone Turesson 141, the recombination-suppressed sex-linked region corresponds to a pericentromeric region of about 2 million bp on *P. trichocarpa* chromosome 19 [4].
In this study, we aim at the identification and further investigation of nucleotide sequence differences between the X- and the Y-haplotype in the sex-linked region of aspen.

Methods

Four different DNA pools consisting of 12 female *P. tremula* individuals, 12 male *P. tremula* individuals, four female *P. tremuloides* individuals and six male *P. tremuloides* individuals, respectively, were assembled and sequenced using Next Generation Sequencing (NGS; Illumina HiSeq 2000; GATC, Konstanz, Germany), achieving a coverage of 24X–38X [5].
The genomic sequence flanked by the markers BDP13 and SRZ21-1 which so far define the borders of the sex-linked region in Turesson 141 [4] was extracted from the *P. trichocarpa* genome sequence of chromosome 19 (version 3.0; Phytozome) and used as reference for subsequent mappings of the trimmed Solexa reads of each pool using CLC Genomics Workbench (version 6.0; CLC bio, a QIAGEN Company, Aarhus, Denmark). The mappings were screened for sex-specific sequence differences using CLC Genomics Workbench [5].
A TOZ19-specific primer pair was used for PCR-testing of TOZ19 in the genomic DNA of an extended set of aspen individuals [5]. The RNA from 13 samples of flower and vegetative buds from male and female *P. tremula* and *P. tremuloides* was extracted for RT-PCR experiments. Specific TOZ19 primers, located in exon sequences, were designed and used for RT-PCR [5].
Moreover, TOZ19 specific primers were used for generation of a DIG-labeled probe that was then applied to screen an arrayed BAC-library of Turesson 141 constructed by Fladung *et al.* [6]. Four BAC-clones including parts of the TOZ19 gene were selected for NGS sequencing (Ion Torrent™ PGM 2, Life Technologies) achieving a coverage of 297X.

Results and Conclusions

The aspen homologue of the *P. trichocarpa* gene Potri.019G047300 (“TOZ19”) was identified to be Y haplotype-specific based on NGS of pooled DNA samples of male and female aspen and subsequent bioinformatic analysis of the NGS data [5]. While the

complete gene is missing in the genome of *P. tremuloides* female plants, a short fragment of the 3´-part of the gene is still present in *P. tremula* females. The male-specific presence of TOZ19 was verified by PCR in 98 unrelated aspen individuals originating from different locations from the natural distribution range of both aspen species [5].
In RT-PCR experiments, a TOZ19-specific fragment was amplified only in cDNA of male individuals, in both vegetative and flower bud samples, indicating that TOZ19 is transcribed in male, but not in female aspens [5].
TOZ19 is potentially involved in early steps of flower development, and represents an interesting candidate gene for an involvement in sex determination in aspen. Based on the male-specific presence of TOZ19, a simple molecular marker (PCR marker) for the determination of the sex of non-flowering aspen individuals or even seedlings was developed [5].
To further investigate the Y haplotype-specific genomic region including TOZ19, we recently sequenced BAC clones including (parts of) TOZ19, which were isolated from a BAC library of Turesson 141 [6]. The bioinformatic analysis of the related NGS data is under way.

Competing interests
The authors declare that they have no competing interests.

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Identification of genetic determinants of the rooting capacity and tolerance to *Mycosphaerella* sp. in *Eucalyptus globulus*

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Key words: Eucalyptus globulus, Mycosphaerella tolerance, rooting capacity, QTLs

Background

Eucalyptus globulus, a fast-growing short rotation tree, is the dominant raw material for the manufacturing of paper pulp and more recently, for biomass and bioenergy production. Two factors are key for *E. globulus* breeding in Spain, the selection of elite clones that show high rooting capacity and that are tolerant to *Mycosphaerella* leaf disease. M*ycosphaerella* sp. causes defoliation, reduces growth of *E. globulus* plantations, and can finally result in dieback and tree death. For this reason, it is of great importance to design breeding programs for resistance to reduce the impact of *Mycosphaerella* leaf disease. Given the long generation times and the labor intensive evaluation of these traits, the availability of molecular markers linked to its major genetic determinants may allow more efficient breeding, accelerating the transfer of traits of interest to commercial clones. Early identification of individuals carrying the most interesting allele combinations allows breeders to grow larger effective populations, which results in decreasing maintenance and evaluation costs. In this study, we have focused on the identification of genetic determinants of rooting capacity and tolerance to *Mycosphaerella* sp. in *E. globulus* using a strategy combining transcriptomics, genetic mapping, phenotypic characterization of targeted traits and QTL detection.

Methods

This study has been carried out using the *E. globulus* intraspecific cross CI-875. This full-sib progeny consisted of 168 individuals obtained by controlled crossing two trees with contrasted phenotypic responses for the targeted traits: Plus819, a tree representing low rooting ability and susceptible to the disease caused by *Mycosphaeralla* spp. used as female progenitor, and Cz-11-281, an elite tree with high rooting ability and tolerant to the disease used as male progenitor.

The complete mapping progeny was phenotyped for both characters during 2 years. Rooting ability (percentage of rooting ability per clone) was assessed by 2,028 rooting trials involving 7,800 cuttings. Individual sensitiveness to *Mycosphaerella* leaf disease was assessed in four one-tree-plot trials (randomised blocks with 6 to 7 replicates in each trial) in three different dates for each trial.

Tree molecular marker technologies were used to genotype all mapping progeny and both progenitors:

- Forty five microsatellites, selected based on their segregation patterns, genomic location, possibility of implementation in multiplex PCR protocols and unambiguous interpretation. They were genotyped in all the progeny using 10 multiplex PCR (Velez-Tebar & Cabezas, unpublished results).

- DArT profiling [1] was performed by Diversity Arrays Technology (DArT P/L, PO Box 7141, Yarralumba, ACT 2600, Australia) in order to rapidly saturate the linkage maps using the high resolution *Eucalyptus* array, which includes 7,680 probes designed for different *Eucalyptus* spp. [2].

- SNP genotyping was carried out using a VeraCode GoldenGate assay for 1,536 SNPs (Illumina Inc., San Diego, CA, USA) designed for genotyping this mapping progeny. The SNPlex targeted 1,134 unigenes, including 16 candidate genes for rooting ability and disease resistance (Díaz-Sala et al., unpublished results). SNPs were detected comparing three cDNA libraries made of RNAs pooled from multiple tissues: one for each progenitor and one from a bulk of 10 progeny individuals. To avoid the non-detection of polymorphisms in sequences with low levels of expression in any of the libraries, SNP calling was performed using alignments developed independently on each of the libraries. This strategy also allowed to infer the segregation type for the markers, which was used in the design of the SNP array.

QTL analyses were carried out on independently on the three genetic maps using multiple QTL model analysis of MapQTL 5®. LOD thresholds were estimated at 99 and 95% confidence for all the genome and for each linkage group using the implemented permutation test with 10,000 iterations.

Results and Conclusions

Genotyping and map construction

Genetic maps of each progenitor and a consensus map for the cross were constructed using the two way pseudo test-cross strategy [3] to study the linkage relationships among a total of 1,919 molecular markers (45 microsatellites, 978 DArT, 896 SNP). The use of DArT and SNP genotyping technologies allowed saturating the linkage maps whereas the inclusion of microsatellite loci facilitated their comparison with previously developed maps. The consensus genetic map covered 1,617.1 cM, including 1,814 markers with an average distance of 0.9 cM. The parental maps covered 1,820 cM for Plus819 and 1,345.1 cM for Cz-11-281, with 1,189 and 1,117 markers included and average inter-marker distances of 1.5 cM and 1.2 cM, respectively. The 7.7 % of the molecular markers included in the consensus map for the cross showed distorted segregation of alleles at $p<0.01$. Most of these markers clustered on a large region covering almost 80% of the LG8 of the male progenitor (Cz-11-281).

Phenotypic evaluation

Percentage of rooting ability by clone followed a normal distribution ranging between 8% and 68%. For disease severity, clonal effect was much more significant than block effect in every trial. Clonal correlation between trials was high (r^2 =0.71, $p<0.0001$).

QTL detection

For rooting ability there were detected six QTLs: three on the female progenitor, explaining up to 21% of total phenotypic variance; and three on the male progenitor, explaining up to 46% of total phenotypic variance. For *Mycosphaerella* disease susceptibility were identified four QTLs, explaining up to 72 % of total phenotypic variance: one on the female and three on the male progenitor. Major effect QTLs for both traits were identified on the male progenitor (37.4 % for root ability and 64.2% for *Mycosphaerella* susceptibility).

Competing interests

The author declares that they have no competing interests.

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Expanding the genomes of the Myrthaceae: Progress towards the guava (*Psidium* guajava) genome

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Key words (max 4): Psidium guajava, genomes, myrthaceae,

Background
The availability of the *Eucalyptus grandis* (Myburg *et al.*, 2014) genome sequence is providing great opportunities for comparative genomic studies within the Mythaceae family. One such family member, Psidium *guajava* (guava) is a prolific subtropical crop that bears high quality and nutritious fruits. This hardy crop is grown in more than 58 countries around the world. Guava is widely used in sustainable farming communities and in rural development programs, and is also one of the crops that are adapted to saline soils. The fruit plays an important role in the diversification of diets to combat nutrient deficiencies, and food security especially in rural communities. Guava wilt disease (caused by *Nalanthamala psidii*) poses a serious threat to guava production in South African guava, and has resulted in extensive losses in production over past years. The advent of high throughput sequencing and genotyping technologies, together with molecular breeding techniques has the potential to greatly improve selection methods for quantitative traits, like guava wilt disease resistance.

Methods
Using the ultra-high troughput Illumina HiSeq 2500 sequencer, we generated an *Allpaths* DNA sequencing library consisting of over 40Gb of data. The *Allipaths* {Butler:ud} library were used to perform an assembly of the the draft genome of *Psidium guajava.* In addition to the *Allpaths* library, we generated long-mate pair (3.5kb) reads to complement the previous dataset and improve the assembly, as well another 10Gb of paired-end (300bp insert size) sequence data.

Results and Conclusions
This work presents the first draft of the *Psidium guajava* genome. In addition to the *de novo* assembly of the genome using a wide array of assemblers (*Allpaths*, SOAP-*de novo*, *VELVET*) and *ab initio* gene prediction software, we made extensive use of the other Mythacea genome resources, like *Eucalayptus* to perform an initial comparative analysis of genome features within the Mythaceae family.

Competing interests
The authors declare that they have no competing interests.

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Effects of primed conifer defences on a tree-killing bark beetle-fungus complex

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Key words: Conifer, Resistance, Ips typographus,

Background

Tree-killing bark beetles are among most destructive pests in conifer forests worldwide, and have huge impacts on forestry, recreation, ecosystem dynamics, carbon cycling and climate change during their episodic eruptive outbreaks. The spruce bark beetle *Ips typographus* is the most destructive pest of mature Norway spruce in Europe. During outbreaks, the beetle and its associated phytopathogenic fungi are able to surpass a critical threshold density and mass-attack and kill living trees [1,2,3,4]. Aggressive bark beetles such as the spruce bark beetle can kill healthy trees during outbreaks because they infect the trees with phytopathogenic bluestain fungi that overwhelm the tree's defensive capacity during coordinated mass-attacks [4].

Ceratocystis polonica, the spruce bark beetle's main fungal associate in Norway, is capable of killing trees in experimental inoculations that mimic mass-attacks [4]. Mass-attacks are coordinated by aggregation pheromones derived from precursors in the tree and/or produced *de novo* by the beetles from metabolic precursors. Long-lived trees depend on effective constitutive and inducible defences to protect themselves against insects and microorganisms. Tree resistance is an important regulator of bark beetle population densities. Resistance controls the initial threshold processes in the interaction between aggressive bark beetles and their host trees, including tree entry, aggregation of local beetle populations and establishment on individual trees [1,3].

Recent research by our group has suggested that priming of tree defences by application of methyl jasmonate (MJ) directly interferes with beetle pheromone production and limits host colonization. This in itself is interesting because it creates opportunities to control bark beetle aggregation through manipulation of host tree resistance. We investigate the tripartite nature of the tree colonization process at the biochemical and transcriptional level, particularly when conifer defences are primed. We use next generation sequencing of the beetle, the fungus and the host tree transcriptome, together with quantification of tree secondary metabolites and beetle pheromones, to generate a molecular ecological portrait of the pheromone interference by the tree, and to probe for the concurrent role of the fungus in the mutualistic co-colonization process by a tree-killing bark beetle-fungus complex.

The results of the priming effect, after MJ treatment, in terms of transcriptome changes in Norway spruce after wounding and inoculation with the pathogen *C. polonica* will be presented.

Methods

Plant material: Mature Norway spruce clones were used, and at least two ramets (trees) of each clone to allow for comparison within the same genotype and between genotypes. MJ primed and unprimed trees were sampled for each clone. Wounded and *C. polonica* inoculated samples were collected from both the primed and unprimed trees.

RNAseq: Illumina HiSeq2000 sequencing. TruSeq TM RNA-seq sample prep. 44 libraries, with separate barcode for each library, were sequenced. ~20 million reads per library.

Bioinformatics: CLC Genomics Workbench and other tools.

qRT-PCR: Selected candidate genes verified with qRT-PCR.

Results and Conclusions

Molecular and anatomical studies of Norway spruce suggest that the hallmark of tree resistance is the speed by which the tree can respond to challenges from pathogens and insects [5]. Molecular defence responses to necrotrophic, beetle-vectored fungi in Norway spruce include local and systemic induction of defensins, peroxidases, chitinases and other pathogenesis related proteins. Thus vectored fungi appear to be key modulators of the defence reaction pathways. Acquired resistance, which may be regarded as a long-term consequence of induced defences, is well known in both angiosperms and gymnosperms, including pine and spruce. In Arabidopsis chromatin modifications and small noncoding RNAs have been found to be involved in acquired resistance [6], but in conifers the molecular mechanisms behind priming are really yet to be unraveled. Through transcriptome and microRNA analysis of Norway spruce we have recently identified transcripts of resistance-related proteins as well as microRNAs that may target and regulate these transcripts. In spruce, many molecular and enzymatic aspects of induced defences are well studied, and these include the molecular biology of terpene synthases and enzyme systems involved in terpene formation. However, but how these and other genes are impacted by priming and later wounding or challenge with the pathogen *C. polonica* is unknown. The results of the priming effect in terms of the resulting transcriptome changes are in progress and will be presented.

Competing interests

No competing interests.

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Using different molecular approaches to resolve the phylogeny and taxonomic complexity of the European black pine, *Pinus nigra* Arnold

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Key words: evolutionary history, taxonomy, phylogenetics.

Background
The European black pine (*Pinus nigra* Arnold) is a widely distributed Mediterranean conifer that covers an area of more than 3.5 million hectares, from North Africa to the northern Mediterranean and eastwards to the Black Sea. It is characterized by a large but highly fragmented distribution area and it presents a strong variability in its morphological, anatomical and physiological traits.
Although one taxonomy is now officially accepted, several others remain in use. The purpose of this work is to clarify the often conflicting classifications of black pine, to help resolve its phylogeny in the Mediterranean basin and to identify genetic markers useful for population genetic studies.

Methods
We analyzed 8 populations with 12 individuals each encompassing the maximum taxonomic diversity of the species. First, we used five DNA Barcoding genes (Cox1, MatK, Rbcl, Trnh and Nad 5-4) as identification tools to characterize the different *Pinus nigra* subspecies. These highly conserved regions are expected to provide species specific and phylogenetically deep information. Second, we used SNP markers in 14 potentially adaptive genes to investigate more recent gene flow and phylogenetic structure.

Results and Conclusions
DNA Barcoding genes could not resolve the taxonomy of black pine: all DNA sequences were identical to each other. On the contrary, results from SNP analyses identified two groups, one eastern group including *P.n. pallasiana* and *P.n. nigra* and one western group including *P.n. mauretanica* and *P.n. salzmanni.*
P.n. laricio was highly admixed, with *P.n. laricio corsicana* related to the western group and *P.n. laricio calabrica* to the eastern group.
Although we can confirm that *Pinus nigra* is better described as a species complex with extensive gene flow rather than as a series of different species, the currently accepted taxonomy (5 subspecies) does not recognize the phylogenetic complexity of the species and the pivotal role of *Pinus nigra laricio*. We suggest that the accepted taxonomy should be revised.

Competing interests
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The involvement of 5-methyl cytosine DNA Demethylases in the dormant-growth transition in poplar

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Key words (max 4): winter dormancy, demethylation, poplar and chromatin-remodelling

Background

Woody species are highly adapted to their habitats. In response to environmental cues woody perennials trigger self-protective developmental programmes, in which signal transduction, transcriptional reprogramming and epigenetic regulation could participate in defining the winter dormancy state. Winter dormancy is the mechanism used by perennial plants to survive the harsh conditions of winter in temperate and cold regions and determines the geographical distribution of tree species (Chuine and Beaubien 2001; Horvath et al. 2003; Allona et al. 2008). Epigenetic control of winter dormancy in woody plants is barely known. Among the important epigenetic marks, 5-methyl cytosine (5mC) regulates gene expression in animals and plants. Global changes in 5mC DNA methylation have been shown in the transition of developmental stages in plants such as chestnut bud set and burst, flowering in azalea, aging in pine trees among other. However, the mechanism and the enzymes involved in the modification of the methylome and its control over those development processes remain to be identified. Our previous results showed higher DNA methylation and less acetylated Lys 8 of histone H4 global levels in poplar stem during winter dormancy compared to active growing season (Conde et al. 2013). In this study we focus in the understanding of the molecular mechanism behind these changes in DNA methylation profile and their role in the control of winter dormancy.

Methods

Analysis of the 5-methyl cytosine levels by the application of the immunofluorescence-based method set up in our lab, in stem vibratome sections cut from hybrid poplar (*Populus tremula x alba*) growing in the field at different stages of winter dormancy process.
To develop a protocol for buds paraffin wax embedding to analyze the level of 5-methyl cytosine by applying our immunofluorescence-based method in poplar apex microtome sections in diferents stages of winter dormancy.
RT-PCR analysis to determine the profile of gene expresion at diferent stages of winter dormancy involved in modification of DNA methylation profile.
Hybrid poplar transformation to obtain transgenic lines with modified expression of a demethylase and phenological experiments with selected lines.

Results and Conclusions

The immunolocalization assays performed in poplar stem sections showed that DNA methylation leves fall suddenly when trees coming from the dormant state are near to restore the growing season. We have determined the spatial distribution of DNA methylation changes in this organ.
We have identified two poplar homologs to Arabidopsis *DME* gene: *PtaDML8/PtaDML10*. The DME protein promotes global DNA demethylation along the genome during endosperm development. Our RT-PCR analyses indicate that the expression of *PtaDML8/PtaDML10* genes increases significantly when trees are near to restart growing after winter dormancy. The phenologycal assays showed that *PtaDML8/PtaDML10* knockdown plants have a delayed in resuming of growth after dormancy.
Taken together, we hypothesize that an active control of the 5mC DNA methylation might play a key role in winter dormancy and that 5mC demethylases would be crucial in this process.

Competing interests

The author declares that they have no competing interests.

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Transposon activation tagging in plants for gene function discovery

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Key words:
Gain of function; mutagenesis; transposition; jumping gene; functional genomics

Background

For black cottonwood (*Populus trichocarpa*) as the first tree, the sequence of the whole genome of was made available (http://www.phytozome.net/poplar) few years ago [1]. In general, sequencing and annotation of the genome of an organism yields a tremendous amount of data and suggestions for putative genes, however, often without any information on their function. Once the whole-genome information is available for an organism, the challenge turns from identifying the structural genomic parts to understanding the function of many genes as possible. The gained functional information can be used to understand how a plant cell genetically coordinates all the different life pathways and consequently, to improve the genetic architecture of a plant cell. A successful approach to unravel gene functions is to introduce a tag in the genome which integrates in or nearby genes. A tag disturbing gene function is useful in many plant species but not appropriate for dioeciously long-lived poplar. Stimulating of gene function by overexpression is known as activation tagging or gain-of-function mutagenesis, and transposons can be applied as tags to do this in a high-throughput, systematic manner. An introduction to transposons present in living organisms as well as to the Ac/Ds transposable element system of maize will be given.

Methods

Transposon tagging was very successful in maize leading to the isolation of different genes. It has been demonstrated that maize transposons could be transferred to and are active in other plant species. We have transferred the maize transposon *Ac* into poplar to check the usability of a transposon-based mutagenesis for the induction of “Knock-out” or “Knock-in” (“gain-of-function” or Activation tagging”) mutant [2], [3]. We clearly could demonstrate that the transposon *Ac* is active in the poplar genome and preferentially reintegrates near or in coding regions [4]. Further, the majority of the re-integrations were found scattered over many unlinked sites on other scaffolds than the one carrying the original integration locus, confirming that *Ac* does in fact cross chromosome boundaries in poplar [5]. Using the *rolC* gene as marker for transposon excision, indeed we could follow transposition of the “Activation Tagging Ds” system (ATDs; kindly provided by Y. Suzuki, University of Tokyo, Tokyo Japan [6], in combination with a transposase gene) on the phenotypic level but had no efficient screen to enrich putative tagged lines [7]. A new approach uses the negative selectable marker gene *tms2* from *Agrobacterium tumefaciens* to screen transposition of the ATD element.

Results and Conclusions

We could demonstrate the application of an efficient activation tagging system for poplar based on a transposon system (the non-autonomous “Activation Tagging Ds” [ATDs]) in a tree species for the first time [7]. Different independent transgenic lines could be obtained carrying a functional HSP::*TRANSPOSASE* construct. Two transgenic lines with highest transposase expression were selected and used for super-transformation with the ATDs-*rolC* construct based on the construct by [7]. In total, 22 double transgenic lines could be

obtained with confirmed mobility of the ATDs element. Four rounds of activation-tagging experiments were conducted yielding in total 12,083 individuals regenerated from putative ATDs transposed calli. From these, 18 different putatively tagged variants could be identified revealing phenotypic variations [7].
A fourth activation-tagging experiment unraveled that approximately only one third of the investigated individuals reveal transposition of the ATDs. Analyses of the new genomic positions of ATDs reveal a very high percentage of tagged genes. To increase the efficiency of the transposon-based activation tagging approach, the *rolC* gene was replaced with the negative selectable marker gene *tms2* from *A. tumefaciens* to obtain only lines with transposition of the ATDs. Different concentrations (10 µM, 25 µM, 50 µM, 100 µM) of naphthalenacetamid (NAM) were added to the regeneration medium. In theory, non-ATDs transposed poplar cells (still expressing the *tms2* gene) metabolize NAM to active IAA leading to callus formation instead of plant regeneration, thus only ATDs transposed poplar cells regenerate plantlets. First experiments indicate the usefulness of this novel activation tagging approach.

Competing interests
The author declares that he has no competing interests.

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Retrotransposon expression in response to *in vitro* inoculation with two fungal pathogens of Scots pine (*Pinus sylvestris* L.)

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Key words: Scots pine, Retrotransposons, *Heterobasidion annosum*, *Lophodermium seditiosum*.

Background

Conifer genomes are characterised by multiple gene families and pseudogenes, contain large inter-gene regions and a high proportion of repetitive sequences. Up to 62% of the loblolly pine genome (*Pinus taeda*) consists of retrotransposable elements (REs) and 70% of these are Long Terminal Repeat REs (Neale *et al.*, 2014). Conifers have genes with large introns (up to 100 kb), that contain various types of mobile genetic elements. Most RE families in conifer genomes are old and inactive, with divergent sequences; however, some highly represented REs have conserved sequences and may be still active (Wegrzyn *et al.* 2014). Evidence of possible transposition was shown by evaluation of somaclonal variation in the Scots pine ramets detected with the IRAP technique (Voronova and Rungis, 2013). Transcription and transposition of REs is associated with stress conditions and/or meristematic tissues in various plant species. However, expression of the RE does not directly imply further transposition. In conifer genomes, it is possible to detect RE sequences co-expressed with stress associated genes as part of their introns. Another option is that RE sequences function as long non-coding RNAs, initiate transcription with their cis-acting elements, or are expressed with other untranslated regions of the genome. It is known that insertions of REs in introns can induce exon shuffling, splicing errors, epigenetic silencing. RE transposition in stress conditions could lead to novel mutations and formation of novel genetic pathways, therefore REs could be involved in genomic plasticity.

Transcripts of various RE-like sequences were detected in Scots pine during infestation with pine woolly aphids and abiotic stress (Voronova *et al.* 2014). This study was aimed to evaluate expression of known REs in the Scots pine genome in response to infection with pathogenic fungi.

Methods

10 Repbase annotated REs from pine species were used to construct real-time PCR primers amplifying the expected polyprotein region. Additionally one unannotated RE and previously studied expressed fragment were analysed. Two fungal pathogens were utilised - *Heterobasidion annosum*, the causal pathogen of root rot and *Lophodermium seditiosum*, the causal pathogen of needle cast. Seeds from 11 Scots pine trees were sterilized and grown in vitro for 20 days. Seedlings were inoculated with *H.annosum* culture suspension by inserting it into the medium approx. 0.5 cm away from the root. 2-4 seedlings from each family were harvested after 7, 14 and 21 days post inoculation, and stored in -80 C°. RNA was isolated from roots and shoots separately.

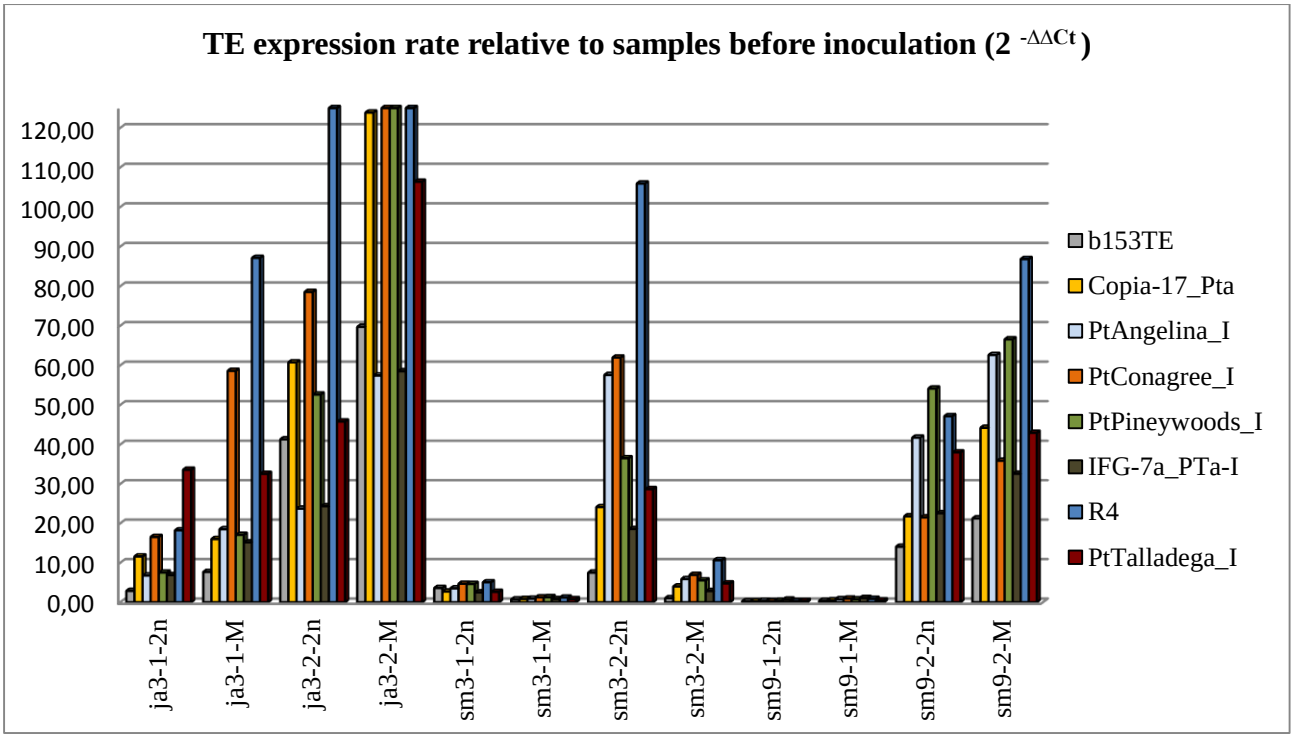
Two year old Scots pine tree ramets (4 clones) were inoculated by spraying them with *L. seditiosum* culture suspension. After one month, typical symptoms of *L.seditiosum* infection were determined in all trees except controls. DNA was isolated several times for detection of infection using real-time PCR and *L.seditiosum* specific primers. Uneven *L.seditiosum* growth within needles prevented the accurate quantitation of infection level by real-time PCR, as Ct-values varied between biological replicates. RNA samples were taken

before inoculation, after two weeks, one month post inoculation. RNA from pine needles was isolated using Rubio-Pina and Zapata-Perez (2011) method. SyberGreen Maxima Master Mix (*Thermo Scientific*) was used for real-time PCR. Seven internal control genes were tested (UBI, rRNA18S, GAPDH, αTUB, EF1α, UEP, APT1), and the two most stable genes for each experimental sample set were used in the subsequent reactions. The relative expression level (ΔΔCt) of REs after infection with *L.seditiosum* was calculated relative to the sample prior to inoculation by StepOne Software v.2.2.2.

Results and Conclusions

In all control ramets, RE expression was lower than in infected ramets, but one control ramet (Ja3-1) showed induction of some REs (Figure 1).

Figure 1. Relative expression rate of nine REs in control ramets (Ja3-1; Sm3-1; Sm9-1) and infected ramets (Ja3-2; Sm3-2; Sm9-2) after two weeks (2n) and one month (M) after inoculation with *L.seditiosum*.



REs *ptConagree-I, ptAngelina-I, ptPineywoods-I, PtTalladega_I, Copia-17_PTa, B153TE*, were upregulated in all infected ramets. RE sequences could be transcribed from different copies in the genome, therefore melting curve analyses can give an insight about RE family activity as melting curves from more highly conserved transcripts will be more specific (Table 1.). RE expression profiles with high Ct values and non-specific melting curves were considered as un-transcribed. This data demonstrates that after infection with *L.seditiosum*, several families of RE are expressed, and that different genotypes have varying RE expression responses.

Table 1. RE product detection parameters (Ct value range, MC- melting curve specificity) and mean relative expression rate increase in *the L.seditiosum* infected trees.

RE name	Ct value range	MC +/-	Mean relative exp. rate increase	
			after two weeks	after one month
Silava_PTa-I	33-35	-	-	-
Copia-17_PTa	21-28	+	35.5	57.4
ptAngelina-I	21.4-29	+	41	42
ptBastroop-I	26.3-33	-	-	-
ptConagree-I	21-30	+\-	54	86
ptOuachita-I	30.7-34.8	-	-	-
ptOzark-I	30.4-36	-	-	-
ptPineywoods-I	18.5-27.3	+	47.7	74.6
PtTalladega_I	23-31	+	37.4	51.4
IFG-7a_PTa-I	17.5-24.6	+	21.8	31.3
R4	25.2-31.8	+/-	117	183.6
B153TE	24-32	+	21	30.67
GAPDH	22.7-30.5	+	-	-

Competing interests
The author declares that they have no competing interests.

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Phenotype dependent differences in the hypomethylome of Norway spruce

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Key words (max 4): Hypomethylome, Norway spruce, Epigenetics, Phenotype

Background
Changes in epigenetic marks, such as DNA methylation and posttranslational histone modifications, mirror morphological and physiological changes associated with growth and development in trees in a wide variety of processes, including aging, phase change, organ maturation, bud set or bud burst. Thus, deciphering the multiple layers of epigenetic regulation that control transcription is critical to understand how plants develop and respond to their environment. DNA methylation in plants occurs to a large proportion in repetitive regions (e.g. transposons), while active gene space is generally hypomethylated. This fact provides an unprecedented opportunity to analyse differential DNA methylation in a genome-wide fashion.

Methods
By applying an optimized methyl filtration protocol followed by next generation sequencing (MREseq) we analysed four phenotypic pools of Norway spruce individuals from natural populations as well as provenance trials to identify differentially methylated genomic regions characteristic for the respective phenotypic trait (early versus late flushing and drought resistant versus drought sensitive). During the analysis, we used a whole-genome snapshot dataset as control. In order to validate the results and test the methylation status of the identified regions on a large sample set in future, a novel, PCR-based detection method was developed, which has the potential to replace a validation via bisulfite sequencing.

Results and Conclusions
Comparing data generated with this MREseq approach to the *Picea abies* genome (v1.0), 67 % of the generated sequence reads identified on average 23 % of the *P. abies* gene models per phenotypic dataset. Due to the improved filtration technique, in each of the MREseq datasets, less than 0,5 % of the *P. abies* transposable elements (2 – 6 % of the sequenced reads) have been detected. On contrary, in the whole-genome snapshot dataset 74 % of the sequence reads hit transposable elements. On average, 24 % of the mapped gene related reads of each phenotypic dataset fall within the coding gene region plus the 1 kb up- and downstream region surrounding it, considered to as regulatory region. In contrast,

in the whole-genome snapshot dataset only 2 % of the mapped reads fall in this +/- 1 kb extended gene region. Further comparative analysis allowed the identification of methylation differences in regulatory regions and within coding regions. In detail, 1.418 and 1.564 regions (+/- 1 kb) were identified in the early flushing and the late flushing dataset only, respectively. In the drought sensitive and drought resistant datasets, 70 and 8.267 regions (+/- 1 kb) were identified, respectively.
First data will be presented on differentially methylated genomic regions possibly being responsible for flushing behavior in Norway spruce.

Competing interests
The author declares that they have no competing interests.

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European Douglas-fir stands and seed sources: Estimation of the varietal and geographic origin, and Comparisons in morphology, bud burst and genetic diversity

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Key words: Douglas-fir, unknown origin, European seed stands, seed

Background

The introduction of non-native forest tree species to Europe, initially focused on fast growing tree species, dates back to the 17th and 18th century when the demands for natural resources to sustain the on-going industrialisation of Europe were enormous. Today´s issues of biomass production and C sequestration as well as the question of whether non-native forest tree species could increase the adaptive capacity of forests to long-term climate change patterns have fuelled a growing interest in non-native tree species in Europe. At present, 5.2% (8.1 Mio ha) of the total European forest area is covered by non-native tree species.

In Europe, Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) is the second most wide-spread non-native conifer. It is a North-American species represented by two hybridizing varieties (the coastal and Rocky-Mountain) within its native distribution range in the US and Canada (1). In the European forestry this conifer is seen as an alternative for native tree species, exceeding them by higher growth potential and growth stability even under dry conditions, which are predicted for the future climate (2). In the 190 year long existence of Douglas-fir in Europe, the importance and the interest in the varietal and geographic origin (provenance) of imported material was neglected during the first 140 years until provenance research revealed a link between provenance and phenotype. Based on this research, the coastal variety and its provenances from Oregon to the Western Cascades (US) are recommended for planting in the majority of European countries, whereas the Rocky-Mountain variety is only recommended for some European countries with continental climate. In general, European Douglas-fir populations (stands) planted up to the 1980s are of unknown origin (3). We cannot also exclude that seed material of both varieties and/or different provenances were mixed when

establishing these stands. Despite the lacking information on their origin, a number of these stands serve currently as European seed sources at national or even international level. The role and contribution of these stands by both natural regeneration and/or seed spread by human for the future European forests is questionable.
Here we i) present results on the estimated varietal and geographic origin of selected European Douglas-fir seed stands of previous unknown origin and ii) compare if the estimated origin matches with the areas recommended by provenance research. In addition, we demonstrate if seedlings from 5 different and commonly on market-accessible Douglas-fir provenances of American and European origin differ in genetic diversity, morphological characteristics, and bud burst timing.

Material and Methods
38 Douglas-fir populations with 766 individuals covering the natural distribution range of Douglas-fir within the US and Canada served as reference populations to which populations of unknown origin were assign to. 18 studied European seed stands were situated in Austria. In the comparison of American and European seed sources, material from a common garden experiment represented by 5 provenances and 852 seedlings originating from different seed stands and different crop years was used. For genotyping, 13 nuSSRs were used (4). Assignment tests (to variety and geographic origin) were conducted in GENECLASS2 (5) and STRUCTURE (6). Genetic diversity measures and differences in morphology (height, diameter) and bud burst were calculated using standard genetic and statistics software packages.

Results and Conclusions
Twelve out of 18 old Douglas-fir seed stands were assigned to areas from Washington (US) and North-Oregon (US) in the Western Cascades, which are recommended seed source areas for planting in Austria. The Rocky-Mountain variety, which is a no-go variety for Austria was present in one “pure” Rocky-Mountain stand and two variety-mixed (both varieties present) stands. In the latter, the Rocky Mountain individuals most probably originate from areas in New Mexico (US) and British Columbia (Canada). Concerning genetic diversities (*He* and allelic richness), Austrian seed stands were very similar to American reference populations. By comparing American with European seed sources, a higher genetic diversity was found in American seeds. No other general difference (height, diameter or bud burst timing) could be detected. One Austrian seed source revealed the largest height and diameter. A high variability in bud burst timing was observed between and within all studied seed sources, with a rather small gradient of bud burst timing (~8–10 days). All but one of European seed sources originate most probably from the areas of the coastal variety from the Western Cascades. In one European seed source, seedlings of both varieties were identified. Results of this research and its implications for management of this species in Europe are discussed in relation to breeding, verification of recommendations based on provenance research, detection of the origin of Douglas-fir stands growing on

“distinct” sites, and further research especially of those stands, where both varieties co-occur and might hybridize.

Competing interests

No competing interests.

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Stucture of genetic diversityty in *Abies alba* Mill. populations from the Czech Republic

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Key words: simple sequence repeats, silver fir populations, genetic variability and differentiation

Backround

Abies alba Mill. is a coniferous species belonging to the family of *Pinaceace*. It plays a very important ecological role as a stabilizing element in the forest ecosystem. This species is located mainly in the mountain forests of southern and central Europe. Silver fir is a species native to the Czech Republic, where it grows in all marginal and inland mountains. Over the past two centuries, however, trees of this species have decreased in number, and especially from the forests of the middle parts of the northern region of its occurrence. A catastrophic decline occurred as a consequence of environmental stress factors and silvicultural preferences for other conifers, mostly Norway spruce, in the second half of the 20th century. In order to reintroduce this species in larger proportions, it is important to acquire more detailed knowledge about the dynamics of genetic diversity within and among silver fir populations. The aim of this study was to provide initial insight into the levels of genetic diversity and differentiation of *Abies alba* populations in the Czech Republic using selected nuclear microsatellite loci.

Methods

Needle samples were collected in spring from eight indigenous populations growing in the Czech Republic. The sample sites were selected to cover the areas of important *Abies alba* populations in the Czech Republic. The populations are designated as JD01 from Babín in Natural Forest Area 16 (Bohemian–Moravian Highlands), JD02 from Velké Polčané, Morávka in Natural Forest Area 40 (Moravian–Silesian Beskids), JD03 from Vodslivy in Natural Forest Area 10 (Central Bohemian Hills), JD04 from Kraslice and Klášterec in Natural Forest Area 1 (Ore Mountains), JD05 from Vsetín in Natural Forest Area 41 (Hostýn–Vsetín and Maple Mountains), JD06 from Hochwald in Natural Forest Area 28 (Hrubý Jeseník Mountains), JD07 from Hojsova Stráž in Natural Forest Area 13 (Bohemian Forest), and JD08 from Kraví hora, Boubín in Natural Forest Area 14 (Nové Hrady Mountains). These populations are growing in gene reserves. Total genomic DNA was extracted using a DNeasy Plant Mini Kit (QIAGEN, Venlo, Netherlands) from 20 mg of dry needles collected from 250 *Abies alba* adult individuals. PCR was optimized with the tested primers, whose oligonucleotide sequences had been developed by HANSEN et al. [1] and CREMER et al. [2].

Eight nuclear microsatellite markers were chosen to assess genetic diversity of the *Abies alba* populations, and they were assembled into two multiplexes (A and B) with specific primers labelled fluorescently (FAM, VIC, NED). The primers in multiplex A

were SF b4, NFH3, NFF3, and SF g6. In multiplex B, these were SF 78, SF 1, NFH15, and NFF7. The PCR profile was as follows: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing temperature at 58°C for 30 s and extension at 72°C for 45 s, with a final extension step at 72°C for 20 min. PCR products were separated by capillary electrophoresis using the Applied Biosystems 3500 genetic analyser. PCR amplifications and sizing of amplification products were repeated at least twice to confirm the fragment lengths. Alleles were sized using GeneMapper® 4.1 software (Applied Biosystems). Calculations of genetic diversity parameters were performed using the statistical programs GenAlEx 6.5 [3, 4], and CERVUS [5].

Results and Conclusions

The eight *Abies alba* populations were evaluated separately. Clear, reproducible PCR products were produced for all eight microsatellite loci. There were 151 different alleles detected at the 8 loci in the 250 silver firs, ranging between 5 and 48 alleles and with an average of 18.9 alleles per locus. Expected heterozygosity ranged from 0.451 to 0.902 with a mean value of 0.753. Shannon's information index calculated for allelic and genetic diversity ranged from 0.723 (at locus SF 1) to 2.627 (at locus SF 78). The numbers of different alleles in the eight investigated *Abies alba* populations at individual loci were detected too. The highest number of alleles (25) was found in the JD03 population at locus SF 78. The lowest number of alleles (3) was found same at all populations at locus SF 1. The genetic diversity expressed as Shannon's information index values was highest in the JD06 population from Hochwald (1.908). Genetic distances among populations were calculated based on Nei´s standard genetic distance [6]. The longest genetic distance (0.232) appeared between the JD02 (Velké Polčané, Morávka) and JD07 (Hojsova Stráž) populations. The closest genetic distance (0.091) was between the JD01 (Babín) and JD03 (Vodslivy) populations. Polymorphic information content (PIC) of selected microsatellite loci was obtained using CERVUS. All observed loci were highly polymorphic (value around 0.8) with the exception locus SF1, which had a much lower PIC value (0.385). This assessment revealed there to be excesses of homozygotes at the SF g6 and SF 1 loci. Genotyping data from 250 *Abies alba* adult trees within eight important populations located in different parts of the Czech Republic imply genetic differences among the studied populations.

Competing interests

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Microsatellite markers using for clonal identification in the Norway spruce seed orchard

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Key words: Picea abies, clonal homogeneity

Background

Seed orchards are an essential component of the most forest tree breeding programs. The main goal of seed orchards is to produce forests tree seeds of a higher genetic value compared to that originating from selected stands [1]. In the Czech Republic the first seed orchard (larch) was established in the 1956 [2]. To date in the Czech Republic have been officially registered 100 recognized seed orchards, mainly 27 seed orchards of Scots pine, 20 seed orchards of larch and 16 seed orchards of Norway spruce. For verification of clone composition and clonal homogeneity of Norway spruce ramets we used SSR markers. Microsatellites are highly variable markers that are commonly used in population genetic studies for analyses of gene flow, parentage analyses, and studies on genetic diversity [3]. In particularly nuclear simple sequence repeat (SSR) markers have proven to be extremely useful for characterizing cultivars and identifying clones [4].

Methods

The clonal identification in model seed orchard of Norway spruce was studied by DNA analyses using the Simple Sequence Repeats (SSR) method. Total genomic DNA was extracted by DNA Plant Mini Kit (QIAGEN) from needles taken from 100 sampled trees of seed orchard. We used five ramets from twenty different clones of Norway spruce. DNA was quantified using an NanoPhotometr (Implen). PCR was optimized for the nine tested primers that have been scanned in publications [5, 6, 7]. Measurement of the size of amplification products was carried out on the genetic analyzer Applied Biosystems 3500. The obtained data were analysed by means the statistical programs CERVUS [8] and GenAlEx 6.501 [9, 10].

Results and Conclusion

There were detected 115 different alleles at 9 loci in the 100 Norway spruce individuals from seed orchard, i.e. 9.4 alleles per locus and population in average. The most polymorphic in our set of samples was locus WS00716.F13. Nine independent nuclear microsatellite loci we used to obtain multilocus individual genotypes. Tab. 1 shows observed heterozygosity, expected heterozygosity, number of alleles, numbers of heterozygotes and Polymorphism Information Content (PIC) of microsatellite loci. Allelic richness (number of alleles) at each locus ranged from 21 – 2. Expected heterozygosities ranged across loci from 0.212–0.928 (average of 0,763) and observed heterozygosities range from 0.24–1.00 (average 0,751).

Table 1 Genetic diversity parameters for the 9 microsatellite loci in the 100 ramets of Norway spruce

Locus	Number of different alleles (Na)	Observed Heterozygosity (Ho)	Expected Heterozygosity (He)	Number of heterozygotes	Polymorphism information content (PIC)
PAAC23	8	0.730	0.675	73	0.646
PAAC19	16	0.660	0.908	66	0.896
$SpAGD_1$	18	0.520	0.928	52	0.918
WS00716.F13	21	0.910	0.926	91	0.916
WS0092.A19	2	0.240	0.212	24	0.189
WS0022.B15	13	0.890	0.765	89	0.745
WS0073.H08	5	0.810	0.742	81	0.691
WS00111.K13	16	1.00	0.882	100	0.868
WS0023.B03	16	1.00	0.865	100	0.850

These results illustrate the utility of the microsatellite loci for assessing spatial patterns of genetic diversity and for individual identification. Declared clone affiliation was confirmed in 94% of sampled trees in tested seed orchard. The identified genetic loci were verified as highly polymorphic and could be further used for clonal identification of Norway spruce trees.

Competing interests

The author declares that they have no competing interests.

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Abstract poster session II

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Single-locus versus Multi-locus Effects Underlying the Genetic Architecture of Local Adaptation in Eastern White Pine (*Pinus strobus*)

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Key words: Genetic basis of local adaptation, Natural selection, Range-wide genetic structure, SNPs in climate-related candidate genes

Background

Understanding the genetic architecture of local adaptation is critical in forest trees, especially in the context of climate change. Natural plant populations are often adapted to their local climate and environmental conditions. Forest tree populations exhibit this pattern, amply evident from their provenance tests. However, the knowledge of genetic architecture of local adaption remains largely unknown for forest trees. This scarcity of knowledge stems partly from the complexity of the underlying genetic architecture of local adaptation in forest trees relative to the types of statistical methods used to document local adaptation from genetic data. Specifically, statistical methods used to detect loci as contributing to the genetic architecture of local adaptation focus on single-locus effects, whereas the underlying genetic architecture of local adaptation for forest trees likely involves the among-population component of intergenic linkage disequilibrium. Outliers detected by single-locus methods typically have large, or at least the largest among the loci surveyed, allele frequency differences among sampled populations. Many causative loci, therefore, are unlikely to be detected using scans for F_{ST} outliers, since the conditions for large allele frequency differences to arise among populations of forest trees may be rare. This is evident from relatively limited lists of putatively causal genes identified in most studies. Little research has focused on understanding the relative contributions of single gene versus polygenic effects to the genetic architecture of local adaptation for forest trees at the molecular genetic level.

Methods

We have examined single-locus versus multilocus contributions to local adaptation in eastern white pine (*Pinus strobus*) by determining the genetic structure of range-wide 29 natural populations in relation to local climatic factors using both putatively neutral SSRs, previously identified outlier SSRs, and putatively climate-related functional SNPs in candidate genes. The candidate genes used have protein products with functional responses to climate factors for model plants. Standard outlier and environmental correlation analyses and a novel implementation of graph theoretical approach were used to examine genetic architecture of local adaptation in response to local climate factors.

Results and Conclusions
Magnitudes of population structure were similar between SSRs and SNPs. Outliers consistent with diversifying selection were rare for both SNPs and SSRs. However, genetic distances based on the multilocus among-population covariances (conditional genetic distances) were significantly more correlated to climate, even after correcting for spatial effects, for SNPs as compared to SSRs. Simulation studies demonstrated that the mutation rate differences between SNPs and SSRs do not affect the differences in the correlation of conditional genetic distances with climate obtained from these marker types. Our study has demonstrated that the genetic architecture of adaptive traits is complex and that the polygenic covariances in allele frequencies among functional loci primarily drive adaptation to local environments in eastern white pine. Therefore, there is a need to take into account the multilocus effects in understanding the genetic architecture of local adaptation.

Competing interests

The author declares that they have no competing interests.

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Does molecular diversity generally decrease from south to north? Contrasting results from a rare and scattered forest tree (*Sorbus domestica* L.)

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Key words: gene flow, genetic bottleneck, molecular diversity, true service tree

Background

During past ice ages, many tree species persisted with limited distributions in southern refugia, where climate conditions had been less severe. As the climate began to warm and ice shields retreated, species started to spread northwards and populations increased in number and size. However, due to numerous founder events during range expansion populations often went through genetic bottlenecks which resulted in loss of alleles and increased homozygosity. This phenomenon is more common known as 'southern richness and northern purity' and was first outlined by Hewitt (1999). In consequence, populations that are located more distantly from their potential refugia should encompass less genetic diversity than those that are nearby. We show here that this pattern must not necessarily hold true for all tree species, especially when other evolutionary important mechanisms are involved that might have helped to cope with small population census sizes and limited availability of mating partners. We investigated true service tree (*Sorbus domestica* L.), one of the rarest European tree species, in consideration of its range-wide genetic make-up and its population structure, focusing also on proportions of historic and recent gene flow on a smaller geographical scale.

Methods

Our study included 15 putative natural populations as well as data from three clonal archives, covering an area from South-west Britain to South-east Bulgaria. We used two kinds of molecular markers (seven nuclear microsatellites and one cpDNA minisatellite). Molecular diversity was assessed in terms of allelic richness to account for unequal sample sizes. Population structure and differentiation was inferred using UPGMA clustering, PCoA

as well as individual-population assignment approaches. For the analysis of historic and recent gene flow between two nearby located populations we used Bayesian inference methods.

Results and Conclusions

Allelic richness was unexpectedly high for both markers within populations (mean per locus: 3.868 for nSSR and 1.647 for cp minisatellite) and we could not find a significant decline in genetic diversity from south to north. Moreover, there was no evidence of inbreeding (mean F_{IS}: -0.047), even though some populations are of small census size and have only a limited probability of random mating. The Apennine Peninsula was characterized as a geographic region with comparatively high genetic diversity for both genomes. Overall population differentiation was weak to moderate (F_{ST}: 0.138) and it was obvious that populations formed three groups in Europe (GB-France, Mediterranean/Balkan, Austria). Historic gene flow between two local Austrian populations was high and asymmetric while recent gene flow seemed to be disrupted. We conclude that molecular mechanisms such as self-incompatibility and high gene flow distances are responsible for the observed level of allelic richness as well as for this weak population differentiation. However, human influence could have contributed to the present genetic pattern, especially in the Mediterranean region. Comparison of historic and recent gene flow may mirror the progress of habitat fragmentation, which might had led to limited movement ranges for both pollinators and frugivores. Additionally, changing of forest management might have resulted in reduced fecundity and fruiting success and thus in lower dispersal probability.

Competing interests

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Genetic structure of relict and isolated Scots pine (*Pinus sylvestris* L.) populations from Central-Eastern Europe: signals of recent population fragmentation?

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Historical evolutionary events and local ecological drivers highly affect the present day genetic structure of natural populations. Scots pine (*Pinus sylvestris* L.) a dominant tree species of the Eurasian taiga communities has survived in only small populations at the southern margin of the distribution where inconsequence of postglacial climate warming occupied the most extreme habitat types such as peat bogs or dry montane rocky outcrops. In our study, we characterize the genetic structure of 421 individuals from 20 natural populations native to Central-Eastern Europe growing mainly in extreme ecological conditions. Microsatellite markers were involved (4 cpSSR and 8 nSSR) in the study. AMOVA analysis revealed almost similar among populations' genetic differentiation in both nuclear and chloroplast markers. Overall 7% of total genetic variance reside among populations, while 93% of variance within populations. Bayesian clustering for genetic assignment evaluated with STRUCTURE, showed separation of populations into two main groups: (1) Western Hungarian with Slovakian accompanied by populations from Transylvanian Western-Island Mountains and the Southern Carpathians, (2) Eastern Carpathians with Bulgarian populations including also the northernmost population from Estonia. BAPS analysis on chloroplast SSR loci identified similar results by clustering Western-Island Mountains with the Southern Carpathians, and differentiating the Eastern Carpathian populations. Spatial distribution of haplotypes (Haplotype Analysis), further strengthen differences among the above mentioned geographical regions. Both marker types showed well balanced genetic diversity and low frequency of private alleles, with some exceptions. Mantel test exhibited no correlation of genetic and geographic differentiation among populations, and low deviations from the Hardy-Weinberg equilibrium. Approximate Bayesian Computation-based analysis method (ABC) revealed a major population divergence from an ancient population, and an admixture event in the species evolutionary history probably in late-Pleistocene, and suggests a recently ongoing population segregation and population fragmentation.

Keywords: nuclear SSR, chloroplast SSR, Scots pine, Pinus sylvestris

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Inga ingoides and *Inga edulis*: how close species are they for agroforestry purposes?

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Key words: introgression, agroforestry, biodiversity conservation, Peruvian Amazon

Background

The Amazon drainage basin containing mainly lowland rainforest habitats is a major component of the Neotropical region with more than 8 million km^2 and about 25 million people (Junk and Piedade, 2011). The Amazon water-level fluctuation up to 16 m causes flooding over a wide floodplain naturally covered with forests, surrounding vegetation and agricultural land (Schöngart et al., 2002). Riparian forests along streams and low-order rivers in the rain forest cover an area of about 1 million km^2, which corresponds to about 50% of the entire wetland area of the basin (Junk and Piedade, 2011 and references therein).

The diversification in the *Inga* genus (Fabaceae) is recent (Richardson et al., 2001) and tropics interspecies strong competition requires strategies for sympatric species to survive in particular habitats. In the Amazon region, different *Inga* species are found in different locations, due probably to flood effect. *Inga ingoides* (Rich.) Willd. is found along the riversides, due to strong flood pulse tolerance, and also upland, and *I. edulis* Mart. preferably upland. The later is the most widely distributed and highly valued species from this genus in this region (Pennington, 1997). This species was improved by human selection focusing on edible fruit and cultivated as fruit tree over millennia. The neglected tree *I. ingoides* could be considered as a multipurpose fruit tree species in agroforestry and other cropping systems practiced on lands affected by periodical flooding. *Inga ingoides* fruit and timber production close to the rivers is expected to be less costly, sustainable and forest-friendly, due to: i) easy accessibility for humans, ii) economy of transport, iii) nutrient income provided by periodical flooding, iv) possibility of its cultivation in forest buffer zones and elimination of the need of colonisation of new forest sites to reach new arable land. Our aims were:1) to verify if *I. ingoides* and *I. edulis* populations from three Peruvian Amazon tributary rivers had diverge and accumulated sounding differentiation, 2) to compare both species populations' genetic diversity estimates, and 3) to assess species' potential introgression.

Methods

The populations were sampled along three Amazon river tributaries, the Pacaya, the Samiria and the Utiquinia rivers and in upland forests, situated in protected areas from the Peruvian Amazon. In total, 139 individuals of *I. ingoides* (77) and *I. edulis* (62) from 8 natural populations were genotyped at four microsatellites (SSRs). Populations were characterized for standard genetic diversity statistics. Genetic structure was explored using analysis of molecular variance (AMOVA), differentiation statistics and Bayesian analysis of population clustering, using STRUCTURE.

Results

Sixty-six different alleles were identified in *I. ingoides* and fifty-eight in *I. edulis* populations, and, on average, both species showed high and similar values of genetic diversity (He ~ 70%). All the *I. ingoides* populations displayed a positive inbreeding coefficient (F_{IS}), unlike the *I. edulis* ones, probably due to biparental inbreeding and consanguinity in the former species, related with potential patches of related individuals' establishment along the rivers. Overall genetic differentiation was weak (G_{ST} = 4.1%). Additionally, no clear isolation-by-distance was found for any of the species, through Mantel tests, which showed very weak correlations (r = -0.30; p = 0.173, *I. edulis*; r = 0.45; p = 0.560, *I. ingoides*). The STRUCTURE identified intense among populations' gene flow and putative strong introgression between species. Those results are, also, supported by the weak among populations within species differentiation (Φ_{SC}= 2.7%; P<0.0001), and the low between species differentiation (Φ_{CT}= 3.6%; P<0.02), indicating, in the later case, at least past gene flow.

Conclusions

Inga ingoides multipurpose agroforestry use in areas affected by periodical flooding is desirable and further genetic improvement could be sought through selection of natural hybrids or artificial hybridization with *I. edulis*. This breeding strategy aims to improve legume size and yield and also maintaining periodical flood tolerance. This new approach in agroforestry could make use of the areas along the river, instead of upland slash and burn to create open areas. Such approach may help to slow down the deforestation and contribute to the creation of sustainable land use practices and biodiversity conservation in the Amazon basin.

Competing interests

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Tickle me tree *"Lagerstroemia indica" :the reason research*

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Key words: **Tickle me tree, *Lagerstroemia indica,* action plant, signal transduction**

Background

In South Korea it is told that Lagerstroemia indica is "tickle me tree" as when tickle the lower part of stem the tips of stem in the same direction move slightly. It has not been reviewed scientifically. The authors are not sure it's phenomenon is world wide or local in Korea. We tried to find out the reasons of it.

Lagerstroemia indica (Crape myrtle, Crepe myrtle) is a species in the genus *Lagerstroemia* in the family *Lythraceae*.

From China, Korea, Japan and Indian Subcontinent *Lagerstroemia indica* is an often multistemmed, deciduous tree with a wide spreading, flat topped, rounded, or even spike shaped open habit. Planted in full sun or under canopy, the tree is a popular nesting shrub for songbirds and wrens.

The bark is a prominent feature being smooth, pinkinsh-gray and mottled, shedding each year. Leaves also shed each winter, after spectacular color display, and bare branches re-leaf early in the spring; leaves are small, smooth -edged, circular or oval -shaped, and dark green changing to yellow and orange and red in autumn.

Flowers, on different trees, are white, pink, mauve, purple or carmine with crimped petals, in panicles up to 9cm.

Lagerstroemia indica is frost tolerant, prefers full sun and will grow to 6 metres with a spread of 6 metres.

Many hybrid cultivars have been developed between *L. indica* and *L. faueri*

Fig. 1 The flower of "Dynamite" cultivar the Crepe Myrtle

Mimosa pudica is well known for its rapid plant movement. Like a number of other plant species, it undergoes changes in leaf orientation termed "sleep" or nyctinastic movement. The foliage closes during darkness and reopens in light.

The leaves also close under various other stimuli, such as touching, warming, blowing, or shaking. These types of movements have been termed seismonastic movements. The movement occurs when specific regions of cells lose turgor pressure, which is the force that is applied onto the cell wall by water within the cell vacuoles and other cell contents.

Methods

1. Comparison of tickling with similar trees(*Chaenomeles chinensis*, *Platanus occidentalis*, *Acer buergerianum*)
2. Comparison of specific gravity with similar trees(*Chaenomeles chinensis*, *Platanus occidentalis*, *Acer buergerianum*)
3. Comparison of electric resistance

Fig 2. Measuring of electric resistance

Results

1. ***Lagerstroemia indica showed response only.***

2. **Specific** Gravity measured are as following table

Table 1. **Specific** Gravity

A: Chaenomeles chinensis, B: Acer buergerianum C: Platanus occidentalis ***D: Lagerstroemia indica***

Replication	A	B	C	D
1	.6211	.7966	.8724	.8715
2	.6616	.7525	.7862	.8450
3	.6679	.7087	.7748	.8972
4	.6344	.9256	.8645	.8950
5	.6440	.7123	.7114	.8012
6	.7148	.6837	.7386	.9121
7	.7445	.7210	.8306	.8543
8	.6367	.7103	.8060	.8888
9	.6990	.7564	.9030	.9088
10	.6860	.7857	1.0638	.8709
Mean	0.67	0.75	0.84	0.87

3. Comparison result with *Platanus occidentalis in other wood properties is as following table*

Table 2.Wood Properties of two species

	Lagerstromia indica	*Platanus occidentalis*
Specific Gravity	0.87 g/cm^3	0.84 g/cm^3
Janka Hardness	4,850 N (1,090 ibf)	3,430 N (770 ibf)
Crushing Strength	64.1 MPa (9,300 ibf/ in^2)	37.1 MPa (5,380 ibf / in^2)
Modulus of Elasticity	10,8GPa(1,566,000 ibf/ in^2)	9.79 GPa(1,420,000 ibf / in^2)
Modulus of Rupture	97.4 MPa (14,120 ibf/ in^2)	69.0 MPa (10,000 ibf/ in^2)

4. For Electric resistance, no differences was detected so far.

Conclusions

Heavier and harder wood property of *Lagerstromia indica* than other tree species could be a cause of tickling phenomenon. But this cannot be sufficient answer. So further research should be continued.

Competing interests

The authors declare that we have no competing interests.

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Chloroplast DNA markers reveal the native locality of origin of *Eucalyptus globulus* at the Tre Fontane Abbey in Rome

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Key words (max 4 domestication, landrace, molecular markers, chloroplast DNA

Background

The plantings of *Eucalyptus globulus* at the Tre Fontane Abbey near Rome have a special place in the domestication history of the Australian eucalypts (Zacharin 1978; Doughty 2000). Trappists of the Cistercian order currently occupy the abbey which has a rich history, including the martydom of Saint Paul. For eucalyptologists the abbey is well-known, not only for the unique eucalypt liquor *Eucalittino* still produced by the Trappists, but also the well-documented plantings of eucalypts in the late 19th century which were linked to the erradication of malaria – a problem which defeated several Roman emperors, even Julius Caesar. Trappists from France occupied the Abbey in 1868, at a time when malaria was rife, and it was not until they drained the nearby marshes and undertook large-scale planting of eucalypts (mainly *E. globulus*; Zacharin 1978) that malaria was virtually eliminated. This no doubt reinforced the reputation of eucalypts as 'fever trees', which was a significant driver in the rapid spread of eucalypts around the world in the 19th century (Doughty 2000).

The main eucalypts growing in the Tre Fontane area today are *E. globulus* and *E. camaldulensis*. Of particular interest is the *E. globulus* (Tasmanian blue gum); one of the most important pulpwood plantation eucalypts (Potts *et al.* 2004). It's native range is on the islands of Tasmania, and adjacent coastal regions of Victoria on mainland Australia. *E. globulus,* one of the first species to be introduced into other countries (1804 France), was rapidly spread around the world, and now there are over 2 million hectares of plantation globally (Potts *et al.* 2004). As part of our study of the origin of the world's landraces of *E. globulus* we sampled trees in vicinity of the Tre Fontane Abbey and these samples are here used to demonstrate the 'forensic' power of the maternally inherited chloroplast DNA.

Methods

Our study was based on haplotypes defined by sequencing the hypervariable JLA+ region of the eucalypt chloroplast genome (Freeman *et al.* 2007). As with most angiosperms, the chloroplast of eucalypts are maternally inherited, providing a means of tracking the maternal lineage. To date we have identified 173 haplotypes across 590 samples from the native range and 197 landraces samples of *E. globulus*. Landraces samples are from *E. globulus* introduced into California, Chile, China, Columbia, Ecuador, Ethiopia, France, Ireland, Italy, Portugal, South Africa and Spain – sampled with a focus on old trees. Within the native range of *E. globulus* the distribution of haplotypes is geographically structured, both in terms of individual haplotypes and groups of related haplotypes (McKinnon *et al.* 2004a). For example,haplotypes of the JS type have only been found in Tasmania and those of the JCg type have not been found on the island of Tasmania. The strong spatial structuring of the genetic diversity, and often localised distribution of specific haplotyes has provided a

powerful tool for identification of the native stand origin of unknown samples of *E. globulus* (e.g. Portugese landrace - Freeman *et al.* 2007).

Results and Conclusions

The three haplotypes found in the six samples of *E. globulus* near Tre Fontane Abbey were of the JS type, clearly indicating a Tasmanian origin. Four samples had haplotype JS43 which is distributed within 60 km of Hobart in south-eastern Tasmania. The other two haplotypes found (JS05 and JS74) have a narrow distribution around the North-West Bay area just south of Hobart, where JS43 has also been found. This area is close to the 1802 anchorage of the French expedition led by Captain Nicholas Baudin which was responsible for the introduction of some eucalypts (probably including *E. globulus*) into France. JS43 is widespread in our landrace samples occurring in virtually all countries including France. JS05 has been found in California, Chile and France. The rarer JS74 has only been found in Portugal. It has been suggested that some of the Tre Fontane trees may have originated from seed directly introduced from Australia via Ferdinand von Mueller (the director of Royal Botanic Gardens, Melbourne from 1857 to 1873) and the explorer Alfred Howitt, whose parents moved to Rome in 1870 (Doughty 2000). The Archbishop of Melbourne is also reported to have presented seed of *E. globulus* to the Trappists during his visit to the Vatican in 1869 (Doughty 2000). However these seedlings reportedly died - as did some early replacements from France (Zacharin 1978). Given that the purported Australian seed introductions were from mainland Australian-based suppliers but all haplotypes identified at Tre Fontane are found in the vicinity of the Baudin anchorage in southern Tasmania, it is more likely that France was the main source of the *E. globulus* used for afforestation at Tre Fontane. All haplotypes found in our 13 samples of *E. globulus* from France originate from the island of Tasmania. By contrast, the other seven Italian *E.globulus* samples we have from Sicily and near Naples have either mainland or Tasmanian origin, consistent with multiple introductions.

Competing interests

The author declares that they have no competing interests.

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Genetic diversity and structure of *Quercus trojana* populations in italy

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Key words: Quercus trojana, genetic diversity ,SSRs, candidate genes

Background

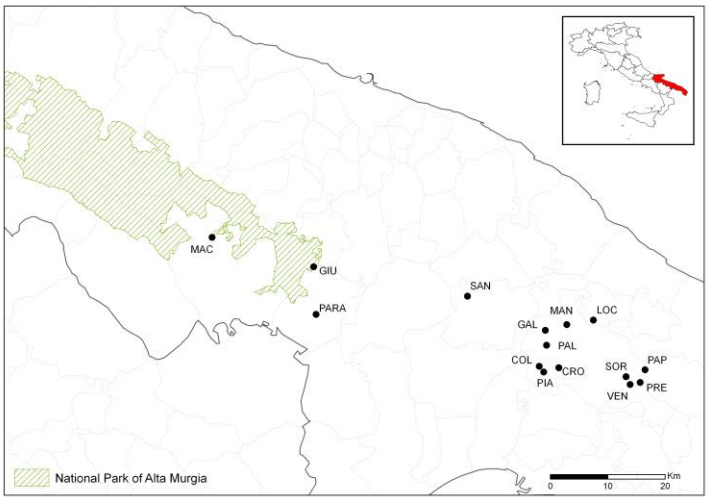
Forests are considered the most complex terrestrial ecosystems due to their high level of biodiversity in term of genetic resources, species and habitat [1]. However, over the past few centuries, the degradation and disappearance of natural forests have caused serious loss of biodiversity leading to a decrease in population's ability to adapt to future changes in biotic and abiotic factors. Thus, the evaluation of the genetic diversity and structure of the current populations is an essential prerequisite for management planning and conservation strategies of forest tree species. Tools as molecular markers are being widely used to characterize genetic variation of long-lived species, since they can greatly facilitate the prioritization of conservation strategies [2]. This work aims to evaluate the potential of genetic diversity studies to identify populations and germplasm to be preserved and used in management programs of genetic resources. We studied *Q. trojana*, an oak species with bulk range centered in the Balkan peninsula. In Italy, its current distribution is restricted to the southern regions (Puglia, Basilicata) and is a remnant of a more widely spread range severely reduced by intensive human exploitation.

Methods

A total of 279 samples from 15 sites were collected. All the sites are located in the Puglia Region (South Italy); two of these in National Park of Alta Murgia.(Figure 1) Total genomic DNA was isolated by grinding 60-50 mg of fresh tissue and using the DNeasy 96 Plant Kit (Qiagen) according the manufacturer's instructions. A set of six polymorphic microsatellites [3][4] were selected and used for the analysis. Polymerase chain reactions were carried out on a GeneAmp 2700 Thermal Cycler (Applied Biosystems, Foster City, USA). The reactions were performed in 20 µl total volume containing 20 ng of genomic DNA following the Qiagen multiplex kit protocol. Cycling parameters were as follows: 15 min at 95 °C; 30 cycles for 30 s at 94 °C , 90 sec at 57 and 1 min at 72 °C; and a final step of 30 min at 72 °C. Amplification products (01-1 µL) were added to 20µL formamide and 0.3 µL LIZ and denatured at 95 °C for 5 min. The samples were run on ABI Prism 3130 Avant DNA sequencer. The resulting raw data were collected applying GeneMapper software (Life Techonogies). A set of measures of intra and inter population genetic statistics (He, Ho, Fis, Fst and Amova) and the Principal Coordinate Analysis were calculated using the software GeneAlEx 6.5 [7]. The structure of populations was analysed using the software STRUCTURE v.2.3.4 [8]. Finally, six candidate genes possibly significant for environmental adaptation [6, 9] were sequenced in a subset of 10 individuals to examine nucleotide diversity. At the same time, HRM

techniques [5] were set up in order to check for signatures of natural selection and assess the adaptive genetic variation across the Italian populations.

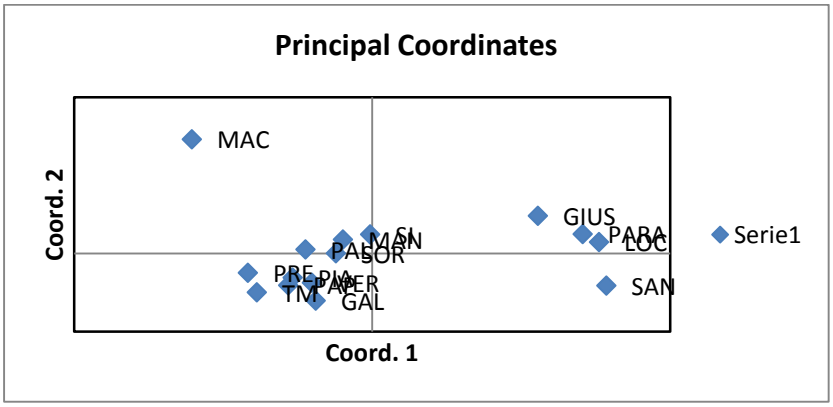
Fig 1. Sampling sites



Results and Conclusions

Our preliminary results indicate a high level of genetic diversity in all populations. The AMOVA analysis shows that the majority of molecular variance (93%) was partitioned within the populations. The molecular variance among the populations was 7%. Two main gene pools and a population (MAC) genetically divergent were identified (figure 2). The first group includes one population collected in the area of Alta Murgia National Park (GIU) and some of the neighboring areas, the remaining populations are clustered in a second group. Four of the six tested candidate genes displayed interesting nucleotide variation and will undergo HRM analyses for a full investigation of the entire dataset. On the basis of the values of allelic richness, divergence among populations and environmental adaptive variation displayed, we will be able to indicate populations to be included in genetic resources conservation programs.

Fig. 2 Principal Coordinate Analysis of the *Q. trojana* populations based on Nei's unbiased genetic distance



Competing interests

The authors declare that they have no competing interests.

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Strawberry biophysical units' ecology to design provenance regions in Portugal using GIS tools

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Key words: Arbutus unedo; provenance design; GIS; multivariate statistics.

Strawberry tree (*Arbutus unedo* L.) displays potential to be successfully businesslike cultured in several regions of Portugal and southern Europe, where it is well adapted to climate and soils. In Portugal, this species has been used by local populations for fruit consumption and spirit production, but remains largely a neglected crop. It has different possible commercial uses, from processed and fresh fruit production to ornamental, pharmaceutical and chemical applications, due to phenolic acids and terpenoid compounds with strong antioxidant activity, vitamin C and tannin content. In addition, due to its pioneer status, it is valuable for land recovery and desertification avoidance, besides being fire resistant. Currently, the demand for improved plants has strongly increased. Under the project ARBUTUS (PTDC/AGR-FOR/3746/2012, *Arbutus unedo* plants and products quality improvement for the agro-forestry sector) 30 trees were selected, in 15 natural populations distributed throughout the country. The stands were ecologically characterized locally, using bioclimatology, lithology, topography, soil type, vegetation and wildfires records, as biophysical units. The data processed was the digital elevation model (DEM), the soil maps, the annual burnt areas since 1975, and the phytosociological and biogeographical maps. In parallel, the climatological data were collected (1981-2010) from various weather stations and bioclimatological indices maps, constructed by Monteiro-Henriques (2010), in order to calculate bioclimatic indices, using geostatistical tools. An exploratory multivariate statistical approach was performed with the collected information: principal component analysis (PCA) and hierarchical cluster analysis (HCA). The obtained information will be used for the provenance regions design in order to select plus trees and for tree species genetic improvement purposes, besides forest tree conservation programs design.

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Relationship between internal morphology and germination of seeds of *Bauhinia longifolia* (Bong.) Steud. - Fabaceae, a Brazilian tree

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Background

Despite the considerable increase of the studies with seeds of forest species in Brazil, the technologists of forest-tree seeds are still difficulties in the establishment of production techniques and quality evaluation of seed due to the large morphological variability of fruits and seeds of different species [1]. Currently, image analysis of seed and seedlings have been shown for the efficient evaluation of physiological, morphological and physical components of seeds, that influence on the quality of the lots. One of the available techniques, x-ray testing has proved efficient in the analysis of the internal morphology of seeds, enabling the study of the relationship between this feature and its potential physiological. The radiographic image analysis shows as an interesting alternative to identification of possible damage and characteristics such as the presence of internal free areas, which can reduce the physiological potential of lots.

The *Bauhinia longifolia* (Bong.) Steudas is a tree of Fabaceae family, subfamily Caesalpinieae; occurs in Gallery forests in areas of Cerrado or Cerrado-forest transition [2], pioneer and autochory dispersion, being considered almost endangered by the environmental secretariat of São Paulo.

The aim of this study was to evaluate the physiological potential of seed of *Bauhinia longifolia* through X-rays and germination at 4 different stages of maturity of the fruits: fruits at the beginning of maturation (green seed); mature fruits (yellow seed); fruits in beginning of the dispersion (brown seeds in the pods open) and after scattering seeds.

Methods

Fruits were collected in remnant native vegetation of riparian forests of São Pedro-SP, and taken to the Analysis of Images Laboratory in Crop Science Department of ESALQ/USP, where seeds were extracted, benefited and manually excluding the malformed and damaged seed. The seeds were separated into 4 different lots: Lot 1: fruits at the

beginning of maturation (green seed); Lot 2: mature fruits (yellow seed); Lot 3: fruits in beginning of the dispersion (brown seeds in the pods open) and; Lot 4: after scattering seeds. Lots of seeds were dried until it reaches 8% of degree of moisture for the beginning of the experiment.

For x-ray analyses were used 10 repetitions of 20 seeds for each lot. The internal morphology viewed in x-rays, was classified and being divided into 6 categories to classify the seeds, in relation to the standard deviation of the free space on seeds and the presence or absence of damage [3].

The study of relationship between the morphology of the seeds and germination was conducted within each class and comparing the classes, whereas seeds undamaged and with differences in internal area occupied by the embryo and endosperm, seed with damage and with differences in the internal area of the seeds and seeds with and without damage to the same area of internal cavity filled.

Later, seeds were submited the test of germination, in paper roll with water amount equivalent to 2.5 times the mass of the dry substrate at 25° C, with continuos light [4]. The evaluations were carried out 15 days after sowing, and the results were expressed as percentage of normal seedlings, abnormal seedlings and dead seeds for each category of each lot.

Results and Conclusions

The relationship between the morphology of the seeds and germination was different for lots examined (Table 1). In category 2, no seeds were found undamaged and with empty spaces, with exception to lot 4 that presented 1.5% of seeds, generating only abnormal seedlings. In Lot 2, there were no seeds with damage without empty spaces, only categories 1 and 4, generally led to higher percentage of normal seedlings.

Table 1. Percentage of seeds in each category (S) of internal morphology obtained by x-ray

Lot	Category	% S
1	**1**	30.5
	2	-
	3	-
	4	55
	5	8
	6	6.5

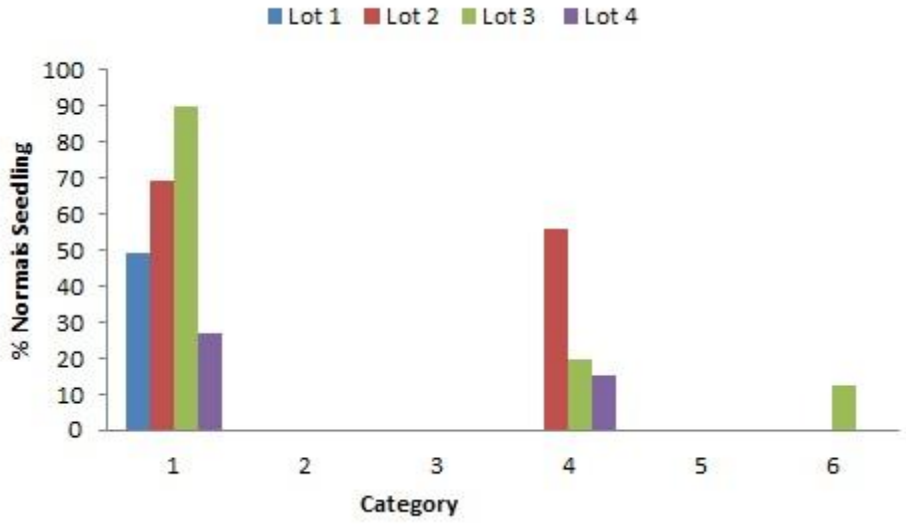
Lot	Category	% S
3	**1**	35
	2	-
	3	-
	4	43.5
	5	9.5
	6	12

Lot	Category	% S
2	**1**	58
	2	-
	3	-
	4	42
	5	-
	6	-

Lot	Category	% S
4	**1**	39
	2	1.5
	3	-
	4	46.5
	5	5
	6	8

Analysis of radiographic images of seeds revealed that there difference between the lots, being that the lot 2 with did not presented seeds with empty space and with minor damage, differentiating it from other lots. It is clear the dimming caused by the presence of decayed tissue, as well as malformation of embryo; examples of cracks in the embryo, which may impair germination. The seeds of category 1 originated higher percentage of normal seedlings seeds of category 4, it is possible that the seeds of category 4 same no damage had lower physiological potential due empty space (Figure 1).

Figure 1. Percentage of normal seedling in each lot separated by categories identified by x -ray analysis of *B. longfolia* seeds



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For *B. longifolia*, lot 2 (mature pods and yellow seeds), showed better physiological potential, resulting in higher percentage of germination and normal seedlings, most being found mostly in category 1.

Competing interests

The author declares that they have no competing interests.

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Genetic identification of maternal and progeny generation of Scots pine (*Pinus sylvestris L.*)

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Key words (max 4): nSSR markers, genetic differentiation

Background
Most of renevals in Polish forests are introduced in an artificial growing way by planting earlier cultivated material in forest nurseries. Investments in infrastructure of highly specialized nurseries are planned, what will meet the demand for high quality planting material. We should bear in mind that possibly rich gene pool of prepared offspring guarantees stable and balancded development of forest ecosystems and conservation of biological diversity.

Methods
The proposed research based on nuclear DNA variation of Scots pine seedlings allowed the recognition of the genetic structure of the maternal trees in relation to the gene pool of the progeny generation produced in nurseries by two ways i) traditional in the fields of production with an uncovered root system, and ii) under controlled conditions in containers with covered root system.
Genetic structure of the maternal and progeny populations was determined by polymorphism analysis of microsatellite sequences of nuclear DNA (nSSR) using three loci SPAG 7.14, SPAC 11.6, SPAC 12.5.
The experiment was set in the Forest District of Olsztynek and Oleszyce because of close location of the experimental units, i.e. traditional and container-based nurseries.

Results and Conclusions
All (maternal and progeny) populations from Olsztynek and Oleszyce are of similar, relatively low genetic diversity at the nuclear DNA level. There has been an increase in the gene pool by 7.8% and 7.1% in terms of nuclear DNA in a whole descendance generation compared to the parental generation in both Forest Districts. Considering the diversity and size of genetic variability within the progeny population examined removably in relation to the maternal generation, the culture of cuttings in containers have a larger gene pool and are more varied than traditionally bred plants, both in populations from Forest District of Olsztynek and Oleszyce. The study group of container bred seedlings in Olsztynek and Oleszyce were enriched by 8.1% and 7.4%, respectively, new genotypes in relation to the maternal gene pool. While the seedlings grown in a traditionnal manner were characterized by 7,6% and 7,1% enrichment of gene pool, respectively.
This investigation confirmed the main thesis of the study that culture of seedlings grown in containers are more genetically diversified and have richer gene pool than seedlings grown in the traditional way. It should be concluded that the differences in the genetic structure of the progeny generation are caused by different conditions of germination, growth and nursing. Greater influence of natural selection takes place in the population of seedlings

grown in the fields of traditional nursery production. The decrease of diversity of the gene pool is the result of adaptation of the population to the prevailing habitat conditions by aliminating “weak” genes.

Competing interests
We declares that we have no competing interests.

Acknowledgements
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Extensive clonal structure and moderate genetic diversity at peripheral populations of *Sorbus torminalis* (L.) Crantz, a scattered, self-incompatible tree species.

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Key words (max 4): clonality, spatial genetic structure, microsatellites,

Background

Wild-service tree (*Sorbus torminalis* [L.] Crantz) is a temperate, insect pollinated tree species with fleshy fruits being dispersed by birds or small mammals. It is relatively common in Southern Europe; however, in Poland the species grows in rare, scattered populations attaining north-eastern species distribution limits. It is considered endangered and therefore being strictly protected. Peripheral populations of such scattered tree species are often expected to experience reduced genetic diversity which might limit their adaptive potential and further spread beyond the current species boundaries, which seems possible in the context of climate change.

Here, we were investigated genetic diversity and its spatial distribution in a peripheral population of *S. torminalis*. Because the species is known to be capable of vegetative reproduction we were also interested to find any evidence of clonal structure.

Methods

Leaf samples were collected from all known adult individuals (172 geo-referenced stems) of *S. torminalis* growing in 5 sub-populations in the Forest District Jamy, Poland. Other nearest populations are located 25 km N-NW. We genotyped all individuals using 13 nuclear SSR loci amplified in two PCR-multiplexes. Besides standard measures of genetic diversity we investigated genetic substructure among sub-populations using STRUCTURE and BAPS software, and spatial genetic structure (SGS) within sub-populations using SPAGEDI.

Results

Among 172 sampled trees, we found only 100 unique multi-locus lineages (MLL), which suggests the existence of 100 individual genotypes. Replicates within the same MMLs were considered ramets of the same clone, however examples of somaclonal variation within clones have been observed. The number of ramets within clones varied from 2 to 17, with the furthest distance between ramets of the same clone ranging from 13 to 21 meters, suggesting that the observed clonal structure results from regeneration based on root suckers.

Microsatellite loci used in this study indicated none or insignificant levels of *null* alleles. Genetic diversity was found to be fairly high (H_e=0.756), with a mean number of alleles per locus equal to 10 (A_e=4.94). The whole population considered as a set of 100 MLLs was found to be in Hardy-Weinberg equilibrium and there were no signatures of inbreeding (F_{IS} = -0.029), which might be expected for self-incompatible plant species.

STRUCTURE and BAPS identified 3 distinct clusters, and the two methods were highly concordant. Regardless of clonal structure, significant SGS was detected within subpopulations based on MLLs. Pairwise-kinship coefficient was significant for individuals

separated by as far as 1000 meters. Average kinship coefficient in the first distance class was high and found to be $f^{(1)}$=0.181 (0.048), while the intensity of SGS was equal to S_p=0.0269. The effective neighborhood size was rather small (N_b= 33.78), but was comparable to the mean census size of sub-populations.

Conclusions

The studied population exhibited fairly high levels of genetic diversity, probably promoted by self-incompatibility system. Nevertheless, if the studied population is expected to be utilized as a seed source for *ex-situ* or *in-situ* conservation purposes, the existence of clonal structure has to be taken into account in order to avoid excessive sampling of particular genets represented by larger number of ramets. Detected spatial genetic structure also suggest sampling from widely spaced individuals.

Competing interests

The authors declare that they have no competing interests.

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Genotypic characterization of cultivated and wild *Prunus cerasus* var. *austera* from Central Italy

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Key words: Prunus cerasus var*. austera*, genetic diversity, SSRs

Background

Prunus cerasus var. *austera* (var. *austera*) is an allotetraploid species (AAFF, 2n=4x=32), thought to result from natural hybridization between *P. avium* and *P. fruticosa* [1]. In central Italy, this sour cherry tree is traditionally cultivated for its fruits as small stands or scattered plants. The cultivated plant material is usually of unknown genetic origin. Genotypic characterization of var. *austera* germplasm provide information for germplasm management to optimize the conservation of diversity and also for use by breeders in developing improved cultivars. The objective of this study was to provide a genetic characterization of the var. *austera* germplasm from Umbria region using microsatellite markers (SSRs).

Methods

Fourteen nuclear polymorphic SSRs, developed for *Prunus* L. sp, were selected from literature [2]. A total of 161 plants of sour cherry (*P. cerasus* L.), morphologically identified as var. *austera*, were collected in Umbria region (Fig. 1). One individual of *P. cerasus* var. *caproniana* (sour black cherry, var. *caproniana*) was added as control. Total genomic DNA was isolated from leaves using the DNeasy 96 Plant Kit (Qiagen) according the manufacturer's instructions. Multiplex PCR was performed following the Qiagen Type-it kit protocol and PCR products were separated using an ABI3130 Genetic Analyzer (Applied Biosystem). The alleles were scored using GeneMapper program (Applied Biosystem). Statistical analyses were performed to calculate the Hardy-Weinberg expected heterozygosity and the Shannon-Wiener diversity index with ATetra program [3] specific for tetraploid species. All the SSR alleles were coded as dominant markers for the estimation of the genetic diversity parameters by GenAlEx 6.5 [4,5] and STRUCTURE 2.3.4 [6,7] programs.

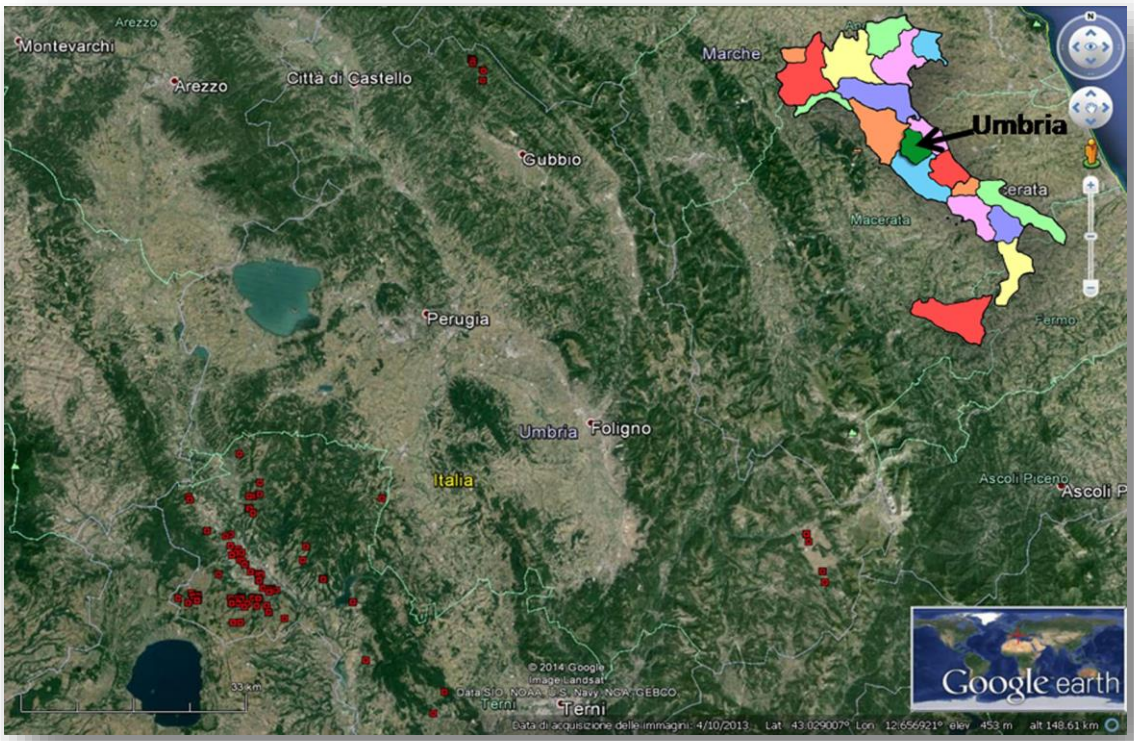

Figure 1: Sampling sites in Umbria Region

Results and Conclusions

All the primer pairs used in the analysis were polymorphic and gave satisfactory amplification products. The number of alleles per locus ranged from 4 (EMPA003) to 24 (PceGA34), with an average of 9.57 alleles per SSR. The expected heterozygosity (He) varied between 0.52 to 0.83, with the highest value corresponding to PceGA34 SSR. Shannon-Wiener diversity index (H') ranged from 0.86 to 2.23 (Table 1).

Table 1: Description and genetic diversity parameters of the 14 SSR loci analyzed.

Locus	Repeat motif	No of Alleles	Size Range (bp)	He	H'
EMPaS01	$(GA)_9(GA)_{11}$	12	217-242	0.69499	1.48027
EMPaS02	$(TTG)_7$ctgc$(TG)_{10}(AG)_8$	10	127-149	0.54576	1.11843
EMPaS06	$(CT)_{12}$	10	191-223	0.52946	0.95767
EMPaS10	$(GA)_{28}$	10	150-192	0.69617	1.48771
EMPaS12	$(TG)_{10}$a$(GA)_{10}$aa$(GA)_{13}$	8	102-146	0.67103	1.17493
EMPaS14	$(TG)_{10}$ccat$(TC)_5$ccat$(TC)_8$	9	168-214	0.67351	1.25185
EMPA002	$(AG)_{13}$	8	105-139	0.6782	1.22726
EMPA003	$(AC)_8$	4	168-179	0.75281	1.38629
EMPA017	$(AG)_{19}$	8	143-245	0.76109	1.49843
UDP96_005	$(AC)_{16}TG(CT)_2CA(CT)_{11}$	5	94-125	0.53588	0.87398
UDP97_402	$(AG)_{17}$	8	113-142	0.67998	1.31065
UDP98_412	$(AG)_{28}$	11	93-128	0.76866	1.72777
PceGA34	-	24	129-181	0.83103	2.23316
PS12A02	-	7	147-171	0.5248	0.86211
All Loci	-	-	-	0.66738	1.32789

He, expected heterozygosity; H', Shannon-Wiener diversity index; bp, base pairs

A first analysis with GenAlEx 6.5 allowed to identify 76 unique genotypes, 52 of them were represented by unique individuals, while the remaining 24 included from 2 to 29 individuals. Sampling of clone individuals geographically close is likely due to vegetative propagation of *P. cerasus*, while the presence of clones with a high number of individuals spread over the region could suggest the dissemination of some genotypes by the farmers. A second analysis with GenAlEx 6.5 and STRUCTURE 2.3.4 was carried out keeping only the unique genotypes. Despite the morphological assignment of all plants to var. *austera*, the genetic results showed six individuals belonging to var. *caproniana* (assignment probability above 0.9), as the control sample, and an hybrid between var. *caproniana* and var. *austera* (assignment probability of 0.64). Therefore, SSRs were efficient to determine the variety of plants which were difficult to distinguish morphologically. Finally, the principal coordinates analysis based on genetic distance of only the var. *austera* genotypes (GenAlEx 6.5) showed that most plants form a main genetic cluster, whereas only few individuals were genetically separated from it (Fig. 2). These results suggest that var. *austera* plants in Umbria share a common genetic background but maintain a relatively high genetic diversity within them.

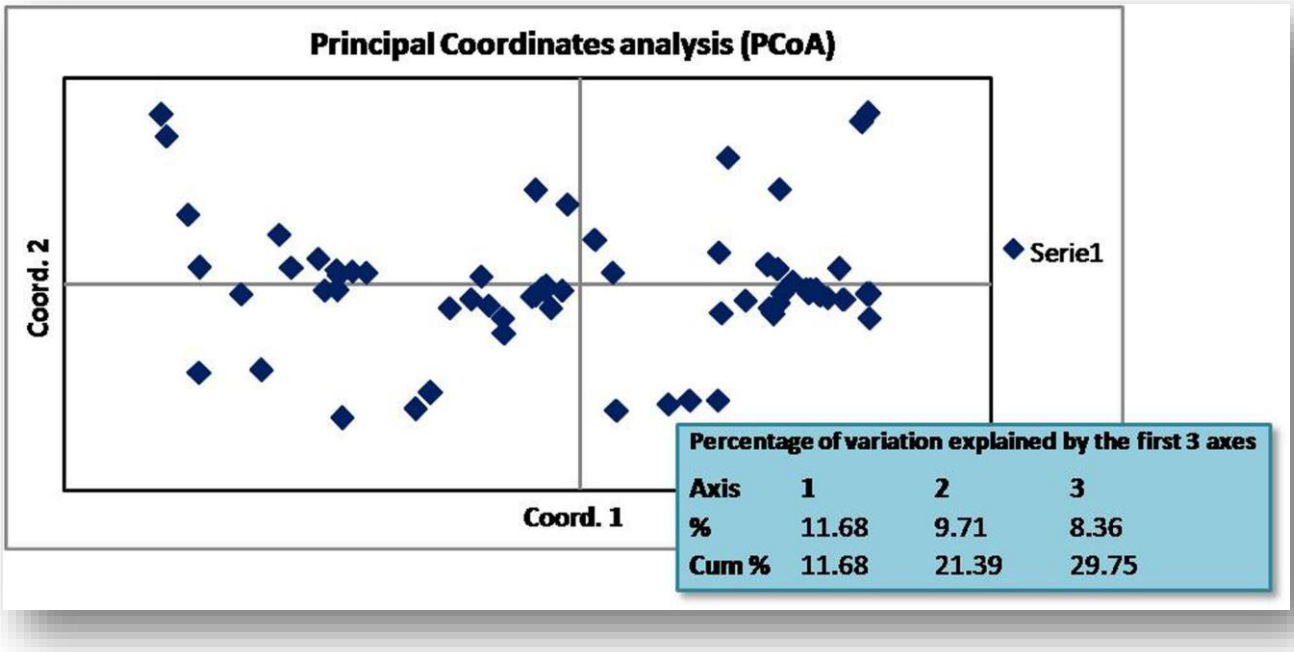

Figure 2: Principal Coordinate Analysis of the *Prunus cerasus* var. *austera* based on genetic distance.

The SSR used in this study allowed the discrimination between the *austera* and *caproniana* varieties of *P. cerasus* and the evaluation of the genetic diversity of var. *austera.* The analyzed plants from the Umbria region present an interesting level of genetic diversity. These results are a promising starting point for future conservation, management and breeding programs of var. *austera* genetics resource in Umbria region.

Competing interests

The authors declare that they have no competing interests.

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Nuclear and chloroplast SNP markers support successful poplar breeding

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Key words: Poplar species, chloroplast, nuclear, SNP markers

Background

Within the genus *Populus,* several species belonging to different sections are cross-compatible. Hence, a high number of interspecies-hybrids occurs naturally and, additionally, has been artificially produced in huge breeding programs during the last 100 years, because high growth rates and a broad range of applicability from wood and paper to energy production, led to a widespread cultivation in Europe and North America [1].
However, determination of the single poplar species by morphological characters is often difficult and represents a great challenge for the use of molecular markers for species identification. But, breeding activities for the registration of new high performing clones necessitate unambiguous species identification. Thanks to the 'barcode of life' initiative [2], several chloroplast (cp) primers are available that already have been used for differentiation of a broad range of plant species [3]. We already identified a couple of species-specific cp SNP-markers for differentiation of 14 most common cultivated poplar species [4, 5]. But, using cp markers, at least we can only identify the maternal part of a crossing. Thus, for identification of hybrids, we actually need nuclear SNP markers. Therefore, the aim of this study was to evaluate nuclear regions for their efficacy to differentiate poplar species and track the genealogy of hybrids.

Methods

Eight nuclear regions have been tested for amplification products in a gradient PCR using annealing temperatures between 53°C and 59°C with several self-designed primer combinations. Promising PCR products have been sequenced. Obtained sequences were aligned and screened for presence of SNPs by using either the software SeqMan 7.1.0 from DNAStar (Lasergene, Madison, WI 53705, USA) or Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, USA). The sequences around the SNPs were checked for restriction sites using the software NEBcutter V2.0 from New England BioLabs Inc (Ipswich, USA).

Results and Conclusions

Four of the eight initially used nuclear regions resulted in promising PCR products. These were the genes gibberellin oxidase (GA20ox), KNOX3, TB1, and LEAFY. From 14 species at least three individuals have been sequenced using these four nuclear regions and were checked for species-specific SNPs and InDels. In total we identified between four and 44 SNPs and between zero and 31 one InDels for each species. Of these, up to six SNPs or InDels per species were species-specific. For only two species, we found no species-specific SNPs and InDels. But, combining seven SNP and InDel markers in a logical progression allows the differentiation of all species. Sometimes, only a small number of individuals per species were available, thus, the investigation of more individuals is necessary. Furthermore, in some cases differentiation of species based on only three SNPs or InDels out of about 150 SNPs/InDels in total. In these cases, the species status might be doubtful.
Especially interspecies-hybrids are well suited for biomass production because of their superior growth and advanced resistance traits. Chloroplasts are maternally inherited in

angiosperms, thus, species-specific cp markers give us the possibility to identify the direction of a crossing. So, the combination of the above described nuclear markers with the earlier identified cp markers [4, 5] leading to the possibility to track the genealogy of hybrids (Fig. 1).

	Chloroplast 1	**Nuclear marker 1**
max (AA) x tri (TT)	A	AT
Possible parents	♀**:** A x ♂**:** T	AA x TT / AT x AT / AT x TT / AA x AT
	Chloroplast 2	**Nuclear marker 2**
max (GG) x tri (CC)	G	GG
Possible parents	♀: G x ♂: C	GC x GC / GG x GC / ~~GC x CC~~ / ~~GG x CC~~

Fig. 1: Example for tracking of the genealogy of a two-species hybrid assumed to be composed of the two species *P. maximowiczii* (max) and *P. trichocarpa* (tri).
The chloroplast markers identified max as mother. Using only nuclear marker 1, no conclusion about parents was possible. Adding chloroplast marker 2 confirmed max as mother. The nuclear marker 2 indicated that at least one of the parents was no pure species, but a hybrid. Thus, the investigated hybrid is probably 'the result of a back-cross. The inclusion of a third/fourth nuclear marker will further unravel the genetic constitution of the parents.

Competing interests
The authors declare that they have no competing interests.

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Cross-species transferability of EST-based SSRs and nuclear SSRs to Mexican white pines.

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Key words: Cross-species transferability; Mexican white pines; Nuclear microsatellite markers.

Background

Mexican white pines (*Pinus chiapensis* (Mart.) Andresen, *P. strobiformis* Engelm., *P. ayacahuite* Ehrenb. Ex Schltdl, and *P. ayacahuite* var. *veitchii* (Roezl) G.R. Shaw, and *P. lambertiana* D.Douglas) represent a valuable natural resource because they play an essential role in ecological processes and are an important source of timber and cellulose [1]. Despite the ecological and economic importance of these Mexican white pines, very few studies have been conducted regarding their genetic diversity and population structure, which would facilitate the conservation and sustainable management of their genetic resources.

Microsatellites or simple sequence repeats (SSRs) provide a rich source of highly informative codominant genetic markers that are suitable for a variety of genetics and breeding studies and applications. Recently, next-generation sequencing of genomic DNA allows the identification of large numbers of microsatellites, however, all of the approaches require library construction and sequencing efforts, which are still not affordable or feasible in laboratories with scarce resources. In such cases, cross-species transferability of microsatellite markers between phylogenetically close species could serve as an adequate interim strategy, particularly when the objective is only to find a limited number of markers to conduct population and conservation genetic studies [2].

The objective of this study was to examine how informative and how transferable are 11 nuclear SSRs developed in *Pinus cembra* L and *P. edulis* and 8 SSR loci from transciptome sequencing developed *in P. contorta* to Mexican white pine species and their informativeness.

Methods

DNA was extracted from a minimum of seven individuals of each species using the CTAB method with minor modifications. DNA quality and concentration were determined visually on agarose gels and by UV spectrophotometry. DNA samples were diluted in MilliQ water to a final concentration of 40 ng/µL. We selected a subset of 8 EST-based SSRs (Pico_61 and 65_contig; Pico_100, 101, 120, 128, 129, 153 and 158_singleton) that contained perfect tetra-nucleotide repeats tested for successful amplification of *P. flexilis* [3]. Also, All eight nSSR markers (Pc 1b, 3, 7, 18, 22, 23, 25 and 35) published for *P. cembra* were tested [4] and we selected three nSSR markers (CAT39, GGT19 and GGT54) designed for *P. edulis* [5]. All 19 SSR loci were amplified using both the Multiplex Master Mix (Qiagen) according to the manufacturer's instruction and the PCR conditions described. For each reaction, we scored results as positive or negative for amplification when PCR resulted in a product in

the expected size range, as detected on 1.5% agarose gels, but also recorded markers that resulted in more than one discrete PCR product. Markers that amplified cleanly at or near the expected size were further characterized to detect size variations among seven individuals in each pine especies. PCR products were separated on 8 % polyacrylamide gel and the alleles at a SSR locus were determined by scoring the PAGE gels with Gel Login 100, Kodak and the Phoretix 1D&10 program (Totallab Ltd) and verified manually.

Results and Conclusions (these last two either separate of together)

We tested 19 SSRs across four species in the genus *Pinus* that have not previously benefitted from thorough marker development. The primers of 11 SSR loci produced amplification products in the expected size range: Pico_101 and 129_singleton; Pc 1B, 3, 18, 22, 23, 25 and 35; CAT39 and GGT54). A marker was deemed suitable when a single locus amplified efficiently at the expected size and with a minimum of stutter bands when assayed on silver-stained polyacrylamide gel. Until now, we amplified four locus: Pico_101, Pc 18, 22 and Pc 23 across seven individuals in each of the above species and determined numbers of alleles (3, 2, 4 and 4, respectively) and allelic size (163-155, 150-148, 390-354 and 225-205 bp, respectively), the results of the rest SSR loci will be present in the meeting. Our results highlight the value of cross-species transferability of EST-based and nuclear SSR SSR markers provide a valuable resource for population genetic studies in *Pinus*.

Competing interests

The author declares that they have no competing interests.

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PROTOPLAST ISOLATION FROM LEAF TISSUES OF HOLM OAK, A NEW TOOL FOR THE STUDY OF DNA INTEGRITY

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Key words: holm oak, enzymatic protoplast isolation, Comet assay

Background

Holm oak (*Quercus ilex* L.) is a sclerophyll tree with ornamental value. For this reason, this species was widely used for the establishment of gardens and parks around historical villas in Italy and today accounts for 9% of the trees in its urban areas. Taking advantage from structural and morpho-physiological traits, holm oak is well adapted to global warming conditions and to elevated concentration of tropospheric ozone, which is a serious problem in urban environment. Nevertheless, in this species a quantitative method for the evaluation of cell injury due to oxidative stress is still lacking. The Single Cell Gel Electrophoresis assay (SCGE), also known as Comet Assay, could be considered a good estimator of DNA damage in individual eukaryotic cell. This method has been mainly employed in animal tissue, because the plant cell wall represents an obstacle for the extraction of nucleus. Therefore the application in plant species is sporadic and often limited to root tissues. To our best knowledge, no data exists for sclerophyll leaf tissues, which are very difficult material due to the presence of sclerenchyma. The establishment of a reliable protocol for the isolation of protoplasts from mature holm oak leaf tissues as well as checking their suitability for the Comet assay are the aims of the present work.

Methods

Mechanical, chemical and enzymatic methods [1-3] were compared in order to fix the best method for protoplast isolation from holm oak leaf tissues. Seedlings in controlled environment and plants selected in the city of Viterbo were considered. Fresh and frozen leaf tissues of different ages (from 2-3 months to 1 year) were tested in comparison with model plants (*Vicia faba*, *Tamarix africana* and *Cydonia oblonga*) after a sterilization procedure. The removal of trichomes from abaxial side of the leaf was necessary in order to avoid an interference with Comet assay and a specific method was set up using liquid nitrogen and dry ice powder. The Comet assay was performed according to Tice [4]. Isolated protoplasts were suspended in 5 µl of 1X phosphate buffered saline (PBS) and kept on ice during subsequent samplings. After cell lysis, electrophoresis was conducted at 25 V and 300 mA for 20 min at 4 °C. Nucleoids were stained with ethidium bromide (20 µg/ml) (Sigma, Italy) and examined at 400x magnification with an automatic image analyser (Comet Assay III, Perceptive Instruments, UK) connected to a fluorescence microscope (Axioskop 2, Zeiss, Germany). To evaluate the yield of DNA damage, computer generated tail moment (TM) values and percentages of DNA damage, such as tail length (TL) and tail intensity (TI), were used.

Results and Conclusions

The best results in terms of number and quality of isolated protoplasts (Figure 1) were obtained using an enzymatic solution modified from Wakita et al. [3], with Cellulase “Onozuka” RS (2%) and Macerozyme (1%) in the presence of antibiotics. The digestion of leaf tissues were carried out at 30 °C for 48h under gentle rotation in dark conditions after a vacuum infiltration period of 30 min. Comet assay results revealed that the isolated protoplasts from mature leaf tissues of holm oak could be handled for this technique. In addition, it was not observed any symptom of DNA damage, with every extraction method used (Figure 2). To our best knowledge, this is the first time that a reliable protocol was provided for the isolation of protoplasts from mature leaf tissues of holm oak. In conclusion, the isolated protoplasts could be suitable for the estimation of DNA damage in plant subjected to environmental oxidative stresses, as well as for the establishment of in vitro protoplast culture and the study of plasma membrane functionality of this important tree species.

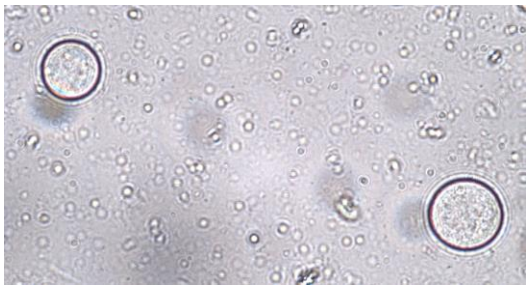

Figure 1: isolated protoplasts from holm oak mature leaf tissues using the reported enzymatic method.

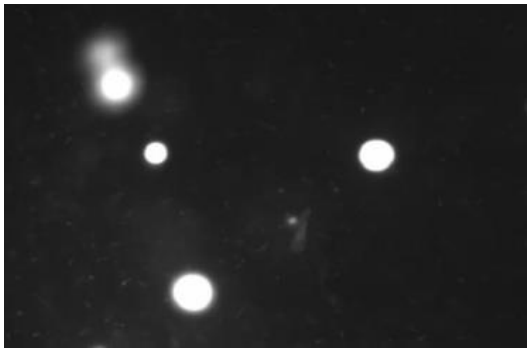

Figure 2: Nucleoids of holm oak stained with ethidium bromide at fluorescence microscope (400x magnification).

Competing interests

The author declares that they have no competing interests.

Acknowledgements

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Genetic structure and diversity of natural black poplar (*Populus nigra* L.) populations along three rivers in Slovenia and Croatia

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Key words: Populus nigra, genetic structure, genetic diversity, Slovenia and Croatia

Background

The European black poplar (*Populus nigra* L.) is one of the most important tree species of alluvial forests in Europe. In Slovenia and northern Croatia, ecosystems, where black poplar is the keystone species, are fragmented and endangered mostly because of river regulations, pollution, urbanisation and felling of vital trees for wood. Black poplar is also an indicator species for the vitality and intactness of alluvial ecosystems. To inventory genetic diversity and structure of the remaining natural populations, a study using nuclear microsatellites was conducted.

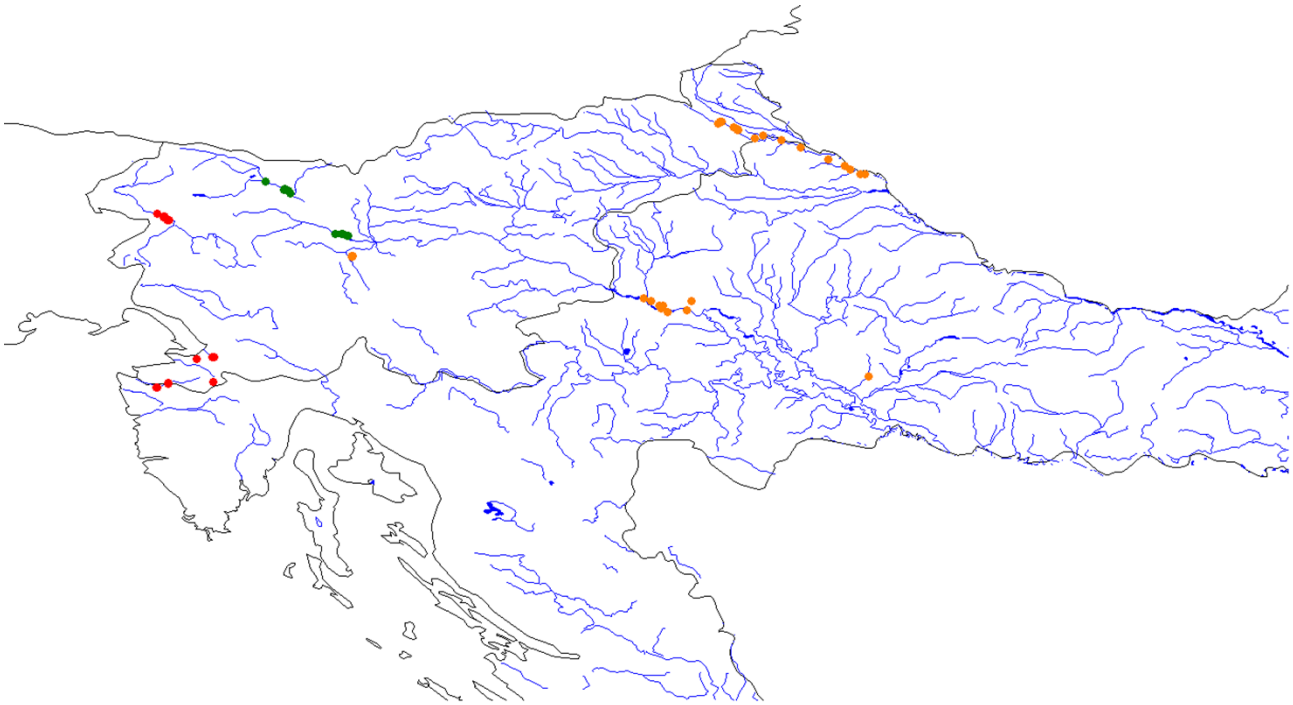
Methods

We analysed genetic diversity and structure of 10 black poplar populations (200 trees) along the main river systems across Slovenia and Croatia (rivers Soča, Sava and Mura) and in the submediterranean using 6 microsatellite loci (WPMS16, WPMS20, WPMS14, PMGC14, WPMS09, WPMS18). Eleven common cultivated clones of *Populus* x *canadensis* were also included in the analysis in order to assess the level of introgression of genes of the American Eastern cottonwood, *P. deltoides,* into the gene pool of native black poplar. Standard methods using programmes GenAlEx, SpaGeDi and Structure were used to obtain estimates of genetic diversity and genetic structure including presence of hybrids.

Results and Conclusions

Three genepools were observed for our dataset, mainly following the river systems (Soča, Mura, Sava with two genepools; Figure 1). No clear accumulation of allelic richness nor gene diversity downstream neither for Sava nor Mura was observed. In natural populations, the introgression of *P. deltoides* genes was very low. The results indicate that the gene pool of the remaining *P. nigra* populations maintains high genetic connectivity along rivers even if fragmented today. The obtained results can be used to further develop dynamic *P. nigra* conservation in endangered forest ecosystems at regional and national levels.

Figure 1: Structure genepools of sampled populations in Slovenia and Croatia denoted by different colours. Populations belonging to the same association stretched along rivers; therefore more than one circle can represent the entire population. Submediterranean populations were pooled due to small sample sizes.



Competing interests

The author declares that they have no competing interests.

Acknowledgements

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Population structure of *Eucalyptus cladocalyx* based on SSR marker analysis

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Key words: genetic structure, Bayesian clustering, microsatellite markers

Background

Eucalyptus cladocalyx F. Muell occurs naturally in a restricted natural range in South Australia: Kangaroo Island, the Eyre Peninsula and the South Flinders Ranges are the three disjunct regions of groups of subpopulations [1]. This specie is capable of growing under relatively dry environments and is grown for bioenergy, shelter belts, sawn timber, naturally durable posts and apiary [1,2]. Given that about 41 percent of the world's land area can be classified as drylands [3] it is clear that there is potential for more extensive use of this species [1,2].

Major selection criteria of *Eucalyptus* breeding programs include productivity and quality wood. Surprisingly, much less attention has been paid so far on drought stress tolerance. Analysis of the amount and distribution of genetic variation within and among populations of a species can increase our understanding of the historical process underlying genetic diversity and provide basic information for breeding programs [4].

DNA marker technology is considered as a cost-effective method that facilitates population genetics studies and would contribute to accelerate breeding programs. In this context, the simple sequence repeat (SSR) markers have been widely used to determine the genetic structure of several species. This study was focused on determine the population structure of *Eucalyptus cladocalyx* in a germplasm collection introduced to Chile from Australia based on SSR marker analysis.

Methods

The patter of population structure was inferred using 91 SSR primer pairs developed by Brondani et al. [5] in the software STRUCTURE [6]. A provenance-progeny trial established in the northern Chile was used as plant material. The sample material consisted of 47 families from five Australian localities and 2 from a local seed source (Illapel, Choapa Province). The Australian trees are from Flinders Chase National Park in the Kangaroo Island; Marble Range and Cowell in the Eyre Peninsula; Mt. Remarkable and Wirrabara State Forest in the Flinders Ranges. A total of 98 trees were sampled in the trial (two trees per family). Posterior probabilities were estimated using the Markov Chain Monte Carlo (MCMC) method. The length of the burn-in period and number of MCMC samples after burn-in were set to 5,000 and 50,000, respectively. Twenty runs were performed by setting K (number of subpopulations) from 1 to 12. The model developed by Evanno et al. [7] was used to determinate the most likely K value. The Bayesian-point estimation and 95% credible intervals of population differentiation (F_{ST}) were calculated using R.

Results

The most probable number of subpopulations in the sample was three (K = 3) estimated based on 91 neutral SSR markers (Figure 1). Cluster 1 contained 44 trees, over

43% of which were collected from Mount Remarkable. Cluster 2 contained 30 trees (30.6 %) of which 12, 10, 5, 2 and 1 are from Mount Remarkable, Cowell, Marble Range, Wirrabara and Illapel, respectively. Cluster 3 contained 24 individuals, over 54 % of which were collected from Flinders Chase.

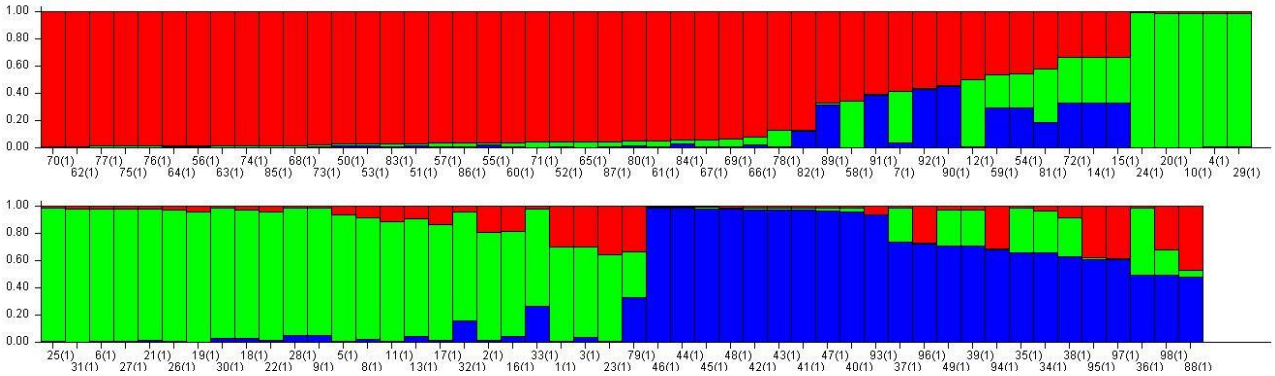

Figure 1 Estimation of subpopulations in a sample of *E. cladocalyx* without prior classification information. The estimated membership probability (Q) for each individual is shown in the vertical axis.

The levels of genetic differentiation (F_{ST} values) are shown in Table 1. Cluster 3 (0.184) showed the highest F_{ST} value, followed by cluster 1 (0.116) and Cluster 2 (0.072). For all cases, Bayesian credible intervals of the F_{ST} confirmed the existence of a significant genetic divergence among clusters (Table 1). This result is in accordance with the Neighbor-joining trees constructed at individual and population level (data not shown).

Table 1 Bayesian point estimation and 95 % credible intervals (CI) of the genetic differentiation (F_{ST}) calculated in each group genetically differentiated of *E. Cladocalyx*

Cluster	N	F_{ST}					
		Mean	Median	Mode*	SD	CI (95%)	
						2.5	97.5
I	44	0.117	0.116	0.117	0.016	0.088	0.150
II	30	0.072	0.071	0.072	0.015	0.045	0.103
III	24	0.184	0.184	0.184	0.025	0.137	0.235

*Kernel density estimates of the marginal posterior distributions. N: numbers of individuals

Conclusions

Three homogenous genetic groups were identified, confirming that the trees of *Eucalyptus cladocalyx* from natural forest are highly and significantly structured. Individuals from Kangaroo Island were the most genetically distant according to phenograms and the Bayesian clustering analysis. In addition, individuals from the local seed source (Illapel) were clustered with individuals from Flinders Chase, indicating their possible origin.

Competing interests

The author declares that they have no competing interests.

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Range-wide analysis of genetic variation of *Pinus mugo* Turra using chloroplast microsatellites

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Key words: dwarf mountain pine; divergence; genetic structure; cpSSR

Background

Pinus mugo Turra (dwarf mountain pine) belongs to the *Pinus mugo* complex – a highly polymorphic European group of closely related pines of the *Sylvestres* subsection. *P. mugo*, common in the Eastern Alps and in the Carpathian Mountains, constitutes a very important component of montane regions since it prevents soil erosion and avalanches. The genetic background of the species belonging to *P. mugo* complex is very similar which indicates that they share the same evolutionary history and have diverged only recently (Wachowiak et al. 2013). It is believed that the divergence of the *P. mugo* species began in the Pliocene when they split into single Pleistocene refugia (Christensen 1987). What is more, it is very likely that the members of the *P. mugo* complex moved to higher altitudes during warmer interglacials, as they do now due to climate warming. Such retreats may have also constituted an important factor in shaping their present genetic structure (Heuertz et al. 2010). Here, we present the genetic structure of *P. mugo* populations within the natural range of the species using chloroplast microsatellites.

Methods

We genotyped 553 *P. mugo* individuals from 21 native populations using 13 paternally inherited chloroplast microsatellites (Vendramin et al. 1996; Provan et al. 1998) in two multiplex PCRs. The populations studied represent the natural distribution range of *P. mugo* as well as its putative refugia from the Alps, Carpathians and Balkans. Genetic diversity parameters were calculated in GenAlEx ver 6.5 (Peakall and Smouse 2012), Arlequin ver 3.5 (Excoffier et al. 2005) and Haplotype Analysis ver 1.05 (Eliades and Eliades 2009). Additionally, the genetic structure of the populations was estimated with Geneland (Guillot et al. 2005) and BAPS ver 6 (Corander et al. 2008).

Results and Conclusions

Populations of *P. mugo* showed high genetic diversity, accompanied by substantial intrapopulation differentiation. We detected 3 to 14 alleles for each locus that together formed 311 haplotypes, out of which 201 (65%) were private. In contrast, differentiation among populations and mountain ranges was low. These results suggest high homogenizing effect of gene flow by pollen. Nevertheless, nearly all pairwise F_{ST} values

were significantly higher than zero, which confirmed the presence of slight population structure.
Populations from the Giant Mountains were highly homogenous, whereas the southeastern-most peripheral and one isolated population from Italy were clearly separated from the others, especially from populations located in the Carpathians and in the Balkan region. The population from Monte Baldo in Italy displayed the lowest values for all calculated genetic diversity parameters. This finding is particularly important for the management of genetic resources of *P.mugo* and conservation strategies in the face of ongoing climate changes.

Competing interests
The author declares that they have no competing interests.

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Sub-structuring of Scots pine across European distribution range based on *cp*DNA SSR markers.

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Key words: Pinus sylvestris, CpSSR microsatellites, population structure, biodiversity

Background
Scots pine (*Pinus sylvestris* L.) is one of the most ecologically and economically important forest-forming tree species in the Northern Hemisphere which has the widest natural distribution range of all conifers. Currently the economic, historical, scientific and recreation importance of the species woodlands has increased and the conservation of native pinewoods has focused both public and scientific interest. Better understanding of the genetic relationships between populations and the genetic basis of population phenotypic and adaptive variation is important in the face of the predicted environmental change (Savolainen et al. 2007, Neale and Kremer 2011). Knowledge about the genetic structure can advance rational and sustainable conservation of populations providing background for adaptive variation studies. In the presented study we investigate the levels of chloroplast simple-sequence repeats (SSRs) variation and relationships between populations in the European distribution of the species.

Methods
Genetic variation within and between 24 native populations (676 individuals) of Scots pine representing the species European range was examined using the set of 13 polymorphic *cp*SSR loci (Vendramin et al. 1996; Provan et al. 1999). Genetic parameters for the loci, haplotype analysis and the genetic diversity within and among populations were estimated using GenAlEx v. 6 (Peakall and Smouse 2006), Haplotype Analysis v.1.05 (Eliades and Eliades 2009) and Arlequin software (Excoffier et al. 2005). The genetic relationships among populations where further analyzed using Geneland software (Guillot 2008).

Results and Conclusions
All *cp*SSR loci were polymorphic, and the number of alleles per locus ranged from 3 to 11. Private alleles have been detected in individuals from populations from Spain, Scotland and Turkey. Haplotype diversity was high He = 0,982. Large amount of genetic variation was found within populations of *P. sylvestris* while a relatively small portion of variation was distributed among the populations (Fst = 0.04). Low genetic structure is expected for highly outcrossing wind pollinated tree species, in which efficient gene flow has a homogenizing effect on the distribution of genetic variation across large geographical ranges (Provan et al.

1998, Robledo-Arnuncio et.al. 2005, Cheddadi et al. 2006). However, four subgroups could be identified in our dataset providing some evidence of heterogenous patterns of genetic differentiation across the studied distribution range.

Competing interests

The author declares that they have no competing interests.

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Does human-induced selection influence the spatial genetic structure diversity and dynamics in beech forests?

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Key words (max 4): Fagus sylvatica L., silviculture, genetic diversity, spatial genetic structure

Background
Forest ecosystems have an "internal capacity" to adapt to environmental disturbances thanks to their genetic variability. The Italian forests are the result of a millenary coevolution between ecological and socio-economic reality. Over the time, the cultivation of the forest has led sensitive modifications to biodiversity of the stands, resulting in a reduction of their complexity.
Therefore, it is interesting to examine the relationship between the silvicultural system and the genetic variability of the forest.

Methods
This study examined the effects of silvicultural systems on the genetic diversity in two beech high forest stands in the Apennines (central Italy). To this end, we did a comparison between a even-aged stand managed by the uniform shelterwood system (named Nature Reserve of “Pian degli Ontani”) and a uneven-aged stand managed with a type of single tree selection felling carried out according to traditional knowledge (named Baldo’s Forest).
All trees were genotyped using four primer pairs of nSSR loci (FS1-15, FS4-46, FS1-25 and FCM5).
General estimates of genetic diversity in the stands from nSSR data were calculated using SPAGeDi 1.3a. The spatial genetic structure of the stands was analyzed by STRUCTURE program and Geneland software.

Results and Conclusions
The comparison between the two stands showed no significant differences in genetic diversity measures, while significant differences were observed on the number of rare alleles.
However, the most significant differences between the two beech forests have been detected in the spatial structure of genetic diversity.
The even-aged stand of Nature Reserve of “Pian degli Ontani” presents a structure of random type, devoid of family groups, characterized by gene flows limited in space and with low recombination rates. In contrast, uneven-aged stand of Baldo’s Forest presents a spatial structure of genetic diversity grouped into families, with wide dispersion gene rates, high rates of recombination and a greater number of rare alleles.
The spatial structure of the genetic diversity found in the uneven-aged beech forest was similar to that observed in previous studies carried out in old-growth beech forests in the Apennines (see Paffetti et al., 2012 [1]).

The single tree selection felling, which is repeated at short intervals of time (every 5-6 years), seems to lead to a faster fragmentation of family groups, thus speeding up the evolutionary dynamics of the managed stand compared to the old-growth forest.

Competing interests

The author declares that they have no competing interests.

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Genotype by Environment Interaction in Radiata Pine for Growth and Biomass Traits in Chile

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Key words: Radiata pine, water stress, genotype by environment interaction, breeding

Background

Radiata pine (*Pinus radiata* D. Don) is one of the conifer species that is most commonly planted as an exotic throughout the world. The species was introduced in Chile in 1893 [1] and has been intensively used in forestation because of its fast growth. It now occupies 1.46 million hectares in Chile, with an annual increase in volume estimated at around 40 m^3 ha^{-1} $year^{-1}$ [2]. Genetic improvement of *P*. *radiata* in Chile has started in the 70's and has been directed towards characters such as volume, form and wood density; however, despite an increase in drought is expected [3], there is a lack of studies on phenotypic response of genotypes across different environments, i.e. genotype by environment interaction (GxE). GxE is of particular concern to tree breeders since it determines critical decisions in developing optimal breeding and deployment strategies [4]. The aim of the present study was to analyze, at the nursery level, the significant and practical importance of the genotype x water interactions in a random subset of the Chilean *P*. *radiata* breeding population.

Methods

Two *P*. *radiata* populations from different regions of Central Chile were selected for study, one interior (coming from sandy soils) and one coastal. From each population, seeds from 49 parents were obtained from plus trees in plantation in coastal and interior areas belonging to the company Forestal Mininco S.A. Seeds were sown in 0.140 L pots filled with a mixture of composted bark of radiata pine and perlite, and slow-release fertilizer. After germination, the seedlings from each family were arranged in a split-plot design with watering regime as the whole plot and family nested within population as the subplots. Two populations, 49 families per population, and two watering treatments were used, based on previous findings [5, 6]. Three 13-day cycles of withholding of water, plus 2 days of watering between each cycle, were applied in the water stress treatment. The two watering treatments, well-watered (WW) and water-stress (WS), were applied to three replicates of the 98 families with 11 seedlings of each family included per sub-plot (i.e., 6,468 seedlings in total). Total height (H) and root collar diameter (D) were measured, and three dry fractions roots (RDW), needles (NDW), and stems (SDW)) were weighed (±0.01 g). The following variables were subsequently derived from the measured variables: total dry weight (TDW=NDW+SDW+RDW), root-to-shoot ratio (RSR= RDW/SDW+NDW), and height-to-diameter ratio (HDR=H/D). Genetic correlations between two watering regimes, were calculated according to [7].

Results and Conclusions

Despite the small number of environments analyzed in this study, the genotype by environment interaction was statistically significant for all traits (Table 1), especially for H and RSR.

Table 1 Type B correlations and their standard errors between watering treatments

	D	H	HDR	NDW	SDW	RDW	TDW	RSR
Type B correlation	0.34±0.21	0.37±0.19	0.10±0.25	0.27±0.22	0.11±0.22	0.21±0.22	0.09±0.23	0.36±0.22

Genotype by environment interaction in radiata pine has been studied extensively (see references in [8]), and agrees with the results of our study. Similar results were found for young radiata pine seedlings in Chile and Spain where family by site interactions were significant for growth and biomass traits [5, 6, 9]. This high genotype by environment interaction found in early nursery tests could be a preliminary argument for regionalization of the radiata pine breeding programs in countries like Chile or Australia, which contain extensive areas of current or future low rainfall. In Chile a predicted increase in drought should result in a more Mediterranean climate [3], which could seriously affect the establishment and productivity of radiata pine. However, breeders have usually aimed to develop genotypes that are satisfactory for a wide range of conditions (i.e., low genotype by environment interaction in field trials). Results of the present study suggests that if the differences between genotypes regarding water availability conditions are pronounced, separate breeding programs for different soil water abailability might be needed. With such interactions, tree breeders must decide whether to select for stability of performance and accept a slower rate of improvement, or to develop populations specifically adapted to each environment in view to maximize gain.

Competing interests

The author declares that they have no competing interests.

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Genetic diversity in candidate genes for drought stress. photoperiod perception and cold tolerance along environmental gradients: evidence of climate-driven local adaptation in *Nothofagus* spp from Patagonia

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Key words: climatic gradients, candidate genes, Patagonian forests

Background

Water availability, temperature and photoperiod are factors of outmost relevance for the modulation of growth and development of tree species in their natural habitats. In view of the possible consequences of climate change, the understanding of the genetic basis underlying the trees' response to these factors is highly relevant.

In the Southern Hemisphere, temperate Patagonian forests are distributed along natural environmental gradients that might be imposing different selection pressures on the native flora. Temperature and photoperiod variations are mainly explained by latitude whereas precipitation regimes vary from west to east. Therefore, phenotypic variation in several plant traits is probably the consequence of divergent selection pressures along species´ distribution areas that might reflect local adaptation.

The aim of this work was to characterize the genetic variation at candidate genes in populations of *Nothofagus* species distributed along natural environmental gradients in the Patagonian Andes. In order to unravel possible climate-driven adaptation we studied patterns of variation in nucleotide sequences of genes potentially associated to (1) drought stress responses in contrasting populations of *Nothofagus nervosa* and *N. obliqua* along the precipitation gradient and (2) temperature and photoperiod perception in populations of *N. pumilio* along its large latitudinal distribution.

Methods

We sampled 13 populations of *N. nervosa* and *N. obliqua* covering the precipitation gradient (3500 mm/year to 1000 mm/year) in Lacar Lake (40ºS 71ºW; Fig. 1) and 20 populations of *N. pumilio* along the latitudinal gradient (36° S to 55º S; Fig 2).

We screened 3000 isotigs from the transcriptome database of *N. nervosa* [1] and designed primers for the selected sequences using Primer 3 (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). PCR fragments of selected candidate genes related to drought stress, photoperiod and temperatures response were re-sequenced and gene identity was confirmed using the blast search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Haplotypic (Hd) and nucleote (π) diversity, number of segregating sites (S), and number of haplotypes (H) were calculated using DNAsp (http://www.ub.edu/dnasp/). Tajima's D neutrality test [2] was performed at gene and population level. In order to separate demographic effects from selection individuals from all the populations were genotyped with nuclear microsatellites and population structure was evaluated using STRUCTURE. Populations with similar genetic structure but located at contrasting pluviometric regimes were selected for candidate gene analysis in *N. nervosa* and *N. obliqua*. In the case of *N. pumilio* SSRs revealed two main latitudinal groups [3], and therefore populations from both groups were selected.

Results and Conclusions

Precipitation gradient:

Genetic variation in drought stress candidate genes was mainly associated to differences between species, being *N. obliqua* more diverse than *N. nervosa* (Table 1). However, single nucleotide polymorphisms (SNPs) were also detected at the intra-species level with the presence of heterozygous individuals. Nucleotide and haplotypic diversity have different trends among populations in each species. While *N. nervosa* displays higher levels of diversity at the east for 6 out of 8 genes, *N. obliqua* is more balanced. Differences in gene sequences might be reflecting local adaptation at the species level since *N. obliqua* is proposed to be more tolerant to drought stress [4]. Departures from neutrality found in some genes and populations would support these results (Table 1).

Photoperiod and temperature gradient

We found a strong decrease in genetic diversity for the sequence of TOC1 gene towards the southern limit of *N. pumilio* distribution, which coincides with the region of extreme photoperiods (Hd=0.84 to Hd=0; π=0.0197 to π=0). On the other hand, we observed a slight decrease in nucleotide diversity for EMB and HSP90 towards the northern limit of the species distribution, which coincides with the warmest area of its range (EMB: North: π=0.00127 South: π= 0.02894; HSP90: North: π=0.00199 and South: π = 0.0023). Tajimas´ D was significant and negative in TOC. The reported genetic patterns for TOC1, EMB and HSP90 might be reflecting adaptive variation related to photoperiod and temperature responses across latitude. These trends in the genetic patterns are promising but preliminary, and we are enlarging the number of populations and genes to confirm these results.

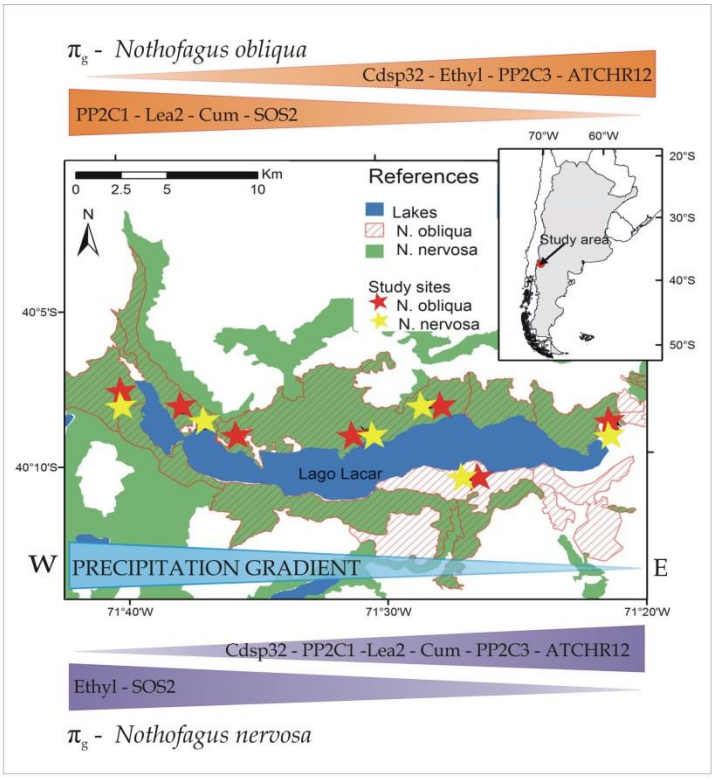

Figure 1: Sampled locations along the precipitation gradient and nucleotide diversity trends.

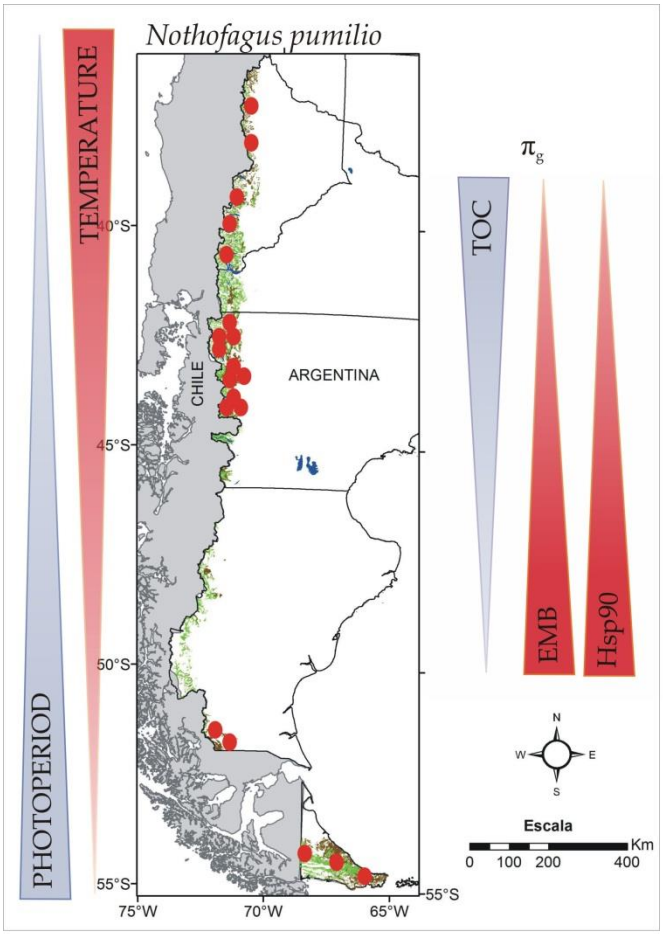

Figure 2: Sampled locations along the latitudinal gradient and nucleotide diversity trends.

Table 1: Haplotypic (Hd) and nucleotidic (π) diversity, number of segregating sites (S), haplotypes (H) and Tajima´s neutrality test (D) by gen and population.

		Total Diversity/Spp.						Total Diversity/Pop.			
CG	*Sp*	N	S	H	Hd (SD)	πt	D	Pop	Hd (SD)	πt	D
cdsp32	*Nn*	36	1	2	0.203 (0.08)	0.0009	-0.23 n.s.	W	0.189 (0.11)	0.0008	-0.59**
								E	0.233 (0.13)	0.0010	-0.45 n.s.
	No	44	5	4	0.555 (0.05)	0.0105	2.78 **	W	0.485 (0.06)	0.0105	2.28*
								E	0.636 (0.07)	0.0109	2.51*
PP2C-1	*Nn*	42	5	7	0.718 (0.06)	0.0025	0.83 n.s	W	0.652 (0.08)	0.0029	1.29 n.s.
								E	0.808 (0.07)	0.0027	0.29 n.s.
	No	46	3	4	0.441 (0.09)	0.0008	-0.60 n.s.	W	0.489 (0.11)	0.0009	-0.80 n.s.
								E	0.403 (0.13)	0.0007	-1.21 n.s.
Ethyl	*Nn*	46	2	3	0.204 (0.08)	0.0004	-0.98 n.s.	W	0.304 (0.12)	0.0006	-0.89 n.s.
								E	0.091 (0.08)	0.0002	-1.16 n.s.
	No	44	2	3	0.280 (0.08)	0.0006	-0.69 n.s.	W	0.159 (0.09)	0.0003	-0.69 n.s.
								E	0.416 (0.12)	0.0009	-0.53 n.s.
Lea2	*Nn*	32	9	9	0.643 (0.09)	0.002	-1.13 n.s.	W	0.632 (0.11)	0.0015	-1.54 n.s.
								E	0.667 (0.14)	0.0027	-0.66 n.s.
	No	36	4	6	0.673 (0.07)	0.0012	-0.28 n.s.	W	0.775 (0.07)	0.0015	-0.40 n.s.
								E	0.574 (0.12)	0.0010	-1.03 n.s.
Cum	*Nn*	38	4	5	0.649 (0.06)	0.0022	0.17 n.s.	W	0.568 (0.09)	0.002	1.59 n.s.
								E	0.680 (0.07)	0.0022	-0.27 n.s.
	No	38	2	3	0.494 (0.07)	0.0011	0.19 n.s.	W	0.595 (0.07)	0.0014	0.43 n.s.
								E	0.366 (0.11)	0.0008	0.49 n.s.
PP2C-3	*Nn*	38	12	9	0.421 (0.10)	0.0044	-1.11 n.s.	W	0.338 (0.13)	0.0029	-1.22 n.s.
								E	0.533 (0.14)	0.0064	-0.11 n.s.
	No	38	8	10	0.858 (0.03)	0.0039	-0.41 n.s.	W	0.758 (0.06)	0.0026	-0.18 n.s.
								E	0.916 (0.03)	0.005	-0.16 n.s.
SOS2	*Nn*	44	4	4	0.586 (0.04)	0.0028	0.81 n.s.	W	0.619 (0.08)	0.0029	0.48 n.s.
								E	0.567 (0.05)	0.0028	0.31 n.s.
	No	48	4	5	0.756 (0.02)	0.0027	0.72 n.s.	W	0.793 (0.04)	0.003	0.61 n.s.
								E	0.743 (0.001)	0.0025	0.92 n.s.
ATCHR12	*Nn*	28	1	2	0.138 (0.08)	0.0002	-0.74 n.s.	W	0	0	-
								E	0.429 (0.17)	0.0006	0.33 n.s.
	No	38	2	3	0.243 (0.09)	0.0003	-0.90 n.s.	W	0.209 (0.12)	0.0002	-0.53 n.s.
								E	0.268 (0.11)	0.0003	-0.09 n.s.

CG: candidate gene; N: number of sequences; *Nn*: *Nothofagus nervosa*; *No*: *N obliqua*; W: west. E: east. n.s. not significant * p<0.05 ** p<0.01

Competing interests

The author declares that they have no competing interests.

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Role of RAV genes in tree seasonal dormancy

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Key words (max 4): RAV, winter dormancy, Populus

Background

Plants from temperate regions adapt to changing environmental conditions along the year. Trees have evolved mechanisms that allow them to monitor and anticipate the seasons, by sensing photoperiod and temperature changes, in order to modulate their growth and development. Trees cycle between growth and winter dormancy states. Dormancy is first initiated by shortening of photoperiod, and is characterized by growth cessation, bud development at the apex, and cold acclimation [1, 2]. In a second step, as a result of a drop in temperature, trees reach a state of endodormancy, the inability of resume growth in response to inductive conditions. Chilling requirement, exposure to low temperatures, needs to be fulfilled in order to release from endodormancy and gain the ability to resume growth in response to good conditions [1, 2].

The molecular and signalling networks that regulate dormancy in perennials are poorly understood. Several studies had shown similarities between the short-day (SD) mediated molecular pathways that control the transition to flowering in Arabidopsis and dormancy establishment in trees [3]. Accordingly, it has been described that Poplar orthologs of Arabidopsis FT and CO are implicated in SD induced growth cessation and bud set [3]. We had previously shown that *CsRAV1,* a chestnut homolog of Arabidopsis *TEM1* and *TEM2* [4]*,* induced sylleptic branching in poplar [5]. In this work we characterize the role of chestnut and poplar *RAV* genes in winter dormancy.

Methods

For the annual gene expression analyses stems were collected from *Castanea sativa* or *Populus alba* adult trees in Madrid, Spain. Total RNA isolation and quantitative RT-PCR analysis were performed as described previously [5]. Hybrid poplar *Populus tremula* x *P. alba* INRA clone 717 1B4 was used to generate the transgenic lines described in [5]. Poplar transgenic lines were screened using the customized arrays designed at the University of Florida [6]. Growth conditions for dormancy induction and release were performed essentially as described in [7]. Arabidopsis (Col-0) were used to generate transgenic lines as described in [8]. Arabidopsis developmental phenotypes were analyzed as described in [9].

Results and Conclusions

In order to determine the implication of *CsRAV1*, *PtaRAV1*, and *PtaRAV2* in the regulation of winter dormancy we have characterized their expression along the year. The results showed that all three genes were induced in early winter and maintained high expression levels until early spring. These data suggested that CsRAV1, PatRAV1 and PtaRAV2 were involved in the regulation of winter dormancy in trees. To test this hypothesis we have used over-expressing *CsRAV1* (*3xHA:CsRAV OX*), and knock-down *PtaRAV1* and *PtaRAV2* (*PtaRAV1&2 KD*) transgenic poplars [4]. The results for growth cessation, bud set and bud burst of the transgenic lines will be discussed.

To gain insight on the molecular function of tree RAV genes we screened *in silico* the promoter region of the homologous FT gene in *Populus trichocarpa* for the RAV1/TEM1 DNA recognition sites described in Arabidopsis (the bipartite sequence CAACA and CACCTG [10]), as it has been reported that Arabidopsis TEM1 binds to the FT promoter [4]. The search revealed that the RAV1 motif was not conserved, pointing to a functional divergence of RAV family members. To check this possibility, we generated transgenic Arabidopsis plants over-expressing *CsRAV1* and looked for the developmental phenotypes described for Arabidopsis *TEM1* and *TEM2* over-expressors [4]. All the Arabidopsis *CsRAV1* over-expressing lines showed WT phenotypes for all the analyzed traits, suggesting that CsRAV1 and Arabidopsis TEM1 and 2 have functionally diverged.

In conclusion, our study reveals a possible function of RAV transcriptional regulators in the control of winter dormancy in trees.

Competing interests

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Evaluation of the adaptive potential of silver fir (*Abies alba*) along altitudinal gradients using reciprocal transplants.

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Key words: Local adaptation, quantitative genetics, forest ecology, global changes.

Background

Global changes, including global warming, alter ecological conditions and may compromise the persistence of ecosystems in their current habitat. Combining plasticity, migration and genetic adaptation processes, species will be able to adapt, to some extent. Although, population genetic differentiation and adaptation have frequently been estimated from common garden experiments at the scale of species distribution range, similar studies were more rarely conducted at local scale.

Methods

In this context, the INRA "Ecology of Mediterranean Forests" Research Unit focus its research on the dynamics of forest tree species such as beech (*Fagus sylvatica*) or silver fir (*Abies alba*) particularly exposed to climate change because of their long life cycle. Combining microdensitometry, association genetics (with SNP markers) and quantitative genetics on adaptive traits (growth, survival, budburst, leaf area, leaf/stem and root biomass) measured on reciprocal transplants and altitudinal gradients, we propose to analyse, at local scale, genetic differentiation and adaptation potential (genetic adaptation and plastic response to climate variation) of silver fir.

The evaluation of plastic and genetic components in phenotypic variability is based on two experimental strategies: (i) long term monitoring of adult trees along three altitudinal gradients (Mont Ventoux, Issole and Vésubie) located at southern limit of the species range in the French Alps and (ii) reciprocal transplants of seeds from 60 mother trees (20 from each gradient) collected at 3 elevations (low, mid and high) along the gradients.

Results and Conclusions

Early trends from the analysis of survival and growth of 5 years-old seedlings in the reciprocal transplants did not reveal any genetic adaptation but showed strong diversity among families. Further analyses will provide information on adult trees adaptation and on the genetic control of the traits under selection, potentially key for adaptation under climate change.

Competing interests

The author declares that they have no competing interests.

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Molecular identification of *Fraxinus excelsior* L. population in terms of ash dieback

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Key words: Ash dieback, DNA markers, microsatellites

Background
For more than 20 years in Poland, we can observe ash dieback phenomenon. This affects not only the individual trees, but also the entire forest stands. Natural regeneration of this species is a serious problem and a challenge not only in terms of timber production, but also in biodiversity protection of the Polish forests. Limiting the European ash dieback has become an important and widely analysed problem. Process of ash dieback is very, complex in the description and this indicates huge complexity in interaction genotype - environment. Analysis of genotypes in the ash stands can enlarge knowledge about the basis of resistance mechanisms and adaptation of *Fraxinus excelsior* (L.) to changing biotic and abiotic factors. One of the approach to estimate the level of genetic variation is based on neutral genetic markers that are not subject to natural selection, but infer the genetic variance of providing information on the pool of genotypes found in the study population. Neutral markers also allow inferences about the level of gene flow between individuals and their degree of relatedness.

Methods
The aim of the study was to analyse 10 molecular markers (6 nuclears and 4 chloroplasts) from 55 samples of ash shoots derived from individuals from forest stand with varying degrees of damage.
The phenomenon of tree dieback is not homogeneous, and it can be described by Roloff's classes. This method is distinguished by 3 degrees of damage depending on the crown size and the potential strength of shoot growth (Dmyterko, 1998).

Results and Conclusions
Nuclear markers used in this study showed very high polymorphism, the number of alleles obtained ranged from 4 to 15, comparing to smaller number of haplotype variants (from 2 to 5) in case of cpDNA markers. The observed level of genetic diversity at all levels of damage was high, but there was no significant genetic distance between the groups of individuals qualified to particular degrees of Roloff. As many as 98% of the genetic variation resulted from the variability observed between individuals. Despite the absence of significant genetic

distance in all groups of different damage levels, private alleles was observed (average from 3 to 4 alleles per group).The high level of genetic differentiation potentially can increase the adaptability of the population, while too low gene pool level may significantly limit this ability.

Competing interests

We declare that we have no competing interests.

Acknowledgements

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Expression profiling of lodgepole and jack pine chitinase gene family in response to inoculation by mountain pine beetle fungal associate *Grosmania clavigera.*

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Background

The current outbreak of mountain pine beetle (MPB) has affected more than 28 million ha. of pine forests in western North America [1]. MPB vectors a number of microbial symbionts, including *Grosmania clavigera*, a fungal pathogen that contributes to tree mortality by growing into the host's xylem tissue and disrupting water transport [2]. Lodgepole pine (*Pinus contorta*) has been the main species affected by the current outbreak. However as MPB range expands eastward and northward beyond its historical habitat, the bark beetle has encountered a novel host: jack pine (*Pinus banksiana*) [3]. Ecological evidence has indicated that host trees from MPB's historic range have lower host quality compared to hosts from novel habitats, suggesting that co-evolved lodgepole pine may have acquired induced and constitutive defenses against MPB that are not present in jack pine [4]. Ecological studies also suggest that trees subjected to abiotic stresses such as drought are more susceptible to MPB attack, particularly at sub-epidemic populations. Conifers invoke an array of defenses, including the synthesis of chitinases, a well-studied family belonging to the pathogenesis response (PR) proteins. Some members of this family act by hydrolyzing chitin, a component of fungal cell walls [5]. Chitinases are highly expressed in response to pathogen challenge, and the timing and magnitude of chitinase expression is important to effective pathogen resistance in conifers [6]. Recent analysis of a large-scale microarray dataset revealed many chitinases that are upregulated in response to inoculation with *G. clavigera*, and whose expression profiles are altered by drought conditions. We are testing the hypotheses that putative orthologs of chitinases in lodgepole pine and jack pine will show differential responses to infection by *G. clavigera*, and that water deficit will exert more effect on chitinase expression in the more drought sensitive lodgepole pine.

Methods

Microarrays and quantitative reverse transcription PCR (qRT-PCR) were performed as described [7] on phloem tissue from two year old lodgepole and jack pine seedlings placed under water deficit or well watered conditions and inoculated with *G. clavigera*. Differentially expressed chitinase genes were selected from the microarray analyses for qRT-PCR. Chitinase gene family members were mined from transcriptome assembles produced on Sanger, Roche (454) and Illumina

platforms. Sequences were classified based on primary structural motifs, and phylogenetic analysis was conducted using Mafft and RaxML. SNPs were identified from a variant analysis of transcriptome assemblies using CLC Genomics Workbench.

Results

In a large-scale microarray study, we identified broad scale, species-specific patterns of response in the transcriptomes of jack and lodgepole pine to inoculation with *G. clavigera*. We identified 47 potential chitinase genes showing significant levels of differential expression in inoculated versus control treatments for both species. To develop a more comprehensive picture of how the chitinase family is implicated in the defense response of lodgepole and jack pine to MPB and *G. clavigera*, we identified putative chitinase sequences in jack and lodgepole pine. These sequences were biochemically classified through protein domain analysis into classes I, II, III, IV,V,VI and VII based on protein alignment with a representative class I chitinase from *Nicotiana tobacum* that exhibits all of the canonical chitinase motifs. Phylogenetic analysis enabled us to identify putatively orthologous pairs of lodgepole and jack pine sequences, and to compare evolutionary relationships with classical biochemical classifications.

Comparing transcript profiles of lodgepole pine and jack pine putative orthologs using qRT-PCR, we found evidence of high levels of chitinase class IV and class I induction in both species by *G. clavigera* inoculation. Multiple class IV orthologs in both jack and lodgepole pine demonstrated peak levels of induction at seven days post inoculation, where as we observed earlier induction of putative class I orthologs at one day post inoculation. There was little change in the expression of class II and VII putative orthologs for either species. Under water deficit conditions, we observed a significant attenuation in chitinase expression for both jack and lodgepole pine.

Comparing different genotypes, we found evidence of both synonymous and non-synonymous substitutions in these chitinase genes. We plan to use allelic resequencing to test the hypothesis that allelic differences may contribute to differential enzyme activity or gene expression in jack and lodgepole pine, which in turn may contribute to differences in host tree defenses.

Conclusions

Our results demonstrate that some members of the chitinase gene family in pine are highly responsive to challenge by *G. clavigera,* with some chitinases responding earlier to inoculation than others. Our results also demonstrate that water deficit causes an attenuation in the magnitude of chitinase induction.

Competing Interests

No competing interests.

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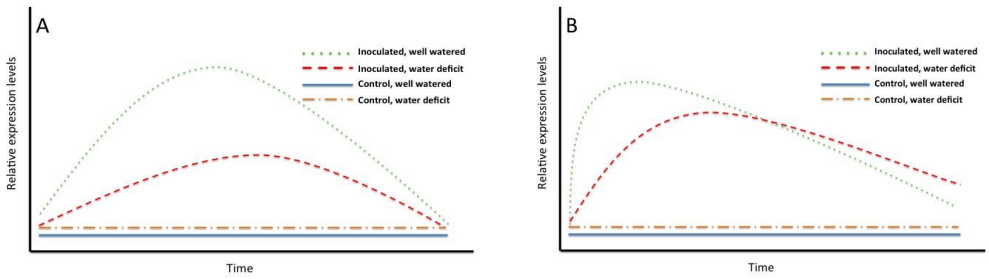

Figure 1. Relative expression patterns of orthologous pairs of jack pine and lodgepole pine chitinase genes observed over time in seedlings under either well-watered control treatment (solid line), water deficit control treatment (dash-dotted line), well watered inoculated treatment (dotted line) or water deficit inoculated treatment (dashed line). **A.** Representative expression pattern of class IV orthologs. **B.** Representative expression pattern of class I chitinase orthologs.

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Transcriptome profile of candidate genes associated with resistance to *Fusarium circinatum* in *Pinus radiata.*

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Background: *Fusarium circinatum* is the causal agent of pitch canker, a serious disease that affects numerous *Pinus* species worldwide. *Pinus* species have varying degrees of susceptibility to this pathogen, with *P. radiata* as one of the most susceptible. Quantitative phenotypic variation and intermediate heritabilities in response to *F. circinatum* have been observed by studies of controlled inoculations in several families of *P. radiata*, suggesting the existence of a genetic component to resistance [1, 2]. Recent studies in plant genomics have discovered and isolated many important genes with functions that confer resistance to various environmental stresses. Currently, advances in massively parallel cDNA sequencing (RNA-seq) provide a cost-effective way to obtain large amounts of transcriptome data from many organisms and tissue types [3]. In this investigation, we report the use of high-throughput mRNA sequencing (RNA-seq) to identify EST of genes differentially expressed during the interaction of *P. radiata* with *F. circinatum* at several time points including the uninfected, 2, 6 and 12 days post inoculation (dpi) in two contrasting genotypes.

Methods: Ten genotypes of *P. radiata* which were previously phenotyped as resistant (R)/susceptible (S) to *F. circinatum* were inoculated with a conidial suspension. Disease symptoms of inoculated, uninoculated and damage control plants were measured at 90 days post inoculation (dpi) and two contrasting genotypes were subsequently selected. Stem tissue from the most R and S genotypes was sampled at 0 (uninoculated), 2, 6 and 12 dpi (inoculated genotypes and damage controls). Three normalized Roche 454 FLX-Ti cDNA stem tissue libraries were sequenced and assembled *de novo* with MIRA3 v3.4.0 [4]. The resulting contigs were functionally annotated using BLASTX against NCBI's plant protein and NR protein databases and Blast2GO (http://www.blast2go.org/) was used to assign gene ontology (GO) terms. Twenty-six non-normalized cDNA libraries were sequenced with Illumina GAIIx (single-end 104bp) and analyzed with the NOIseq R package [5] to identify differentially expressed genes (DEG). All DEGs

were characterized functionally via sequence alignments and associated GO terms assigned to contigs in the *de novo* assembly.

Results: Significant differences between the resistant and susceptible genotypes lesion length were observed, where the most contrasting genotype corresponded to R1 (9.95 ± 8.34 mm) and S5 (63.35 ± 11.35 mm) (Figure 1a). The 470,612 454-HQ reads assembled into 26,215 unigenes with an average length of 1,523 bp. Functional annotation was completed for unigenes: 86% of these assembled sequences had significant protein alignments and 23,897 (91%) could be assigned to one or more GO term. Top hit species for homology based annotations of unigenes include: *Vitis vinifera* (24%), *Citrus clementine* (15%) and approximately 5% of the contigs had aligned *Populus trichocarpa* and *Arabidopsis thaliana* proteins. It is known that stress by injury or damage to plants activates the same metabolic pathways and genes that are also involved in stress responses to pathogens. This is why it is necessary to evaluate the up-regulated genes that are expressed only in the resistant genotype inoculated with *F. cicinatum*. Pairwise comparisons of the number of DEGs up-regulated and down-regulated between inoculated genotype (R1 and S5), damage control for resistant genotype and uninoculated control for resistant genotype at time 2, 6 and 12 dpi are shown in Figure 1b. The total number of genes differentially expressed related to pitch canker resistance is also shown (Figure 1c-e). We identified 39, 242 and 51 commonly up-regulated genes in response to pitch canker infection at 2, 6 and 12 dpi respectively, during 3 pairwise comparisons. To validate the bioinformatic analysis, qRT-PCR was performed for 5 selected genes (*FFP*, *PR1*, *NDR1*, *PR5* and *PR3*) showed that a greatest fold change at three time points related to the pitch canker resistance response. The analyses were consistent with those predicted by the *in silico* analysis.

Conclusions: This RNA-Seq study identified 293 putative candidate genes and validated a set of genes involved in cellular processes and defense mechanisms thereby providing the first platform for studying molecular mechanisms of *F. circinatum* resistant in pine.

Competing interests: The author declares that they have no competing interests.

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Genetic diversity of *Heterobasidion*, the causal agent of conifer root and butt rot

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Key words: Abies, fungal pathogen, pine, population studies

Background

Heterobasidion annosum s.l. is a root and butt rot fungus that cause losses in timber production. This pathogen is relatively common and can be found in saprophytic habit on stumps and wood logs in the forest (Woodward et al., 1998).
In Italy, within the complex *H. annosum* s.l., three European species (*H. annosum s.s*., *H. abietinum* and *H. parviporum*) have been described (Capretti et al., 1998). Furthermore also the invasive *H. irregulare,* introduced from North America, was found along the Tyrrhenian coast (D'Amico et al., 2007; Gonthier at al., 2014).
By using molecular markers the diversity of *H. annosum* s.l. populations have been studied in Italy (Petta et al., 2001; D'Amico et al., 2007; Zamponi et al., 2007). Genetic divergences in *H. annosum* and *H. abietinum* populations were found (Zamponi et al., 2007; Luchi et al., 2011). These variations within each fungal population, could be mainly related to the host population but also to the morphology of Italian peninsula that reduced the gene migration among fungal strains.
Objective of this work is to study the population structure and spatialization of genetic diversity in *H. abietinum* and *H. annosum* populations in Italy by using minisatellites.

Methods

Fungal isolates collected along the Italian peninsula from different hosts, have been use to study the genetic diversity of *H. annosum* s.s. and *H. abietinum,* respectively collected from *Pinus spp.* and *Abies alba* in Italy. Mycelium from fungal isolates was grown in vitro and DNA was extracted according to Vainio et al (1998). PCR amplification using M13 was carried out according to Luchi et al. (2011). The electrophoretic amplified profiles transformed in presence/absence vectors were used for following analyses.
General estimates of genetic diversity in the populations of *H. annosum* and *H. abietinum* isolates were calculated using SPAGeDi 1.3a. The spatial genetic structure of the populations was analyzed by Geneland software.

Results and Conclusions

The population of *H. annosum* isolates shows the higher levels of gene diversity (Nei 1972) than the *H. abietinum* population (D_i = 0.37 and D_i = 0.26, respectively).
The morphology of Italian Peninsula may have influenced the rate of spread of *Heterobasidion* populations (from pine and silver fir). The evolution of *Heterobasidion* species have probably similar spreading history as tree host species, reflecting genetic variation of fungal populations (Johannesson and Stenlid 2003). However the genetic differences between *H. annosum* and *H. abietinum* populations reflect different history of plant host.

In case of *Heterobasidion* subpopulations from Silver fir, like its main host, were never completely isolated showing a diffuse spread from scattered nuclei, such as the glacial refuges. In fact, the considered population of *H. abietinum* isolates appears divided into 4 clusters, but they do not are isolated as demonstrated by the maps of posterior probabilities and by the pairwise genetic distances (D_p, Nei 1972) between the clusters.
The other hand, *Heterobasidion* from pine, host isolation reflects establishment of isolated fungal clusters.
Studies on *Heterobasidion* population of can been useful to understand possible differences in pathogenicity, that may also reduce the biodiversity of conifer stands along Italian peninsula

Competing interests
The author declares that they have no competing interests.

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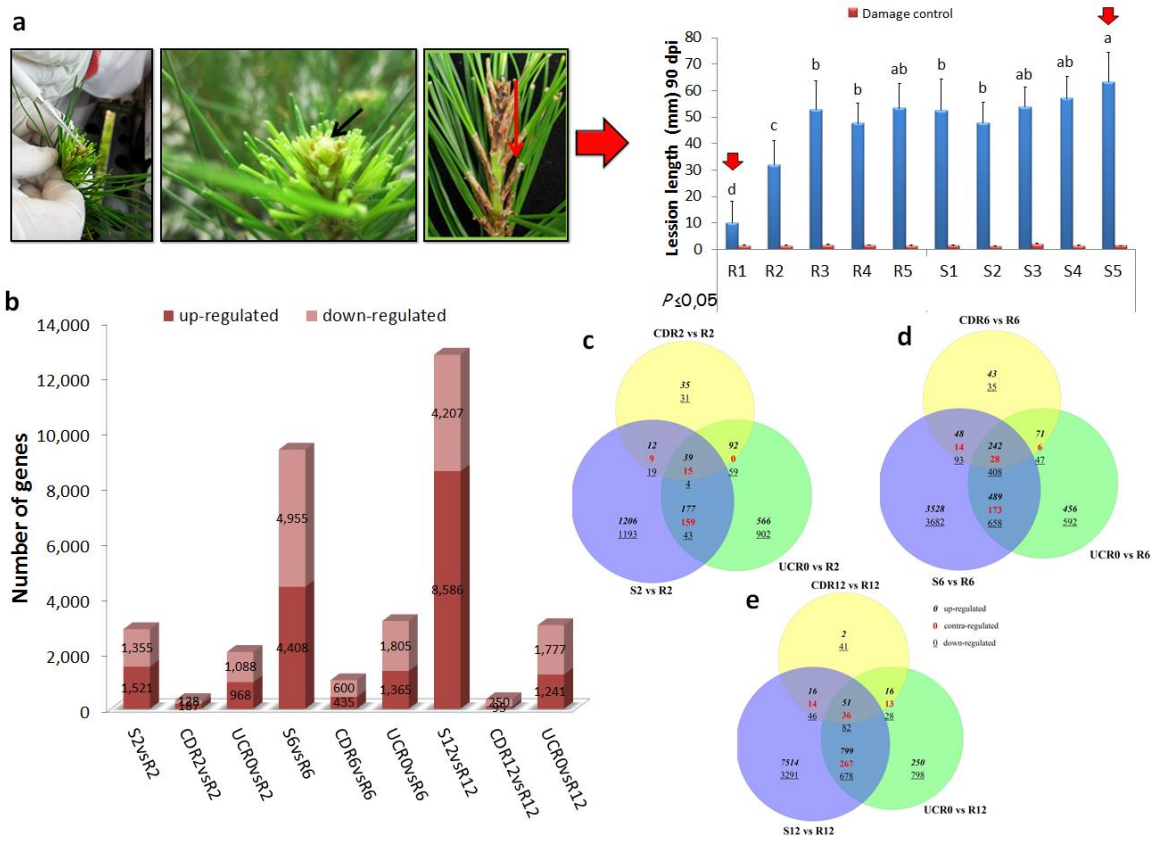

Figure 1: Expression profiling of defense-related genes from contrasting genotypes of *P. radiata* infected with *F. circinatum*. **a)** Inoculation, phenotypic measurements and selection of contrasting genotypes in response to *F. circinatum* infection. **b)** Summary of DEGs between nine pairwise comparisons. **(c-e)** Venn diagram depicting the overlap and discrepancies between differentially expressed genes at the three time points post inoculation. The *italic numbers* of numerical categories represent the number of up-regulated genes; the *underlined numbers* represent the down-regulated genes; the *red numbers* represent the contra-regulated genes.

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Genetic and spatial structure of natural populations of *Ziziphus joazeiro* Mart. – strategy for seed collecting

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Background

Ziziphus joazeiro Mart, known as juazeiro, is a native species of the Biome Caatinga in Brazil, being distributed in several states of the northeast region of the country. Is has an economic and ecological importance mainly in Ceará State and due the overexploitation it has been considered an endangered species. To date, no information is available regarding the genetic diversity for the species and this knowledge would assist the production of seedlings for conservation and reforestation programs of Biome Caatinga Project. This project is part of the Biome Project that has been conducted in Brazil with the aim to extend the use of trees in rural properties to diversify the productive systems with economic and environmental benefits.

Methods

Samples of 31 individuals were collected in a population of *Z. joazeiro* located in an area of natural preservation named “Não me Deixes” (population 1), and 47 individuals were collected in a population located in a farm named Triunfo (population 2). Both populations are located in Ceará State, Brazil. The genetic structure was evaluated with 12 ISSR markers and the attributes polimorphism information content (PIC), marker index, resolution power and optimum number of markers were calculated. The genetic diversity of each population was evaluated using the software POPGENE version 1.32 and the parameters number of observed alleles (n_a), effective number of alleles (n_e), genetic diversity of Nei (H_e), Shanon Index (I), percentage of polymorphic loci (%P) and indirect estimation of gene flow (Nm) were calculated. The genetic diversity inside and between populations was analysed by AMOVA. Jaccard coefficient was calculated using the software GENES version 2014.6.1, and the individuals were clustered with UPGMA. The spatial structure was analysed with the software SPAGeDi version 1.4, calculating the kinship coefficient for ten distance classes.

Results and Conclusions

In the population 1 was observed 220 loci, in population 2 was observed 74 loci and the optimum number of markers for each population was 210 and 65, respectively. This no appreciable difference between the original number and the optimum number of loci probably is due to the dominant character of the ISSR markers. The average PIC for the ISSR set used for populations 1 and 2 was 0.25 and 0.41, respectively, which are of medium informativeness [1]. The markers UBC808, UBC812, UBC815, UBC817 and UBC818 showed the highest values of PIC, marker index, resolution power, being the most informative for both populations. Population 1 showed a low genetic diversity compared to population 2. The number of observed alleles and the number of effective alleles for population 1 were 1.17 and 1.67, and for the

population 2 were 2.0 and 1.98. The genetic diversity was high in population 2 (H_e= 0.38, *I*=0.56) compared to population 1 (H_e= 0.13, *I*=0.25). According to AMOVA results there is a high genetic diversity inside populations, as the specie is undomesticated and with outcrossed mating system [2]. Gene flow (Nm) between populations was 2.17 which is considered low [3], insufficient to overcome allele loss and probably due to the distance from each population that was of 26km. The individuals of population 1 were clustered in ten groups and the individuals of population 2 in fourteen groups. In relation to spacial structure, populations 1 and 2 showed positive coancestry values from the distance classes of 196 and 132 meters, respectively (Figure 1). These results indicate that from these distances the individuals are lees related and useful for seed collecting for seedlings productions in programs of reforestation and conservation.

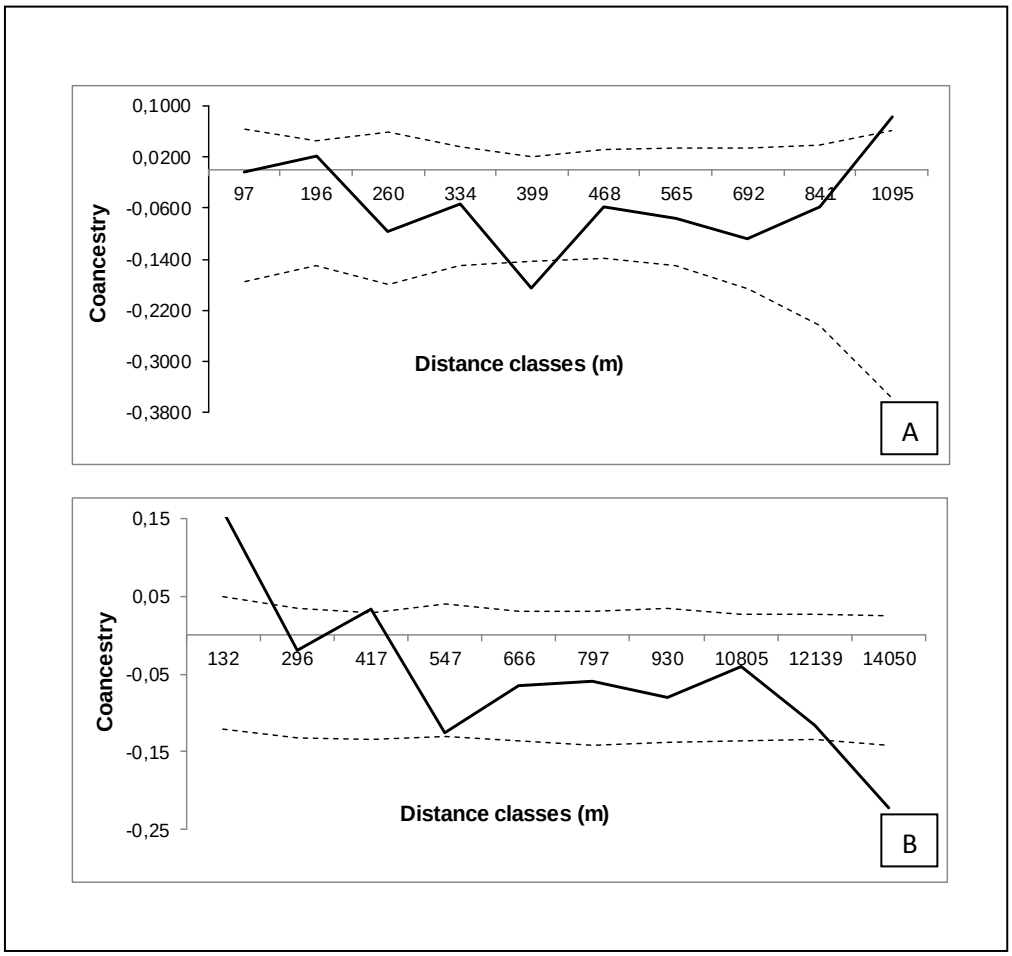

Figure 1 Spacial structure of the populations 1 (area of natural conservation called Não me Deixes – A) and 2 (productive farm called Triunfo - B) calculated using the kinship coefficient and ten classes of distance.

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Abstract poster session III

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Development of Candidate Gene-based KASP SNP Assays and their Application for Genetic and Association Studies in *Eucalyptus camaldulensis*

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Keywords: Association, Kasper, SNP, Eucalyptus

Background

Eucalyptus camaldulensis (*Ec*) is an important tree species for pulp and paper industry. There is an immediate need to apply advanced genomics technologies such as next generation sequencing and targeted or whole-genome SNP discovery for *Ec* improvement [1,2,3]. A few reports have used SNPs to test phenotypic association as well [4]. To further our earlier efforts of SNP discovery [2], we developed SNP assays based on K-bioscience allele specific PCR (KASP) and employed them to find phenotypic association with pulp yield and growth in a natural *Ec* population with a reasonable success.

Methods

Around 250 families from four provenances (Kennedy River, Morehead River, Laura River and Petford; Northern Queen's Land, Australia) of *Ec* were sourced from CSIRO, Australia to generate an association mapping population. A total of 338 individuals were planted in three replications as single-tree plot at a field station near Coimbatore, India. The growth was recorded as diameter at breast height (DBH) at approximately two years after planting. The pulp yield (PY) estimates were recorded by indirect near infra-red (NIR) based technique [5, 6].

From the earlier discovered 1,200 SNPs in ~40 candidate genes in *Ec* [2], the best 527 were used for designing assays, screened at LGC Genomics, UK and analyzed using Klustercaller (LGC Genomics). The observed heterozygosity (H_o), expected heterozygosity (H_e) and Hardy-Weinberg equilibrium (HWE) were calculated using Arlequin 3.5 [7] and tested at p<= 0.05 (corrected for false discovery rate, FDR). The population structure was studied using Structure 2.3.4 [8] with 50,000 burnins, 100,000 Markov Chain Monte Carlo iterations using admixture model with k=1 to 30 followed by Evanno's strategy to estimate k [9]. Linkage disequilibrium (LD) heat-maps were generated using R package LDheatmap [10]. The phenotype-SNP associations were analyzed using TASSEL 3.01 [11] using mixed linear model. The q-matrix was calculated using MSA 4.05 [12] as 1-kinship coefficient. The 216 HWE SNPs, which had a minimum genotype frequency and minimum allele frequency of 0.01 were used to test phenotypic association. The *p*-values for R^2 were FDR corrected for all the SNPs in gene-wise manner at p= 0.05.

Results

From 527 assays, 477 produced scorable data (90.5%) and 438 turned out to be polymorphic. There was a high heterozygosity with almost similar levels of Ho of 0.22 (SD: ± 0.15, SE: ± 0.007) and He of 0.25 (SD: ± 0.15, SE: ± 0.007) and 137 SNPs showed deviation from HWE. Structure analysis did not indicate any subpopulation as expected. LD survey indicated that from 166 possible neighboring pair-wise SNP combinations within genes, 14 had r^2 >=0.8 with smallest block being 5 and largest being 867 bases.

Thirty one SNPs showed R^2 within the rage of 0.05 to 0.2 with DBH, and three of them showed significant association after FDR correction (TTL_200Y, R^2: 0.07, p= 0.01, q= 0.04; UBP15_228Y, R^2: 0.08, p= 0.03, q=0.03 and UBP_344R, R^2: 0.08, p= 0.03, q=0.03). For PY, 18 showed R^2 within the rage of 0.05 to 0.2 and only one showed significant association after FDR correction (TOTKin_1703K, R^2: 0.12, p= 0.001, q=0.02). Figure 2 shows the Manhattan plot for $-\log_{10}(p)$ values with chromosomal locations of SNPs and figure 3 shows box and bar plots for class means of the SNP classes.

Conclusions

This study proves importance of targeted candidate gene approach to find SNP-phenotype associations with a high success rate (4 associated SNPs/438 genotyped SNPs, ~1%). Further it also proves that KASP technology can be very efficient and successful method to employ for SNP survey in species with high heterozygosity and high SNP density.

Competing interests

The authors declare that they have no competing interests.

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Figure 1: Manhattan plot showing $-\log_{10}p$ values of R^2 with SNP positions over chromosomes. A and B shows association data for DBH and PY respectively. The marked SNPs show significant associations post FDR correction.

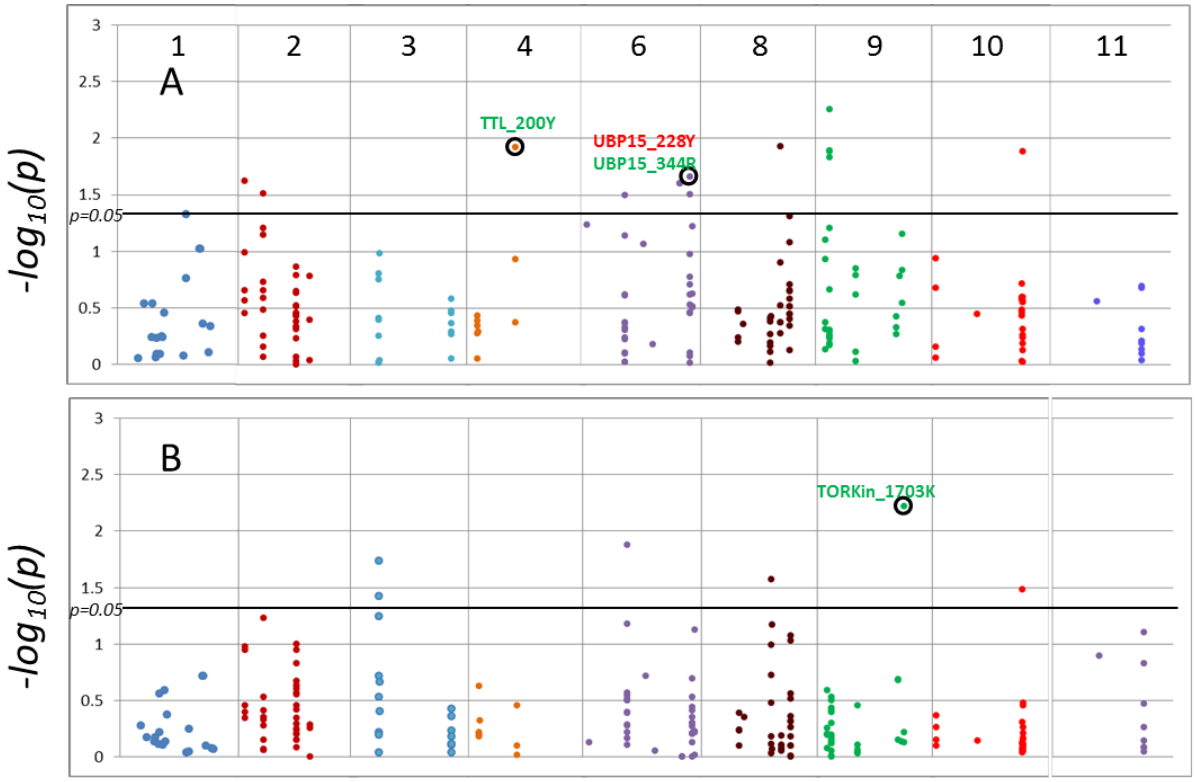
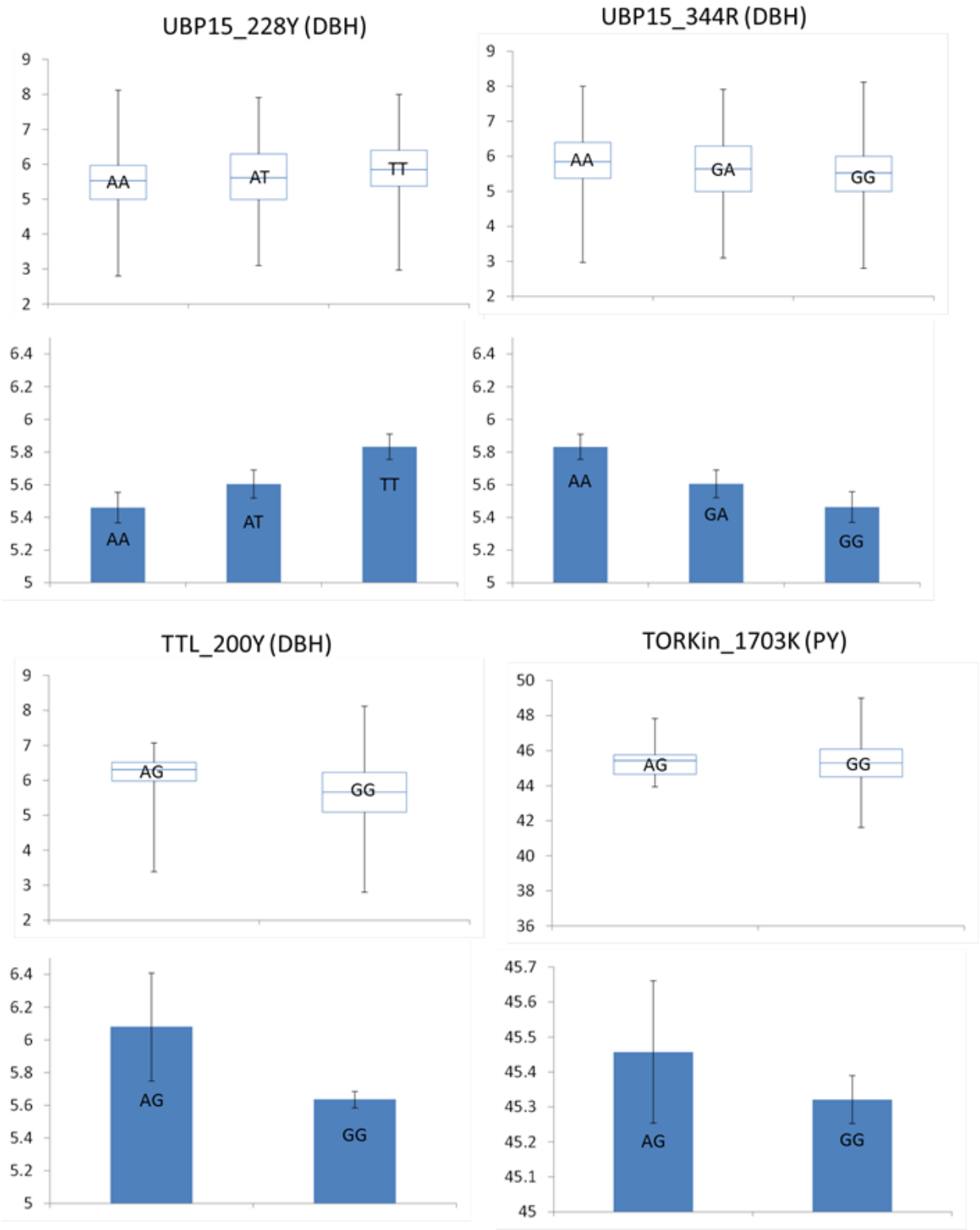


Figure 2: Box plots and bar plots for significant SNPs with DBH and PY. X-axis: SNP classes, Y-axis: phenotypic value, DBH in cm and PY in %.



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Biotechnologies and tree improvement for forest tree farming in highland areas in Italy

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Key words (max 4): valuable species, molecular markers, improvement, forest tree farming

Background

Italy is a mountain country, characterized by the North-South line of Apennines and the East-West line of Alps in the northern part. Highland areas about 70% (23 million ha). The climate is Mediterranean along the coasts and in the islands, but relatively temperate in the interior, where precipitations are higher. Local administrations have to cope with the management of 2 – 3 Millions ha of abandoned land areas with important soil and water resources. Wide programs were funded by the European Commission (UE) and by the national government for developing intensive forest tree farming activities in marginal areas the last 30 years . The aim was stocking higher quality wood resources and reducing the future import from eastern and the tropical countries. The Italian furniture industry is one of the most powerful in the world and covers 80% of the European import of valuable wood. Italian forests cover 10 Millions ha are, but are mostly protective. Breeding and improvement were initially developed in the framework of national programs and then in the framework of cooperative programs (Euforgen) or EU research projects focused on walnuts and wild cherry (Brains, Always, Trebreedex, Trees4Future).

Methods

Breeding and improvement Strategies were based on a holistic vision, from the first survey of the wild resource variation (genetic analysis) towards the phenotypic selection of wild basic materials and breeding. Wide comparative tests and trials were established to know the value of materials as well as the interaction *genotype x environment* interaction and nowadays for evaluating *plasticity* for many phenotypic traits of commercial. Molecular markers and other biotechnology tools as *in vitro* techniques and vegetative propagation were widely used.

Results and Conclusions

Nowadays, the changing climate evidence, but also the extension of plantations out of the natural range of most of those temperate mixed forest species pushed to focus attention on adaptive traits as physiology of stress resistance/tolerance, the variation of phenology, become focal for developing an adaptive management of plantations with species, unknown from this point of view. Methods for evaluating materials are developed, improved, standardized at European level in order to select new materials and to develop MAS (Marker Assisted Selection), able to facilitate future actions. It has been also focal developing European data bases and developing a common legislation on trading reproductive materials.

Competing interests

The author declares that they have no competing interests.

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Breeding for browsing "resistance" in Norway spruce?

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Key words breeding, spruce, browsing, resistance

Background

Red Deer (*Cervus elaphus*) browsing on Norway spruce (*Picea abies*) seedlings in Austria can result in delayed establishment of regeneration, plantation failure and subsequent economic losses for the forest owner [1]. Observations in natural and artificial regenerations show high variation in browsing damage among individual trees and suggest that Red Deer may have preferences for certain phenotypes. Mechanisms of browsing "resistance" have been discussed to be related to plant secondary metabolites (PSM). Spruce may contain both beneficial and deleterious phytochemicals that impact palatability and diet selection for ungulates [2].

Methods

In order to test for phenotypic variation in browsing damage, four forest observation sites were established in the province Carinthia on elevations from 1280 to 1629 m a.s.l. At each site, 200 Norway spruce seedlings with heights between 40 to 231 cm are exposed to high deer pressure. Within the first vegetation season all individuals were phenotyped for physical characters, browsing, flushing, overall assessment (high vitality and no damage) and other damages caused by biotic or abiotic factors. Most extreme phenotypes were selected as putatively browsing "resistant" and susceptible genotypes, respectively. Needles from selected genotypes were sampled and frozen for DNA extractions. All phenotypic assessments will be repeated throughout the next two vegetation periods. In addition, browsing "resistant" and susceptible genotypes will be propagated by rooted cuttings.

Results and Conclusions

By now, a total of 120 individuals have been identified as being either putatively browsing "resistant" (40 individuals) or highly susceptible (101 individuals) (Figure 1). However, data from the following years are going to fine tune this draft selection. To test for genetic causes of the phenotypic variation in browsing damage, the phenotypic differences will be related

to candidate genes putatively associated with browsing “resistance”. The huge spruce genome and its low linkage disequilibrium makes a targeted candidate gene approach using Next Generation Sequencing the best among other genomic approaches [3]. Because whole exome sequencing kits for spruce are not yet available in an accessible commercial solution, targeted enrichment of gene space using sequence capture, i.e. “customized targeted sequencing” has been envisaged to re-sequence candidate genes [4]. Potentially candidate genes comprise the most important genes involved in PSM pathways (terpenoids, phenypropanoids), anti-nutritional enzymes and proteins (proteases inhibitors, polyphenol oxydases, arginases, threonines deaminases, chitinases, trypsine inhibitors) and defense signaling routes (Jasmonates, Octadecanoids, Ethylene, Salicilic acid, MAP Kinases, Peptide signals…)[5, 6].

The long-term objective is to test for genetic variation of browsing damage patterns and to identify genes related to browsing “resistance”. If such genes can be identified, the resistance of Norway spruce to deer browsing can be improved by marker-assisted selection. Also those genes could be utilized to screen a large number of individuals by next-generation Ecotilling [7] for rare variants; these could be potentially useful in future browsing “resistance” breeding programs.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

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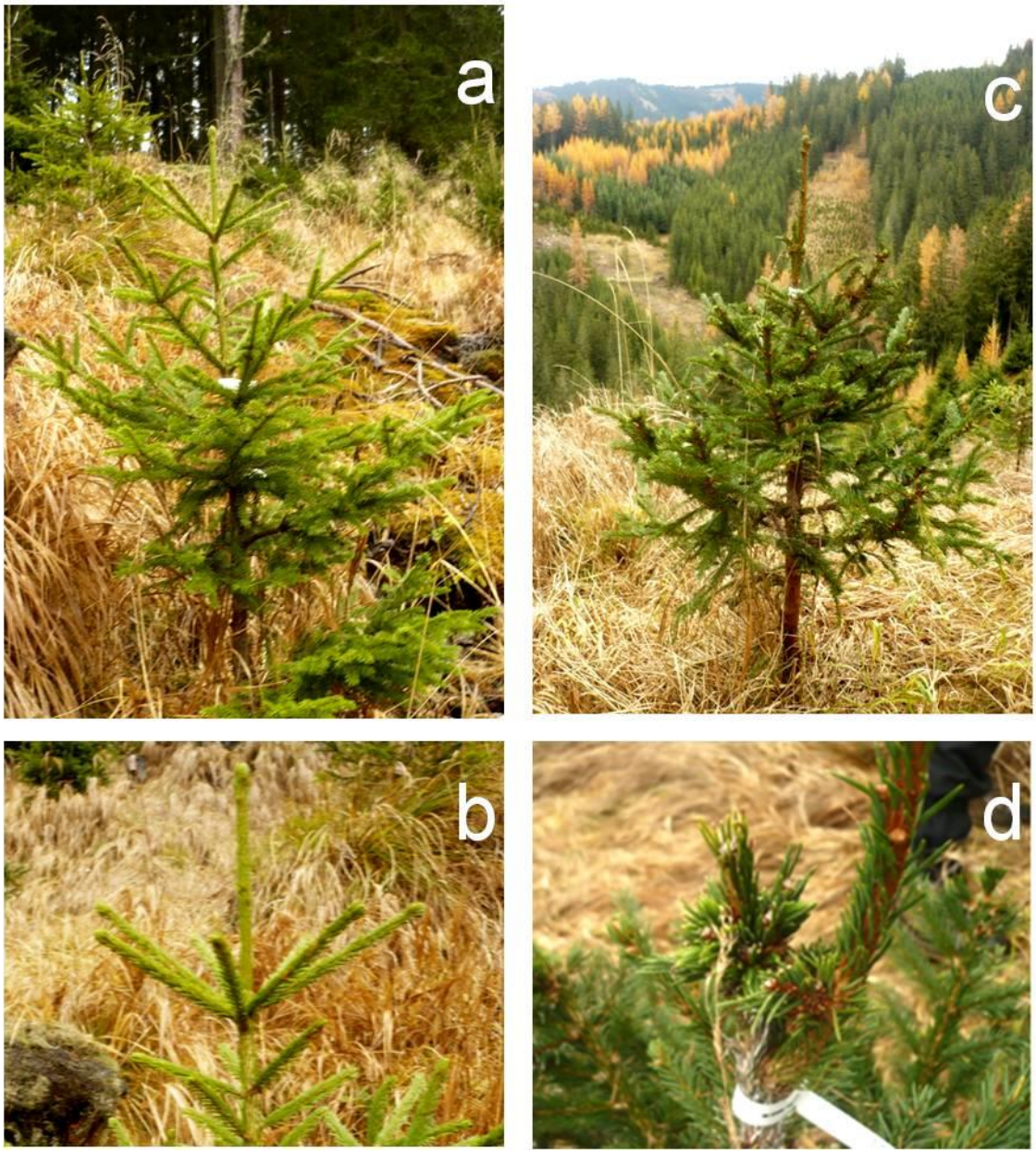

Figure 1. Putatively browsing “resistant” individual (a) with intact terminal shoot and side shoots (b). Susceptible individual (c) with terminal shoot and side shoots completely browsed (d). New leader shoot substitute the former browsed leader (d).

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Annual Variation of Mating System in Seed Orchard of *Pinus thunbergii* Revealed by Microsatellite Markers

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Key words: Pinus thunbergii, seed orchard, outcrossing rate, reproductive success

Background

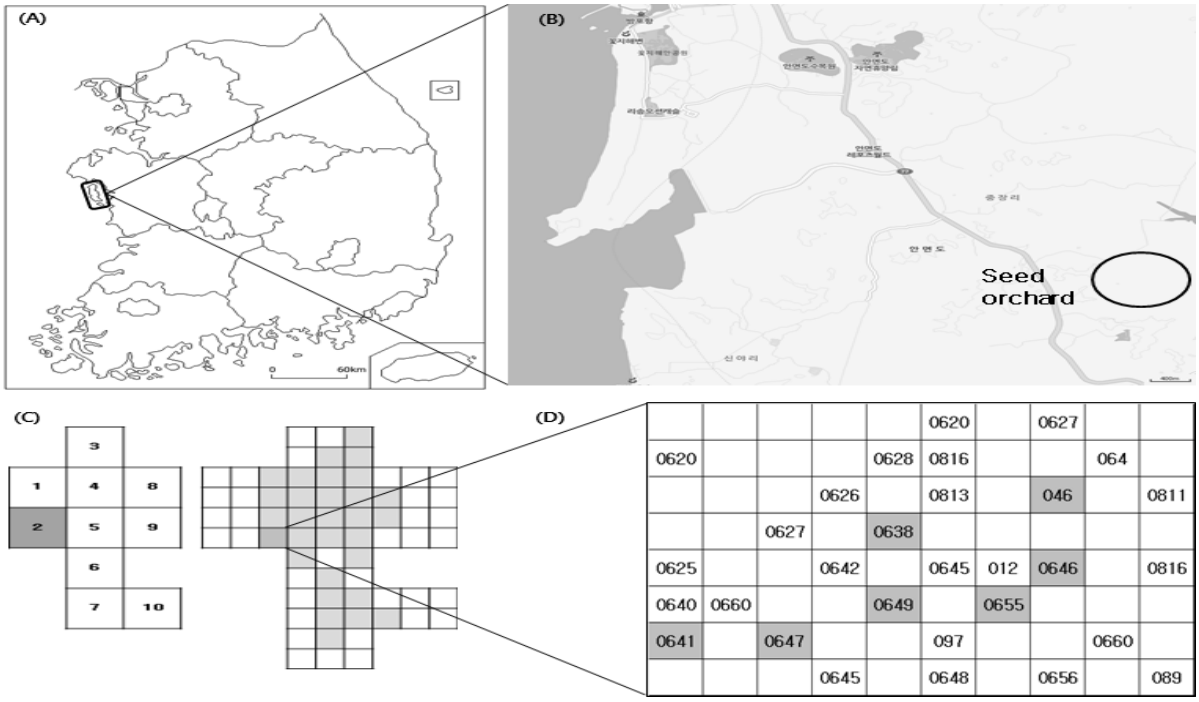
It is necessary to understand on mating system in seed orchard in order to improve managing skill, maximize potential of constituent clone, and obtain genetic gain. It is because of that mating circumstances were influenced by meteorological factor in each year. Comparing parameters of mating system on seed, produced in consecutive years, is essential to understand a general tendency of mating dynamics of seed orchard [1]. In this study, to provide useful information for improving seed orchard and establishing advanced generation seed orchard and to secure forestry management, the parameters of mating system were assessed.

Methods

To assess parameters of mating system in a seed orchard such as outcrossing rates,

degree of pollen contamination, and correlation of paternity (r_p), seeds were collected in the years of 2008, 2009, and 2010 from produced in '81plot of the *Pinus thunbergii* seed orchard at Anmyeon island, South Korea (Figure 1) and needles were obtained from constituent clones of that including 7 mother trees as well.

Figure 1. Location of (A) Anmyeon island in South Korea, (B) *Pinus thunbergii* seed orchard ’81 plot (N 36° 27′48″, E



126 °23′24″), (C) the study block in seed orchard, and (D) clone of 7 mother trees designated and others in the study block.

Microsatellite loci were amplified using 11 primer pares (pdms009, pdms065, pdms221, [2]; pde5, pde7, pde14, [3]; RPS2, *Pinus strobus*, [4]; SPAG7.14, *Pinus sylvestris*, [5]; B4535-2, B4630, B4637, [6]) with DNA extracted from embryo and needles. Genotypes were scored according to PCR product length at each locus using a 3130 *xl* Genetic Analyzer (Life Technolosies Corp., USA) and gene mapper ver. 4.0 analysis software (Life

Technolosies Corp., USA). From genotype data for the paternal candidates of the constituent clones, seed, and 7 mother trees, genetic diversity of each generation was calculated with GenAlEx ver. 6.41 [7]: observed heterozygosity (H_o), number of alleles (A), effective number of alleles (A_e), Shannon's diversity index (I). Self-fertilized seeds and pollen donor clones of seeds that had not been produced by contaminated pollen were determined using exclusion method with CERVUS ver 3.0.3 program [8].

Results and Conclusions (these last two either separate of together)

The observed heterozygosity (H_o) was 0.639 in mother trees and the range of 0.611 to 0.634 in seeds for three years. Shannon's diversity index (I) was 1.608 in mother trees and the range of 1.472 to 1.508 in seeds (Table1).

Table 1 Genetic diversity of clones and seeds from *Pinus thunbergii* seed orchard '81 plot in Anmyeon island, South Korea, using 11 microsatellite markers (The standard deviation is in parentheses.)

	N	A	A_e	I	H_o
Clone	102	12.5 (2.254)	4.2 (0.669)	1.608 (0.197)	0.639 (0.053)
Seed	809	15.9 (2.849)	3.8 (0.640)	1.502 (0.203)	0.620 (0.066)
Total	910	16.8 (2.941)	3.8 (0.651)	1.526 (0.204)	0.622 (0.064)
06_41^{08seed}	40	7.6 (1.309)	2.4 (0.330)	1.058 (0.169)	0.555 (0.079)
06_46^{08seed}	40	7.6 (1.351)	2.5 (0.277)	1.125 (0.152)	0.586 (0.063)
06_47^{08seed}	40	7.99 (1.282)	2.5 (0.259)	1.158 (0.145)	0.614 (0.072)
06_49^{08seed}	39	6.8 (1.212)	2.5 (0.269)	1.084 (0.154)	0.555 (0.073)
06_55^{08seed}	40	7.6 (1.239)	3.0 (0.397)	1.222 (0.176)	0.677 (0.080)
04_60^{08seed}	32	7.5 (1.268)	3.0 (0.372)	1.283 (0.164)	0.707 (0.072)
06_38^{08seed}	40	7.8 (1.464)	2.4 (0.321)	1.103 (0.172)	0.600 (0.089)
$total^{08seed}$	271	12.3 (2.149)	3.8 (0.670)	1.484 (0.205)	0.611 (0.066)
06_41^{09seed}	40	8.4 (1.521)	2.7 (0.397)	1.182 (0.181)	0.627 (0.087)
06_46^{09seed}	40	7.3 (1.047)	2.4 (0.251)	1.088 (0.140)	0.577 (0.075)
06_47^{09seed}	36	6.5 (1.012)	2.4 (0.270)	1.082 (0.134)	0.606 (0.073)
06_49^{09seed}	30	6.0 (0.831)	2.3 (0.219)	0.994 (0.135)	0.524 (0.068)

06_55^{09seed}	40	8.3 (1.236)	2.9 (0.367)	1.262 (0.162)	0.664 (0.074)
04_6^{09seed}	39	7.8 (1.524)	3.0 (0.372)	1.270 (0.165)	0.685 (0.073)
06_38^{09seed}	39	7.8 (1.143)	2.4 (0.351)	1.107 (0.150)	0.594 (0.081)
total^{09seed}	264	12.9 (2.318)	3.7 (0.607)	1.472 (0.197)	0.614 (0.064)
06_41^{10seed}	40	8.1 (1.498)	3.0 (0.503)	1.237 (0.194)	0.664 (0.094)
06_46^{10seed}	40	7.5 (1.098)	2.4 (0.246)	1.062 (0.144)	0.543 (0.064)
06_47^{10seed}	37	7.9 (1.297)	2.7 (0.276)	1.227 (0.144)	0.668 (0.077)
06_49^{10seed}	39	7.6 (1.479)	2.7 (0.304)	1.122 (0.165)	0.604 (0.079)
06_55^{10seed}	40	7.9 (1.310)	3.2 (0.393)	1.328 (0.169)	0.686 (0.073)
04_6^{10seed}	39	8.3 (1.421)	3.1 (0.406)	1.311 (0.165)	0.690 (0.081)
06_38^{10seed}	38	7.5 (0.976)	2.4 (0.355)	1.063 (0.148)	0.586 (0.089)
total^{10seed}	273	13.1 (2.451)	3.8 (0.625)	1.508 (0.203)	0.634 (0.068)

^{08seed} =seeds produced in 2008, ^{09seed}=seeds produced in 2009, ^{10seed}=seeds produced in 2010, *N*= number of sample, *A*= number of alleles, A_e= number of effective alleles, H_o= observed heterozygosity, and *I*= Shannon's information index.

There was no significant genetic difference between generations and among production years ($P > 0.05$). Estimates of outcrossing rates and contamination rates ranged from 87.7%~92.0%, 66.0%~69.1%, respectively (Table 2).

Table 2. Outcrossing rate and contamination rate in *Pinus thunbergii* seed orchard '81 plot in Anmyeon island, South Korea, using 11 microsatellite markers

Mother	2008			2009			2010		
	N	outcrossing rate(%)	contamination rate (%)	*N*	outcrossing rate(%)	contamination rate (%)	*N*	outcrossing rate(%)	contamination rate (%)
06_41	40.0	72.5	65.0	40.0	90.0	80.0	40.0	97.5	82.5
06_46	40.0	92.5	90.0	40.0	77.5	57.5	40.0	75.0	62.5
06_47	40.0	100.0	70.0	36.0	100.0	61.1	37.0	100.0	86.5
06_49	39.0	87.2	66.7	30.0	56.7	50.0	39.0	76.9	56.4
06_55	40.0	100.0	60.0	40.0	97.5	52.5	40.0	100.0	77.5
04_6	32.0	100.0	56.3	39.0	100.0	97.4	39.0	100.0	56.4
06_38	40.0	90.0	70.0	39.0	92.3	59.0	38.0	94.7	71.1
Mean	38.9	91.7	67.9	37.7	87.7	65.4	39.1	92.0	70.4

Frequency of contribution of clone in most frequently contributing pollen to 7 mother trees as paternal tree was 0.25 (2008), 0.17 (2009), and 0.13 (2010), respectively, which revealed

uneven mating success (Figure 2).

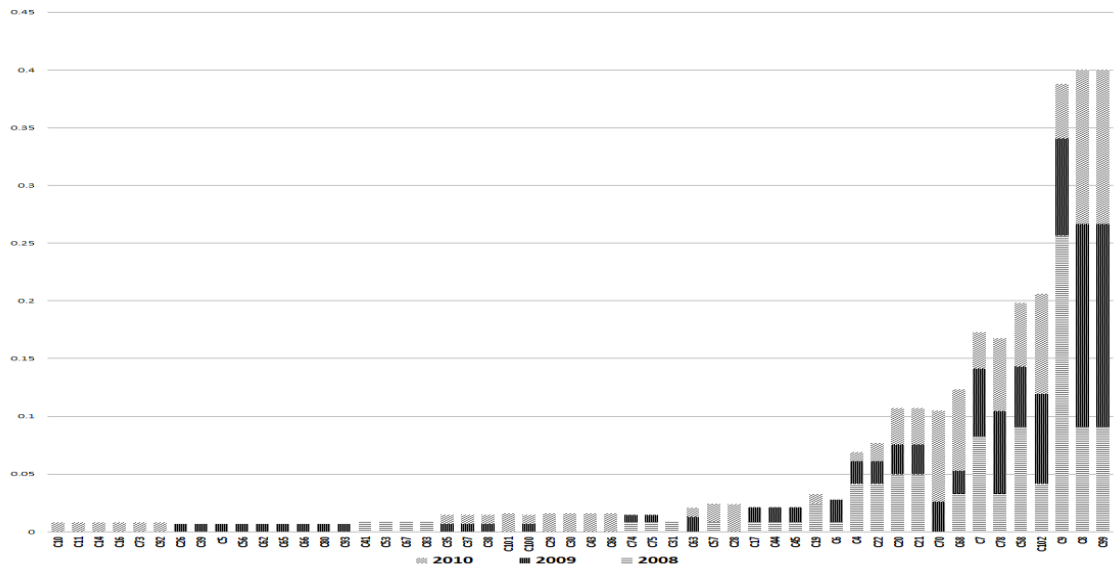


Figure 2. Reproductive success frequency among clones constituting *Pinus thunbergii* seed orchard ’81 plot, South Korea, over three years. X-axis is genotype of clones within seed orchard ’81 plot.

In conclusion, high level of outcrossing rates and a lot of putative pollen contributors were observed in seeds. However, high level of pollen contamination from outside of seed orchard and incomplete random mating among clones within seed orchard were estimated which might result in the maintenance of genetic variation. Taking the level of outcrossing rate and putative pollen contributors, genetic gain of seed produced from seed orchard, inbreeding depression by self-fertilization was expected to have less of an effect on genetic gain of seed produced from seed orchard seed produced from seed orchard was expected to have genetic gain on which inbreeding depression by self-fertilization had less of an effect and to be conveyed genetic variation. Although frequency of contribution of clone inside seed orchard as paternal tree was appeared uneven, there was no significant genetic difference between generations. Cause of that seeds maintained genetic diversity was not expected

by random mating among clones within seed orchard but by alleles originated from natural population which were flowed into seed orchard by pollen. To make an accurate estimate of genetic gain and to improve genetic quality of seeds produced in seed orchard, control of pollen contamination from outside of seed orchard may be the first option and selection of clones showing high general combining ability (GCA) from progeny test may be the second option for proceeding to the next generation seed orchard.

Competing interests

The authors declare that we have no competing interests.

Acknowledgements

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Screening of 19.2 Mb of genomic sequence surrounding DArT markers associated to wood quality traits in *Eucalyptus globulus*

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Key words: GWAS, lignin, genotyping.

Background

Genome-wide association studies (GWAS) aim at the identification of markers linked to genomic regions involved in the control of traits of interest [1]. A genotyping array with 7,680 DArT marker probes was developed for *Eucalyptus* [2]. Sequences obtained for 6,896 of them revealed that DArT markers preferentially target the gene space and display a homogeneous distribution across the genome [3], providing

good and targeted coverage for association studies in the *E. grandis* genome [4]. DArT markers showing significant associations with important traits and underlying genes are potentially useful to accelerate breeding and improve our understanding of the genetic control of complex traits.

Methods

Plant material and phenotypic traits

A mapping population of 134 *E. globulus* (Labill.) individuals was randomly sampled from an open-pollinated INTA trial located in Balcarce (Buenos Aires province, Argentina 37° 45′ S, 58° 17′ W) involving a total of 4200 trees that included 70 families (from 1 to 2 trees per family) from eight Australian origins and four landraces. Several growth and wood quality traits were evaluated: diameter at breast height, straightness of the bole, total height and wood density. In addition, wood chemical components were estimated using Near-Infrared (NIR) spectroscopy as described earlier [5]: lignin content (TL: Total Lignin and KLASON), Syringyl/Guaiacyl ratio (SG) and ethanolic extractives (ET EXT). The phenotypes were corrected for normality and the effect of large-scale environmental variation was removed by exporting residuals from an analysis where replicate was included as random effect in the model using ASREML [6].

DNA extraction and genotyping

Total DNA was extracted from young leaves using the CTAB method and DArT genotyping was carried out at DArT Pty as described earlier [2].

Association mapping

The analysis was carried out using TASSEL [7] under a mixed linear model (MLM) [8]; calculated as: $\mathbf{y}=\mathbf{S}\alpha+\mathbf{Q}\mathbf{v}+\mathbf{Z}\mathbf{u}+\mathbf{e}$ where **y** is the phenotype edited by environmental effects within each trial, **α** is the fixed marker effects related to **y** through the **S** matrix (DArT markers), **v** is the vector of fixed effects that estimates the proportion of each individual to the population structure and which is related to **y**

by the population structure (**Q**) matrix, **u** is the polygenic random effect (not captured by the markers) that is related to **y** through the **Z** matrix and **e** are the residual effects. The **Q** and **K** matrices were estimated as described before [5]. Positive associations were declared using a false discovery rate (FDR) for multiple comparisons ($p<0.05$) [9].

Mapping and annotation of DArT markers associated with target traits

Sequences for associated DArT probes were aligned to the sequence assembly of the *E. grandis* genome (version 1.1, http://phytozome.jgi.doe.gov). Mapping was carried out using the BWA-SW (version 0.6.2) component of the Burrows-Wheeler Alignment tool using default settings. A custom perl script was used to annotate the mapping positions with genes within a 600 kb window corresponding approximately to a 1.2 cM recombination distance [3]. Further annotation information available from Phytozome was used to predict gene function by *Arabidopsis* best hit.

Results

A total of 2,354 high quality dominant DArT markers were polymorphic and therefore used in the association analysis. Genetic structure carried out with a set of 400 random DArT markers revealed four subpopulations, consistent with the Australian origins present in the population, and was included in the association model. After correction for multiple testing ($p<0.05$), 44 positive marker-trait associations were found, all of them for lignin related traits as follows: 20 for TL, 14 for ET EXT, 7 for KLASON and 3 for SG.

Thirty three DArTs marker probes were mapped to single positions on chromosomes 1 and 7 (4 DArT each); 2 and 3 (5 DArT each); 4, 6, 8 and 9 (3 DArT each); 11 (2 DArT) and 10 (1 DArT); allowing the scanning of 19.2 Mb of annotated sequences.

A total of 1,137 annotated *E. grandis* genes were located in the scanned genomic segments and the function of 1,107 of them was predicted using the best *Arabidopsis* TAIR 10 hit. Based on the work of Myburg *et al.* [4] 60 genes were found in the following *E. grandis* categories: two genes for “Predicted cellulose and xylan genes” (CesA genes and glycosyltransferases), three genes each for “Predicted

transcription factor MYB" and "Predicted terpene synthase" and one gene each for "MADS and K-Box genes" and "Predicted peroxidase and laccase genes". Fifteen and 35 genes were found respectively in "SDRLK genes" (S-domain-Receptor-Like Kinase) and "Interpro domain in 968 genes unique to *Eucalyptus"*.

Conclusions

Only associations for lignin related traits were found and no significant association could be detected for growth and form. Despite the low power of the association mapping population used, these results corroborate previous reports in *Eucalyptus* [5] indicating that growth related traits are considerably more complex and likely controlled by several hundred or thousands of small effect regions that will hardly be detected in its entirety, even using a very large mapping panel, likely following what has been recently shown for human height [10]. Our results therefore suggest not only that lignin related traits are more tractable by GWAS, but also that genes with putative functions that are consistent with the measured traits can be detected providing clues for further molecular studies.

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Assessing patterns of genotype-by-environment interactions to aid planning for integrating genomic selection into tree breeding programs

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Keywords: tree breeding, genotype-by-environment interaction, genomic selection, linear mixed models

Background

Important genotype-by-environment interaction occurs when we observe rank changes of genotypes across environments. In practical terms, if an optimum set of genotypes in one environment is no longer optimum in contrasting environments, genetic gain is compromised. Genomic selection models attempt to characterize the additive effects of markers that are linked to QTL, and the values of each marker/allele combination can be added to estimate a breeding value for each individual. When the values of alleles change due to GxE interactions then the genomic selection model prediction accuracy is reduced. Without good information about GxE, a separate genomic selection model is required for each breeding zone. Therefore, if we can understand the patterns of GxE accurately, we may be able to strategically sample geographically to produce a model that will retain a high level of accuracy across a larger area.

The goal of this study was to characterize patterns of GxE for the lodgepole pine breeding program in British Columbia, Canada, and make recommendations about the minimum number of genomic selection models required for the larger program.

Methods

Sample Population and Experimental Design

The lodgepole pine breeding program in British Columbia, Canada, is composed of 41 progeny test sites with varying levels of genetic connectedness (i.e. common families tested among sites). Of these 41 sites, 33 had adequate family representation and connectedness for analysis, and represent two generations of progeny testing. The first generation is composed of 18 test sites from six initial breeding zones and the second-generation progeny tests are composed of 15 test sites from five of the initial six breeding zones. All sites were established using a randomized complete block design with families planted in row plots and measured for height growth at ages 5 and 10 from planting.

Statistical Analysis

The height at age 10 data was analyzed using a linear mixed model analysis and an approximate reduced animal model (ARAM) (Cullis et al. 2014):

$$y = X\tau + {}^{1}/_{2}\left(F_{op} + M_{op}\right)u_{ap} + Z_p u_p + e \qquad [1]$$

Where X is an incidence matrix for fixed effects, F_{op} and M_{op} are the incidence matrices for females to offspring and males to offspring, respectively, u_{ap} is a vector of additive genetic effects for the parents with var(u_{ap}) = σ^2_a, Z_p is an incidence matrix assigning data to the random interaction terms and design features with var(u_p) = Iσ^2_p where σ^2_p is the variance component for factors, and e are residual effects and var(e) = σ^2. The ARAM ignores Mendelian segregation and in this case results in a model that will give identical (co)variance estimates to the full animal model. The advantage of the ARAM is a significant reduction in equations that must be solved and computing time which increases the success rate for convergence of complex models and large datasets (Quaas and Pollak 1980). A factor analytic model was employed to estimate additive genetic (co)variance among sites and genotype–by-environment interactions (Cullis et al. 2014). Microsite variation was modelled using first-order autoregressive matrices for columns and rows and the following R matrix (Ye and Jayawickrama 2008):

$$R = \sigma^2_\xi [AR1(\rho_c) \otimes AR1(\rho_r)] + \sigma^2_\eta I \qquad [2]$$

where $AR1(\rho_c)$ and $AR1(\rho_r)$ are the first-order autoregressive correlation matrices for columns and rows, respectively, I is an identity matrix, σ^2_ξ is the spatial variance, and σ^2_η is the non-spatial variance.

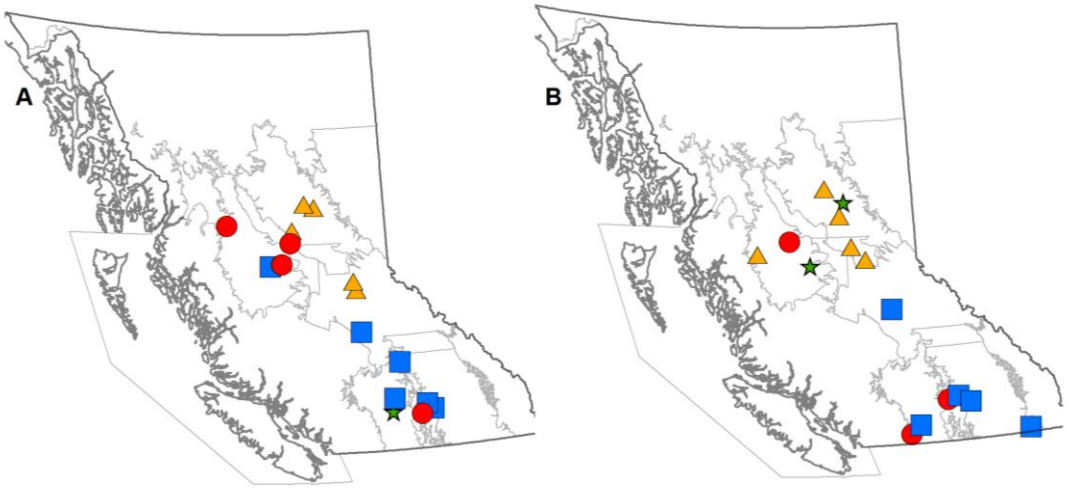

Figure 1: First-generation (A) and second-generation (B) test sites grouped according to Type-B genetic correlations. Triangles = northern group; squares = southern group; circles = overlap group; stars = outlier sites.

Results

The best model was achieved using a factor analytic of order 3 for the first-generation series and order 2 for the second-generation series. On average, genetic correlations among sites in the first-generation series were higher than those of the second generation which may be a result of bias due to varying levels of connectedness. Correlations among sites in the first-generation series ranged from 0.43 to 0.99 with a mean of 0.79 while correlations among sites in the second-generation ranged from -0.50 to 1.0 with a mean of 0.52. Heritability estimates for height growth were much lower for second-generation sites with a mean of 0.09 compared to the first generation with a mean of 0.25. The reduction in heritability was expected given the removal of provenance effects by selecting a subset of parents from the first generation. A heat map clustering algorithm was used to re-order the correlation matrix into groups of sites with high genetic correlations. Sites in the first-generation series clustered into two main groups and one small outlier group with two sites (Figure 1). The two main groups separate nicely between sites for breeding zones in northern and southern regions of the province. A group of four sites have strong correlations with sites in the northern and southern groups and can be considered overlap sites. Patterns of genetic correlations among sites in the second-generation series are similar to the first-generation series. The sites cluster into two main groups which separate sites in northern and southern breeding zones.

Conclusions

The results from this study show that there is relatively little GxE among sites in the three northern breeding zones and two southern breeding zones, but significant GxE among sites between these two larger geographic regions. This suggests that two main breeding

zones are more appropriate for lodgepole pine in British Columbia. Furthermore, the minimum number of genomic selection models required to adequately support the five major breeding zones for lodgepole pine in British Columbia appears to be two. In the absence of information about the patterns of GxE, a separate genomic selection model would be required for each zone resulting in a minimum of five required.

Competing interests

The authors declare that they have no competing interests.

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Genomic selection in a multi-generation conifer breeding population : maritime pine as a study case

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Keywords: maritime pine, genomic selection, multi-generation population

Background

Genomic selection aims to predict genetic merit of individuals based on a large number of DNA markers covering the whole genome to exploit the linkage disequilibrium between markers and any QTL. Several genomic selection proof-of-concepts have been published on conifers (Beaulieu *et al.* 2014, Resende *et al.* 2012, Zapata-Valenzuela *et al.* 2012).
On maritime pine, a first genomic selection study has been carried out based on a population with a large genetic base (communication submitted by Isik et *al.* entitled "Linkage Disequilibrium and Genomic Selection in Maritime Pine"). Average predictive ability reaches between 0.43 and 0.56 depending on trait considered; which is promising considering the low marker coverage.
Following this first genomic selection proof-of-concept on maritime pine, we have carried a second study based on a smaller effective size population from three successive generations. The goal of this study was, first, to increase the predictive ability and, second, to evaluate the efficiency of genomic selection models over generations.

Methods

We have sampled a genomic selection population with a limited effective size over three generations from the French maritime pine breeding program (the base population G0, the first and second generation G1 and G2, respectively). Given the physical size of the genome (24 Gb/C) and the low number of genetic markers available, the aim of this study was to increase predictive ability by retaining significant linkage disequilibrium between markers and QTLs in a small effective size population. The population was designed following a two-step strategy. First, we selected G2 trees in polycross trials based on their performance and genetic diversity criteria (to reach similar contribution from G0 trees). Then, we recovered the paternity identity to select 710 G2 trees and all their ancestors from the base population (46 G0 and 62 G1 trees). This population

(Ne=24) with high breeding values for growth and stem straightness was genotyped with 4,100 informative SNPs. The pseudo-phenotypes considered for genomic selection analyses consisted in the breeding values evaluated from a global analysis of the breeding population (more than 350,000 trees phenotyped).

Results and Conclusions

Three different genomic selection methods were considered: genomic BLUP, BAyesian Ridge regression and Bayesian LASSO. Genomic selection predictive models were established both within G2 generation (training set with 80% of the G2 and the 20% remaining G2 for the validation set) and between generations (training set with G0 - G1 trees and validation with the G2 generation). In both cases, predictive ability was high (>0.7). This study suggests that, even with low marker coverage, genomic selection could be efficient over generations if a population of low effective size is considered.

Competing interests

The author declares that they have no competing interests.

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Zapata-Valenzuela J, Isik F, Maltecca C, Wegrzyn J, Neale D, McKeand S, Whetten R: SNP markers trace familial linkages in a cloned population of Pinus taeda—prospects for genomic selection. *Tree Genet Genomes* 2012.

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Efficient method to extract high quality DNA from dried Eucalyptus leaves

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Keywords: Eucalyptus, purification, high-quality genomic DNA.

Background

Since the 70s, FCBA is working on Eucalyptus species and his research program is focusing on wood quality, biomass production and frost resistance. The Eucalyptus species under study are *E. gunnii*, *E. dalrympleana* and their hybrid *E. gundal*. Today several thousand hectares are planted in south of France for paper pulp or biomass production.

In various research projects, we need high quality DNA in order to explore genetic diversity or compare our species with other studied eucalyptus species such as *E. grandis* and *E. urophylla*. Eucalyptus species are typically more demanding than other trees to extract pure, high quality DNA for molecular genetics. And it is even more crucial for leaves which contain a lot of impurities such as terpenes, polyphenols and polysaccharides, which tend to co-purify with DNA (1).

DNA yield and quality often vary amongst species of the same genus and amongst different sources of tissues from the same tree (2). In order to respond to these issues, FCBA has developed a DNA extraction method from a commercial kit to obtain large amounts of high quality DNA within the shortest time possible.

The use of molecular markers in breeding programs cannot be done without an optimized management of costs and time devoted to DNA extractions. In this study, a mixer mill is used for tissue disruption with liquid nitrogen first, then the Plant Prep Adem-Kit (Ademtech) is used with paramagnetic beads to bind DNA during purification in 96-well plate. Some supplementary steps were also added to efficiently remove impurities.

Methods

Plant material

The foliage from several hundred individuals of Eucalyptus (*E. gunnii* and *E. dalrympleana*) were collected from a FCBA conservatory of Eucalyptus genetic resources, and stored dried with silicagel.

DNA extraction

The DNA extraction was performed according to the protocol provided by Ademtech but some steps were modified or added. The improved protocol includes the seven following operations:

1) Grinding: Two stainless steel beads and 50 mg of dried leaves were placed in microtubes with PVPP (Polyvinylpolypyrrolidone) (3) and were grinded for 1 min at 30 Hz in a mixer mill.

2) Lysis: 400 µl of preheated Ademtech lysis buffer, 4 µl of RNase A and 2% of PVP (Polyvinylpyrrolidone) (w/v) (3) were added in each microtube. The plates were vortexed and then incubated and mixed for 15 min at 65°C and 1 500 rpm. Lysates were centrifuged 15 min at 6 500 g and the supernatant (around 250 µl) was transferred into new plates.

3) Cleaning: 250 µl of dichloromethane (4) were added in each microtube. Plates were mixed gently by inversion. Then plates were centrifuged 5 min at 6 500 g and the lysates were once again transferred into new plates.

4) Binding: 250 µl of isopropanol and 15 µl of silica-Adembeads were added. Plates were mixed by inversion and incubated 5 min at 1 000 rpm.

5) Washing: Plates were magnetized on magnetic stands. Supernatant was discarded carefully without disturbing the pellet of magnetic particle. 500 µl of washing buffer were added. The washing step was repeated four times with different buffers (first washing buffer I, then washing buffer II, then ethanol 70% and to finish ethanol 90%).

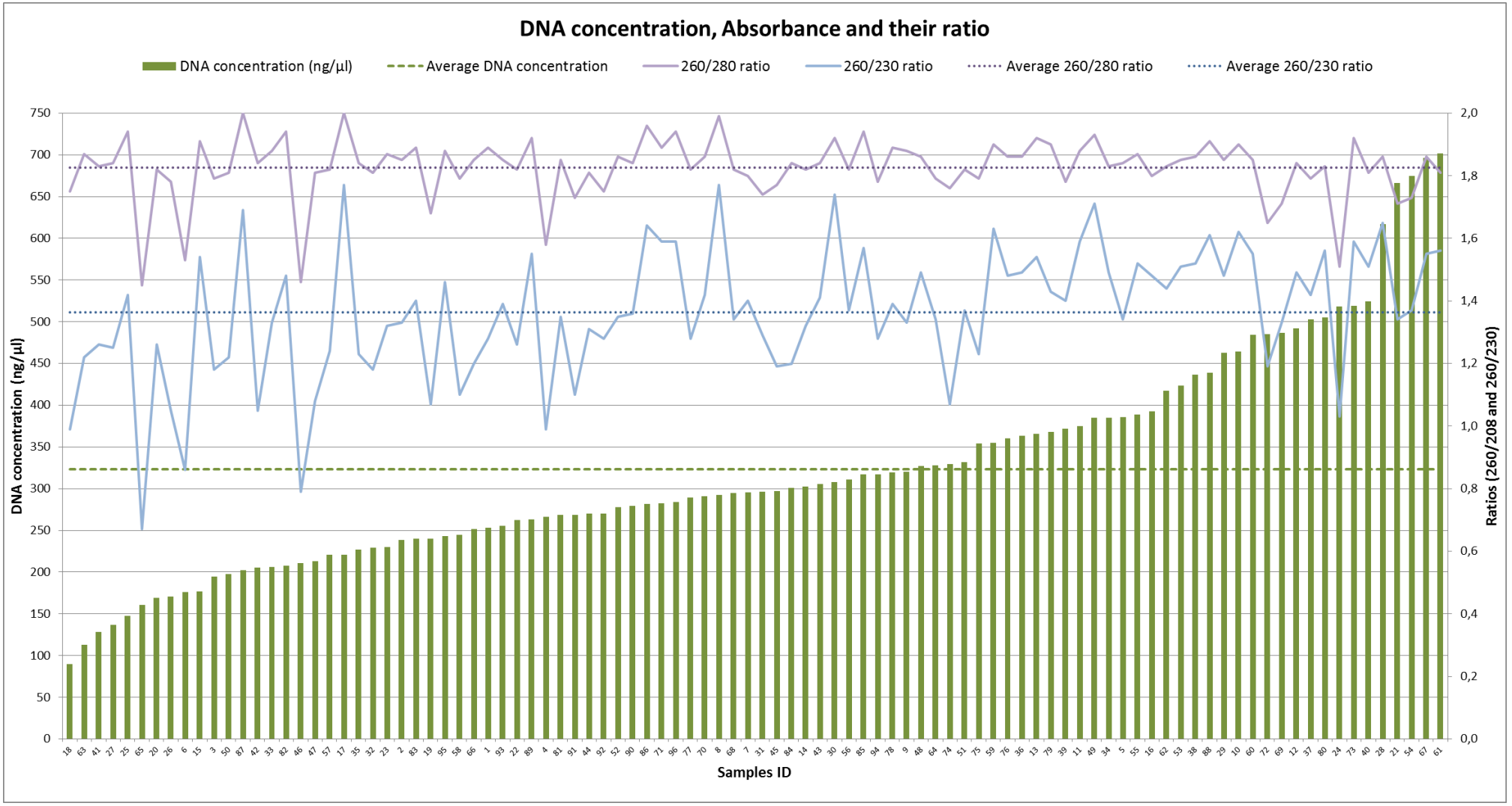
6) Drying: Plates were magnetized and supernatant was discarded. The pellets were dried for 5 min.

7) Elution: Pellets particles were resuspended in 60-100 µl of elution buffer. Plates were incubated at 50°C and 1 000 rpm for 5 min. Eluates were magnetized for 5 min and then transferred into microplates for the storage at – 20°C.

Results and Conclusions

Genomic DNA was quantified by spectrophotometric measurement of sample absorbance at 260 nm and 280 nm, and qualified at 230 nm. Graph 1 shows some of the results such as the obtained purity of DNA extracts. Actually the average ratio 260/280 and 260/230 was respectively 1.8 and 1.3. The PVPP and the PVP were crucial components of the protocol in order to favor yield and purity of DNA isolates. These substances are adsorbents commonly used to eliminate polyphenols. With regard to the dichloromethane, this substance was used to allow the removal of proteins and lipidic compounds. Results show that this protocol effectively enable the extraction of high DNA concentrations of high purity; the average DNA concentration was around 323 µg/µl. DNA quality was further evaluated by amplifying several microsatellite loci (5).

Graph 1: Example of spectrophotometric measurements for one 96-well plate



Competing interests

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GENETIC VARIATION BETWEEN PROGENIES of *Pinus caribaea* Morelet var. *caribaea* BASED ON MICROSATELLITE MARKERS

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Background: Species of *Pinus* genus are widely used in homogeneous reforestation projects world wide, especially for their good adaptation to different climates and the wide use of their products (wood, resins, pulp, Sawn wood, paper, etc…) (1). Morphological and silvicultural traits of *Pinus* besides the introduction of *P. caribaea*, tropical species, contributed to geographical expansion of planted forests in Brazil. Reforested areas, previously restricted to the South, have been expanded, reaching the Southeast and Midwest beyond some areas of North and Northeast (2) regions. Currently microsatellite is the most polymorphic among the available markers. The first microsatellites were developed for *Pinus radiata* (3). Nearly 86 % of the genome of this species consisted of repetitive DNA elements (4). The aim of this study was to estimate genetic diversity within and among three progenies of *P. caribaea* var. *caribaea* classified according to their breeding values performed from diameter at breast height (d.b.h) based on SSR markers.
Material and methods: For the present study, 96 individuals of *P. caribaea var. caribaea* progenies were selected and tested in an experimental area located at the Farm Teaching and Research facilityfrom – Universidade Estadual de São Paulo (UNESP), campus Ilha Solteira, in municipality of Selvíria, Mato Grosso do Sul State, Brazil. Values were predicted for total trees, based on the dbh at 14.3 years old. Individuals were classified into three groups (subpopulations) according to their breeding values: superior, intermediate, and inferior. Thirty inferior individuals were selected, 36 intermediate, and 30 superior. ADN of these groups was extracted following the protocol proposed by (5), and modified by (6). Genetic parameters were estimated using Tool for Population Genetic Analyses (TFPGA) software. Variation between and within subpopulations was performed through Wright's *F* statistics (8).

Results and discussion: For individuals from superior group variation in allele frequencies ranged from 0.5192 (allele 1 primer RPTest 09) to 0.9318 (allele 1 primer PtTX 2037). The highest and smaller allele frequencies were observed for allele 2. For intermediate group, allelic frequencies varied from 0.6667 (primer RPTest 09) to 0.9000 (primer APC13) for allele 1 and from 0.0571 (primer APC13) to 0.3333 (primer RPTest 09) allele 2. For inferior group alleles frequencies varied from 0.5352 (primer RPTest 09) to 0.9783 (RPTest 01) for allele 1; and from 0.0217 (01 RPTest) to 0.4348 (RPTest 09) allele 2. Average observed and expected heterozygosity values were very similar to three groups. The variability for superior group was slightly higher (0,3725 and 0,3363) than that for intermediate (0,3690 and 0,3207) and inferior (0,3430 and 0,2840) groups. According to the F statistics, there is fixation of alleles analyzing average of all loci, between groups (subpopulations) F_{ST} was near zero (FST = 0.0075), furthermore a heterozygotes

excess of (FIS = -0.1489). This means that the selection in the progeny test is favoring individuals heterozygous over homozygous.

Conclusion: Genetic variation within progenies was maintained, despite of intense selection either for subpopulations with inferior, intermediate and higher performance for dbh.

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Measuring telomere length in forest trees and its relevance to tree breeding

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Key words: Telomere length, terminal restriction fragment (TRF), quantitative real-time PCR (qPCR), *Populus*.

Background
Telomeres are specialized nucleoprotein complexes located at the ends of chromosomes to protect them and ensure cell viability. In humans, telomere length research has gained great relevance because of its key role in aging and in aging-associated illnesses such as cancer, or cardiovascular diseases. However, very little is known about the functional significance of telomere length in plants and trees. Until now, the availability of a practical method for measuring telomere length in plants and trees has been rather lacking.

Methods
Plant samples consisting of mature and young leaves, light green and yellow leaves as well as *in vitro* leaves were collected from *Populus* spp., *Zea mays* and *Arabidopsis thaliana*, and stored at – 80°C. Measurement of telomere length by the Southern blot analysis of terminal restriction fragment (TRF) lengths and of T/S by qPCR were performed on genomic DNA. In this study approximately 30 samples from 3 different plant species were analyzed. A comprehensive decision-making process for telomere length research in trees was also provided. Furthermore, a regression analysis between this newly standardized qPCR method and the conventional Terminal Restriction Fragment (TRF) analysis by Southern blot was established to validate the data, and to determine the average length of the subtelomeric region. All statistical analyses were performed using the R software (version 2.15.2, A Language and Environment Copyright, 2012).

Results and Conclusions
A robust measurement of telomere length was developed and used to demonstrate the heterogeneity of telomere length in several plant and tree species and tissues. This newly developed qPCR method will facilitate the analysis of tree telomere length (TTL) from low amounts of tissues. This method involves the amplification of single copy sequences as DNA quantity reference, and was initially described by [1] in humans. It can be further exploited to study tree aging and responses to environmental stress in order to determine if telomere length can be used as potential biomarker of stress [2,3]. Together, these data will

make a significant contribution to telomere biology in trees and can have implications on plant breeding and improvement programs.

Competing interests

The author declares that they have no competing interests.

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Prediction Accuracy of Growth and Wood Attributes of Interior Spruce in Space Using Genotyping-by-Sequencing

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Key words: Interior Spruce, Genomic Selection, Genotyping-by-Sequencing

Background: Genomic selection (GS) in forestry can substantially reduce the length of breeding cycle and increase gain per unit time through early selection and greater selection intensity, particularly for traits with low heritability and late expression. Affordable next-generation sequencing technologies made it possible to genotype large numbers of trees at a reasonable cost.

Methods: Genotyping-by-sequencing was used to genotype 1,126 Interior spruce trees representing 25 open-pollinated families planted over three sites in British Columbia, Canada. Four imputation algorithms were compared (mean value (MI), singular value decomposition (SVD), expectation maximization (EM), and a newly derived, family-based k-nearest neighbor (kNN-Fam)). Trees were phenotyped for several yield and wood attributes. Single- and multi-site GS prediction models were developed using the Ridge Regression Best Linear Unbiased Predictor (RR-BLUP) and the Generalized Ridge Regression (GRR) to test different assumption about traits architecture. Finally, using PCA, multi-trait GS prediction models were developed.

Results: The EM and kNN-Fam imputation methods were superior for 30 and 60% missing data, respectively. The RR-BLUP GS prediction model produced better accuracies than the GRR supporting traits' complex architecture. GS prediction accuracies for multi-site were high and better than those of single-sites while multi-site predictability produced the lowest accuracies reflecting type-b genetic correlations and deemed unreliable. The incorporation of genomic information in quantitative genetics analyses produced more realistic heritability estimates as half-sib pedigree tended to inflate the additive genetic variance and subsequently both heritability and gain estimates. Principle component scores as representatives of multi-trait GS prediction models produced surprising results were negatively correlated traits could be concurrently selected for using PCA2 and PCA3.

Conclusions: The application of GS to open-pollinated family testing, the simplest form of tree improvement evaluation methods, was proven to be effective. Prediction accuracies obtained for all traits greatly support the integration of GS in tree breeding. While the within-site GS prediction accuracies were high, the results clearly indicate that single-site GS models ability to predict other sites are unreliable supporting the utilization of multi-site

approach. Principle component scores provided an opportunity for the concurrent selection of traits with different phenotypic optima.

Competing interests

The authors declare that they have no competing interests.

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Novel and known microRNAs identified in vascular tissues in Scots pine and Maritime pine

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Key words: Pinus pinaster; Pinus sylvestris; smallRNA; post-transcriptional regulation

Background

Among conifers, Pines have high economic importance mainly due to wood and resin production. In the last decade, several transcriptomic and proteomic resources were made available providing opportunities to identify major molecular players involved in xylogenesis and biotic and abiotic responses (e.g. [1,2]). Nevertheless, the understanding of post-transcriptional regulation mediated by miRNA in Pines remains scarce. MicroRNAs are small non-coding RNAs (21-24bp) that act by down-regulating mRNA expression either by cleavage or by translational repression, through direct base-pairing to target sites [3]. Gemome-wide identification of miRNAs and the perception of the impact of their regulatory roles on plant development and stress responses is susceptible to benefit the definition of strategies for wood quality improvement or stress response strategies.

Next generation sequencing (NGS) technologies have been commonly used, in recent years, on the identification of novel and known miRNAs. Besides, miRNA target identification is also benefiting from these new technologies. High-throughput methods such as the parallel analysis of RNA ends (PARE), also known as Degradome-Seq or genome-wide

mapping of uncapped transcripts (GMUCT) [4], have contributed for the identification and validation of miRNA-directed target cleavage. Indeed PARE is most suitable for plants and coupled with high-throughput sequencing allowed for the identification of widespread mRNA cleavage events regulated by miRNAs in different species [5].

Here, we will present the first results on the identification of miRNA present in vascular tissues (developing xylem and phloem) and needle epidermis of two pine species (*P. pinaste*r and *P. sylvestris*) of major economic and ecological importance in European forest.

Methods

Total RNA was extracted from developing xylem and phloem from adult trees of *P. pinaster* and *P. sylvestris*, and also from needle epidermis of *P. pinaster*, following the protocol of Carvalho *et al*. (2015). RNA samples were sent to LC Sciences (Houston, TX, USA) to generate five smallRNAs libraries each corresponding to a tissue/species combination, and sequenced with the high-throughput Illumina Solexa system. A dedicated LC Sciences pipeline was used for the identification of miRNA candidates. For the same tissue/species combinations, five degradome libaries were prepared and sequenced by LC Sciences. CleaveLand pipeline [6] was used to identify miRNA:target iteractions, based on *P. pinaster* and *P. sylvestris* transcriptomes available at www.plantgenie.org.

Results and Discussion

Non-coding transcriptome were successful assessed by NGS for vascular tissues and needle epidermis of both *P. pinaster* and *P. sylvestris.* High-throughput sequencing of the five small RNAs libraries generated a total of 6,4M raw reads, from which 5.6M are considered mappable, with a size modal distribution (21nt, 32% of the reads). Among these, 4.8% of all mappable reads allowed us to identify 5,832 miRNA candidate loci, including 44 known miRNAs already identified in other species (1,3 M reads) and 1,291 new high-confidence miRNA candidates (9,9M reads). The majority of the known miRNAs was already identified in *P. taeda and P. densata*. Seventy four miRNA sequences with highest abundance (number of reads higher than the average of 1059 reads) were selected for testing their presence in the several sampled tissues and genomes. The analysis of melting curves, allowed us to confirm the presence of both pre- and mature miRNAs in 41 out of 74 miRNAs analysed. Additionally, degradome sequencing analysis allowed us to detect 357, 312, 534, 1790 and 428 interactions between target genes and putative miRNAs for *P. pinaster* and *P. sylvestris*. The genomic resources presented here stand out with great

relevance for the understanding of post-transcriptional regulation underlying the development of the vascular tissues and stress responses in conifers.

Competing interests

The author declares that they have no competing interests.

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Hairy root transformation of *Eucalyptus grandis*, a tool to investigate the function of genes involved in wood formation

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Keywords: Eucalyptus, hairy roots, secondary cell wall, Cinnamoyl CoA Reductase (CCR)

Background

Eucalyptus are among the world’s leading sources of woody biomass because of their rapid growth rate, broad adaptability to diverse edaphoclimatic conditions and their multipurpose wood properties. Wood is formed of secondary cell walls (SCWs) mainly constituted of cellulose, hemicelluloses and lignins. The composition and the structure of the SCWs relying among interactions between these biopolymers are major determinants of industrial processing efficiency. The huge economic importance of Eucalyptus wood has been a driving force that led to the sequencing of the *Eucalyptus grandis* genome [1]. This invaluable tool has

allowed genome-wide characterization of many gene families, notably those involved in the lignin biosynthetic pathway [2] as well as transcription factors families containing members known to regulate SCWs' formation such as the MYB [3], NAC [4], ARF [5] and Aux/IAA [6] families. These studies combining comparative phylogeny to large-scale expression profiling in a wide panel of developmental and environmental conditions have underscored new candidates potentially regulating wood formation. To get further insight into their roles, these candidates need to be functionally characterized preferably using a homologous transformation system. Although stable transformation protocols have been established for several *Eucalyptus* species [7, 8 and references therein], they are not suitable for medium/high throughput functional characterization since they are tedious, time consuming and present low efficiencies. To overcome these limitations, we decided to develop an alternative stable transformation system using *A. rhizogenes* to functionally characterize genes involved in xylogenesis.

Methods

We adapted an *A. rhizogenes*-mediated transformation protocol developed for *E. camaldulensis* [9], and optimized it to transform *E. grandis* seedlings using the A4RS strain and binary vectors harboring fluorescent reporter genes (GFP or DsRED). One month after infection, non-fluorescent roots were excised from *in vitro*-grown composite plants that were then acclimatized and grown for six more weeks. Xylem anatomy and lignin content were assessed by histochemistry. Lignin content and composition are being evaluated by thioacidolysis.

Results and discussion

Using this optimized protocol, we obtained good transformation efficiencies, 62% in average, reaching up to 75% is some cases. This percentage reflects the number of co-transformed plants (containing at least one co-transformed root), over the total number of plants inoculated by *A. rhizogenes*. This transformation rate is far higher than the *A. tumefaciens*-mediated stable transformation of Eucalyptus spp [7 and references therein]. Forty five days after acclimation, the length of the composite plants' transgenic roots reached 20 cm in average.

The primary and secondary xylems' structures in transgenic roots were similar to that of wild type roots suggesting that *E. grandis* hairy roots can be used to perform functional characterization of genes involved in wood formation. As a proof of concept, we transformed the roots with a cinnamoyl CoA reductase (EgCCR1) antisense construct. This enzyme catalyses a limiting step regulating the carbon flux towards lignins [2, 10]. The *E. grandis* hairy roots with down-regulated *EgCCR1* showed the presence of an irregular xylem with collapsed vessels as shown previously in tobacco [11]. The lignin content was reduced as suggested by the faint phlorogucinol staining in agreement with previous studies in other angiosperms.

Conclusion

We described an efficient and rapid stable transformation system for *E. grandis* using *A. rhizogenes* and fluorescent proteins allowing easy selection of hairy roots. The development of such a powerful tool is timely since it is suitable for medium throughput functional characterization of genes from the recently sequenced Eucalyptus genome [1]. Last but not least, we have shown that the hairy roots are able to develop secondary xylem similar to non transgenic roots, and as a proof of concept, we demonstrated that down regulation of *EgCCR1*, a key lignin gene, produced similar SCW phenotypes to those reported previously in stems of other species. These results support the usefulness of the hairy roots as a homologous and versatile system to study genes involved in wood formation in *Eucalyptus*.

Competing interests

The author declares that they have no competing interests.

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Molecular understanding of nitrogen-fixing nodule development in the tropical actinorhizal tree *Casuarina glauca*

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Keywords: Casuarinaceae, Frankia, nitrogen fixation, symbiotic genes

Background

Casuarinaceae trees are fast-growing multipurpose species which do not require chemical fertilizers due to their symbiotic association with the nitrogen-fixing actinomycete *Frankia* and with mycorrhizal fungi that contribute to improve phosphorous and water acquisition by the root system. As a consequence, these actinorhizal trees are capable of growing in poor and disturbed soils and are important elements in plant communities worldwide. They are used in forestry for timber and pulpwood production; they are also planted to stabilize desert and coastal dunes, protect field crops and restore degraded soil sites [1].

Because nitrogen is a critical element for tree growth and development, the understanding of the molecular basis of actinorhizal root nodule symbiosis is a major issue. Recently, the development of genomics in some actinorhizal trees such as *Casuarina glauca* and *Alnus glutinosa*, together with the possibility to obtain transgenic *C. glauca* plants following *Agrobacterium* gene transfer, has offered new approaches to understand the molecular basis of the actinorhizal process [2]. The current knowledge regarding the early stages of the symbiotic process in *C. glauca* will be highlighted.

Methods

Genetically transformed *C. glauca* plants were used for dissecting the roles of candidate genes in plant root infection and nodule development [3]. Two different protocols were developed, one depending on the disarmed *A. tumefaciens* strain C58C1(pGV2260; pBIN19GUSint) [4] and the other one relying on a quick procedure to obtain composite *C. glauca* plants with co-transformed hairy roots [5].

The reporter genes *GUS* and *GFP* were used to characterize the spatiotemporal expression conferred by promoter regions of symbiotic genes. RNAi constructs were introduced by *A. rhizogenes* in the root system of *C. glauca* in order to down-regulate the targeted plant genes [6].

Nodulation of transgenic and control plants of *C. glauca* was achieved with the strain of *Frankia* CcI3 [4].

Results and discussion

The understanding of the molecular mechanisms involved in the actinorhizal symbiosis has benefited from the recent breakthoughs obtained on the model Legumes *Medicago truncatula* and *Lotus japonicus* [7]. In Legumes, the receptor kinase DMI2/SymRK encodes a leucine-rich-repeat receptor kinase that is required for nodulation and endomycorrhization. Downregulation of *CgSymRK* isolated from *C. glauca* revealed that the frequency of nodulated RNAi-*CgSymRK* plants was reduced 2-fold compared to *C. glauca* control plants [8]. Additional experiments showed that CgSymRK was also necessary for the establishment of the symbiosis with *Glomus intraradices*, thus revealing the key role of CgSymRK in root endosymbioses in *Casuarina*, and the conservation of SymRK function between legumes and actinorhizal trees [8].

The symbiotic gene *CgCCaMK* that encodes a Calcium and Calmodulin-dependant kinase homolog to the *DMI3/CCaMK* gene from legumes, was then characterized [9]. Functional analysis of the *CgCCaMK*-RNAi plants revealed that this gene was necessary for appropriate root infection by *Frankia* and nodule development in *C. glauca*, thus suggesting that the early signal transduction pathway required for actinorhizal nodulation is at least partially common with rhizobial and arbuscular (AM) symbioses.

The actinorhizal symbiosis is a highly controlled process, involving specific *Frankia* factors recognized by plant roots [2]. Using transgenic *C. glauca* plants, we demonstrated that *CgCCaMK-GUS* was induced after 24 h of contact with the still unknown *Frankia* factors. This finding is of particular interest since it provides a biological test for the isolation and further characterization of *Frankia* molecules perceived by the actinorhizal tree.

Conclusion

Ongoing research on the actinorhizal tree *C. glauca* has led to major advances in our knowledge of symbiotic genes involved in the interaction with *Frankia* [2]. The major challenge in the coming years will be the isolation of the receptor for the signal molecules emitted by the actinobacteria in response to contact with the root system of the host, and the biochemical characterization of these molecules.

Competing interests

The author declares that they have no competing interests.

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Tree selection including several QTLs related to growth and wood quality traits in *Eucalyptus grandis*

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Key words: QTL – *Eucalyptus grandis* – Wood quality – Marker assisted selection

Background

A large number of QTL maps have been developed in the genus *Eucalyptus* [1]. However the application of this tool in forest species is often limited by the high diversity in trees, preventing transfer of information obtained in a mapping population to other individuals of the breeding program. However, when controlled crosses made from elite trees are generated, selection within their progeny would yield superior individuals, as potentially some will carry favorable regions for traits of interest from each parent, which may have synergistic effects.

Wood density, is a convenient indicator of mechanical properties for sawn timber production and also determines pulp yield. For paper production, low lignin [2] is desirable to reduce the pollution caused during removal, whereas for sawn timber production, high lignin content confers hardness. Furthermore, a low content of extractives for paper production is required due to the difficulty of removing lignin,

whereas in solid applications both lignin and extractives could offer protection against pathogens [3, 4].

Previously, QTLs for wood density, modulus of elasticity, tree height, diameter at breast height, volume, lignin content (Klason and total) and composition (Syringyl/Guaiacyl); and ethanolic extractives in an intraspecific cross of *Eucalyptus grandis* were mapped [5].

In this paper the allelic phase of the markers that flank the QTLs were used to identify individuals from the cross that contained favorable QTLs for each trait.

Materials and methods

Plant material. An intraspecific cross of *E. grandis* with parents contrasting for wood density (Clone EG-INTA-161 x Clone EG-INTA-152) consisting of 131 full siblings (with three to seven clones) of a population planted on a single site.

Genotypic data. Maps were developed with DArT, SSR, EST-SSR and GC-SSR and each QTL flanking DArT markers are described earlier García *et al.* (2011) [5].

Individuals that presented the favorable configuration for each QTL for each trait and those that accumulate a greater number of QTLs were manually identified.

Mapping and annotation of DArT markers. The WGS sequence assembly of *E. grandis* genome (version 1.1, http://phytozome.jgi.doe.gov) was used as reference for mapping of all the DArT sequences [2]. The gene location and GO annotation in *E. grandis* genome were obtained with biomaRt package, and the gene identity was complemented with the information obtained from Myburg, *et al.* (2014) [6]. For each QTL with highest LOD, flanking markers were identified and +/- 0.5Mbp regions (recombinations distance of approximately 1cM each side [7]) were explored.

Results and discussion

Those individuals carrying favorable QTLs alleles for nine economically important traits studied (**Table 1**), and the individuals that accumulate several QTLs for each trait (**Figure 1**) were identified.

Table 1. Number of individuals with favorable alleles for QTLs associated for nine traits.

Physical and mechanical properties	**Wood density**	**QTL1 (R^2=0.131)**	**QTL2 (R^2=0.083)**	**QTL3 (R^2=0.290)**	**QTL4 (R^2=0.068)**					
	Number of individuals	38	70	50	34					
	Modulus of elasticity (MOE)	**QTL (R^2=0.094)**								
	Number of individuals	51								
Growth	**Tree heigth (TH)**	**QTL1 (R^2=0.178)**	**QTL2 (R^2=0.098)**	**QTL3 (R^2=0.109)**						
	Number of individuals	7	56	14						
	Diameter breast height (DBH)	**QTL1 (R^2=0.228)**	**QTL2 (R^2=0.065)**							
	Number of individuals	2	7							
	Volume (VOL)	**QTL1 (R^2=0.085)**	**QTL2 (R^2=0.223)**							
	Number of individuals	57	62							
Chemical properties	**Klason lignin**	**QTL1 (R^2=0.006)**	**QTL2 (R^2=0.005)**	**QTL3 (R^2=0.002)**	**QTL4 (R^2=0.204)**	**QTL5 (R^2=0.002)**	**QTL6 (R^2=0.005)**	**QTL7 (R^2=0.003)**	**QTL8 (R^2=0.094)**	**QTL9 (R^2=0.039)**
	N° individuals with high content	38	60	66	69	5	57	30	51	69
	N° individuals with low content	57	61	63	62	9	50	31	62	37
	Total lignin	**QTL1 (R^2=0.052)**	**QTL2 (R^2=0.136)**	**QTL3 (R^2=0.023)**	**QTL4 (R^2=0.020)**	**QTL5 (R^2=0.020)**	**QTL6 (R^2=0.029)**	**QTL7 (R^2=0.030)**		
	N° individuals with high content	48	68	20	3	2	48	5		
	N° individuals with low content	46	62	8	9	2	37	5		
	Lignin composition (S/G)	**QTL1 (R^2=0.164)**	**QTL2 (R^2=0.083)**	**QTL3 (R^2=0.213)**						
	N° individuals with high ratio S/G	68	3	54						
	Ethanolic extractives	**QTL1 (R^2=0.105)**	**QTL2 (R^2=0.120)**							
	N° individuals with high content	47	3							
	N° individuals with low content	53	9							

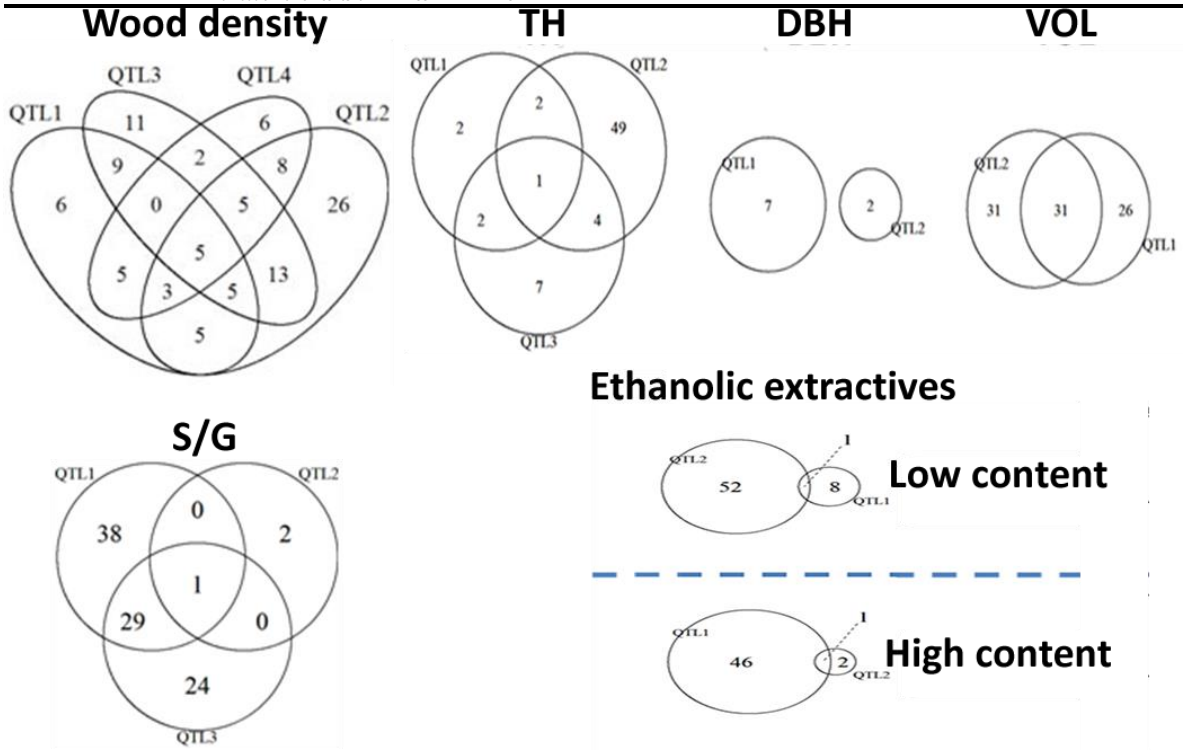

Figure 1. Venn's diagrams representing the number of individuals with favorable QTLs for wood density, tree height (TH), diameter (DBH), volume (VOL), high ratio for S/G, and for ethanolic extractives (low and high content).

As a result for mapping and annotation of QTL regions, a total of 1,581 genes with 2,560 GO Id's were found (**Table 2**).

Table 2. Genes and GO terms in a range of +/- 0.5Mbp from DArT flanking QTL obtained from E. grandis reference genome and known genes involved in lignin, cellulose and xylan biosynthesis, peroxidases and S-domain receptor-like kinase (SDRLK).

QTLs	Scaffold	Genes	GO id's	Lignin Biosynthesis	Cellulose and xylan	Peroxidases	SDRLK
Wood density	1,5,6,9	299	445	nd	1	nd	4
MOE	6	26	81	nd	nd	nd	nd
TH	5,6	355	518	nd	3	2	23
DBH	6	168	283	nd	4	nd	13
VOL	5,6	330	485	nd	4	1	19
Klason	2,3,4,5,6,7,8	340	563	5	1	nd	nd
Lig-Tot	2,5,6,7	353	505	nd	1	nd	4
S/G	2,6	217	294	nd	2	1	4
Ext_et	5	67	71	nd	nd	nd	nd
Total		**1,581 ***	**2,560 ***	**5**	**6 ***	**4 ***	**29 ***

NOTE: nd: non-detected.
* Several QTLs co-located for many traits.

It is important to highlight that five genes involved in lignin biosynthesis were found at Klason QTLs.

Conclusions

This work reveals the applicability of QTL mapping for selection of genetically superior individuals in tree breeding programs. This analysis could be useful for INTA's breeding programs, by the incorporation of individuals of this population that accumulate favorable QTLs.

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Novel winter-associated regulators of the circadian clock in poplar

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Key words: winter dormancy, Populus sp., circadian clock.

Background
Winter dormancy is an adaptive mechanism that allows trees from temperate and cold regions to survive the harsh conditions of this season. Critical steps of this process are strongly influenced by environmental cues, mainly daylength and temperature. The mechanism that integrates these signals is the circadian clock. Despite the importance of the correct functioning of the clock for the healthy state of the plant [1], low temperatures cause the disruption of the circadian clock in trees, which consists in a transcriptional activation followed by an arrhythmic expression [2-5].
In this work we uncover winter-associated regulators of the circadian clock in poplar.

Methods
Firstly, we made a transcriptional fusion with the promoter of *LHY2*, a circadian clock gene, and the luciferase gene. This construct was used to generate transgenic poplars (717-1B4, INRA clone). With these events we characterized the expression of this promoter under different conditions of photoperiod and temperature. To this aim we have set up a circadian luminiscence assay registering luciferase activity from leaf discs with a luminometer. Then we carried out a Yeast One Hybrid (Y1H) screening with a library enriched in winter-associated factors and using this promoter as bait. Candidate regulators are tested *in vivo* using Golden Braid technology [6] and transient assays in poplar, by which we overexpressed and silenced the candidate genes.

Results and Conclusions
Here we present the characterization of the *Populus tremula x alba LHY2* promoter under three different photoperiod conditions. Our results indicate the selected promoter region contains the circadian elements as well as the luciferase activity shows the expected expression under both long and short days.
In the Y1H screening, we found several candidates that are classified either as transcription factors or chromatin remodelers. We will discuss the possible role of these proteins as regulators of the poplar circadian clock.

Competing interests
The author declares that they have no competing interests.

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GWAS for growth and wood quality traits in *Eucalyptus*: a SNP segment based-approach enhances the proportion of variance explained by associated SNPs.

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Keywords: association genetics, lignin, *Eucalyptus*

Background

Decoding the connection between sequence polymorphisms and complex phenotypes has been one of the main drivers of plant genetics research, both to understand the underpinnings of biological processes and to enhance directional selection in breeding programs. In eucalypts, QTL mapping studies in bi-parental populations revealed several QTLs, although their numbers and magnitudes of effects were under and overestimated respectively. Association genetics (AG) studies in forest trees based on candidate genes suffer from bias introduced by the *a priori* choice of such genes and could never account for more than just a few percent of trait variation [1]. The first GWAS study in *E. globulus*, highlighted the merits of integrating population structure and relatedness in AG studies but had low power due to limited marker density and sample size [2].

We have recently developed a high-density multi-species SNP genotyping platform, the EuCHIP60k [3]. It provides a density of one SNP/12–20 kb, opening unprecedented opportunities for GWAS studies in the genus. Additionally, the EuCHIP60K design sampled SNPs across the site frequency spectrum, mitigating ascertainment bias. By sampling rare genetic variants, a GWAS is expected to capture larger proportions of the 'missing' heritability [4]. Moreover, the single SNP marker approach typically does not describe the complete distribution of gene effects. By using multiple SNP markers combined into a chromosome segment the likelihood to capture the complete effect of the QTL is increased. In this study we carried out the first truly genome-wide study in *Eucalyptus* and one of the few

existing in trees. We compared the conventional single-marker GWAS analysis with a segment-based test of association using multiple SNP markers.

Methods

*Population, growth data and SNP genotypes***.** A full-sib progeny trial consisting of 58 *E. grandis x E. urophylla* hybrid families was used. Diameter at breast height (DBH) and height were assessed at age 4 and transformed into a measure of volume. A randomly stratified sample of 999 trees across 45 full-sibs families was selected for the study. SNP data were obtained with the EuCHIP60K.

Wood chemical and physical phenotyping. Near-infrared spectra of wood flour sampled at age 5 were obtained for all 999 samples. A representative subset of 350 samples was selected based on NIR spectra for lab chemistry and physical analyses including: cellulose content (%), hemicellulose (%), S:G lignin ratio, soluble, insoluble and total lignin, wood density, microfibril angle, fiber length and width and coarseness. NIR calibration models were built for these traits and used to predict in the remaining 649 samples. For MFA and fiber traits NIR did not provide usable calibration models and, as such, were not used in the GWAS analysis.

*Association analyses***.** Single-marker associations were performed using a mixed linear model with SNP effects as fixed and additive genetic effects as random. Analyses were conducted using TASSEL 5.0. Associations were corrected for multiple testing using false discovery rate from 0.01 to 0.2. The proportion of genetic variance explained by a segment of SNP markers was estimated following [5] using segments of 200kb size. A likelihood ratio test (LRT) with Bonferroni correction was used to test the significance of the largest effect segment of each chromosome for each trait.

Results

After removing family structure effects, 367,378 association tests were performed with 33,398 SNPs for eleven traits. This resulted in 19,812 associations (p-value < 0.05). After correction, 298, 119 and 65 SNP-traits associations remained at q-values <0.2, <0.05 and <0.01 respectively. Around 80% of the significant associations in every FDR range were for lignin content and composition 10, with the most notable one for S:G ratio closely flanking the ferulate 5-hydroxylase (F5H) gene on chromosome 10, corroborating previous reports [2]. No significant associations survived at q<0.05 for growth traits, highlighting their higher complexity. The amount of phenotypic variation explained by each SNP marker estimated one at a time was small, accounting between 1.2% and 5.7% and likely overestimated. When effects were estimated simultaneously as random effects with BLR, the proportions of phenotypic variance were even lower. Six significant SNP segments of 200 kb were found for DBH, Volume, MAI, S:G ratio and Soluble lignin, explaining considerably larger fractions of the variance, the largest being for S:G, explaining 9.3 and 14.4% respectively on chromosomes 1 and 10. Segments on all other chromosome for all traits were not statistically significant.

Conclusions

Despite the larger sample size used when compared to all previous GWAS studies in trees, and a genome-wide coverage with high marker density, our results show that the complexity of most traits with the exception of lignin related ones is such that only a small proportion of the variation could be explained the significant SNP-trait associations. Combining SNP markers into segments considerably increased the amount of phenotypic variance explained. We expect, however, that a genomis prediction approach, where all marker effects are estimated and fit simultaneously into predictive models without rigorous significance tests, should prove more efficient to capture larger proportions of the variation for molecular breeding applications.

Competing interests

The authors declare that they have no competing interests.

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FAP-DF and CNPq for grant support and J.L. Gualdrón Duarte and J.P. Steibel for helpful discussion and advice on the statistical analysis.

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Genetic structure and genome-wide association mapping for vegetative propagation traits in *Casuarina*

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Key words: *Casuarina*, DArT-seq, Association mapping

Background

Casuarina species is a most widely planted trees in the coastal regions in India, tropical and subtropical regions around the world, due to their fast growth, short rotation, high productivity, ability to fix atmospheric nitrogen and suitability for pulp and paper production (1). Despite the apparent advantages, marker development and molecular breeding of *Casuarina* have not been initiated due to lack of genetic and genomic information and till now only few studies have been conducted to assess the genetic diversity using dominant gel based markers (2). Keeping this in view, we have characterised two species of *Casuarina* using thousands of single nucleotide polymorphic (SNP) markers derived from Diversity Array Technology based genotyping by sequencing approach (DArT-seq). In addition, genome wide association study (GWAS) was performed for rooting ability in *Casuarina*.

Materials and Methods

The materials used in this study were composed of 250 individuals of *C. junghuhniana* (*Cj*) and 112 individuals of *C. cunninghamiana (Cc)*. Of these, *Cj* accessions were established at Mettupalayam, southern India (11º19 ' N, 77º56' E) as mother stock plants for vegetative propagation. Stem cuttings of 8-10cm were placed in the growth medium of vermiculite with application of rooting hormone (4-indol-3yl-butyric acid

,IBA) used as a basal dip. Final rooting assessment was conducted after 12 weeks. The percentage of rooting was measured at the ratio of number of rooted plants to the total number of cuttings. Genotyping was carried out using genotyping by sequencing approach performed by DArT Pty Ltd (DArT P/L) as described previously (3) using *PstI+MseI* complexity reduction method. The genome wide SNP markers were used to estimate the genetic structure of the accessions using three different approaches: Bayesian admixture (4), measuring genetic differentiation (F_{st}) among populations and Principle Coordinate Analysis (PCoA, 5) using 2080 SNP markers common between the two species. SNP markers associated with rooting ability in *Cj* was determined using mixed linear model (MLM) using kinship matrix and population structure as covariate at false discovery rate (FDR) adjusted P<0.05.

Results

Using genotyping by sequencing (GBS) approach, we have characterised ~58,000SNPs and ~88,000in-silcoDArT markers in 250 accession of *Cj* and 112 accessions of *Cc*. After applying stringent criteria (Call Rate >0.90, MAF >0.05 and missing genotype <10%) GBS approach used yielded 32496 markers (22158DArTs and 10,338 SNPs) in *Cj* and 32382 markers (21168DArTs and 11214 SNPs) in *Cc*, of which 2080 SNP markers were common between the populations and approximately 60% of the markers were in gene rich region. Polymorphic information content (PIC) of the markers ranged from 0.05 to 0.489 with an average of 0.342.Observed heterozyosity ranged from 0.02 to 0.996 with an average of 0.270 and expected heterozygosity ranged from 0.05 to 0.749 with an average of 0.253. Population structure using model based approach showed only peak of ΔK, for K = 2, suggested the presence of two main populations (Figure 1). Pair-wise population F_{st} value(0.353), indicated significant genetic differentiation between *Cj* and *Cc*. PCoA analysis showed that the first principal coordinate accounts for 52.2 % of total variation and separates the *Cc* accessions from *Cj* accessions. The second principal coordinate (Figure 2, 3.5% of total variation) separated *Cj* individuals into two groups. Analysis of molecular variance (AMOVA) indicated that most of the variation occurred among population (71%) than within population (29%) in *Casuarina* (Table 1).

Vegetative propagation is widely used for deployment of improved genotypes. GWAS identified 3 DArT markers and 34 SNPs that were significantly associated with rooting ability after FDR correction in *Cj*. The phenotypic variation explained by each SNPs associated with rooting ability ranged from 5.8 % (Cj_5656322) to 15.1% (Cj_5669155).

Conclusion

Present study suggests that GBS based on DArT-seq can effectively detect thousands of genome-wide SNP markers and provide effective tool for the exploration of *Casuarina* accessions. Both model based ancestry analysis and genetic distance based analysis strongly supports that clear delineation between *Cj*and *Cc*. Significant DArT and SNP markers detected in this study for rooting ability can be used in marker assisted selection programs in *Casuarin*a. In addition SNP markers identified in this study provides a basis for dissecting growth and wood quality traits and will aids in *Casuarina* improvement programs through marker assisted selection and genomic selection.

Competing interests

The author declares that they have no competing interests.

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Table 1. Analysis of molecular variance (AMOVA) for the two species of *Casuarina* based on 2080 SNP markers.

Source	df	SS	MS	Variance component	Total variation (%)	P value *
Among Pops	1	197780.6	197780.6	1275.1	71%	0.001
Within Pops	360	186522.6	518.1	518.1	29%	0.001
Total	361	384303.2	198298.7	1793.2	100%	

Figures

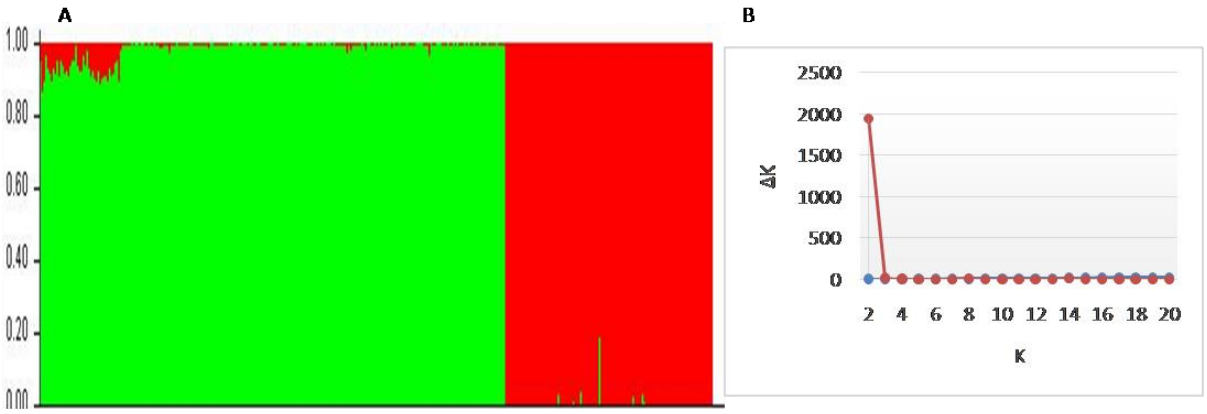

Figure 1a.Two subgroups inferred from structure analysis. The genotype of each individual accession is represented by a vertical line divided into colored segments. **b**. STRUCTURE estimation of the number of population for K ranging from 1 to 20 by ΔK values.

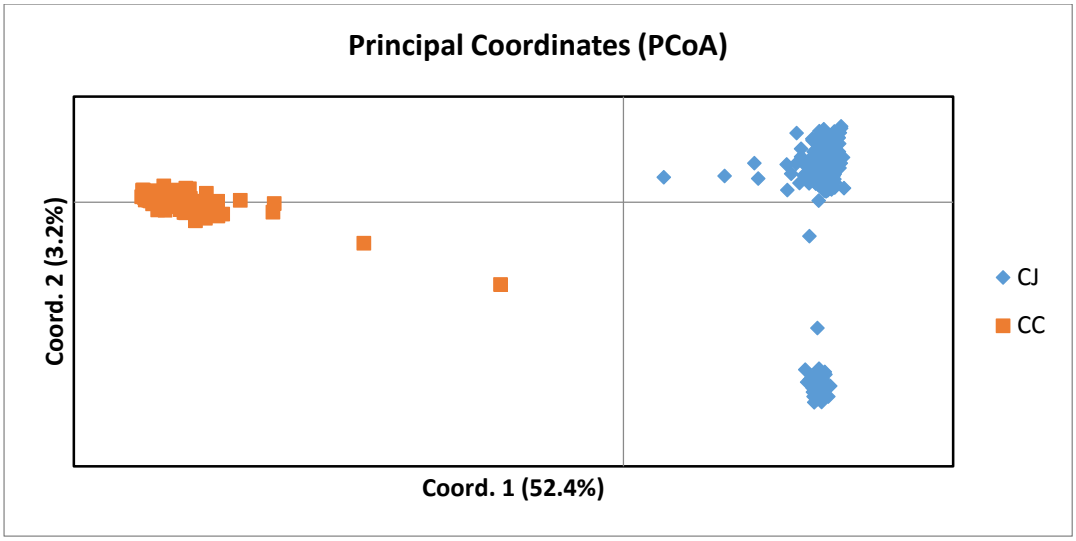

Figure 2. Principal coordinate analysis (PCoA) of SNP markers showing the clustering of *Casuarina* accessions.

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Genomic prediction of growth traits in *Pinus taeda* using genome-wide sequence-based DArT-seq markers

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Background

Commercial loblolly pine (*Pinus taeda* L.) plantations in Brazil supply raw material for a large array of wood based industries and businesses.The increasing local pine wood demand has encouraged development of seed production programs by private companies as well by public institutions. Continuous supplies of genetically improved seed have enhanced productivity and turned intensive pine silviculture into a sustainable and attractive activity. However, pine breeding cycles of 15 to 25 years are long even in tropical and subtropical regions of Brazil. Furthermore, breeding decisions are traditionally made using only growth traits in most programs due to the challenges in measuring wood quality traits in large progeny trials. This limits the genetic gains that could be obtained if additional wood quality traits were contemplated.

With the advent of more accessible DNA marker technologies it is now economically and logistically possible to consider applying molecular breeding to pine breeding programs in order to select indirectly for multiple traits. Despite of the advances made in QTL mapping and association genetics in loblolly pine [1, 2], no effective application to tree breeding has been achieved to date. Genomic Selection (GS) promises to bridge this gap by predicting phenotypic performance based on a genome-wide panel of markers whose effects on the phenotype are estimated simultaneously in a large and representative 'training' population of individuals without applying rigorous significance tests [3]. This approach has been proposed as a way to shorten tree breeding cycles and allow indirect selection while increasing selection intensity for all traits simultaneously, consequently enhancing genetic gains per unit time [4]. The power demonstration of this approach was pioneered in loblolly pine [5] and *Eucalyptus* [6] soon followed by other studies in loblolly pine [7] and other conifers [8]. All these studies have shown positive prospects for the adoption of GS in tree breeding.

In loblolly pine, however, due to the complexity of its genome, there are still some regarding the development of a standard large-scale marker genotyping platform. Although SNP chips have been developed [9] and used for GS experiments [5] these were limited in genomic coverage to genic regions and not readily accessible to the breeder´s community. Sequence based genotyping methods based on genome complexity reduction have been successfully used in loblolly pine. Genotyping by exome sequence capture was used to build a linkage map covering 2,841 genes,. A linkage map with 2,469 DArT-seq markers [10] was used to anchor a portion of the loblolly pine genome assembly [11], and the standard GBS protocol was used to generate ~17,000 markers in a small set of *Pinus contorta* accessions [12]. In this work we used 16,355 sequence-based DArT-Seq markers covering both genic and non-genic regions to genotype a genomic selection in a training population of 960 loblolly pine trees and to develop predictive models for growth traits.

Material and methods

Population, genotyping and phenotyping. Phenotype and genotype data were obtained for 960 trees of a progeny trial established in Ponta Grossa (PR) (25°25'S 49°15'W) involving 35 maternal open pollinated families of elite trees from a clonal seed orchard. This population constitutes the breeding population of EMBRAPA Forestry. Assessments included total height starting at age 2, and every year thereafter until age 6 and DBH (stem diameter at breast height) from age 4 to 6. DBH and height data were used to estimate tree volume. DNA extractions were carried out from pine needles and total genomic DNA sent to DArT Pty (Yarralumla, Australia) for DArT-seq genotyping. DArT-seq in *P. taeda* was carried out by complexity reduction using double digest with PstI_ad/TaqI/HhaI_ad. PstI adapter was tagged with 96 different barcodes enabling multiplexed sequencing into a single lane. FASTQ files were quality filtered at ≥ 90 % confidence and for the presence of barcode sequences. All 960 DNA samples were genotyped in duplicate in separate lanes providing fully replicated sequencing data with minimal lane effect. Sequences were aligned against a pseudo-reference consensus sequence created by assembling all the sequence reads generated for all the DNA samples at each locus. The output files from the alignment generated using BWA were processed using an analytical pipeline developed by DArT Pty to produce tables for the dominant PAV markers and co-dominant SNPs.
Genomic predictions. BLUP/REML was the mixed linear model used to analyze phenotype data. All phenotypes were corrected for fixed effects and deregressed for parent effects and genomic values (GEBV) predicted using Random Regression-Best Linear Unbiased Predictor using the RR-BLUP R package [13]. The linear mixed model was adjusted for the estimation of marker effects. A Jack-knife cross-validation method [14] was used to estimate the prediction accuracy.

Results and discussion

A total of 14,306 DArT-seq markers were obtained and used in the analyses. Using all markers, predictive accuracies from cross validation varied between 0.27 at age 2 up to 0.40 at age 6 for DBH, height and volume traits. Such predictive accuracies were obtained relating individuals in the training and validation subsets. This result indicates that the predictive models are likely capturing relatedness. Furthermore, these results suggest that fewer markers could be tentatively used for the practice of GS, if the candidate selection population consists of progeny of the training population. However, while such marker selection might work well in such conditions, models developed from a selected set of markers based on their effect might suffer from over-fitting and therefore display a reduction in prediction accuracy in later generations [15]. In *Picea glauca*, removing uninformative loci from predictive models also seemed to have a positive effect on cross-validation, with a slight increase in accuracy [16]. Like in our study, this increase could be partly accounted because these marker subsets were not identified in an independent dataset [15] upwardly biasing accuracy estimates. In fact, the linear regression coefficients between the observed and predicted values were greater than 1, indicating that the marker effect was biased. In conclusion, these results show that DArT-seq markers provide a useful platform genotyping of loblolly pine providing large numbers of high quality markers. In line with our previous reports in loblolly pine and eucalyptus [5, 6], these results further support that genomic prediction might soon become an operational tool in the practice of tree breeding. However caution should be taken when developing prediction models to avoid over fitting that might mislead the perspectives of potential gain of this method especially as one intends to predict in generations removed from the training set.

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Genomic technologies to select for resistance to *Phytophthora* disease

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Key words: Phytophthora, Radiata pine, Breeding, Resistance

Background

Phytophthora diseases have the potential to devastate entire industries especially with the threat of new incursions through increasing globalisation. The *Phytophthora* group of plant pathogens is renowned worldwide as the causal agents of forest and tree-crop diseases. In New Zealand, the most widely planted forest tree species, *Pinus radiata* D. Don (radiata pine), is affected by red needle cast (RNC) caused by the aerial pathogen *Phytophthora pluvialis* [1]. Selection, including early selection with DNA markers, and breeding for resistance to this disease is one possible solution. Recent work in radiata pine has indicated a genetic component for resistance to RNC [2]. Quantitative methods usually rely on the presence of the disease in existing field tests. Environmental factors also impact disease, with the presence/absence or even severity of disease varying within and among sites. This affects our ability to accurately phenotype disease responses. Furthermore, the geographical distribution of existing trials does not always reflect that of the disease. Therefore, a more reliable system for quantifying resistance is required.

In this paper we present evidence for heritable resistance to RNC in radiata pine. We also describe an in vitro assay system, using detached needles, that has been developed to determine the level of resistance in a controlled environment, and the generation of a SNP marker resource suitable for generating genomic breeding values (GeBVs) for resistance to RNC. We discuss how these will be brought together to find an integrated and rapid solution to this new needle disease in New Zealand.

Methods

Disease Evaluation Trial 1

One clones-within-families genetics trial was assessed at Wharerata forest on the east coast of New Zealand, as described in [2]. Visual assessments were performed to measure two main crown symptoms of disease progression: a percentage of crown that was “red” called (RNC), and a percentage of crown from which needles had been cast (NC).

Disease Evaluation Trial 2

Clonal material derived from sixty three families from the Radiata Pine Breeding Company (RPBC) was assessed for resistance to RNC using an in vitro detached needle assay. Freshly picked needles were exposed to a solution of *P. pluvialis*

zoospores for 18 hours before incubation in humid chambers for 14 days. Average lesion length and total number of lesions were determined and individuals ranked. Experiments were repeated with a subset of genotypes to determine reproducibility of ranking scores.

Marker Development
A total of 17 transcriptomic libraries, encompassing 8 genotypes and 10 tissues, were sequenced using Illumina HiSeq2000 (Illumina, San Diego, USA) and assembled into a single multi-genotype reference using Trinity [3] The assembly was compared to the *Pinus taeda* L. v. 1.01 genome assembly [4] to identify exomes. A total of 80,000 exome capture probes were designed for exome capture genotyping-by-sequencing (GBS) [5]. A pilot set of 24 diploid and 6 haploid samples were genotyped to determine the efficacy of exome capture-GBS as a genotyping platform for genomic selection in radiata pine.

Results & Conclusions
Disease Evaluation
Both of the traits used to assess RNC resistance, were found to be heritable ($\hat{h}^2$ 0.21-0.31) when assessed in a single clones-within families genetic trial [2]. Selecting for resistance to RNC is, therefore, likely to deliver healthier trees. More assessments across a number of sites and seasons are required to confirm this result, although optimisation of the in vitro assay may prove a more effective and accurate method of assessing disease response.

Individual clones representing all 63 RPBC families have now been assessed with the in vitro assay. An initial subset of 48 clones, representing the lowest (putative resistant) and highest (putative susceptible) levels of lesion development was repeatedly screened and ranks were consistent within quartiles.

Marker Development
From our multi-genotype transcriptome assembly we predicted 449,951 exons using the *P. taeda* reference genome [4]. These were the basis for designing 80,000 exome capture probes. High levels of successful exome capture observed in the pilot study indicate that sequence assembly and exon prediction was very accurate. Although as a consequence of highly efficient probe capture, much more of the exome was captured than anticipated thereby reducing the overall read depth across all probes. This resulted in lack of reproducibility in SNP calling between technical replicates. We determined a minimum read depth of 20 x will be required for accurate genotyping in future populations. With optimisation of the probe set, including removal of probes which capture little or no polymorphism, this resource can now be applied to the RNC-screened clones within the RPBC breeding population.

In combination, evidence for heritable resistance, a quantitative and robust phenotyping system, and a suitable SNP resource and genotyping system will enable us to breed for resistance to *Phytophthora* diseases. The application of the markers in a structured training population will facilitate the use of genomic selection for disease resistance in radiata pine.

Competing interests
The author declares that they have no competing interests.

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Exploring Poplar GH5 Enzymes

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Key words: Glycoside hydrolase, GH5_11, GH5_14, mannanase

Background

The glycoside hydrolase family 5 (GH5) contains numerous cell wall degrading enzymes with diverse activities. Recently, the GH5 family was divided into 51 subfamilies [1]. Sequences from higher plants can be found in three of those subfamilies: GH5_7, GH5_11, and GH5_14. Plant GH5_7 enzymes have been reported to act as *endo*-β -mannanases (EC 3.2.1.78), but mannan transglycosylase activity has also been occasionally claimed [2-3]. The single characterized enzyme in subfamily GH5_14 is a β-1,3-glucosidase (EC 3.2.1.58) from rice [4], whereas the substrate specificity for enzymes in subfamily GH5_11 is currently unknown. Subfamily 14 contains exclusively plant enzymes, but GH5_11 also comprises fungal representatives, and in GH5_7 enzymes from all major organism kingdoms are present. Genes encoding for enzymes belonging to the three subfamilies are present in sequenced genomes of trees such as poplar, *Eucalyptus* and spruce. The activity of plant GH5_7 mannanases are important for developmental processes such as xylem differentiation, flower formation, fruit ripening and seed germination, but little is known about the biological role of enzymes belonging to the other two GH5 subfamilies. The up to now sole characterized poplar GH5 enzyme is a GH5_7 mannanase expressed in xylem tissues, and hypothesized to via hydrolysis of cell wall mannans generate signal molecules affecting xylem differentiation [5]. Here we present bioinformatic analyses of all these enzymes in combination with quantitative PCR (qPCR) and *in silico* gene expression analyses, in order to unravel the function of other poplar GH5 enzymes.

Methods

Poplar GH5 protein sequences were retrieved from the Phytozome server (http://phytozome.jgi.doe.gov) for bioinformatic analyses. Catalytic modules and appended unknown modules were analyzed separately. Phylogenetic analysis was performed using Phylip 3.69 (http://evolution.genetics.washington.edu/phylip.html). Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2) was used for protein structure prediction. RNA was extracted from various tissues of wild-type hybrid aspen *Populus tremula x tremuloides*, and cDNA was prepared from the RNA samples, which was used as template in quantitative PCR (qPCR) experiments. Primers were designed for all hybrid aspen GH5 genes and tested to ensure proper amplification. Two reference genes (*TUB* and *CYP*) were used for normalization, and *GT43* was used as a positive control.

Results and Conclusions

In the *Populus trichocarpa* v3.0 genome we were able to identify nine subfamily GH5_7 full-length genes, three GH5_11 members and four genes encoding GH5_14 enzymes. A previously unreported GH5_7 gene was named *PotriMAN9*. Poplar GH5_7 enzymes contain a single catalytic module, whereas the modular architectures of GH5_11 and GH5_14 enzymes reveal putative carbohydrate-binding modules (CBMs) appended to the catalytic GH5 modules. In GH5_14 proteins the non-catalytic module is located in the N-terminal part, while the observed CBM-like module in GH5_11 enzymes is found in the C-terminal region. The GH5_11 CBM-like domain shows distant similarity to CBM13, a CBM also classified as a lectin and present in, for example, ricin and agglutinin. However, our phylogenetic analysis indicates that the GH5_11 putative CBM does not belong to family CBM13 (Figure 1). Furthermore, a closer inspection reveals that while cysteins probably important for structure stabilization are present in the plant attached modules, the typical three QxW repeats found in CBM13 are missing. Homology models of the putative plant GH5_11 CBMs adopts a β-trefoil similar to CBM13 modules.

We determined the transcript profiles of GH5 genes in 10 different tissues of wild-type hybrid aspen. Most of these tissues were wood-related. For three genes, one from each subfamiliy, we were not able to detect any transcripts in the sampled tissues. The expression pattern could not be determined for *PotriMAN9* due to unspecific primers. All remaining genes were down-regulated in the apical shoot compared to the upper part of the stem, except *PotriMAN5,* which was instead up-regulated*,* and *PotriMAN3*, which was expressed at the same level in the apical shoot and the stem. Three GH5_7 mannanase genes were up-regulated in xylem and opposite wood. The same three genes, *PotriMAN4*, *PotriMAN6* and *PotriMAN8,* has previously been reported to be expressed in xylem [5]. The other GH5_7 genes were instead down-regulated in these tissues. *PotriMAN5* was generally up-regulated in leaves.

Since flower, seed and root tissues were not included in our qPCR experiment, we also performed *in silico* gene expression analyses as a complement. Data mining of published gene expression experiments from poplar and Arabidopsis indicated that subfamily GH5_11 genes were mainly expressed in flower organs and dormant cambium, whereas poplar genes coding for subfamily GH5_14 enzymes were up-regulated in roots and young leaves. RT-PCR showed that Arabidopsis GH5_11 transcripts are expressed in flower tissues. The gene expression data presented here indicate a role for poplar GH5 enzymes in developmental processes such as the formation of flowers and wood, but the function and mode of action of these enzymes are not well understood. Future work will include heterologous expression of poplar GH5 enzymes in order to determine their biochemical and catalytic properties.

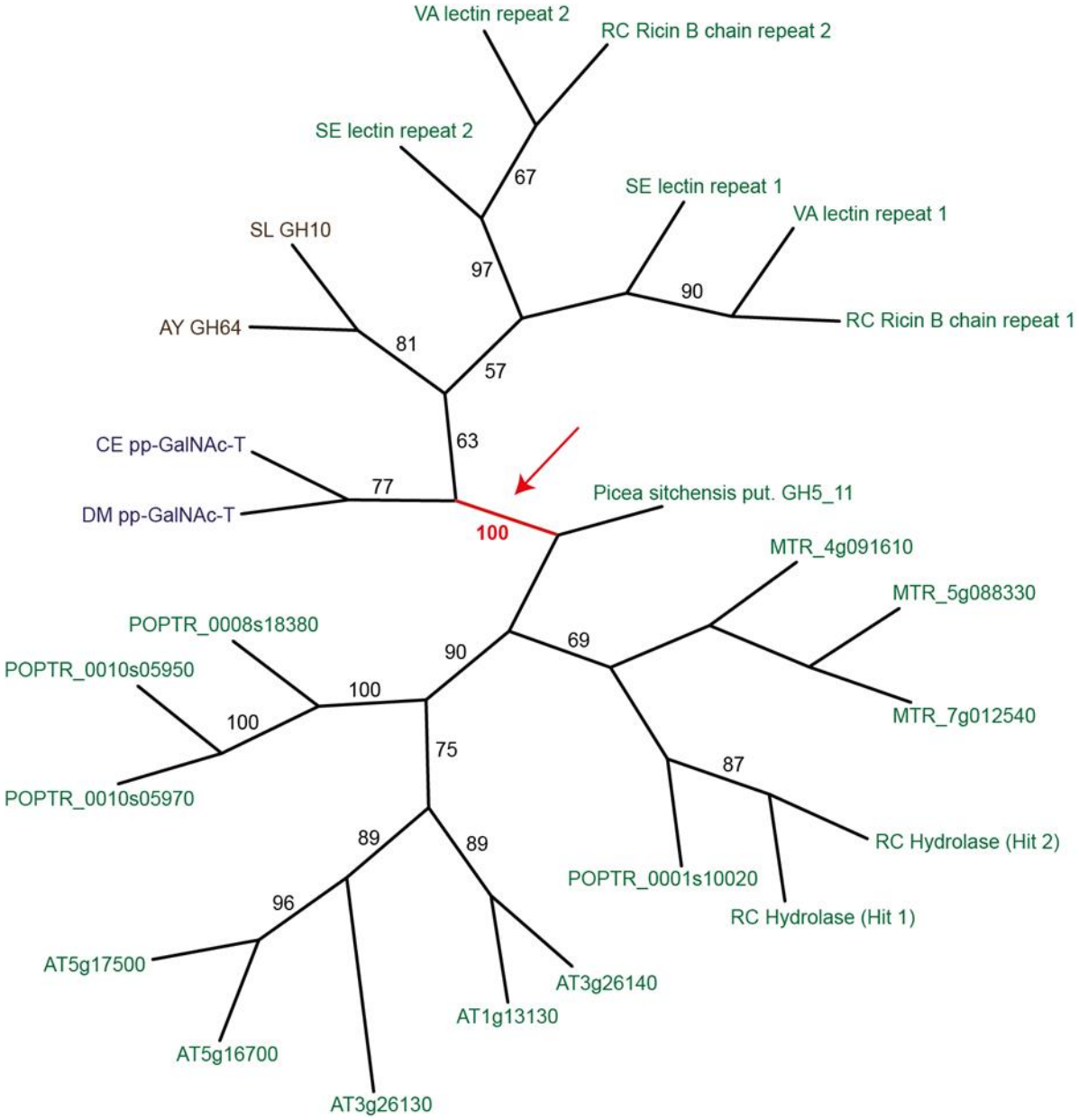

Figure 1 Phylogenetic analysis of putative plant GH5_11 CBMs and CBM13 modules from plants, animals and bacteria. Note the bootstrap support of 100% for the separation of CBM13 and the GH5_11 type CBM.

Competing interests

The author declares that they have no competing interests.

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Characterization of the cork oak transcriptome dynamics during acorn development

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Key words: *Quercus suber*, fruit, transcription factor, response to water

Background

Seed protection and dispersal are the main functions of the fruit. Fruit initiation and development play a crucial role in plant adaptation, and successful fruiting strategies are important drivers of colonization of new niches. Cork oak (*Quercus suber* L.) has a natural distribution across western Mediterranean regions and is a keystone forest tree species in these ecosystems. Despite the especially critical role of the fruiting phase for oak regeneration, the molecular mechanisms underlying the biochemical and physiological changes during cork oak acorn development are poorly understood. To address this issue we have characterized the transcriptome dynamics of the cork oak acorn development, paying special attention to transcripts involved in transcriptional regulation and response to water. This work was performed in the context of the Portuguese cork oak EST consortium project [1].

Methods

Acorns were collected between mid June and late November 2009 from cork oak trees growing in six different locations in the South and Centre of Portugal. In each collection, acorns and isolated embryos were carefully observed for evaluation and documentation of the developmental stage, before freezing in liquid nitrogen. Total RNA was extracted from frozen samples of acorns covering early developmental stages up to the maturation stage using a protocol described by Reid et al. with minor modifications [2, 3] and further purified for removal of any DNA contamination. Two normalized and 5 non-normalized cDNA libraries were prepared and sequenced using Titanium GS-FLX (454-Roche) at Biocant (Cantanhede, Portugal).

Results and Conclusions

A total of 80,357 ESTs were *de novo* assembled from RNA-Seq libraries representative of the several acorn developmental stages. Approximately 7.6% of the total number of transcripts present in *Q. suber* transcriptome was identified as acorn specific. A total of 2,285 differentially expressed (DE) transcripts were clustered into six groups according to their expression profile. The stage of development corresponding to the mature acorn exhibited an expression profile markedly different from other stages. Approximately 22% of the DE transcripts putatively code for transcription factors or transcriptional regulators, and were found almost equally distributed among the several expression profile clusters, evidencing their major roles in the regulation of acorn development. Transcripts related to response to water, water deprivation and transport were mostly represented during the early and the last stage of acorn development, when tolerance to water desiccation is possibly critical for acorn viability. This work provides new insights into the developmental biology of cork oak acorns, highlighting transcripts putatively involved in the regulation of the gene expression program and in processes likely essential for adaptation to environmental conditions.

Competing interests

The author declares that they have no competing interests.

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Isolation and characterization of four dehydrin genes from *Eucalyptus nitens*

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Background

Eucalyptus is a forestry genus grown world-wide for solid timber as well as for pulp and paper production. *Eucalyptus globulus*, because its rapid growth and excellent wood quality characteristics has shown significant expansion in the forestry industry and it is cultivated in subtropical areas with temperate climate with absence of severe frost. On the other hand, *E. nitens* is a species suitable for planting on high-altitude sites where severe frosts and snow occur, having a greater resistance to cold and being less susceptible to freezing than *E. globulus.*
The mechanism underlying cold tolerance in several plants has been studied in recent years, being well known that there are many genes involved in this process. Among these genes, the COR (cold- regulated), have been widely studied in plant acclimation to low temperatures, being dehydrins (DHNs) one of the most analyzed genes in several plants. DHNs play a key role in plant response and adaptation to abiotic stress, they are involved in seed desiccation in response to low temperatures, drought, salinity and abscisic acid, having an important role during cell dehydration, and cell membrane stabilization. In *Eucalyptus*, it has been shown that DHNs could have an import role in frost tolerance after plants are submitted to a cold acclimation process (Fernández et al 2012). The fact that *E. nitens* presents a greater tolerance to cold than *E. globulus* could be due to the role of dehydrins in this species. This work reports the identification of four new DHNs in *E. nitens* and examines their responses under low temperature and compares them with those previously reported in *E. globulus*.

Methods

Two *E. nitens* families, represented by eighty rooted seedlings previously characterized as resistant and susceptible to frost (RF and SF) and one genotype of *E. globulus* (80 ramets) characterized as frost resistant were placed in a cold chamber and subjected to a cold acclimation process. Plants were sampled at different time points and tRNA, cDNA was synthesized by using High Capacity kit and used for transcript abundance studies (qRT-PCR), Ubiquitin C and α-Tubulin were employed as housekeeping genes. For sequence analysis, DNA as purified by CTAB, and primers designed previously (Fernandez et al, 2012) were employed by conventional PCR, the fragments obtained were purified and sequenced by using Sanger (Macrogen, Corea). The *EniDHNs* putative promoter regions were analyzed for plant regulatory elements using PlantCARE.

Results and Conclusions

Four putative dehydrins were identified in *E. nitens* (*EniDHN1*, *EniDHN2*, *EniDHN3* and *EniDHN10*) and compared with those previously described in *E. globulus*. The analysis of the promoter region showed that the four *EniDHNs* contained several cold- or dehydration inducible cis element. In general these elements can be classified mainly into hormone-responsive and stress-responsive elements. When comparing the number of cis-elements present in *EniDHNs* and in *EuglDHNs* significant differences were found between ABRE elements especially in *DHN2* where ABRE and G-box elements, being higher in *E. nitens* than in *E. globulus*. Transcript abundance of the four dehydrins described in both species was measured by qRT-PCR, increasing when comparing the acclimated condition with the non-acclimated state, being higher in a RF family of *E. nitens* than in the SF family.

When comparing the transcript abundance of all four DHN genes of *E. globulus* with those of *E. nitens*, under the same conditions, results showed higher expression levels in *E. nitens* than in *E. globulus* (figure 1). As well, high expression levels of *DHN2* in both species could be observed, being higher in *E. nitens* than in *E. globulus. EuglDHN2* and *EniDHN2* were the only genes showing a significant transcript accumulation in resistant plants at the acclimated state.

The analysis in the genomic sequence of DHN genes from *E. globulus*, and *E. nitens*, in combination with gene expression under cold acclimation, could partially explain the differences of these species to frost tolerance.

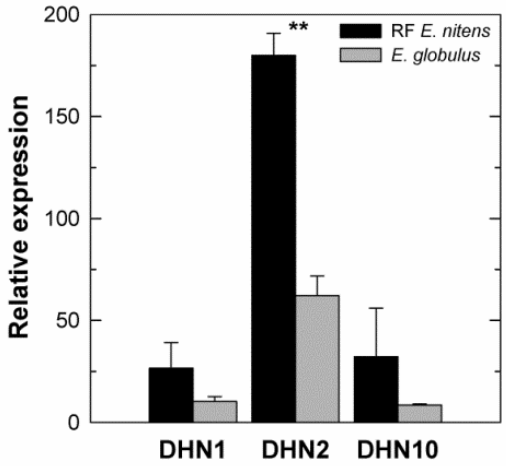

Fig. 1 Gene expression of three DHN genes in leaf of *E. nitens* and *E. globulus* under low temperature using two internal controls UBC and α-TUB. The asterisks on top of the bars (mean + SE) indicate statistically significant differences among RF of *E. nitens* and *E. globulus* in the CAAF treatment (**p≤0.01).

Competing interests

The author declares that they have no competing interests.

Acknowledgements

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African Orphan Crops Consortium (AOCC): a Global Partnership to Address Food and Nutritional Requirements in Africa through Tree Genomics Applications

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Background

Optimal nutrition is critical for human development and economic growth, but sub-Saharan Africa's efforts to mitigate malnutrition, lag behind rest of the world. There are several important crops, indigenous or naturalized in Africa, which have potential to improve resilience of food systems and raise farmer's income. A wide portfolio of these crops requires promotion, but new methods are required to do so to overcome some of the traditional boundaries for wider use. Rapidly developing genomics technologies which are already being applied to major crops can also be applied to Africa's orphan plants for their improvement.

The African Orphan Crops Consortium (AOCC), a public-private international partnership seeks to address this gap by undertaking intense genomics research on neglected 101 African trees and crops. The AOCC has decided to sequence genomes of 101 species of African interest, re-sequencing 100 accessions from each of them and to develop fast, economical and easy germplasm screening tools based on SNP genotyping. The species include 43 trees, herbs, shrubs, lianas vegetable, legumes and grain crops. Partners of the consortium comprise the World Agroforestry Centre (ICRAF); NEPAD (New Partnership for Africa's Development); Biosciences eastern and central Africa (BecA/ILRI); Mars Incorprated; BGI; Life Technologies; LGC Genomics; Google; University of California, Davis (UCD); Agriculture Research Council (ARC), South Africa; University of Ghent; iPlant Collaborative and World Wildlife Federation (WWF). A growing network of collaborators includes CGIAR centres, crop consortia, Agriculture Research Council and Illumina Inc.

The AOCC has planned to work on collaborative model to translate its outcomes through network of African breeders nurtured through the African Plant Breeder's Academy (AfPBA). The AfPBA is managed by UC Davis and hosted by ICRAF and will train 250 breeders from African national and regional institutes to deploy these advanced resources for crop improvement.

Methods:

The Africa-specific crops were listed and prioritized through online surveys and individual interactions with breeders, farmers, academicians, socialists, businesses and policymakers from Africa. It includes 43 tree species with varied applications (table 1). Apart from these AOCC also works on numerous annuals which are traditionally part of agro-forestry ecosystems in Africa.

The short listed trees will be sequenced primarily using next generation sequencing (NGS) technology at BGI. Initially k-mer survey will be carried out on a representative panel of a few accessions/species for estimation of heterozygosity and genome size [1]. These findings will be used to decide about sequencing strategy, expected depth and bioinformatics pipeline. Generally, a standard pipeline based on SOAP *de novo* standardized by BGI will be employed for generating assemblies and contigs [2].

The transcriptome sequencing will be carried out at ARC, South Africa using Illumina NGS and trancripts will be generated and annotated using standard RNA assembly and annotation tools at iPlant.

Re-sequencing panel from each species will consist of 100 public accessions which are nutritionally important to Africa which also represent extant diversity. The re-sequencing will be performed by multiplexing on Ion Proton using P1 or P2 chips (Life Technologies) at ICRAF. The SNP discovery will be performed using standard Ion pipelines at iPlant Collaborative, USA.

The discovered SNPs will be screened as per breeder's requirements primarily targeting yield, nutrition, resistance to abiotic and biotic stress to prioritize SNPs for assay design. Low to high density SNP panels will be developed for each species for candidate gene-based association studies, genome wide association studies (GWAS), genome selection (GS) and bi-parental mapping.

The phenotyping will be carried out by network of breeders primarily focusing on nutritional quality, yield potential and resistance to diseases and pests etc. over bi-parental mapping populations and association mapping panels. This will be performed using standard set of practices such as structure analysis, association analysis and genomic selection (GS) wherever possible. The results will be employed for population improvement, pre-breeding and line improvement through breeding tools such as back-crossing and/or recurrent selections using directional approach.

Results and Conclusions

The AOCC has been successful in bringing together researchers and individuals from private and public institutes of diverse background and specializations. There has been generous funding in cash and kind from various stake holders with common intention to uplift the standard of living of under-privileged people of Africa through food and nutrition interventions. The sequencing and re-sequencing activities have already begun for several crops. Due to diverse and complex nature of these activities, the AOCC would like to share its resources and expertise with any group with overlapping interests to avoid redundancy. Thus, the consortium is looking forward for active collaborations for germplasm collection, funding, specialist suggestions and expert guidance to achieve the goal of food and nutrition for all with emphasis on Africa.

Competing interests

The authors declare that they have no competing interests.

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Table 1: List of tree species targeted for sequencing and re-sequencing under AOCC.

	Species name		Species name
1	*Adansonia digitata*	23	*Irvingia gabonensis*
2	*Adansonia kilima*	24	*Lannea microcarpa*
3	*Allanblackia floribunda*	25	*Macadamia ternifolia*
4	*Anacardium occidentale*	26	*Mangifera indica*
5	*Annona senegalensis*	27	*Moringa oleifera*
6	*Annona reticulata*	28	*Morus alba*
7	*Artocarpus altilis*	29	*Parinari curatellifolia*
8	*Artocarpus heterophyllus*	30	*Parkia biglobosa*
9	*Balanites aegyptiaca*	31	*Persea americana*
10	*Boscia senegalensis*	32	*Pistacia vera*
11	*Canarium madagascariense*	33	*Psidium guajava*
12	*Casimiroa edulis*	34	*Ricinodendron heudelotii*
13	*Cassia obtusifolia*	35	*Sclerocarya birrea*
14	*Chrysophylum caimito*	36	*Strychnos spinosa*
15	*Cocos nucifera*	37	*Syzygium guineense*
16	*Davryodes edulis*	38	*Tamarindus indica*
17	*Detarium senegalense*	39	*Uapaca kirkiana*
18	*Diospyros mespiliformis*	40	*Vangueria infausta*
19	*Elais guineense*	41	*Vangueria madagascariensis*
20	*Faidherbia albida*	42	*Vitellaria paradoxa*
21	*Garcinia livingstonei*	43	*Vitex doniana*
22	*Garcinia mangostana*		

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Identification of differentially expressed genes from Spanish *Ulmus minor* genotypes with contrasted tolerance to *Ophiostoma novo-ulmi*

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Key words: Ducth elm disease, microarray, resistance genes, *Ulmus minor*

Background

Dutch elm disease (DED) is a vascular wilt disease caused by two fungi, *Ophiostoma ulmi* and the more pathogenic *Ophiostoma novo-ulmi*. The spores of DED fungi germinate within branches where the fungus grows and the hyphae spread through xylem vessels inducing their blockage and cavitation, resulting in foliar wilting and the subsequent tree death. The outbreak of two pandemics during the last century severely affected North American and European elm populations. Nowadays, numerous trees are still dying due to the difficulties to control this highly virulent disease. *Ulmus minor* and *U. americana* are the species most drastically affected by DED. Since 1928, in an effort to conserve the genetic resources of native *Ulmus* species and to obtain DED tolerant genotypes several breeding programmes have been carried out in the United States and Europe. Initially, some Asian elms that present a high degree of tolerance to *O. novo-ulmi* were used as source of genetic resistance by crossing them with native species. Recently, the Spanish elm breeding programme obtained seven highly tolerant *native U. minor* genotypes that are registered as forest reproductive material [1]. Using three *U. minor* genotypes with contrasted tolerance to *O. novo-ulmi* (susceptible, moderate and highly tolerant) a massive transcriptome in response to abiotic and biotic stress was constructed. Different replicates of theses genotypes were inoculated with *O. novo-ulmi*, *O. ulmi* or the endophye fungi *Daldinia concentrica* as biotic stress factors and other plants were subjected to a water stress treatment as an abiotic stress factor. A sample containing RNAs pooled from all genotypes and treatments were 454 pirosequenced obtaining a final transcriptome with 58,395 unigenes.

Methods

A selection of unigenes was included in the microarray design (Agilent 8 x 60K, Agilent Technologies, CA, USA). Four Spanish elms with remarkable differences in tolerance to DED were selected for this study. Their degree of tolerance to *O. novo-ulmi* was evaluated by assessing the percentage of wilting leaves at 28, 60 and 120 days post inoculation (dpi) with the pathogen. Two genotypes, M-DV1 (Dehesa de la Villa, Madrid) and VA-AP38 (Arrabal del Portillo, Valladolid), showed high degree of susceptibility to *O. novo-ulmi* whereas the other two genotypes, AB-AM2.4 (Almansa, Albacete) and M-DV2.3 (Dehesa de la Villa, Madrid, registered genotype), were highly tolerant. Five years old plants were inoculated with a highly pathogenic local strain of *O. novo-ulmi* (Z-BU1). The inoculation was carried out about 15-30 days after full leaf development following the protocol described by Solla *et al.* [2]. Control plants were inoculated with distilled water. Two-year-old twigs were collected from a height of 2 meters at 1, 3, 7, 14 and 21 dpi from both control and infected trees. Leaves were removed from the branches, keeping only the stem, immediately frozen in liquid nitrogen and stored at -80º C. RNA from the tissue sampled was hybridised to the microarrays

Results and Conclusions

Tolerance of specific *U. minor* genotypes to *O. novo-ulmi* may be determined, in a great extent, by induced plant defence mechanisms. The activation of a molecular response during fungal colonization should result in the activation of chemical and/or anatomical defence mechanisms; for instance, the accumulation of fungitoxic phenolics or the formation of suberized barriers of parenchyma cells that prevent the spread of the pathogen. In the present work, a total of 2,279 unigenes significantly modified their level of transcripts for any sample point or genotype during the treatment. A set of 236 genes out of the previously mentioned group were identified in the four studied genotypes which is indicative of an expression regulation during infection. An enrichment analysis of this group showed an increase of Gene Ontology (GO) terms related to “response to biotic stimulus”, “cell communication” or “extracellular region”. Genes usually related to tolerance to *O. novo-ulmi* as *PAL* (phenylalanine ammonia lyase) or *CHT* (chitinases) showed significant upregulation. However these genes would not be directly related with tolerance since they changed their expression in both, susceptible and resistant genotypes. In contrast, a consistent group of genes significantly modified the level of transcripts exclusively in tolerant genotypes; 157 genes were identified in AB-AM2.4, 88 genes in M-DV2.3 and 18 genes in both genotypes. In both cases, time course analysis highlighted two main patterns: genes differentially expressed at 1 dpi and genes differentially expressed from 14 to 21 dpi. Notable increases in level of transcripts of *LRR* (Leucine rich repeat), *MYB* and *BHLH* transcription factors among others were identified. These results suggest that *U. minor* tolerance to *O. novo-ulmi* is related to the expression of these differential genes.

Competing interests

The authors declare that they have no competing interests.

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Screening for candidate genes of stress response in pine

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Keywords: conifers, drought, functional genomics, gene expression

Background

Plants are exposed to fluctuations in the environmental conditions that have short- and long-term effects in their physiology. Extreme unfavorable conditions such as low water availability can have a strong impact in fitness and productivity [1]. Plant mechanisms to respond to drought have been characterized in herbaceous plant species [2], and key regulatory genes have been successfully used to improve tolerance [3, 4].Experimental approaches that can be used for functional analysis in model plant species are not easily adapted to recalcitrant tree species with large genomes. Using genetic and transcriptomic approaches, sets of genes associated to drought stress have been identified in conifers [5, 6, 7]. However, the regulatory processes underlying the mechanisms for drought response and long-term adaptation in conifers are still unclear. Here, we used a sequential approach to identify candidate genes for drought response in pine. Genes with differential responses in pines with contrasting drought response phenotypes were identified and tested for response to dehydration and ABA treatments.

Methods

A collection of 1,000 cDNA pine sequences was used to select an initial set of 330 genes annotated as stress- or secondary metabolism-related, or encoding putative transcription factors or regulatory proteins. Quantitative RT-PCR expression analyses were carried out in sequential rounds, using samples from pine trees exposed to osmotic stress, pine clones with contrasting drought-response phenotypes [8] exposed to dehydration, and 21-day-old pine seedlings exposed to dehydration or ABA treatments. The analyses were carried out using either whole plants or separate organs (root, shoot and brachyblasts in pine clones;

root, hypocotyl, cotyledons and shoot apex, including developing needles, in pine seedlings).

Results and Conclusions

An initial screening of 330 genes was carried out using samples of plants exposed to osmotic stress. Overall, 19% of the genes, including a significant number of secondary metabolism-related genes, were down-regulated while, for 10% of the genes, transcript levels increased in response to the treatments, the most extreme values corresponding to stress-related genes. No significant changes were observed in response to the treatment in 46% of the genes. Transcript levels were undetectable for 25% of the genes.

A total of 100 genes, including genes showing extreme responses and non-responsive genes encoding putative transcription factors and regulatory proteins, were selected for a second round of expression analysis to test response to dehydration in two pine genotypes with contrasting drought-related phenotypes. This approach led to the identification of 24 genes that showed genotype-specific basal expression and/or response to dehydration.

Six genes encoding dehydration-related proteins were selected for detailed analysis in different organs of plants exposed to dehydration and seedlings exposed to dehydration or ABA treatments. The stress-responsive gene expression of three genes encoding embryogenesis-related proteins and two genes encoding dehydrins showed a correlation with the expression measured in the ABA-treated tissues in a gene- and tissue-specific manner, indicating that water stress-responsive gene expression might be regulated by ABA-dependent and ABA-independent pathways. A gene encoding an RD22-like protein, expressed exclusively in pine clones with a high-tolerance phenotype and showing a negative response to both dehydration and ABA treatments, was identified as a candidate gene for further studies on the regulation of drought responses in pine.

Competing interests

The authors declare that they have no competing interests.

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De novo assembly and annotation of a transcriptome during xylogenesis in *Pinus canariensis* Chr. Sm. ex DC

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Key words: Pinus canariensis, Transcriptome, Annotation, Next-generation sequencing

Background

Nowadays, there is a great amount of genomic and transcriptomic data available about forest species, including ambitious projects looking for complete sequencing and annotation of different gymnosperm genomes [1, 2]. *Pinus canariensis* is an endemic conifer of the Canary Islands with re-sprouting capability and resilience against fire and mechanical damage, as result of an adaptation to volcanic environments. Additionally, this species has a high proportion of axial parenchyma compared with other conifers, and this tissue connects with radial parenchyma allowing transport of reserves. The most internal tracheids stop accumulating water [3], and get filled of resins and polyphenols synthesized by the axial parenchyma; this is the so-called “torch-heartwood” [4], which avoids decay. This wood achieves very high prices due to its particular resistance to rot. These features make *P. canariensis* an interesting model species for the analysis of these developmental processes in conifers. In this study we aim to perform a complete transcriptome annotation during xylogenesis in *Pinus canariensis*, using next-generation sequencing (NGS) -Roche 454 pyrosequencing-, in order to provide a genomic resource for further analysis, including expression profiling and the identification of candidate genes for important adaptive features.

Methods

Differentiating xylem was collected at two points of seasonal growth, spring and summer, in order to cover all anatomical events. Total RNA was extracted based on Chang *et al.* [5], and two separate libraries were constructed through 454 pyrosequencing and *de novo* assembled using Newbler 2.5 (Roche454; [6]). Preliminary assemblies were pooled and meta-assembled using CAP3 [7], to remove redundancies and achieve larger contigs. The quality of the assembling was assessed quantitavely by computing the length of contigs, the GC% content and the N50 using Quast 2.3 [8]. Contigs were launched in a local version of BlastX [9] against the Viridiplantae section of RefSeq database (NCBI), with a threshold *evalue* of 0.00001. BlastX output was imported into Blast2GO [10] to assign Gene Ontology (GO) information. Contigs were also aligned to available transcriptomes of *Pinus pinaster* and *Pinus halepensis* [1, 11].

Results and Conclusions

The two libraries constructed at two stages of vegetative growth of *P. canariensis* bring 458,498 reads in spring and 474,393 reads in summer. After assembly, the final transcriptome resulted in 45,509 contigs and 103,764 singletons (i.e. short reads not asembled into contigs), having a total length of 34,024,623 bp, and with a GC% of 42.4 and N50 size of 1,078 bp. BlastX search gave 27,474 hits (60.37 % of contigs), and 172,687 GO terms were obtained after annotation, distributed among different levels for Biological Process (BP; 93,157 GOs, 54%), Molecular Function (MF; 32,994 GOs, 19%), and Cellular Component (CC; 46,536 GOs, 27%). These data were similar to those obtained in *P. halepensis* through RNAseq [11] and Douglas fir with 454 methods [12]. Finally, 92.66% and 79.27% of the contigs aligned to unigenes of *P. pinaster* and *P. halepensis*, respectively.

This work is the first comprehensive analysis of the *P. canariensis* transcriptome, and our data may be a useful resource for further analysis in the identification of candidate genes for a better understanding of adaptive patterns in conifers, such as cell-types differentiation, re-sprout, wound-healing or “torch-heartwood” formation.

Competing interests
The author declares that they have no competing interests.

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Proteomic and transcriptomic analysis of carbohydrate metabolism involved in somatic embryogenesis of Brazilian Pine (*Araucaria angustifolia* Bertol. Kuntze)

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Key words: Somatic Embryogenesis, starch, callose, Araucaria angustifolia

Background

Brazilian Pine (*Araucaria angustifolia*) is a native conifer currently classified as critically endangered species [1]. An alternative propagation approach for conservation is the establishment of a successful somatic embryogenesis system, which has also been used as an experimental system to better understand totipotency in higher plants [2]. Therefore, in order to establish an appropriated indicator of cell lines with highly embryogenic potential, it is necessary to know stimuli and conditions for a correct somatic embryo formation [2]. The comprehensive knowledge about the expression profile of transcripts and proteins has become a powerful tool to understand protein dynamics or molecular and physiological events, as well as to better elucidate the regulation of *in vitro* embryogenesis process [3]. Additionally, qualitative and quantitative variations in carbohydrates and proteins (including the accumulation of storage reserves) are key factors contributing to the whole embryogenesis process, especially embryo development and maturity [4]. In order to investigate the participation of carbohydrate metabolism, which represents one of the most important aspects of plant cell processes, we identified and quantified starch and the expression of carbohydrate-related proteins and transcripts present in two *A. angustifolia* somatic cell lines.

Methods

Two *A. angustifolia* embryogenic cultures (ECs) with different embryogenic potential were used, being SE1 responsive and SE6 blocked cell line to maturation medium (ABA and osmotic agents). For analysis, SE1 and SE6 cultures were grown for 21 days on MSG medium (in the dark, at 25ºC ± 2) before harvesting [2]. A total of three biological replicates (composed of a pool of four cell cultures each) were collected and stored at -80°C until further processing. Starch was measured according to [5] and was visualized by optic microscopy staining with lugol. Proteins were quantified using an online nanoflow liquid chromatography tandem mass spectrometry (LC-MS/MS) [6] and identified by searching against Araucaria transcriptome database using MaxQuant software [7]. Futher, *in silico* analyses of the genes corresponding to the identified proteins was performed, and the abundance of the genes was obtained by RPKM (number of reads which map per kilobase of exon model per million mapped reads for each gene, tissue or sample) providing a correlation between gene expression and protein function.

Results

SE6 line exihibits three times higher starch content than SE1.High starch quantities were also visualized by microscopy, using Lugol's stains, which showed a large amounts of starch granules mainly present in the SE6 suspensor cells. Among the differentially expressed carbohydrate-related proteins identified by LC-MS/MS *phosphoglucan chloroplastic-like phostate (AaGWD*) was expressed exclusively in SE1, and *glucan endo-beta-glucosidase-like (AaPDCB)* was overexpressed in SE6. Transcript levels related to these two proteins were identified in both cell lines, being *Aagwd* and *Aapdcb* significantly higher in SE1 and SE6, respectively. *GWD* is a protein directly related to starch hydrolysis [8]. Its presence only in SE1 suggest that this cell line carries out starch hydrolysis continuously, while SE6 lacks the capacity to hydrolyze this carbohydrate. Moreover, *PDCB* increases the deposition of callose in the plasmosdesmatas edges, causing a narrow in cell communication [9] and its overexpression in SE6 might indicate an isolation of the cells, contributing to accumulation of substances (e.g. starch). The presence of callose deposition at plasmodesmata of the embryogenic cells were confirmed by fluorescence microscopy using a staining with blue aniline.

Conclusions

These results suggest a possible correlation between the embryogenic potential of somatic cell lines of *A. angustifolia* with carbohydrate metabolism, especially with the starch biosynthesis and degradation. The absence of expression of *AaGWD* protein with a concomitant overexpression of *AaPDCB* protein as well as the accumulation of starch in SE6 indicates that in this cell line storage is predominant. Therefore, it is crucial change the SE6 lines to a metabolism centered on growth and development (as observed in SE1) in order to enable them to maturation.

Competing interests

The author declares that they have no competing interests.

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Transcriptome sequencing and *de novo* assembly of the cold acclimated *E. nitens* using Ion Torrent

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Key words: *de novo* assembly, RNA-Seq, Ion Torrent.

Background

Most temperate plants have evolved a mechanism to enhance tolerance to freezing during exposure to periods of low, but non-freezing temperatures. This adaptive response is called cold acclimation and is a result of a combination of physiological and metabolic changes depending on transcriptome modification. Several studies have been focused on determining the molecular basis of freezing tolerance in different species of *Eucalyptus* spp. *Eucalyptus nitens* is planted for timber and pulp production and has been described as being frost tolerant. In recent years, the sequencing industry has been dominated by technologies that utilizing fluorescently labeled reversible-terminator nucleotides. The Ion Torrent PGM detecting the protons released as nucleotides are incorporated during synthesis. This work presents a transcriptome analysis of cold acclimated *E. nitens* data to identify the genes associated to cold acclimation using Ion Torrent sequencing data.

Methods

Twelve expression libraries were prepared from leaves of plants exposed to four acclimation conditions, simulating the annual seasonal variation in a growth chamber (NA: Non-acclimated, CABF: Cold acclimated before frost, CAAF: Cold acclimated after frost and DA: De-acclimated). The bioinformatics analysis included: i. data preprocessing: trimming of the sequences in 3' –ends, filtering and homopolymer sequences removing, ii. *de novo* assembly of biological replicates per condition using MIRA4 assembler and clustering replicates to obtain the reference transcriptomes using a CD-HIT tool iii. annotation of transcriptomes to identify the genes involved in *E. nitens* cold acclimation using BLASTN and a related species (*Eucalyptus grandis*) and iv. mapping reads to the four references transcriptomes using Bowtie2.

Results and Conclusions

For each library, low-quality bases and the sequencing adapter were trimmed. Short reads (<100pb) and homopolymer sequences were removed, which are known to be most common error in Ion Torrent sequencing. After filtering, a total of ~17 million

high-quality transcriptomic reads were obtained per library, giving rise to average of 120 bp per read. For *de novo* assembly ~12 million of reads per library were obtained. The four transcriptomes were aligned to the transcriptome of *E. grandis* using BLASTN similarity searches for the entire transcriptome. An *e-value* 1e-10 was considered significant and maximum of 1 hit was taken into account per query. The comparison of ~600,000 assembled contigs per cold acclimation condition with the *E. grandis* transcriptome database using BLASTN matching 32,838, 32,070, 31,823 and 31,705 genes in the NA, CABF, CAAF and DA transcriptome, respectively. Approximately 40% of reads were mapped to the NA, while 50% of reads were mapped to the CAAF transcriptome.

We conclude that Ion Torrent sequencing allows *de novo* transcriptome assembly and a fast, cost-effective, and reliable method for development of transcriptomic tools for non-model plant species. The present analysis can help to understand the cold acclimation mechanism used by *E. nitens*.

Competing interests

The author declares that they have no competing interests, giving the consent to publish.

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HSFA2 TRANSCRIPTION FACTOR RESPONDS TO COLD ACCLIMATATION AND MIGHT BY RELATED TO DEHYDRIN GENE IN *Eucalytus nitens.*

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Background

Cold acclimation is a process by which plants acquire freezing tolerance upon prior exposure to low non-freezing temperature [1]. This process involves numerous biochemical, physiological and metabolic changes which allow hardy plants to trigger the mechanisms needed for the acquisition of freezing tolerance (FT), which determines their capacity to survive winter. During cold acclimation, dehydration may partly contribute by preventing ice formation and ameliorating the effects of freeze-induced cellular dehydration [2]. Extremely hydrophilic proteins such as dehydrins (DHN) are known for its protective role in dehydration. The classical view therefore considers DHNs as membrane stabilizers and structural stabilizers of target proteins and nucleic acids [3]. Although the precise function of DHNs has not been elucidated, their consistent accumulation in various plant groups during conditions that cause cellular dehydration, and presence in tissues that have low water content, strongly supports their role in dehydration tolerance of plants [4]. At the transcriptional regulation level, promoter motif-based regulation remains an extensively studied topic of importance for eukaryotic gene expression. DNA regulatory motifs are 5–15 base pair (bp) long nucleotides within the promoter region that function primarily as transcription factor (TF) binding sites. The interaction between TFs and their target motifs can lead to induction or repression of gene expression [5]. Several transcription factor families are involved in complex and overlapping responses under different stress conditions. Among these transcription factor families, heat shock factors (HSFs) are of particular interest because their functions in heat stress response and thermotolerance are highly conserved across all eukaryotes. It was reported that might play a crucial role in the development of cross-adaptation to temperature stress [6]. The aim of this work was to study the *Hsfa2,* a plant HSF, transcription factor as candidate in *dhn2* gen regulation in *Eucalyptus nitens* in response to cold acclimation.

Methods

Plants from a half-sib family of *E. nitens* were placed in a growth chamber with controlled temperature and photoperiod. Temperature and photoperiod were changed in each treatment. For treatment 1 nonacclimated (NA) plants were maintained under a 14-h day length at 20/12°C day/ night temperature, leaves were collected and the condition of the growth chamber was changed. For treatment 2, leaves for cold-acclimated plants in short days (10h day) at 8/4°C before night frosts of −2°C (CABF) were collected. Treatment 3 comprised of cold acclimated after night

frosts of −2°C (CAAF); after 1 week of CABF, four night frosts of −2°C (one per night) with a decrease of 2°C/h were applied, and leaves were collected after the last frost (at 8°C). For treatment 4, deacclimated (DA), plants were exposed to long day length (14 h day length) and 12/6°C day/night temperature for 1 week, and leaves were collected on day 28. Leaves collected were sampled for RNA extraction and qRT-PCR for exploratory analysis of *dhn2* and *hsfa2* gene expression. RNA was extracted from leaves using the CTAB method. To qRT-PCRs reactions, two housekeeping genes (*Gapc2* and *Eif4a1*) were used. Three biological replicates for each sample were used, and three technical replicates were analyzed for each biological replicate. In order to identify regulatory motifs present in the promoter region of the DHN gene, *Eni.dhn2*, the PLACE database of previously characterized motifs was used and searched for presence of these motifs in the promoter region of the gen [7]. The gene relative expression values were subjected to analysis of variance to test the effect of cold treatments. Data were checked for normality and homogeneity of variances. A Tukey test ($n=3$ $p \leq 0.05$) was used to identify those values with significant differences with SAS 9.00 software.

Results and Conclusions

Heat shock transcription factors (HSFs) specifically bind to *cis*-elements termed heat shock elements (HSEs), which consist of alternating units of the sequence (5'-nGAAn-3'), and subsequently activate the transcription of inducible genes [8]. We identified the sequence 5'-TAAATTTTTT-3' in the *Eni.dhn2* promoter in the 5'-upstream region about 500pb from the transcription start site (TSS). This sequence was predictable as a motif by the consensus sequence 5'- AGAANNTTC-3' reported [9]. The target sequence (HSE) of *Hsfa2* gen was frequently observed near 1 Kb upstream of the ATG start site [10, 11].

The relative expression of gene *Eni.dhn2* increased up to 3-fold after CABF and reached a maximum level after CAAF (6-fold). The same results were observed with *Eni.hsfa2* gen but with a higher transcript accumulation (13-fold) after CAAF treatment. In both cases, the transcript accumulation decreased at DA treatment.

This increment after CABF and CAAF could be related to a decreased of free water, as a consequence of cold acclimation. It is known that the *Hsfa2* gen is not only related to heat temperature, it is also up-regulated by salt and osmotic stress [11]. Studies on the relationship of this gene with the *Eni.dhn2* gene may be useful for understanding the responses involved in cold acclimation and furthermore his regulation.

Key words: Cold acclimation, dehydrins, HSP.

Competing interests

The author declares that they have no competing interests.

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Identification of four dehydrin genes in *E. nitens*

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Background

Low temperature is an environmental factor limiting plant growth, affecting their development and reducing their productivity. Cold resistance is one of the main mechanisms studied in plants, in some species it can increase their cold tolerance in response to environmental stimuli such as low non-freezing temperatures, a phenomenon known as cold acclimation. During the acclimation state a large number of genes are induced, thought to be responsible for the increase in cold tolerance. In cold-tolerant plants, several dehydrins are expressed in response to cold treatment. *Eucalyptus nitens* has a rapid growth and adaptability, being resistant to cold temperatures. In this work four dehydrin genes (EniDHN1, EniDHN2, EniDHN3 and EniDHN10) were identified in *E. nitens*.

Methods

DNA was extracted from leaf tissue of *E. nitens*. For the identification of DHNs genes in *E. nitens* primers were designed using as reference the dehydrin sequences in *E. globulus* (Fernández et al 2012). The full-length DHNs nucleotide sequences were translated using ExPASy (http://web.expasy.org/translate/). The S-, K- and Y segments of DHNs proteins were identified using the ExPASy prosite server (http://prosite.expasy.org/). The comparison of DHN proteins sequences from different species were aligned using CLUSTAL-W version 2.1 and a phylogenetic tree was generated using PAUP version 4.0, with Neighbor-Joining method on 1,000 bootstrap iterations.

Results and Conclusions

Four dehydrins were identified in *E. nitens*. The full length of *EniDHN1* was 1,325 bp, with a coding sequence of 537 bp corresponding to 179 amino acids. For *EniDHN2* the fragment amplified was 1,892 bp with a coding sequence of 885 bp corresponding to 295 amino acids. For *EniDHN3* it was possible to isolate one fragment of 1,473 bp with one coding sequence of 441 bp corresponding to 147 amino acids. For *EniDHN10* the sequence was 552 bp with a coding sequence of 297 bp corresponding to 99 amino acids.

To compared the four sequence isolated with DHNs described for other species of *Eucalyptus* we found high percentage of identity, *EniDHN1* has 98% sequence identity with the dehydrin describe for *E. globulus* (AER27687) and a high identity with DHNs sequences of *E. grandis* (93% with KCW56702 and a 59% with KCW56700), *EniDHN2* presented 84% sequence identity with *E. globulus*

(AER27688) and 50% sequence identity with *E. grandis, EniDHN3* has 96% sequence identity with *E. globulus* (AEY77126) and 90% sequence identity with *E. grandis* (KCW53080). The predicted *EniDHN10* polypeptide has 98% identity with *E. globulus* (AER27689) and 88% sequence identity with *E. grandis* (XP_010036245). A phylogenetic tree including all *EniDHNs* protein isolated and dehydrins described for other species of *Eucalyptus* was created. Based on the phylogenetic results, the *EniDHN*s proteins were clustered in different groups, the *EniDHN1* clustered with the *Eucalyptus* SKn-type, the amino acid sequence showed one S segment and two K, for *EniDHN2* the results showed a cluster with the *Eucalyptus* SKn-type, the amino acids sequence showed one S segment and two K. *EniDHN3* was clustered with the Eucalyptus YnSKn-type *EniDHN3* protein contained three Y segments, one S segment and two K segments, while for *EniDHN10* was cluster in the KS-type, the amino acid sequence showed a K segment and one S segment (Figure 1).

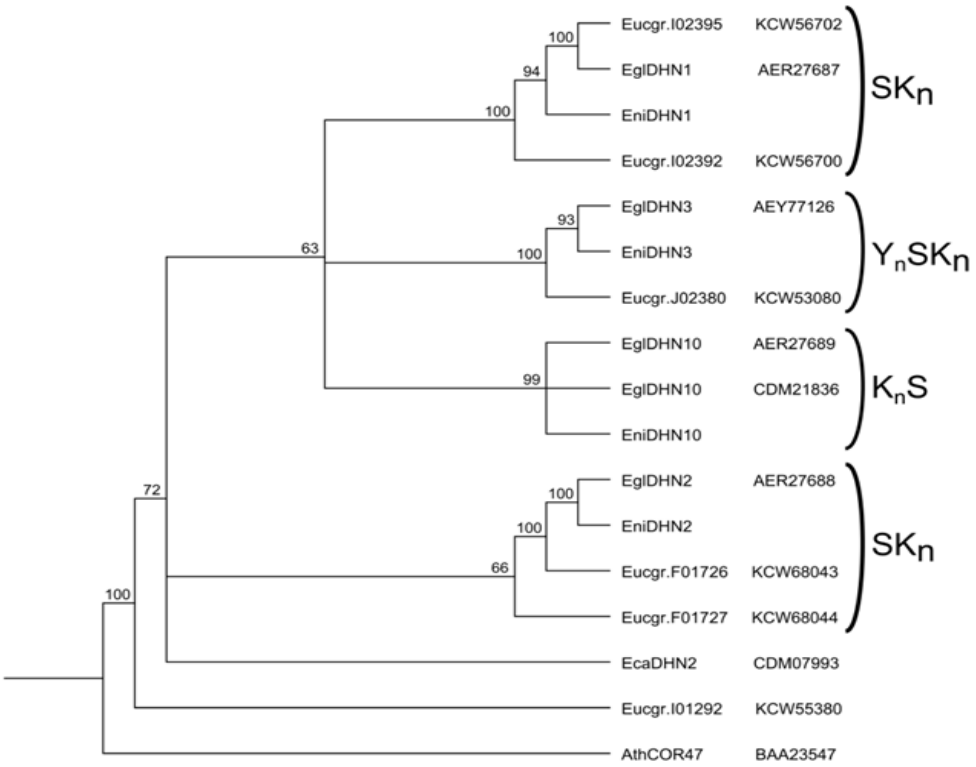

Fig.1 Phylogenetic analysis of dehydrins proteins from *Eucalyptus* species. Phylogenetic tree was generated using PAUP program. Proteins were arranged according her sequence similarities.

Competing interests

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Towards elucidating the role of DREB1 and DREB2 transcription factors in *Eucalyptus* whole plants under abiotic stresses: gene regulation in leaves, stems or roots

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Key words: Eucalyptus, abiotic stresses, DREB factors, spatial gene expression

Background

Abiotic stresses such as drought and extreme temperatures affect the survival, growth and reproduction of plants and finally influence their spatial distribution. Considering its tropical and subtropical origin and ever-growing habit, *E. grandis* is reported to exhibit a surprisingly high level of frost tolerance as a result of a very rapid hardening capacity. *E. gunnii* is one of the most freezing tolerant species of *Eucalyptus* genus which is also known as heat-tolerant.

To face adverse environmental conditions, the plant have evolved complexe adaptative processes which include developmental, physiological and biochemical changes, controlled through gene regulation pathways. The AP2/ERF (APETALA2/Ethylene-Responsive element binding Factor) family, recently annotated in eucalyptus [1], includes DREB (Dehydration Responsive Element Binding) subfamily, known as essential in the regulation of abiotic stress response. With regard to the E. grandis DREB subfamily, an obvious feature is the presence of 17 DREB1/CBF genes, the maximum reported to date for dicotyledons, in contrast to the moderate number of DREB2 (6 genes) which is similar to the other plants.

The DREB factors recognize the cis-acting sequences CRT (C-Repeat) or DRE (Dehydration Responsive Element) located in the promoters of abiotic stress responsive genes. The DREB1/CBF genes are known as cold-responsive when DREB2 genes are more generally associated with water stress or heat-shock. Our previous analyses on eucalyptus DREB1/CBF overexpressors [2] and leaf cold transcriptome [3] provided evidences for the involvement of the DREB1/CBF genes in leaf cell protection under cold. More recently, a preliminary study [1] indicated that both DREB1 and DREB2 are stress-responsive without strict group specificity in eucalyptus leaves under cold, heat and drought. However, in the literature, very little is known about plant regulation of DREB1 and DREB2 genes in other organs and their possible involvement in stress adaptive responses.

Methods

Here we show a comprehensive analysis of the DREB1 and DREB2 gene expression in stems, roots or leaves of two contrasted *Eucalyptus* species (*E. gunnii* and *E. grandis*) under moderate stresses (drought, heat or cold). In silico analyses of promoter sequences of the 23 genes under study provide predictions on DREB1 and DREB2 differential regulation.

Results and Conclusions (these last two either separate of together)

The presented data reveal in both species a differential regulation of these genes according to the organ and the stress. The comparison of resulting expression profiles allow identifying groups of DREB genes preferentially responsive to one stimulus and more regulated in one organ. The striking amplification of DREB1 groups in *Eucalyptus* genome would be associated to a sub-functionalization of the duplicated genes resulting in a differential regulation and would complete the limited number of DREB2 genes for regulating multiple abiotic stress responses. In conclusion, DREB1/CBF and DREB2 may be key players of cross-talks between cold, heat and drought responses which provide both efficiency and plasticity to the *Eucalyptus* response to environmental stresses. This study provides significant information for selecting DREB candidate genes towards improving stress tolerance in *E. gunnii* and *E. grandis*.

Competing interests

The author declares that they have no competing interests.

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UNDERSTANDING WOOD FORMATION IN EUCALYPTUS SPP: ANATOMICAL AND TRANSCRIPTOMICS APPROACH

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Key words: Resistograph, basic density, fiber biometry, transcript abundance

Background. In Chile, *E. globulus* is the mainly commercial hardwood for pulp and paper production due to its fast growth, high basic wood density, high pulp yield and good fiber and handsheet properties, but it has a poor frost tolerance. *E. nitens* has been introduced to the country for forest plantation and pulp production because of its frost tolerance, but its wood has a poor quality and low basic density. Currently, efforts of the main Chilean forestry companies are focused on developing *E. globulus* x *E. nitens* hybrids presenting additive traits, combining frost tolerance and high wood quality. Trees of 6 year-old *E. globulus* and *E. nitens* were analyzed, determining basic wood density and fiber quality of both *Eucalyptus* species using a fiber analyzer equipment and micro-drilling tool. Transcript abundance of genes involved in wood formation were evaluated by qRT-PCR to determine any correlation between transcript abundance and anatomical and fiber biometry features, in order to generate information for further selection of trees in genetic improvement programs.

Methods. *Wood material*. One hundred 6-year-old trees of each *E. globulus* and *E. nitens* growing in field plantation were studied by pre-screening wood density with a IML-Resistograph micro-drilling tool at breast height (BH). After ranking the data, 30 *E. globulus* and 30 *E. nitens* trees with high and low density were selected. Incremental cores were sampled at BH for further anatomical and fiber biometry analysis. *Basic density determination.* Incremental cores were sampled to determine basic density of each tree according to the Tappi Standard Method T258 om-94. *Anatomical analysis.* Blocks of 2 cm^3 taken and transversal micro-sections of 30 µm thickness were obtained (Aguayo *et al.*, 2010). *Fiber biometry analysis*. Matchsticks obtained from longitudinal cuts of sub-samples taken along the incremental core were macerated and treated using Franklin solution and analyzed in a Fiber Tester equipment (Carrillo *et al.,* 2015). *Resistograph measurements*. Trees were drilled bark-to-bark at BH using IML Resistograph PD400 and processing using PD-Tools Pro software (Isik et al., 2003). *Transcript abundance. CesA3*, *XTH1* and *PME2*. The forward and reverse primers employed, were obtained from Goulao *et al.,* (2011). Transcript abundance was determined by using a Real Time PCR System according to Elissetche et al., (2011).

Results and Conclusions. *E. globulus* showed a basic density range of 408.6 to 540.8 kg/m^3, with an average of 477.6 kg/m^3, while *E. nitens* ranged 432.8 – 558.1 kg/m^3, with an average of 490.3 kg/m^3. Mean amplitude of *E. globulus* genotypes was 27.1%, while for *E. nitens* was 31.9%, being *E. nitens* amplitude average significantly superior to *E. globulus* amplitude average. For both species a positive correlation was observed between basic density and mean amplitude. *E. globulus* correlation index was 0.84 (p<0.001), and for *E. nitens* was 0.85 (p<0.001). No

significant differences were observed between both species in cell wall thickness, lumen fiber width and vessel width mean, but when comparing between early and latewood significant differences were observed. Fiber length and fiber width of *E. globulus* ranged 0.51 – 0.88 mm and 17.8 – 20.1 µm, respectively, while coarseness ranged between 49.4 and 82.4 µg/m. Fiber length and fiber width of *E. nitens* ranged 0.55 – 0.73 mm and 17.1 and 20.7 µm, respectively, while coarseness ranged 47.9 and 77.3 µg/m. Correlation analysis showed positive and significant relations between fiber length and fiber width with coarseness of *E. globulus* and *E. nitens*, which is expected due that coarseness is a parameter that involve both variables. CesA3, XTH1 and PME2 was evaluated and correlation with anatomical and biometry of fiber was determined. Transcript abundance of Cesa3 and XTH1 genes presented significant correlation with fiber length, cell wall thickness, vessel width, DBH and wood density. PME2 no significant correlation with evaluated parameter was determined.

Morpho-anatomical features show correlation with transcript abundance in two genes and provide correlation with wood density indicator of wood quality, could be used in improvement selection programs.

Competing interests

The author declares that they have no competing interests.

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Impact of potassium and sodium fertilization on the leaf transcriptome of Eucalyptus grandis submitted to water deficiency

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Key words: Eucalyptus, fertilization, water deficiency, transcriptome

Background

Fast growing Eucalyptus tree is well adapted to various soils and climate environment, but its growth varies strongly according to these factors (Merchant et al. 2007). In the south of Brazil, Eucalyptus clones selected from breeding program are adapted to ecological conditions characterized by water deficiency during the dry season. Thanks to potassium fertilization to supply deficient soils (Almeida, 2010), plantation productivity is one of the highest in the world. However, Brazilian Eucalyptus plantations productivity is altered by climate change due to a greater rainfall variability leading to longer dry periods, increased temperature, and vapor-pressure deficits (Gonçalves et al., 2013). On the other hand, with the increasing of wood demand, potassium consumption is rising with negative consequences from an economic point of view. One alternative to adapt to this context is to select more efficient hybrids or clones in terms of water and fertilizer uses. Potassium could potentially be replaced by low amount of sodium. Its interest has been shown in drying soils for some plants, especially when there is limited access to other nutrients such as potassium (Munns and Tester, 2008). Eucalyptus trees fertilized with sodium increased above-ground biomass by 56%, and 130% with potassium, in comparison with the control without fertilization (Almeida, 2010). The possibility of using partially sodium to fertilize Eucalyptus plantations opens up new perspectives. Fertilizer amount could be reduced in plantations near the coast, and unpurified fertilizers containing sodium such as sylvinite could be used at reduced cost by small producers in poor tropical regions. The development of new cultural practices more sustainable requires an improved understanding of crop response to water stress in interaction with mineral nutrition.. The aim of this study is to evaluate: (1) the role of potassium and sodium fertilization on Eucalyptus response to drought and (2) the impact of these fertilizations on wood properties.

Methods

An experimental design was set up on field with a highly productive Eucalyptus grandis clone planted in a complete randomized block design, with 2 factors tested in interaction: the water availability with 100% rainfalls (+H2O) or 66 % of rainfalls (-H2O) set up with rainfall exclusion system, and the fertilization with +K, +Na or Control (-K-Na). RNAs were extracted from leaves, collected from four 2-year-old trees for each of the 6 treatments at the end of the rainy season. The construction of the RNA-seq libraries and sequencing were performed accordingly to Illumina High Seq protocols. The alignment and assembly was done with TopHat (Trapnell, 2009) based on the E. grandis reference genome from

Phytozome (Myburg, 2014). Then differential expression was analyzed by using the R package “DESeq2” with multi-factors design (Love, 2014). For selecting factors (fertilization, water availability, and interaction between both) for which genes present a significant differential expression, a model selection procedure based on the likelihood ratio test was applied. A false discovery rate (FDR) procedure was used for multiple testing correction with a threshold at 0.01. Finally, we performed functional annotation and gene enrichment by using Blast2GO (Conesa, 2005). Gene expression profiles

Results and Conclusions (these last two either separate of together)

After assembling and alignment, we identified 36,376 genes in our RNA samples. From them, we highlighted 4159 genes presenting a differential expression, divided in 4 groups, related to the fertilization only (964 genes), the water availability only (2568 genes), or both factor without interaction (326 genes) and with interaction (301 genes). We annotated 89% of these genes, and a gene enrichment analysis allowed to identify specific biological process related to each group of genes. For example, the analysis of the group “water availability” showed that 100% rainfalls samples were enriched in genes related to response to light stimulus and cation transport, while there is fewer genes related to translation compared to the 4159 genes as reference set. For 66% rainfalls samples, there was enrichment in genes related to tRNA aminoacylation for protein translation and embryo development.

This leaf transcriptomic analysis allowed identifying groups of genes differentially expressed in function of the fertilization, water availability, and both factors with and without interaction. Moreover, for each group of genes, we identified specific biological process pattern. This analysis will help to dissect the mechanisms involved in the response of Eucalyptus to water deficiency compared to 100% rainfalls when trees are fertilized with K or Na. This knowledge will be important to address new demands for adjusting mineral fertilization for trees less demanding in water content.

Competing interests

The author declares that they have no competing interests.

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Using induced resistance in *Eucalyptus grandis* to identify broad spectrum defence mechanisms

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Key words: Forestry, systemic resistance, fungus

Background

Eucalyptus species are used in forestry worldwide because their rapid growth and desirable properties render them well suited to the wood and paper industries. *Eucalyptus* products are also used in pharmaceuticals and textiles. However, disease caused by a variety of pests and pathogens that affect different parts of the tree continues to reduce yields by damaging plantations [1]. Broad-spectrum resistance could be a very useful countermeasure against this problem. Plant basal resistance is often general and broad-spectrum, but may not be sufficient to prevent disease. Additional defences may be activated upon attack, but this may be too late to provide resistance. Induced resistance results from exposure of the plant to a stimulus such as a microbe (systemic acquired resistance or SAR; induced systemic resistance or ISR), or wounding (systemic induced resistance or SIR) [2]. Generally, IR appears to confer resistance by enhancing basal defences systemically prior to attack. IR can be long-term and broad-spectrum, but like basal defences, IR may not provide complete resistance. Additionally, the timing of IR activation relative to secondary exposure is important [3]. Systemic acquired resistance has been demonstrated in herbaceous plants such as *Arabidopsis* [4], but has not been studied widely in trees.

Chrysoporthe austroafricana is a fungal stem canker pathogen that occurs in Africa [5]. *E. grandis* clones with different levels of resistance to *C. austroafricana* have been identified [6], and previous studies showed that the local transcriptional responses differ between moderately resistant and highly susceptible clones. Differential production of the monoterpene cymene distal to infection has also been observed in these clones, suggesting that their systemic responses to infection may differ. The purpose of this study was to exploit the interaction between *E. grandis* and *C. austroafricana* as a model system to explore the systemic responses to infection to identify processes that may confer broad-spectrum resistance to *Eucalyptus.*

Methods

Stems of *Eucalyptus grandis* clones TAG5 and ZG14, which are moderately resistant (MR) and highly susceptible (HS) to *Chrysoporthe austroafricana,* respectively, were inoculated with a virulent isolate of *C. austroafricana* (CMW 2113). After three days, leaves were

harvested and subjected to RNA-Sequencing (Beijing Genomics Institute, China). Leaves from wounded plants were used as controls. The transcriptome data were analysed using the Tuxedo suite of tools on the Galaxy platform to identify differentially transcribed genes. Processes affected by infection were visualised using REVIGO, MapMan (3.6 0RC1) and BiNGO (BiNGO 3.0.2).

Results

All RNA-Seq sequences were of high quality with Phred scores above 30. Approximately 74% of the mRNA reads mapped to the *Eucalyptus grandis* genome for all groups. The moderately resistant plants had about ten times more differentially expressed genes (724 genes, 658 transcripts) than the highly susceptible plants (72 genes, 69 transcripts). Within the down-regulated gene sets, gene ontology (GO) terms associated with cellular metabolism were over-represented or unchanged in both. Some GO terms related to defence responses were over-represented in the up-regulated gene sets, while others were over-represented in the down-regulated gene sets or unchanged. Within the up-regulated gene sets, GO terms related to defence responses, such as biotic stress signalling, secondary metabolism, and responses to fungus, herbivore and bacterium, were over-represented in MR plants. In HS plants, GO terms related to response to herbivore are over-represented, while GO terms related to responses to fungus and bacterium are over-represented in the down-regulated gene set or unchanged (Figure 1).

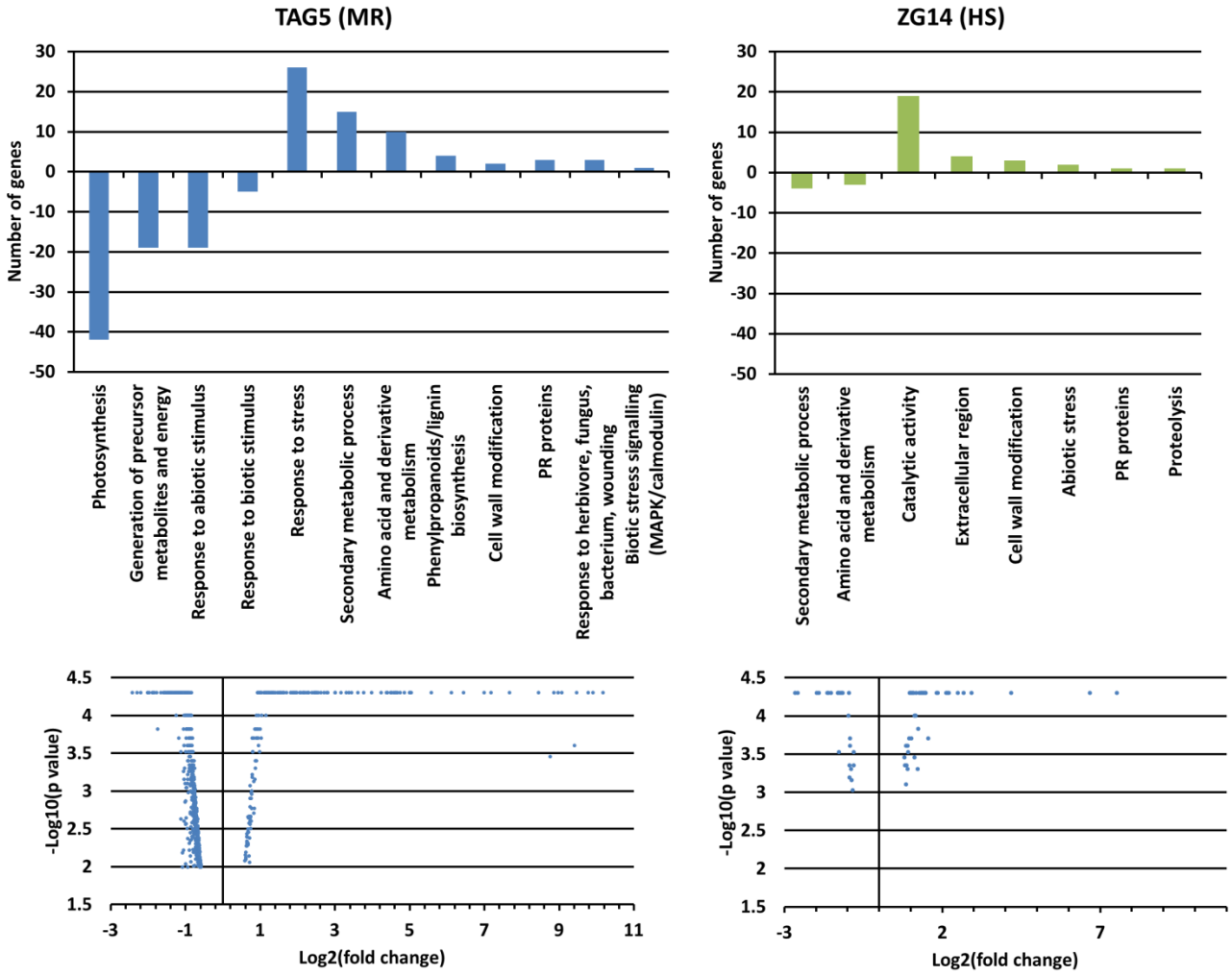

Figure 1 Systemic responses to fungal inoculation in moderately resistant and highly susceptible *Eucalyptus grandis* plants. A. Over-represented GO terms in the up-regulated (positive values) and down-regulated (negative values) gene sets of MR and HS plants. **B.** Volcano plots of significant differentially transcribed genes in MR and HS.

Conclusion

The findings of this study suggest that MR, but not HS, plants are able to strongly activate broad-spectrum responses to fungal inoculation within three days after inoculation. The timing and type of systemic responses may be important during IR in *E. grandis*. Many of the defence processes that appear to be involved in the systemic responses of *E. grandis* are known to be involved in basal defence [2, 7], consistent with the notion that IR results from enhanced basal defences. Secondary challenge with other pathogens will reveal whether these transcriptional responses result in functional systemic resistance, and may demonstrate which responses confer the most effective IR. Ultimately, strategies for producing trees with enhanced resistance to a range of pests and pathogens may be identified.

Competing interests

The author declares that they have no competing interests.

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Genomic resources in *Casuarina* sp L.

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Background

Of the few commercially important *Casuarina* species, *C. equisetifolia* (*Ce*), *C. junghuhniana* (*Cj*), *C. cunninghamiana* (*Cc*), *C. glauca* (*Cg*) are the most widely planted trees in Southeast Asia, China and Australia, due to their fast growth, high productivity, short rotation period, soil fertility augmentation and suitable for pulp and paper production. In India, Casuarina is still not very popular among farmers due to poor adaptability and lack of coppicing ability in Inland and dry zone. These issues can be addressed through informed tree breeding approach, however due to the limited genome information, recent technologies such as association studies, genomic selection are yet to be established in Casuarina. Therefore, an attempt was made to understand the genome of *Casuarina* by deciphering the drought genes, coppicing genes, transcriptome derived simple sequence repeats (SSR) to advance in genetic and genomic research in *Casuarina*. Besides trait specific markers, sex specific and species specific markers were developed to add the genomic resources of *Casuarina*.

Methods

Trait specific markers

DNA was isolated from different species of *Casuarina* sps. Each species panel was screened with 35 randomly amplified polymorphic markers (RAPD). Specific PCR fragments were eluted and ligated to pJET1.2 vector. Ligated mixtures were transformed into *E.coli* (DH 5α) using TransformAid bacterial transformation kit (Fermentas). Products were sequenced, trimmed vector sequence and primers were designed to obtain SCAR markers.

EST - SSRs

Expression sequence reads used in this study was developed from high throughput transcriptome sequencing of drought tolerant and susceptible genotype of *Ce* and *Cc* (Unpublished). Unigene sequences were derived using CD-HIT (http://weizhong-lab.ucsd.edu/cd-hit). Microsatellites have been mined using MISA tool (http://pgrc.ipk-gatersleben.de/misa/) and primers were designed using the Primer3 software. A total of 18 microsatellite markers were synthesized and used for genetic diversity and population structure analysis (1) in a set of 349 individuals belongs to *Cj, Cc* and *Cg* accessions.

Transcriptome

Contrasting genotypes of *Ce* seedling were selected based on drought and coppicing ability. Total RNA was extracted from tissues from contrasting plants using Trizol (Invitrogen) and the quality of RNA checked by Bioanalyzer. Transcriptome libraries (single library with average insert size of 250bp) were constructed according to the Illumina protocol by barcoding tissues separately. Next

generation sequencing (NGS) was carried out using Illumina Genome Analyzer II with the read length of 72 bp. De-novo assembly and read alignment were carried out using Bowtie V0.12.7 and SAM 0.1.7. Expression value was calculated using custom Perl code, based on which fold change. Transcripts with a fold change of < -1 were considered as down-regulated, and > +2 as up-regulated. Genes were characterized into biological processes, cellular process and molecular function based on gene ontology.

Result and discussion

Designed primer (IMC3015) was screened in population containing 20 female and 8 male individuals collected from Ongole, India (15° 30' 0" N, 80° 3' 0" E). Female individuals showed 1100bp amplification and male individuals showed 900 bp amplification. This marker (IMC3015) differentiates both male and female individuals. Similarly, the SCAR markers, developed in four species of Casuarina (IMC3050 for *Ce*, IMC3053 for *Cg*, IMC3064 for *Cc* and IMC 3067 for *Cj*) were confirmed by screening one bulk and one individual from each species. These markers are currently being used in culling out admixtures and selection hybrids in open seed orchards.

A total of 7454 primers were designed from 11502 identified SSR of which 18 primers were used in the current study. The population structure analysis showing only peak of ΔK, for $K = 3$, suggested the presence of three main populations (Figure 2). The genetic differentiation (Fst) values were derived using Arlequin software [2] showed there is no allele sharing between the populations and Fst values ranged from 0.28 to 0.32 (Figure 2), demonstrating all the three populations are genetically differentiated [4], however they are inter crossable.

Drought transcriptomics study of stress and control condition revealed that, 247 genes were up-regulated and 379 genes were down-regulated out of 626 differentially regulated genes. A total of 263 genes were up regulated in roots which are slightly different because of more activity of genes related to lipid, amino acid and carbohydrate metabolism, supporting important membrane and osmotic adjustment mechanisms in this tissue, which are essential for plants to survive under drought stress. In case of coppicing ability, totally 186 genes are up regulated of which, several of up regulated genes in this category belongs to cell wall associated proteins, heat shock proteins, auxiliary bud initiation pathway, auxin response pathway. Down regulated genes mainly belongs to strogolactone biosynthetic pathway, as this is directly involved in bud growth through MAX2, RMS4 and D3 proteins.

Conclusion

In the present study, we report comprehensive genomic resource essential for crop improvement of tropical tree for which almost no prior genetic information existed. Several novel genes identified for drought and coppicing ability and validated, which can be deployed through association studies to hardwire commercial traits into desired genotypes. The result of this study will facilitate the development of comparative genetic maps, study of genetic diversity, gene mapping, marker-aided selection and positional cloning of useful genes in Casuarina.

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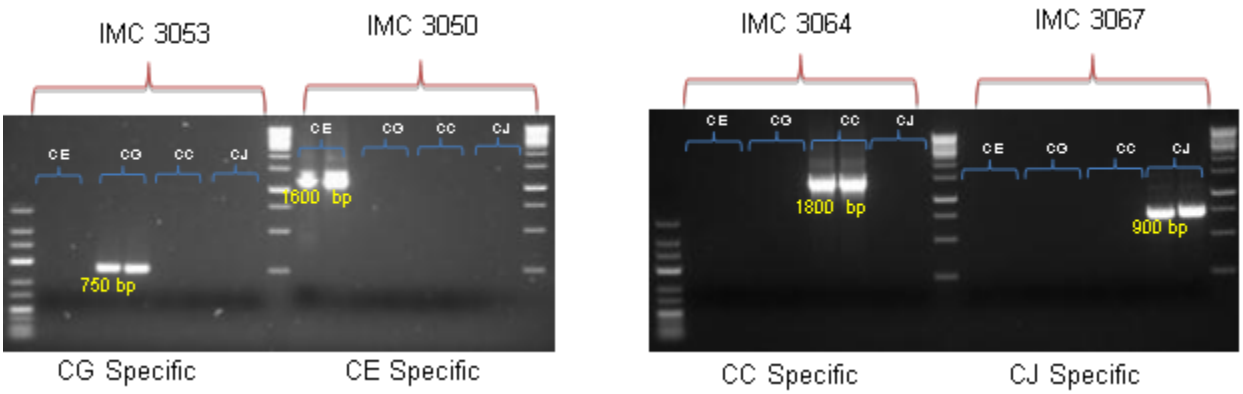

Figure 1. shows the electrophorogram showing confirmed SCAR products in four *Casuarina* species

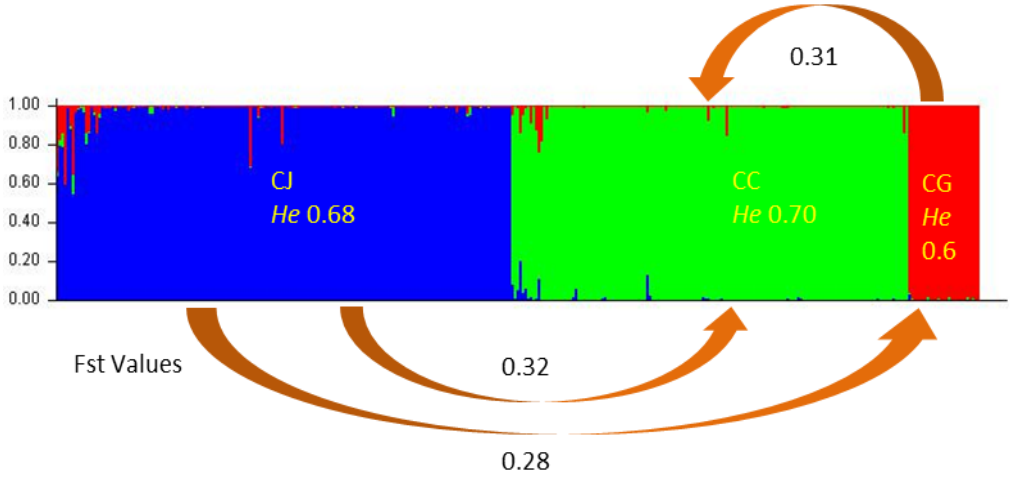

Figure 2. Three subgroups inferred from structure analysis. The vertical coordinate of each subgroup indicates the membership coefficients for each individual. The colored spots represent the three species.

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Identification, characterization and gene expression analysis of three *CBF* transcription factors during cold acclimation of *Eucalyptus globulus*.

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Key words: cold acclimation, c-repeat binding factor, real time PCR.

Background

Low temperature is an environmental factor that limits plant growth, affecting their development and productivity. In Chile, *Eucalyptus globulus* is the most important hardwood species for the forest industry and it is mainly used for pulp production. However, this species is highly sensitive to freezing temperatures and its establishment is difficult on areas with temperatures below -5 °C. Cold tolerance studies in non-hardened *E. globulus* plants have determined a LT_{50} between -5,3ºC and -5,6ºC [1]. Cold resistance is one of the main mechanisms studied in plants. In some species, the previous exposure to low non-freezing temperatures can increase their resistance to freezing temperatures, a phenomenon known as cold acclimation [2]. During this state of acclimation, many genes are induced, producing biochemical changes traduced on a greater cold tolerance [2]. Among these genes, the *CBF* transcription factors family have shown a quick response to cold, because they are induced within 15 minutes of the exposure of plants to cold temperatures [3]. In relation to species more freeze tolerant as *E. gunnii*, four groups *CBF* genes have been reported, Egu*CBF1a* - *b* - *c* - *d*, all of them with different expression profiles to cold [4]. *CBF* gene search in *E. grandis* genome indicate the presence of 17 homologous sequences, including a pseudogene [5]. In *E. globulus* only one sequence of *CBF*, Eg*CBF1* have been reported [6]. Therefore, in this work the identification of other *CBF* homologous sequences on *E. globulus* and its expression profile related to cold acclimation is reported.

Methods

Two different genotypes of *E. globulus*, one resistant and one sensitive to freezing temperatures, were exposed to cold acclimation with four different treatments [7] (Fig. 1). An expression library was generated based on cDNA samples of both genotypes, exposed to non-acclimated (NA) and acclimated after frost at -2ºC (CABF) treatments. The reads obtained were mapped and identified from the *E. grandis* genome (unpublished data). To isolate *CBF* genes in *E. globulus*, we identified distinctive sequences between *CBF* genes reported in *E. gunnii*, and used these regions to design specific primers to each a - c - d sequences groups (Table 1).

The CTAB method was used to extract RNA from *E. globulus.* High Capacity cDNA Reverse Transcription Kit (Life technologies) was used for cDNA synthesis. PCR amplified fragments corresponding to coding regions were isolated and cloned on pGEM-T vectors (Promega) for subsequent sequencing. BLASTN algorithm was used for sequence identification (http://blast.ncbi.nlm.nih.gov/).

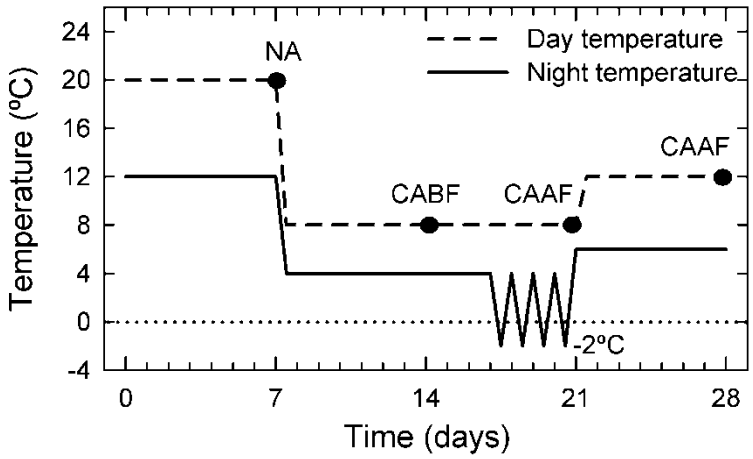

Fig. 1 Cold acclimation treatments: **NA** non-acclimated, **CABF** acclimated before frost at -2ºC, **CAAF** acclimated after frost at -2ºC and **DA** de-acclimated. Extracted from Fernández *et al.* 2010.

Table 1 Sequence of primers used for *CBF* gene sequencing on *E. globulus*

Gene	Forward	Reverse
Egl*CBF1a*	5'-ATGAACCCTTTCTCTTCTCATTCCCAT-3'	5'-TCAGATCGAATAGCTCCATAATGACGT-3'
Egl*CBF1c*	5'-ATGAACCACTTCTTCTCTTCTTACTCAGA-3'	5'-CTACATGGAATAGTTCCATAACGACACG-3'
Egl*CBF1d*	5'-ATGGCAGCCCCCGGGAAC-3'	5'-CTACAGGGAATAGCTCCATAACGACA-3'

For relative expression analysis, 6 months-old plants were used. These plants were exposed to cold acclimation treatments, and leaf material was collected 7, 14, 21 and 28 days after treatment. Primers and probes were designed for each isolated sequence (Table 2). Real time PCR technique used TaqMan® Gene Expression Master Mix Kit (Life technologies).

Table 2 Primers and probe sequences for gene expression analysis

Gene	Forward	Reverse	Probe
Egl*CBF1a*	5'-TCGGAAGAGGGAGTGTTCTACG-3'	5'-TCCAAATTCTCCTCGTCCTCTCC-3'	5'-ATCTCCGCCCCATTTT-3'
Egl*CBF1c*	5'-AGACGTACTCCAATGAACCACTTC-3'	5'-CACCTCCTCATCAGAAAAGTTCCC-3'	5'-ACCATAGCACTCCT-3'
Egl*CBF1d*	5'-GATGGAGTGCGGATGAAAGGC-3'	5'-CATCAGAGGCCCAACATCAGAAG-3'	5'-CGTGCAGTCTCTATT-3'

Results and Conclusions

A total of 15 *CBF* homologous sequences were identified in *E. globulus* from a cold treatment expression library. The genes were classified in four groups: a, b, c and d, based on *CBF* genes previously described in *E. gunnii*. Three *CBF* sequences were isolated, corresponding to one gene of the groups a, c and d. The full length of Egl*CBF1a* was 736 bp, with a coding sequence (CDS) of 660 bp corresponding to 220 amino acids. For Egl*CBF1c*, the fragment amplified was 996 bp, with a CDS of 687 bp corresponding to 229 amino acids, and for Egl*CBF1d*, the sequence was 1,285 bp, with a CDS of 588 bp corresponding to 196 amino acids.

The gene expression analysis in two different *E. globulus* genotypes showed that acclimated plants increased transcript accumulation of Egl*CBF1a*, Egl*CBF1c* and Egl*CBF1d* in leaf tissues, compared to non-acclimated plants. The highest transcript accumulation was observed in Egl*CBF1a* and Egl*CBF1d* genes of the resistant genotype, with average expression levels of 1,311 and 823-fold, respectively. The same values have been reported in *E. gunnii* [4]. In the case of Egl*CBF1c* gene, a minor transcript accumulation (28-fold) was

observed in the acclimated condition of the resistant genotype, versus the non-acclimated plants. Finally it was observed that the expression of Egl*CBF1c* gene is constitutive, unlike Egl*CBF1a* and Egl*CBF1d* genes, which are induced by cold temperatures.

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De novo **assembly and characterization of the leaf transcriptome from young rubber trees (*Hevea brasiliensis*)**

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Keywords: *Hevea brasiliensis*, rubber tree, leaf, transcriptome

Background

Natural rubber exhibits unique properties including flexibility, impermeability to liquids and abrasion resistance. Due to these characteristics, more than 40,000 products have natural rubber in their composition and it has great importance in the tire industry [1].

The rubber tree [*Hevea brasiliensis* (Willd. ex Adr. De Juss.) Muell-Arg.] is the major source of natural rubber in the world due to the high quality and production. *H. brasiliensis* is a diploid (2n=36, n=18) species with a haploid genome size estimated in 2.1 Gb.

The Amazon rainforest is the species center of origin and presents a suitable climate to rubber tree growth due to its warm and humid climate. However, this region also offers optimal growth conditions to the fungus *Microcyclus ulei* (P. Henn) v. Arx, which causes the South American Leaf Blight (SALB) disease. SALB remains a permanent threat to the entire rubber industry [2].

The disease has led to the expansion of rubber tree plantations to suboptimal areas, such as southern plateau of Brazil, where *M. ulei* development is restrained [3]. These areas provide new stressful conditions, such as low temperatures and high altitudes, which affect rubber production. Thus, rubber tree breeding is seeking for new varieties that are resistant to these new stress conditions

Rubber tree breeding is time-consuming and expensive. Generally, large areas are required for the experiments and it can take more than 20 to obtain a new variety. Molecular biology techniques can optimize and reduce the time expended in rubber tree breeding.

RNA sequencing (RNA-seq) has become a widespread technique because it provides the high-resolution characterization of transcriptome in non-model species, such as rubber tree, enabling the detection of thousands of single nucleotide polymorphisms (SNPs). These markers can be useful for functional saturation of genetic maps, leading to the identification of markers that are directly related to economic traits.

The goal of this study is to investigate the leaf transcriptome of two young *H. brasiliensis* clones, which are recommended for plantation in several countries and develop functional molecular markers.

Material and Methods

Leaf samples from young GT1 and RRIM600 clones (6 months) were collected at the Agência Paulista de Tecnologia dos Agronegócios/SAA, Votuporanga, São Paulo, Brazil. The samples were frozen on dry ice and stored at -80ºC. The total RNA extraction was conducted according to Oñate-Sánchez & Vicente-Carbajosa (2008) [4] protocol with modifications. RNA integrity was checked using denaturing 1% agarose gel.

The paired-end library was prepared following the protocol of the Paired-End Sample Preparation Kit (Illumina Inc., San Diego) using 3ug of total RNA. The samples were sequenced at GAIIx Illumina platform with 72 bp reads length.

The raw reads were filtered using NGS QC Toolkit 2.3 [5] with default parameters. The *de novo* assembly was performed with CLC Genomics Workbench package (v4.9, CLCBio, Cambridge, MA) with the following parameters: maximum gap and mismatch count were set to 2, the insertion and deletion costs were set to 3, the minimum contig length was set to 300 bp, the length fraction and similarity parameters were set to 0.5 and 0.9, respectively and the word size (k-mer) was set to 29.

The contigs were searched against UniProtKB/Swiss-Prot protein database using BLASTx with a cut-off e-value of 1e-10.

Results and Conclusions

In total, it was generated 51,736,736 raw reads for GT1 and 47,077,990 for RRIM600 clones. To exclude low quality sequences, the reads were trimmed using NGS QC Toolkit [5]. After data filtering, 44,040,812 (85.12%) and 37,820,708 (80.33%) high quality reads were obtained for GT1 and RRIM600 genotypes, respectively.

These high quality sequences were used for the *de novo* assembly using CLC Genomics Workbench. To perform the *de novo* assembly, the data obtained from

the two genotypes was used. In total, 81,861,520 reads assembled, generating 34,718 contigs.

The contigs length varied from 176 to 20,555 bp with an average length of 999 bp. The N50 was 1,350 bp and the GC content was 42.10%.

From the 34,378 contigs, 3,119 contigs presented high similarity to non-plant sequences, suggesting that these sequences are contaminant sequences from other organisms, such as fungi and bacteria and were removed from further analyses. A total of 31,259 contigs were obtained, which 20,432 (65.36%) had lengths between 176 bp to 1 kb and 8,469 (27.09%) were between 1 to 2 kb and 2,698 (8.63%) were longer than 2 kb.

The 31,259 contigs were annotated against UniProtKB/Swiss-Prot database. A total of 18,775 contigs presented similarity to plants proteins at the UniProtKB/Swiss-Prot database.

The proportion of sequences with significant BLASTx hits was higher for longer contigs. The BLASTx yielded significant hits for 9,972 (48.80%) contigs that were 176 bp to 1 kb in length, while the contigs with lengths between 1 to 2 kb and longer than 2 kb presented 6,588 (77.78%) and 2,215 (82.09%) annotated contigs, respectively.

The contigs obtained in the *de novo* assembly will be annotated in different databases such as NCBI non-rendudant (nr), Gene Ontology (GO) and Kyoto Encyclopedia Genes and Genomes (KEGG). Putative SNPs will be searched and validated for each genotype, aiming the development of functional markers for rubber tree.

Competing interests

The author declares that they have no competing interests.

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Root tip response to drought is altered in SUT4-RNAi poplar plants

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Background

In leaves of many herbaceous species, sucrose passively accumulates in the vacuole during the day while being simultaneously and actively effluxed into the cytosol until its depletion at night (1). In Populus leaves, sucrose levels are sustained at high levels throughout the diel, and only starch is severely depleted at night (2). The need for high leaf sucrose in order to sustain export to sink organs in Populus means that sucrose compartmentalization and metabolism must be strictly coordinated.

The control of sucrose homeostasis in any metabolically dynamic and sucrose-rich tissue potentially bears osmotic or hydrodynamic consequences. Sugar concentrations were significantly perturbed not only in leaves, but also in bark and xylem of PtaSUT4-RNAi poplars (3). That finding raises the possibility that altered tonoplast sucrose trafficking can impact osmotic and metabolic status along the leaf to root continuum. Water uptake was reduced in SUT4-RNAi poplar during soil drying, while water uptake as proxied by plant transpiration was normal in well-watered or drought-adapted SUT4-RNAi plants (4). A plant-wide transcriptomic analysis coupled with sucrose and hexose profiling was therefore carried out to investigate the mechanisms by which sucrose subcellular compartmentalization might condition water utilization in Populus during soil-drying and subsequent recovery.

Methods

PtaSUT4-RNAi transgenic lines of Populus tremula x alba (clone 717-1B4) were described previously (3,4). Two genotypes, wild type (WT) and transgenic line 'G' were used for comprehensive transcriptomic characterization. Water was withheld from drought-treated plants until turgor loss became apparent in sink-source transitional leaves (plastochron index LPI-3 to LPI-5) (~3 days). Source leaves (LPI-15), developing xylem, phloem with bark, and root tips were harvested from well-watered, wilting and recovered plants.

Sucrose, glucose and fructose levels were estimated by GCMS. RNA was isolated from frozen tissue aliquots, and RNA-Seq library preparation with HiSeq-2000 (Illumina, San Diego, CA) sequencing were performed at BGI America (Cambridge, MA). Self-organizing-map (SOM) clustering analysis was performed to visualize treatment and

genotypes effects on the expression of DE genes. Functional annotation of signaling and metabolic pathways was obtained from KEGG (http://www.genome.jp/kegg/pathway.html) and PoplarCyc v3.0 (http://plantcyc.org). Gene ontology (GO) enrichment analysis was performed using the R package topGO. The WGCNA (v1.29) package in R was used to construct a co-expression network for all root DE genes, and then for two sub-networks (WT and SUT4-RNAi) using an established pipeline (5).

Results and conclusion:

Underpinning the differing transcriptional responses of the two genotypes was an abnormally high shoot-sucrose to root-sucrose concentration ratio in RNAi plants. Constitutively increased transcript levels of ABA biosynthetic genes and bark storage proteins suggested the existence of latent stress in root tips of well-watered RNAi plants. During drought and recovery, aquaporins responsible for cross-membrane water trafficking exhibited larger transcript oscillations in xylem and more sustained transcript reductions in root tips of SUT4-RNAi than WT plants. Transcriptome network analysis revealed enhanced sucrose-independent as well as altered ABA-sensing/signaling and stronger ethylene and jasmonate sensing/signaling activities in SUT4-RNAi root tips, but stronger fatty acid and suberin biosynthesis in WT root tips during soil drying. Larger transcript level increases of stress-inducible genes such as late-embryogenesis-abundant proteins in RNAi roots were consistent with increased shock and altered functioning during soil drying. The data integrates sucrose transport, compartmentalization and stress signaling with the idea that water uptake in Populus can be conditioned by a combination of SUT4 function, xylem sugar status and the efficiency of sucrose delivery to root tips during and shortly after a drought stress.

The alterations in AQP, LEA, VSP/BSP, HSP expression in transgenic root tips are more consistent with increased stress, or stress perception, and not decreased stress in roots as might be expected if the decrease in water uptake capacity originated in transgenic leaves. Sucrose compartmentalization may be linked with stress-sensing pathways for optimizing water uptake and distribution throughout the plant under conditions of declining water availability. Altered compartmentalization may lead to increased root injury during a decline in soil water if water uptake by roots and water loss through leaf stomata become uncoupled.

Competing interests:

The authors declare that they have no competing interests

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The role of cork oak Myb1 transcription factor in phellogen development – an approach for the in vivo identification of the involved regulatory networks.

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Quercus suber, cork, transcription factor, ChIP-SEQ

Background

Cork oak (*Quercus suber* L.) is a well-adapted Mediterranean evergreen tree species. It is spread over the western and coastal region of the Mediterranean area, under an Atlantic influence, occupying its largest area in Portugal and Spain [1]. The economic importance of *Quercus suber* (Qs) is due to its thick and characteristic bark – the cork, a noble raw material. However, the knowledge of the processes underlying cork formation and development, although fundamental, is still scarce.

Cork or phellem is formed by the meristematic activity of phellogen and is composed of cells enriched in suberin and lignin. The reported studies that attempted to explain cork formation and differentiation have focused on the biosynthesis of these two polymers.

Transcription factors (TFs) regulate gene expression of many crucial biological processes. These are proteins that recognize DNA in a sequence-specific manner and

regulate the frequency of initiation of transcription upon binding to specific sites in the promoter of target genes.

Among TFs are the MYB proteins mainly represented in plants by the R2R3-MYBs that largely regulate plant-specific processes. TFs act in networks in which a protein may regulate the expression of another to control directly or indirectly the expression of a particular gene, in a temporal and spatial fashion way. Modifications in gene expression patterns required for coordination of various biological processes such as the normal development or environmental induced responses are in part a consequence of the changes in DNA binding status of various TFs. Therefore, the accurate identification of the interacting elements for TFs *in vivo* is crucial for elucidating gene regulatory networks.

This work is an attempt to identify the molecular mechanisms involved in cork formation and differentiation focusing on QsMYB1, a transcription factor up-regulated in cork forming tissues [2].

Methods

Qs somatic embryos were induced from eleven adult trees by two distinct hormone mediated protocols [3], [4]. Generated cell lines were characterized for kanamycin resistance as a selective agent for transformants. Genetic modified (GM) cell lines overexpressing fusion QsMYB1::3xFLAG® were generated by *Agrobacterium tumefaciens* co-cultivation [5]. In parallel, QsMYB1::3xFLAG® protein functionality was evaluated in a *Saccharomyces cerevisiae* (Sc) mutant for QsMYB1 ortholog, bas1. GM embryo clusters were selected in selective medium and overexpression was confirmed by Real Time – PCR and Western Blot. Transformed somatic embryos were subjected to chromatin immunoprecipitation (ChIP) in order to perform a ChIP sequencing (ChIP-SEQ).

Results and Conclusions

The best treatment to induce somatic embryogenesis from adult cork oak trees was studied in eleven different genotype cell lines. Results indicate a genotype-dependent response for each hormonal treatment. There is an interaction between genotype and treatment showing that these factors do not completely explain the source of variation. Each genotype may be analysed independently to choose the best somatic embryogenesis induction treatment.

To determine the effect of kanamycin on somatic embryogenic lines proliferation, three genotype embryogenic cell lines, were cultured on proliferation medium supplemented

with different concentrations of kanamycin. Kanamycin inhibits the proliferation of somatic embryos and may be used as a selective agent of transformants in a concentration ranging between 37.5 $mg.L^{-1}$ and 100 $mg.L^{-1}$, depending on the cell line.

GM lines were generated by embryo masses co-cultivation with *Agrobacterium tumefaciens,* carrying proper plasmids. After six months of embryogenic masses selection, clusters were subjected to Real Time – PCR and Western Blot analysis. Results indicate overexpressing of QsMYB1 transcripts and presence of the QsMYB1::3xFLAG® protein.

In parallel, a yeast functional complementation assay was performed in the Sc strain Y06015, a bas1 mutant. QsMYB1::3xFLAG® encoding sequence was inserted into the pVV208, pVV209, pVV214 and pVV215 Gateway™ yeast expression vectors. bas1 mutant cells were transformed with these expression vectors and functional complementation was checked visualizing cells growth in an adenine bradytrophic medium. QsMYB1::3xFLAG® proteins showed to complement bas1 transformed mutant cells since cell growth in adenine bradytrophic medium was observed, meaning that QsMYB1::3xFLAG® proteins complement the lack of BAS1.

Chromatin extraction and shearing optimization was performed from embryos masses overexpressing QsMYB1::3xFLAG®. Protein/DNA fragments were imunoprecipitated and enriched population of DNA fragments were the input for indexed sequencing libraries. Sequencing is being conducted on the Illumina HiSeq Platform.

This work is an approach to identify the DNA targets of MYB1, a TF related with cork formation. To achieve the objective, stable Qs cell lines were successful established, characterized and genetically modified to overexpress a QsMYB1::3xFLAG® fusion protein. In yeast, QsMYB1::3xFLAG® protein revealed to be functional. The 3xFLAG® epitope allowed us to track the protein and localize its DNA targets by ChIP-Seq. This work is a successful attempt to develop a system that may be used to study *in vivo* Qs regulatory mechanisms at a cellular and molecular level.

Competing interests

The authors declare that they have no competing interests.

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Development and use of embryo culture for micropropagation of threatened woody species

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Key words: *Amygdalus georgica, Castanea sativa, embryo culture, micropropagation.*

Background: The *in vitro* technology is considered as one of the most efficient methods of *ex situ* conservation. The *in vitro* plant reproduction has a great application potential in conservation of rare and endangered species with the purpose of maintaining the genetic resource diversity and for accelerated growth of biomass. Clonal propagation via somatic embryogenesis (SE) and auxiliary shoot development is effectively used for micropropagation of several genera, but its efficiency still needs further improvement. Establishment of efficient clonal mass propagation methods through zygotic embryogenesis of forest tree species will likely contribute to better approaching over vegetative multiplication and somatic embryo development *in vitro*.
The European chestnut tree *Castanea sativa* Mill. is one of the dominant tree species in the western Georgia. It occupies approx. 75% of area covered with forests. The distribution area and the number of chestnut individuals are significantly being reduced due to the low self-renewal and diseases. *Castanea sativa* Mill. is included in the Red List of Georgia (2006) with an IUCN Red List regional assessment of Vulnerable [1,2].
The almond (*Amygdalus communis* L.) is an oldest commercial nut crops native in the frontal and central Asia. Out of the 35-40 species of genus *Amygdalus* widely diffused in the world, Georgian almond (*Amygdalus georgica* Desf.) represent endemic species of eastern Georgia. The main reason for the population reduction is uncontrolled, uncultivated soil brake. In the Red List of Georgia *Amygdalus georgica* is assessed as Endangered [2]. The conservation of the threatened hardwood species would have great value to Georgian forest ecosystems for maintaining genetic resource diversity. At this point, the goal is to further optimize feasible *in vitro* protocols for micropropagation of rare and endangered woody plants for offsetting the pressure on the natural populations.
Methods: Zygotic embryo axes were excised from mature chestnut seeds and cultured at standard culture conditions on a defined medium supplemented with different concentrations of (1, 2.5, 4.4 µM) 6-Benzylaminopurine (BAP) and kinetin. The optimal concentrations were determined for development of axillary shoots from both embryonic axes and subcultured shoots. After multiplication shoots were maintained successively for 6-8 weeks by sequential subcultures in proliferation and elongation media with significant acceleration in the proliferative rate. Rooting and acclimatization of elongated shoots was developed with indole-3-butyric acid (IBA) or 1-Naphthaleneacetic acid (NAA) at concentrations of either 1 µM or 15 µM to induce root primordial. Approximately 2 cm long shoots were cultured in rooting medium. The rooted shoots were relocated for adaptation in controlled chambers over the following three weeks and maintained to 60±5% moisture content and 23±1°C. Micropropagated plantlets were then transplanted to plastic pots filled with mixture substrate.
Results and conclusions: Development of axillary shoots was induced when isolated embryonic axes were cultured on a defined medium containing 6-Benzylaminopurine (BAP) and kinetin at different concentrations. The optimal concentrations of cytokinins were determined for development of shoots from both embryonic axes and subcultured shoots.

The size and the number of shoots produced were evaluated. The low concentrations of BAP (1 and 2.5 µM) used on the zygotic embryos helped to create several lateral shoots in auxin-free medium. Kinetin was not as effective as the BAP treatments however, it promoted of rapid elongation of apical shoot. By the same way, the best results were obtained when use BAP for multiple shoot initiation [3,4]. For *in vitro* rooting of shoots different concentrations of indole-3-butyric acid (IBA) and 1-Naphthaleneacetic acid (NAA) were used. IBA at 15 µM was successfully used to induce root primordial and identified as the most suitable auxin for rooting seedlings. Our result is in agreement with observations that IBA and NAA are the most effective for rooting of different almond species *in vitro* [5]. At the end of 7 weeks of culture, 70% of all explants have developed long primary and secondary roots.

Embryo culture is valuable *in vitro* tool for breeding. The technique has been developed to reduce the long dormancy period of seeds and to shorten the breeding cycle of plants by growing excised embryos *in vitro* [6,7]. In this regard propagation of threatened woody plants using embryonic tissues as healthy rootstocks is beneficial for both, rapid mass production and the maintenance of genetic resource diversity that allow conservation of selected valuable lines.

Competing interests

The author declares that they have no competing interests.

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Somatic embryo maturation using a high concentration of gellan gum promotes germination of somatic embryos of *Pinus armandii* Franch. Var. *amamiana* (Koidz.) Hatusima, an endemic and endangered species in Japan

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Key words: Gellan gum, Pinus armandii, Somatic embryogenesis, Yakutanegoyou

Background

Yakutanegoyou [*Pinus armandii* Franch. var. *amamiana* (Koidz.) Hatusima] is an endangered five-needle pine species endemic to the islands of Yakushima and Tanegashima in the southwestern region of Japan (Environment Agency of Japan 2000). Yakutanegoyou populations have recently markedly decreased due to pine wilt disease, which is caused by the pinewood nematode *Bursaphelenchus xylophilus* (Akiba and Nakamura 2005). Therefore, the development of an efficient and stable plant regeneration system is essential for the large-scale propagation of resistant clones. Somatic embryogenesis is the most attractive technique for mass propagation of selected trees, for *ex situ* conservation of genetic resources by cryopreservation, and for plant regeneration in genetic transformation.

Methods

Somatic embryogenesis was initiated from megagametophytes containing immature zygotic embryos. Embryogenic cultures were maintained and proliferated on 1/2 EM medium (Maruyama et al. 2000) supplemented with 3 µM 2,4-dichlorophenoxyacetic acid, 1 µM 6-benzylaminopurine, 30 g l^{-1} sucrose, and 1.5 g l^{-1} L-glutamine. The pH of the medium was adjusted to 5.8 before sterilization.

Somatic embryo maturation experiments were performed in darkness at 25°C. Embryogenic tissues were cultured on EM medium containing 50 g l^{-1} maltose, 2 g l^{-1} activated charcoal, 100 µM abscisic acid, and 100 g l^{-1} polyethylene glycol (PEG; Average Molecular Weight: 3000; Wako Pure Chemical, Osaka, Japan) or 10 g l^{-1} gellan gum (Gelrite®; Wako Pure Chemical, Osaka, Japan) without PEG.

After maturation, somatic embryos were transferred to a germination medium containing basal salts at concentrations similar to those used for maintenance and proliferation, but without plant growth regulators, and supplemented with 30 g l^{-1} glucose and 2 g l^{-1} activated charcoal. Cultures were kept at 25°C under a photon flux density of approximately 65 µmol m^{-2} s^{-1} provided by white fluorescent lamps (100 V, 40 W; Toshiba, Tokyo, Japan) for 16 h daily. The numbers of somatic embryos that germinated and developed into plantlets were recorded after 6 and 12 weeks, respectively.

Results and Conclusions

Table 1 shows that when somatic embryos matured with PEG were placed directly on the germination medium, the root emergence of embryos and subsequent plantlet conversion occurred at low frequencies (an average of 41% and 33%, respectively). In contrast, somatic embryos matured on the medium containing a high concentration of gellan gum (10 g l^{-1} without polyethylene glycol) germinated at an average frequency of 81%, and 80% of total somatic embryos tested developed into plantlets. In addition, somatic embryos matured on the medium containing a high concentration of gellan gum germinated faster

and were more synchronized than those matured on the medium supplemented with PEG (data not shown). We observed the start of germination (root emergence) 1–2 weeks after transfer into the germination medium, and embryos were subsequently converted into plantlets (emergence of both root and epicotyl) after 4–8 weeks of culture.

Table 1. Germination and plant conversion from somatic embryos of yakutanegoyou (*Pinus armandii* var. *amamiana*) after maturation with polyethylene glycol (PEG) *1 or a high concentration of gellan gum*2

	Germination frequency (%)		Conversion frequency (%)	
Cell line	PEG	Gellan gum	PEG	Gellan gum
HR-17A	46 (92 / 200)	83 (374 / 450)	31 (62 / 200)	82 (369 / 450)
HR-10A	40 (79 / 200)	91 (186 / 205)	35 (70 / 200)	90 (185 / 205)
HR-10B	32 (16 / 50)	77 (127 / 164)	28 (14 / 50)	75 (123 / 164)
HR-10C	36 (18 / 50)	82 (167 / 203)	34 (17 / 50)	82 (166 / 203)
I01-1	51 (38 / 75)	97 (239 / 247)	40 (30 / 75)	95 (235 / 247)
I25-5	16 (4 / 25)	51 (101 / 200)	12 (3 / 25)	50 (99 / 200)
Total	41 (247 / 600)	81 (1,194 / 1,469)	33 (196 / 600)	80 (1,177 / 1,469)

Values in parentheses represent (germinated or converted somatic embryos / total somatic embryos tested).
*1 Somatic embryos generated on maturation medium with 100 g l^{-1} PEG (Av. Mol. Wt.: 3000).
*2 Somatic embryos generated on maturation medium with no PEG and 10 g l^{-1} gellan gum.

Several improved pine embryo maturation protocols that involve reducing the availability of water to cultured cells by increasing the medium gel strength (with a high concentration of gellan gum) to produce mature somatic embryos with low water content have been reported (Klimaszewska et al. 2007).

In conclusion, when somatic embryos were matured on the medium containing a high concentration of gellan gum, the germination frequency and subsequent plant conversion frequency of yakutanegoyou somatic embryos were improved by about twofold (41% to 81%) and more than twofold (33% to 80%), respectively, compared with those in somatic embryos matured on the medium supplemented with PEG. This improvement represents a promising perspective for an efficient mass propagation of this species.

Competing interests
The author declares that they have no competing interests.

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In vitro induction of tracheary elements in calli of *Eucalyptus* to study genes related to xylogenesis

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Key words: Cell differentiation, Lignification, Xylogenesis, Secondary cell wall

Background

Eucalyptus (*Eucalyptus* spp.) is the most commercially important tree in Brazil, with a remarkable role in the pulp and paper industry. The increase in pulp yield and the reduction in production costs are the main objectives of eucalyptus breeding programs. The delignification process is critical for the extraction of cellulose, and therefore, the study of the lignin biosynthetic pathway is important to understand the genetic and biochemical aspects involved in wood formation (xylogenesis). The xylem tissue of vascular plants contains two main cell types: tracheids and vessel elements, collectively called tracheary elements (TEs). The differentiation of TE is characterized by the formation of a visible secondary cell wall and autolysis, this process is considered as a plant cytodifferentiation model system. The process of TE differentiation including secondary cell wall formation, lignification and programmed cell death, will be better understood by employing *in vitro* cell culture approaches [1]. As the differentiation of tracheary elements is characterized by the deposition of lignin in the final stages, gene expression studies involving cell culture, have contributed enough to provide a range of candidate genes with important functions in the differentiation of xylem cells and towards the biosynthesis of lignin in plant cells.

Methods

E. grandis seeds were germinated *in vitro* in glass vials containing SP culture medium [2] at 26 °C in the presence of light. After 45 days we observed the development of seedlings about 5 cm, the leaves were removed and segments about 1 cm^2 were used as explants. These segments were maintained in SP culture medium containing 6 µM of Picloram (4-Amino-3,5,5-trichloropicolinic) for induction of calli, at 26 °C in absence of light. After 30 days, the calli were transferred to culture media containing 3 µM of Picloram and 1 µM BAP (6-Benzylaminopurine) and maintained for 30 days at 26 °C. The friable calli were used in cell suspensions. For this, about 3 calli (0.8 to 1.0g) were transferred to 100 ml Erlenmeyer flask containing 50 ml of SP liquid medium. The calli were kept overnight in the respective cell suspensions at 26 °C, under agitation (100 rpm). Tracheary elements were separated using sieves of 500 microns, 100 microns and 50 microns. The observation of the cells was performed using a 100x optical microscope Olympus BX53 allowing the visualization with white light and fluorescence.

Total RNA was extracted from cells isolated at 3 different stages of induction (meristematic cells, elongated cells and tracheary elements) according to the optical microscope observation. The extraction of total RNA was performed using the RNeasy Plant

Mini Kit (QIAGEN), according to manufacturer standards, adapted to optimize the process. The protein extraction was performed according to the phenol extraction method [3].

Results and Conclusions

It was possible to obtain a high percentage of friable calli of *E. grandis* and dense cell suspensions, with no contamination. The occurrence of tracheary elements formed in calli was initially detected under agitation in liquid medium. This event was also observed by Yamagishi et al. [4], which may be explained by hormonal action and cell-cell interaction, both essential for *in vitro* formation of tracheary elements. In this case, the tracheary element was presented in clusters and it was possible to visualize lignin deposition in the inner wall forming a darker secondary beams inside the cell. Tracheary elements observed in our work were significantly larger than spherical meristematic cells, with about 400 nm in length. The cell staining with safranin allowed a clearly visualization of the well-defined secondary wall inside the cell, characterizing the process of thickening and lignification. These tracheary elements obtained in the calli and cell suspension showed a very similar patterns to those described in the literature, particularly the occurrence of a well-defined secondary wall deposition. This secondary wall thickening characterized by different patterns of lignin deposition was confirmed by observing the fluorescence microscopy, wherein the lignin autofluorescence was displayed accurately in the tracheary elements, allowing detailed observation of the cell internal structure.

The RNA extraction was performed using meristematic cells, elongation cells and mature tracheary elements before the autolysis process. Our results showed that we were able to succesfully extract RNA from these cells. The extraction efficiency as around 0.38 µg/µL of RNA in samples of meristematic and elongated cells, and in the case of tracheary elements, concentrations were somewhat lower (0.2 µg/µL RNA), due to the fact that these cells are in programmed cell death. Likewise, we were able to extract proteins from these three cell fractions, with varying concentrations of 2.16 µg/µL for meristematic cells and 1.53 µg/µL for tracheary elements.

To our knowledge to date there is no report in literature about *in vitro* occurence of Eucalyptus tracheary elements. Thus the results obtained so far are interesting and promising for the understanding of gene expression involved with the complex mechanisms of lignin formation during xylem differentiation.

Competing interests

We declare no competing interests.

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Cryopreservation of *Araucaria angustifolia* embryogenic cultures: cryotreatment optimization and cell viability evaluation

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Key words: Brazilian Pine, In vitro conservation, improvement, somatic embryogenesis

Background: *Araucaria angustifolia* (Bert.) O. Kuntze is a dioecious conifer native form in south and southeastern Brazil. Drastic population decline and habitat reduction culminated in inclusion of *A. angustifolia* on the IUCN international list as critically endangered. In this context, *A. angustifolia* is a species with great potential for studies to support genetic conservation, and the application of tissue culture tools in this species, such as somatic embryogenesis (SE), which is one of the most promising techniques for its conservation and mass propagation.. In addition, SE offers several advantages when compared to other micropropagation systems, including their high multiplication rate, the potential for embryogenic cultures (EC) scale-up by suspension cultures, the use of bioreactors and the application of cryopreservation techniques [2].

Cryopreservation is defined as the conservation of biological material at ultra-low temperatures, usually at -196ºC, the temperature of liquid nitrogen (LN). Cryopreservation of EC can avoid the detrimental effects of long-term subculture such as contamination, somaclonal variation or loss of embryogenic competence. In addition, the integrated use of SE and cryopreservation will enable the storage and efficient recovery of clones obtained from young reactive tissues and elite genotypes [3]. In this sense, the objective of this study was to evaluate the best cryotreatment time for EC cryopreservation of *A. angustifolia* by means of slow cooling protocol. The effects of the cryotreatment for 60 min were also evaluated by determining post-thaw viability based upon fluorescein diacetate (FDA) staining. Recently, a protocol for cryopreservation was reported [4], however its efficiency is quite restrict.

Methods: EC were induced according to the methodology described by dos Santos et al. [5]. Immature female cones, with the early globular-staged zygotic embryos, were collected in December 2013, from an *A. angustifolia* natural population in Lages, Santa Catarina –

Brazil (latitude 27° 49′ 0″, longitude 50° 19′ 35″, altitude 930 m). After 30 days of induction, the EC were subcultured in Petri dishes containing 25 ml of proliferation culture medium with the same composition as the induction culture medium, except that PGRs concentrations (2 µM 2,4-D, 0.5 µM BAP and 0.5 µM KIN). Subcultures were performed every 21 days for 4 cycles in gelled culture medium for the EC scale-up. After that, EC were transferred for proliferation in liquid culture medium with the same composition as described above without gelling agent. The cell suspension was established in 250 ml Erlenmeyer flasks containing 50 ml of liquid culture medium, kept in dark conditions with permanent agitation (90 rpm) in orbital shaker with temperature of 22ºC (±2ºC).

To optimize cryopreservation protocol, different incubation times in cryoprotectant solution were tested. The cultures were pre-incubated in an orbital shaker at 90 rpm for 2 days in the pretreatment solution consisting of proliferation culture medium plus mannitol (180 g l^{-1}). After this period, the pretreatment solution was replaced by cryoprotectant solution [6] and different incubation times were tested (0, 30, 60, 120 and 240 min). The cryopreserved EC were transferred to petri dishes containing 2 filter paper disks on proliferation medium gelled with agarose (7.5 g l^{-1}) and incubated in the dark at 22 ± 2°C for 30 days. The cultures were weighed at 2, 9, 16, 23 and 30 days in culture and fresh weight gain calculation was performed for different incubation treatments.

After the establishment of the most suitable cryoprotection time, the EC were submitted to the complete cryopreservation protocol based on that described by Mustafa et al. [6]. The procedures were the same described above, using the EC incubation time for 60 min in cryoprotectant solution at 90 rpm at 4°C. Cell viability was assessed by FDA staining at each point of the protocol (EC not cryotreated; EC submitted only to the cryotreatment; EC after cryotreatment and slow cooling in Mr. Frosty not immersed in N_2; EC cryotreated and subjected to cooling in N_2).

Results: In the results of cryoprotection times it was observed that, after 9 days in culture, EC not subject to cryotreatment had the highest fresh mass increasing ratio (1.94 mg mg^{-1} FM), followed by the EC submitted to 30 and 60 min of cryotreatment (1.56 and 1.41 mg mg^{-1} FM, respectively), and finally the EC submitted to the higher cryoprotection times (120 and 240 min). The fresh mass increasing ratio found in other weighing dates (16 and 23 days) were not significantly different among the treatments. The results of the FDA analysis indicated the maintenance of cell viability of certain cell clusters in all points evaluated, especially in regions with greater abundance of embryogenic cells, which may be an

indication that these cells may be more suitable to survival after cryopreservation compared to suspensor cells.

Conclusions: These results indicate that cryotreatment affects the initial increasing ratio, performing a retarding process for the beginning of the cell multiplication exponential phase. However, after a given time in culture, the increasing ratio are equal, indicating that the cryoprotection process, for the incubation times tested, does not affect the proliferation and viability of *A. angustifolia* EC. In addition, the results indicate that the cryopreservation protocol proposed for EC of *A. angustifolia* is efficient and allows its long-term conservation, opening new perspectives for the species conservation programs and basic studies of physiology, biochemistry and genetics.

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Fractioning and cell tracking of *Araucaria angustifolia* embryogenic cultures

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Background: Somatic embryogenesis (SE) is an ontogenetic process by which mitotically quiescent somatic cells can recover their embryogenic potential and produce new viable embryos, by reprogramming of gene expression [1]. An expression of totipotency, SE involves dedifferentiation of a nonzygotic cell and subsequent redifferentiation (reprogramming) [2]. *Araucaria angustifolia* is a dioecious perennial conifer native exclusively to South America in south and southeastern Brazil. Drastic population decline and habitat reduction culminated in inclusion of *A. angustifolia* on the IUCN international list as critically endangered. In this context, *A. angustifolia* is a species with great potential for studies to support genetic conservation, and the application of tissue culture tools in this species, such as SE, is one of the most promising techniques for its conservation and mass propagation.

More specifically, in *A. angustifolia*, SE is characterized by the development of embryogenic cultures (EC), which, in turn, are multiplied as pro-embryogenic masses (PEM) during the early stages of SE. This step is successfully achieved; however, further protocol optimization is required because an unknown factor hampers further maturation of somatic embryos from PEM. The successive stages of development leading to the formation of mature somatic embryos must be accomplished, ideally, by building a fate map of SE, including an adequate number of morphological markers specifying different stages in the whole process. In this sense, the aim of this work is to characterize the process of proliferation of different embryogenic cell fractions of *A. angustifolia* EC through time-lapse tracking technique.

Methods: Embryogenic cultures were induced according to the methodology described by dos Santos et al. [3]. Immature female cones, with the early globular-staged zygotic embryos, were collected in December 2013, from an *A. angustifolia* natural population in Lages, Santa Catarina – Brazil (latitude 27° 49′ 0″, longitude 50° 19′ 35″, altitude 930 m). After 30 days of induction, the EC were subcultured in Petri dishes containing 25 ml of proliferation culture medium with the same composition as the induction culture medium,

except that PGRs concentrations (2 µM 2,4-D, 0.5 µM BAP and 0.5 µM KIN). Subcultures occurred every 21 days for 4 cycles in gelled culture medium for the EC scale-up. After that, EC were transferred for proliferation in liquid culture medium (cell suspension) with the same composition as described above without gelling agent. The cell suspension was established in 250 ml Erlenmeyer flasks containing 50 ml of liquid culture medium, kept in dark conditions with permanent agitation (90 rpm) in orbital shaker with temperature of 22ºC (±2ºC).

After the establishment of cell suspension, it was performed a cell fractioning according to Filonova et al. [4] to assess the dynamics of cell aggregates proliferation. Single cells and few- and multi-celled aggregates were obtained from the <80, 80-200, 200–400, and >600, µm fractions, respectively. These fractions were inoculated into either 0.5 ml aliquots of liquid proliferation medium held in 12 well plates, and subsequently immobilized by mixing with the same volume of 1.2% (w/v) low melting point agarose containing proliferation medium at 35°C. In either type of culture the plating density was set at 25–30 cells or aggregates ml^{-1}. The cultures were incubated in the darkness at 22±2°C. The plates were monitored at 0, 2, 4, 6, 8, 10, 12, 14 culture, with 4 wells per class of cell fraction, in inverted microscope (Olympus IX81), equipped with a computer-controlled digital camera (Olympus DP71).

Results: In general, the cell aggregates of *A. angustifolia* observed by cell tracking showed two types of cells, i.e. highly vacuolated cells, which are more or less elongated (the suspensor cells), and rounded densely cytoplasmic cells, called embryogenic cells. Most of the cells aggregates that passed through the 80 µm pore size started to proliferate starting from day 2 of culture. From the eighth day in culture, the suspensor cells started to proliferate more noticeably, and after 14 days in culture, numerous PEM III were observed. For the fraction 80-200 µm, it was observed a greater presence of suspensor cells as compared to <80 µm fraction, however, the pattern of PEM multiplication was very similar. In addition, it was noticeable that the transition of PEM II to PEM III occurs mainly from the day 10 in culture, and an increased number of PEM III was observed at the end of the 14 days of culture for this cell fraction. For the 200–400 and >600 µm fractions it was observed a large number of PEM II and III and a lower abundance of PEM I. Over the days of culture it was mainly observed a process of the PEM III fragmentation into smaller cell aggregates, and greater proliferation of embryogenic cells in PEM II. However, a large number of cell aggregates showed no proliferation over the evaluation dates.

Conclusions: The results of this study show that a cell fractioning process should take place before the EC transfer for maturation, allowing the cell aggregate selection by size more

suitable for the polarization process and subsequent somatic embryo development and maturation. Furthermore, the cell fractions derived from the smaller pore size presented a greater abundance of viable cell aggregates and in extensive proliferation, which can favor and optimized and high yielding of somatic embryos in the maturation step.

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Simplified droplet vitrification protocol for eazy long term preservation of plant material for functional genomic research and genetic resources management.

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Key words: functional genomics, tissue culture, cryopreservation, conservation

Background
A performing cryopreservation protocol is requested for assisting both genetic resources conservation and securing model lines used for basic research studies (Tsai and Hubscher, 2004). In the frame of the Tree For Joule project, partners in charge of functional gene studies were in need of a way to secure quickly newly generated transgenic lines in order to be able to characterize them on the long run. We thus aimed to develop a simplified but performing protocol for easy cryopreservation of minute amount of lines from various reference Poplar and Eucalyptus clones.

Methods
We revisited our protocols for cryopreservation of dormant and non dormant buds initialy developped respectively for wild forest broadleved tree genetic resources (Harvengt et al 2004) and elite eucalyptus (Paques et al 2002). We developped and tested variations around the droplet vitrification protocol (Sakai and Engelmann 2007).

Results and Conclusions
We started by asking staff who were unfamilliar with the methods to use the in-house pre-existing protocols with standard clone plant material of elm (simplified freezing of dormant buds from field-growing trees; Harvengt et al. 2004) and Eucalyptus (encapsulation-dehydration of actively growing buds; Pâques et al 2001). Since dormant bud cryopreservation proove easy with many species when properly dormant buds are used, appropriate materials were increasingly difficult to obtain due to the huge climatic variation observed along recent years. Moreover, dormant buds does not exist in species with continuous growth as eucalyptus. Regarding encapsulation-dehydration , poor reproducibility, frequent total failure and intensive manpower requirement were observed with unfamiliar staff while familiar staff performed better but with still an average low sucess. Stimulated by the brilliant demonstration of work with potato, rose and bananna hold at a crymcept workshop (Leuven, Belgium in 2005), we thus then decided to concentrate on the establishment of a new procedure based on droplet vitrification.
Initial trials were attempted with 5 clones corresponding to three Eucalyptus species and hybrids.
No significant correspondance was seen between species or clone cold hardinness and plant material survival after cryopreservation. We only got nice correlation between cold tolerance of individual transgenic lines (tranformed with cold-tolerance-related genes, Navarro et al. 2011) and their ability to sustain cold preculture treatment attempted upstream of cryopreservation.
The rather good performance obserevd made us attempting to cryopreserve other broadleaved species with particular interrest for reference poplar clones used in functionnal genomics studies. We therefore tested our protocol on the INRA hybrid clone 717-1B4 (Populus tremula x alba, Leple et al; 1992). No particular difficulty were encountered and

highly reproducible results were obtained with routine cryopreservation of several tens of lines.

The proper performance of the new protocol is being validated and extended on further Eucalyptus and Elm species and hybrids.

Competing interests

The author declares that they have no competing interests.

Acknowledgements

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In silico identification and characterization of genes related to polyamines biosynthesis in embryogenic cultures of Brazilian pine

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Key words: Araucaria angustifolia, polyamines, somatic embryogenesis, transcriptome

Background

Polyamines (PAs) are multifunctional aliphatic nitrogen compounds present in all living cells [1]. In plants the most common PAs are putrescine (Put), spermidine (Spd) and spermine (Spm) [2]. They are involved in many physiological processes, for example, acting as growth regulators in organogenesis, embryogenesis, cell division and differentiation [3,4]. In Brazilian pine (*Araucaria angustifolia*), a native endangered conifer of South America, PAs and amino acids is related to the regulatory mechanisms that are responsible for induction of embryogenic tissues and early somatic embryo formation [5]. The establishment of a successful somatic embryogenesis process, as an alternative propagation approach for conservation, requires the comprehensive knowledge about the expression profile of morphogenetic-related genes in the zygotic counterpart [6]. Recently, a comparative transcriptome analysis of early somatic embryo formation and seed development in Brazilian pine has been reported that constitutes the start point for further gene expression studies [7]. Besides, information regarding the metabolism of PAs in conifers is scarce and its potential use as growth regulator during somatic embryogenesis is still underestimated. In order to improve our understanding about conifer embryogenesis aiming to increase the somatic embryogenesis efficiency, there were carried out investigations about PAs biosynthesis pathway in embryogenic cultures of *A. angustifolia*

Methods

For identification of putative genes involved in PAs biosynthesis pathway, two cell lines (SE1 – responsive and SE6 – blocked to maturation medium (ABA and osmotic agents)) and S1M (SE1 on maturation medium) [5] were analyzed. A search by available sequences in Phytozome (www.phytozome.net), NCBI (http://www.ncbi.nlm.nih.gov) and SustainPineDB (http://www.scbi.uma.es/sustainpinedb), using the tBLASTx program, was performed [8]. Subsequently, the protein sequences obtained from *Arabidopsis thaliana* and *Prunus persica* as query were submitted to tBLASTn on the *A. angustifolia* transcriptome database [7]. The sequences were aligned using the MUSCLE package available in the MEGA 6.0 software with default parameters [9]. The alignment was analyzed using the Neighbor-Joining method with 1500 bootstrap replications. *In silico* prediction of the protein function was performed using Blast2GO [10] and Kegg databases (http://www.brenda-enzymes.org/). In addition, to characterize the PAs biosynthetic pathway we performed a comprehensive *in silico* gene expression analysis by RPKM (number of reads which map per kilobase of exon model per million mapped reads for each gene, for each sample) providing a correlation between gene expression and gene function.

Results

Eight putative PAs genes were identified, six of which showed one copy and the spermidine synthase showed two copies.These genes were named as *AaADC* (Arginine decarboxylase)*, AaODC* (Ornithine decarboxylase), *AaAIH* (Agmatine deiminase), *AaCPA* (*N*-carbamoylputrescine amidase), *AaSAMDC* (*S*-adenosylmethionine decarboxylase), *AaSPMS* (Spermine synthase)*, AaSPDS-like 1* (Spermidine synthase-like 1), *AaSPDS-like 3* (Spermidine synthase-like 3)*.* All protein sequences analized exhibited at least 50% of protein identities and 70% of positives matches with their respectively orthologous. The results indicated that the genes have a high conservation of motifs, except to *AaODC* that only a fragment (161 aa) was retrieved.

Regarding the gene expression, SE1 and SE6 cell lines showed similar profile during the proliferation phase. *AaADC* and *AaSAMDC* had the highest RPKM values in all samples analyzed, followed by *AaSPDS-like 1*, *AaCPA, AaAIH* and *AaSPDS-like 3*, while *AaSPMS* had the lowest. However, transcripts of *AaODC* were not detected in both cell lines. The development of globular somatic embryos in cell line SE1 (after 90 days on maturation medium) was characterized by barely modifications in the expression of *AaADC* transcripts. For *AaAIH*, *AaCPA*, *AaSAMDC*, *AaSPDS-like 1* and *AaSPDS-like 3* we observed a decrease in RPKM values whereas for *AaSPMS* was higher than in the proliferation phase.

Conclusions

The results suggest that, although the embryogenic potential is different in cell lines SE1 and SE6, transcript levels of genes related to PAs metabolism are similiar during the proliferation phase. However, transcript profiles of genes related to spermidine and spermine biosynthesis were altered during early somatic embryo development. The results will be discussed to understand the involvement of PAs metabolism on embryogenesis process aiming the optimization of somatic embryogenesis protocol of *A. angustifolia*.

Competing interests

The author declares that they have no competing interests.

Acknowledgements

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IMPROVED LIGNOCELLULOSIC BIOMASS YIELD OF RAV1 ENGINEERED POPLARS IN A SRC FIELD TRIAL

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Key words: poplar, syllepsis, biomass, RAV1

Background

Plantations of *Populus spp*, *Salix spp.* or *Eucalyptus spp.* are established to produce wood in a reduced space and a short time. Poplars are cultivated with cycles of 15-18 years to obtain saw timber and peeler logs, and when grown for biomass production as short-rotation coppice (SRC), cutting back/coppicing cycles are reduced to 2–5-years intervals. Syllepsis is among the valuable traits that can be targeted to enhance biomass yield of SRCs. Syllepsis, i.e. the outgrowth of lateral buds into branches the same season in which they form without an intervening rest period, increases carbon fixation and allocation in the shoot and hence the general growth of the tree. A high degree of sylleptic branching is known to be positively correlated with biomass yield when these plantations are grown under optimal conditions [1]. In 2012 we established in Madrid (Spain) a SRC field trial with genetically engineered poplars, previously shown to develop sylleptic branches when cultivated in growth chambers, under optimal conditions [2]. The aim of starting up this field trial was to test whether a plastic trait as syllepsis was maintained over time under natural conditions and eventually resulted in an enhanced biomass production

Methods

In vitro culture rooted cuttings were initially potted in 3.5L containers with blond peat and grown in the greenhouse as previously described [2]. The field trial was established in July 2012 in the experimental plot, and included five groups of hybrid poplar *Populus tremula x P. alba* INRA clone 717 1B, the wild-type genotype as control, transgenic events #37 and #60 carrying the 35S::3xHA:CsRAV1 cassette (3xHA:CsRAV1 OX), and events #1 and #22 carrying the 35S::PtaRAV1-hpiRNA cassette (PtaRAV1&2 KD). 30 individuals per group were planted into three blocks of 10 plants each. The experimental plot area was 204 m^2, and the plantation density 10000 trees/ha. It consisted of 12 x 17 rows with a tree spacing 2 x 0.5 m. The border rows were occupied by *P. x euramericana* clone I-214 individuals, planted as 25 cm-long cuttings. Irrigation and weed/pests control were applied, and the first coppicing cycle was done after the second growing season [3]. Several productivity determinants (stem height and diameter, syllepsis and phenology) were monitored, wood anatomy and chemistry analyzed, and aerial biomass yield and calorific value determined.

Results and Conclusions

CsRAV1 over-expressing event #60 showed an advantageous performance in the field regarding stem diameter and biomass production after the first coppicing cycle. In this event, sylleptic branches grew from the main shoot during the first growing seasons, after the plantation establishment and after coppicing. None of the other traits under study such as phenology, wood anatomy and chemistry were noticeably altered when compared to the wild type genotype. These results show that in woody species RAV1 is a highly valuable target gene that can be used as biotechnological tool to enhance biomass yield of poplar SRC plantations without detrimental side-effects in tree development and characteristics.

Competing interests

The authors declare that they have no competing interests.

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Development of Epigenomics based Agri-nutrigenomics: Study of Genome-wide Differential Methylation Patterns in *Eucalyptus camaldulensis* in Leaf and Secondary Xylem upon Fertilizer Application by *de novo* Genome Sequencing and Whole Genome Bi-sulfite Conversion

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Keywords: Agri-nutrigenomics, Epigenomics, Methylation, Eucalyptus

Background

Most of the *Eucalyptus camaldulensis* (*Ec*) plantations in India are grown by small holders under subsistence farming, where balancing the nutrient budget of soil is a least concern. While finding an economically viable fertilizer combination to maintain soil nutrient balance, it is also important to study this phenomenon at molecular level to help in developing nutrient responsive clones. “Agri-nutrigenomics” is an emerging, science area, which studies the effect of nutrient applications on epigenome, transcriptome, proteome or any other part of cell/genome [1]. Nutri-genomics helps in understanding events, post application of fertilizers to plants to device ways to increase its fertilizer use efficiency. The present study explored the effect of optimal fertilizer application package on epigenome to find out genomic regions under differential methylation control. This was attempted by *de novo* genome sequencing of *Ec* and then mapping bi-sulfite converted short reads of DNA from leaves and secondary xylem.

Methods

A three year old *Ec* experimental plot (Clone # ITC 411) with different fertilizer combinations in 16-tree plot was sampled for leaves and secondary xylem from control

and selected treatment plot (N_{200}:P_{100}:K_{100} kg/ha). DNA from each of them was isolated using standard procedures. Native DNA was used to prepare NGS libraries, which were run on Illumina-HiSeq 2000 (101 base, PE), 454 (GS-FLX and GS-FLX+) and SOLID (60 bases mate pair) as per the manufacturer's instructions and standard QC prcedures. Hybrid assemblies were generated over the primary assemblies made from Illumina reads (k-mer: 65) by aligning with 454 long reads using Velvet *de novo* (v 1.2.08). Final scaffolding was done by aligning these assemblies with SOLID mate pair reads using SSPACE Basic 2.0. Final scaffolds with minimum size of 1000 bases were threaded on the chromosomal scaffolds of *E. grandis* (*Eg*) genome using Contiguator tool to generate pseudo-chromosomes of *Ec*. The genome annotations were applied to the scaffolds by BLATing with known gene annotation of *Eg*. The completeness of the assembled genome at gene level was tested using CEGMA tool and *de novo* gene prediction was carried out by AUGUSTUS tool following Arabidopsis gene model. To study differential methylome, the DNA from leaf and secondary xylem were methyl converted using standard protocol [2], NGS libraries made (101 bases, PE) and run over Hi Seq-2000 as per manufacturer's instructions. The bisulfite converted reads were mapped onto the assembled *Ec* genome using Bismark v0.13.0 with Bowtie v1 aligner [3,4]. Only the reads with minimum coverage depth of 10x were used for any inference.

Results

The most economical fertilizer treatment as found from the wood yield was (N_{200}:P_{100}:K_{100} kg/ha), which gave a significant yield benefit (27.5 tons/ha, SD: 3.476, SE: 1.738) over the control (15.6 tons/ha, SD: 3.584, SE: 1.792).

A total of ~58GB of high quality data was generated from Illumina HiSeq (46 Gb), Roche-454 (~ 1.2 Gb) and ABI-SOLID (11.5 Gb) platforms. Final scaffold assembly resulted in 82,569 contigs (min >1kb) corresponding to 742.1 Mb of genomic sequence, of which 493.1Mb sequences were anchored to the 11 linkage groups (LGs) of *E. grandis* genome (Table 1). CEGMA analysis resulted in approximately 160/248 of the core eukaryotic genes (CEGs) aligned as complete gene copies to the assembled *Ec* contigs indicating 64.5% completeness of the genome. From 46,315 genes annotated over *Eg* genome 21,665 were mapped to full length and 9,965 were mapped partially, whereas 14,685 could not be mapped. From *de novo* prediction, 85,752 genes were predicted of which 70% were assigned to 11 pseudo-chromosomes (Figure 1).

Bi-sulfite sequencing generated a total of ~15GB sequencing data for each samples, of which approximately 35 % was mapped uniquely to the reference genome (Figure 2). Differential methylation analysis performed on control vs treated samples identified 2,116 differentially methylated regions in 663 unique nearby transcription start sites (TSS) in leaf, and 2,543 differentially methylated regions in 734 unique nearby TSS sites in

secondary xylem (Table 2). Genes involved in processes such as growth regulations and cell cycle progression, were differentially methylated.

Conclusions

This is one of the unique studies that was conducted in tree species for identifying potentially regulatory regions including genes associated with fertilizer responsiveness. *De novo* assembly was successful in assembling ~92% of *Ec* genome, which is significant, considering the method (i.e, shot gun library) employed. Candidate genes identified in this study will be further validated using candidate gene based or whole genome based association mapping approach and will aid in genetic improvement of *Eucalyptus* using marker assisted selection and transgenics.

Competing interests

The authors declare that they have no competing interests.

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Table 1. Summary of final draft assembly of *Ec* with contig size > 1 kb

Assembly metrics	
Total number of scaffolds	82569
Assembly length (in Mb)	742.06
Min. length (in bp)	1000
Max length (in bp)	57,777,245
Mean length (in bp)	8987.11
Median length (in bp)	1840
N50 value length	41,011,173
N90 value length	2,300

Table 2. Methylation sites observed between control and treated samples of leaf and xylem in *Ec.*

Samples Compared	**Leaf vs leaf treated**	**Samples Compared**	**Xylem vs xylem treated**
Hyper methylated	890	Hyper methylated	1117
Hypo methylated	1226	Hypo methylated	1426
Unchanged	32010	Unchanged	32523
Leaf specific methylated sites	73329	Xylem specific methylated sites	48129
Leaf treated specific methylated sites	37878	Xylem treated specific methylated sites	51544
Type	**Unique TSS Count**	**Type**	**Unique TSS Count**
Hyper methylated	298	Hyper methylated	334
Hypo methylated	365	Hypo methylated	400
Leaf specific methylated sites	13113	Xylem specific methylated sites	7745
Leaf treated specific methylated sites	7619	Xylem treated specific methylated sites	8435

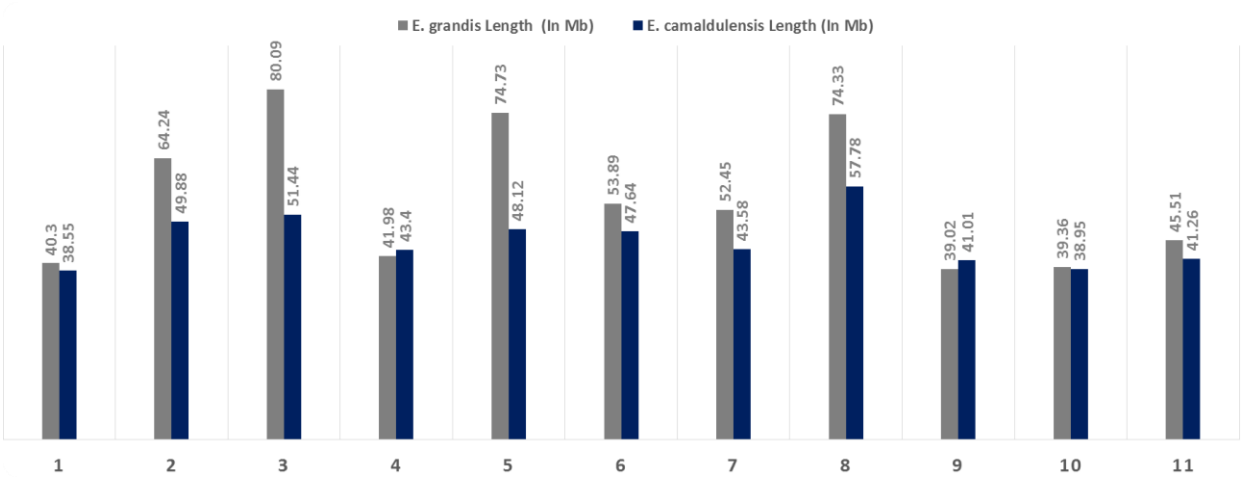

Figure 1. *Ec* chromosome anchoring of *Eg* scaffolds: x-axis shows the individual chromosome and y-axis shows the comparative scaffolds size of *Ec* anchored to *Eg*.

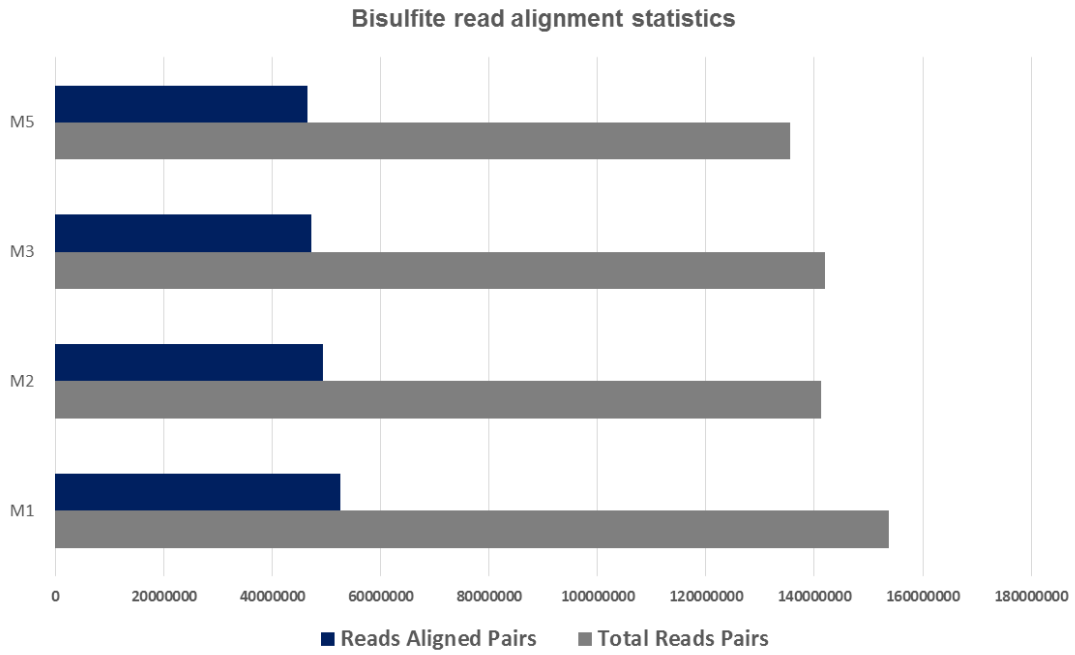

Figure 2. CpG methylation distribution among different sample data set. M1: Leaf control, M2: Leaf treated, M3: Secondary xylem control, M4: Secondary xylem treated

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Strategies for improving the induction of fertile flowers in male and female early flowering poplar

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Keywords: early flowering, Populus, gene stacking, crosssings

Background

Forest tree species reach the reproductive phase only after many years or even decades of juvenile growth. Special treatments, based on plant hormones, growth inhibitors, manipulation of growth environment or physical methods, have promoted flower development in juvenile plants from several forest trees species but not in poplar. Only genetic engineering with some “flowering genes” has allowed inducing early flowering in juvenile poplar plants. Genetic transformation of poplar with genes genes *AtLFY* (Weigel and Nilsson, 1995), *AtFT* (Zhang et al., 2010), *PTLF* (Rottmann et al., 2001), *PtFT1* (Böhlenius et al., 2006), *PtFT2* (Hsu et al., 2006) and *MdFT2* (Tränkner, C. *et al.* 2010) promoted flower development. First successful crossings were reported recently in our group with male early flowering HSP::*AtFT*-poplar (Hoenicka et al., 2014). However, flowers obtained still showed a high level of sterility.

The improvement of early flowering systems in poplar may require development of more reliable systems based on other “flowering genes”. The identity of the shoot meristem in plants is controlled by multiple genes. The *FLOWERING LOCUS T* (*FT*) of *Arabidopsis thaliana* and *FT*-homologous genes from other plant species are key floral promoter genes. However, there are also many genes involved in the regulation of flowering like *CONSTANS (CO)*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), the CO transcriptional activators *FLOWERING BHLH* (*FBH1*

and *FBH2*) (Ito et al., 2012), *CONSTITUTIVE PHOTOMORPHOGENESIS 1* (*COP1*), *CRYPTOCHROME 1* (*CRY1*), *APETALA 1* (*AP1*), *APETALA 3* (*AP3*), *LEAFY* (*LFY*) and many others (rev. Amasino, 2010). COP1 acts as a repressor of flowering by promoting the ubiquitin-mediated proteolysis of CO in darkness and that *CRY*-mediated signal may negatively regulate *COP1*, thereby stabilizing CO, activating *FT* transcription, and inducing flowering (Liu et al., 2008). In *Arabidopsis thaliana*, the blue light photoreceptor cryptochromes (CRY) act to promote photomorphogenic development and the transition from vegetative to floral development (Liu et al., 2008, Exner et al. 2010). The *cry1*-L407F allele of *Arabidopsis thaliana* shows early flowering under short day conditions (Exner et al., 2010). Arabidopsis plants carrying the *cry1*-L407F point mutation have elevated expression of *CONSTANS* and *FLOWERING LOCUS T* under short-day conditions, leading to very early flowering (Exner et al., 2010).

MicroRNAs play an important role on the regulation of the reproductive phase in plants. The miR172 promotes flowering, at least in part, by repressing APETALA 2-like repressors of *FT* (Aukerman and Sakai, 2003). The vegetative phase change in *Arabidopsis* is regulated by *mir156*, a microRNA that promotes the expression of the juvenile phase and represses the expression of the adult phase (Yang et al., 2011).

In this study, several gene constructs and gene stacking strategies were evaluated aiming an improved development of fertile flowers in poplar. Flower development was strongly influenced by gene stacking. First successful crossings were performed with female early flowering HSP::*AtFT*-poplar.

Methods

Crossings with female early flowering HSP::FT-poplar

Crossings were carried out between female HSP::*FT* (hybrid aspen *Populus tremula* x *P. tremuloides*, clone Esch 5) and male wild-type poplar (*Populus tremula* L., clone W52) using the method described before (Hoenicka et al., 2014).

Evaluation of alternative models for early flowering induction in poplar

Different gene constructs were used for genetic transformation of wild type poplar (*Populus tremula* L. clone T89) and hybrid poplar (*P. tremula x P. alba* clone P1):

- Single transformations:

35S::*miR172a*, 35S::*CRY1-L407F*, 35S::*FBH1*, 35S::*FBH2*, HSP::*LFY*, HSP::*AP1*, HSP::*AP3*, 35S::*AP1*, 35S::Mir172, Mir156 (RNAi), 35S::*cry1*-L407F and *COP1* (RNAi).

- Improvement of flower development in HSP::*FT* poplar through gene stacking

HSP::*FT* poplar was transformed with gene constructs HSP::*AP1* and HSP::*LFY*, and the poplar orthologous genes from *AG* and *AP3* (HSP::*PTD* and HSP::*PtAG1*)

Results and Conclusions

Crossings with female early flowering HSP::FT-poplar

Crossings carried out with female poplar were successful. Viable seeds were obtained and young plants were brought into the greenhouse.

Evaluation of alternative models for early flowering induction in poplar

Transgenic poplar lines carrying the different early flowering constructs have been transferred to greenhouse. No phenotypic alteration including early flowering was hitherto observed after single genetic transformation with all “flowering time” genes tested in this study. Phenotyping of these transgenics will be continued in future.

Transgenic lines obtained with the HSP::*LFY* gene construct show a disturbed growth but no early flowering.

The gene stacking approach based on transformation of early flowering HSP::*FT* poplar with 35S::*SOC1* promoted a three-fold increase in the number of catkins obtained under *in vitro* conditions. Further gene stacking approaches with gene constructs HSP::*AP1* and HSP::*LFY,* and the poplar orthologous genes from *AG* and *AP3* (HSP::*PTD* and HSP::*PtAG1*) influenced catkin development (Fig. 1). *PtAG1* promoted an improved catkin development more similar to that of wild type poplar. The *LFY* gene promoted sex change in flowers (Fig. 1).

A recently developed strategy (not shown) allowed a reliable induction of fertile flowers in one-year old male and female poplar as well as successful crossings in both directions (manuscript in preparation).

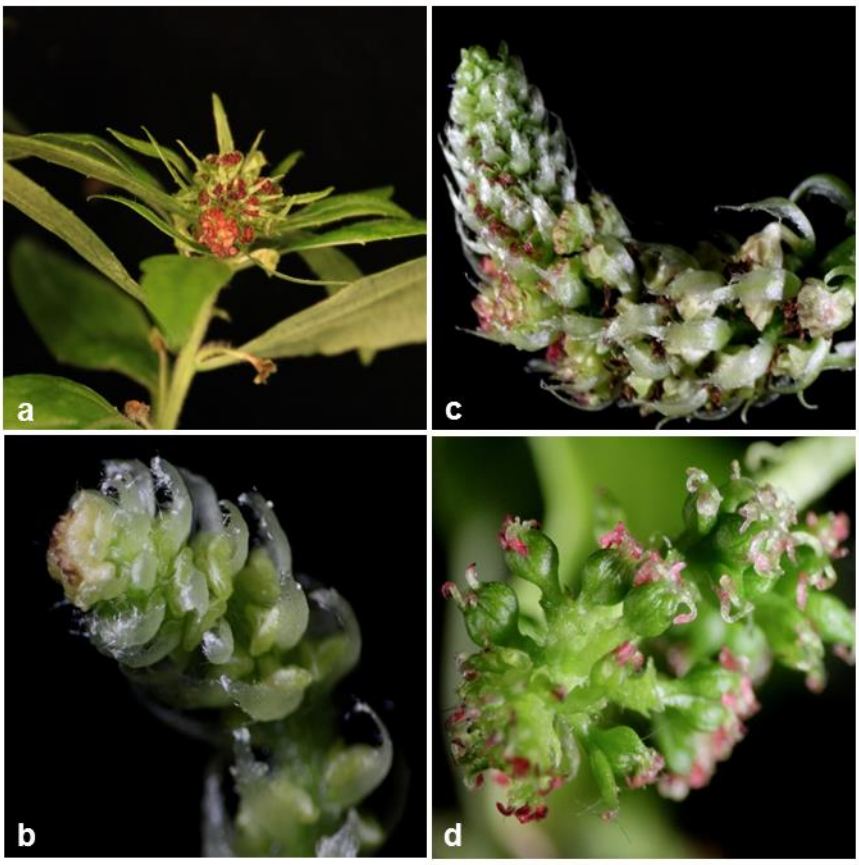

Figure 1. Catkins obtained after genetic transformation of early flowering HSP::*FT* poplar with a second gene construct (gene stacking). (a) HSP::*AP1*: leaves develop between flowers; pollen grains were obtained, (b) HPS::*PTD*, (c) HSP::*PtAG1*, (d) HSP::*LFY*: strongly disturbed flower development and gender change (female instead of male flowers were obtained)

Competing interests

The author declares that they have no competing interests.

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Organization of cis-acting regulatory elements in the promoters of four dehydrins genes of *E. nitens*

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Background

Cis-acting regulatory elements are important molecular switches involved in the regulation of gene activities controlling various biological processes such as abiotic stress and hormonal responses and development processes. Dehydrins are typically induced in response to stress conditions that cause cellular dehydration, such as low temperatures, high salinity and drought. In this work we identify the cis-acting elements in the promoter region of four dehydrin genes (*EniDHN1*, *EniDHN2*, *EniDHN3* and *EniDHN10*) of *E. nitens* and compared them with those previously described for *E. globulus* (*EuglDHNs*).

Methods

DNA was extracted from leaf tissue of *E. nitens*. For the identification of DHNs genes in *E. nitens* primers were designed using as reference the dehydrin sequences in *E. globulus* (Fernández et al 2012). Once identified sequences cis-acting regulatory elements were deduced from PlantCARE. In general the potential cis-elements identified can be classified mainly into hormone-responsive and stress-responsive elements.

Results and Conclusions

For *EniDHN1* three ABRE (ABA responsive elements) elements, one G-box and four CRT (C-repeat) regulatory elements were identified, in the case of *EniDHN2* four ABRE elements, seven G-boxes and two CRT were found, while in *EniDHN3* three ABRE elements, five G-box and four MYC elements were identified and for *EniDHN10* only one ABRE element was found. The results showed that the four *EniDHNs* contained several cold-or dehydration inducible cis-element and these elements are involved in both the ABA-dependent and ABA-independent pathways of gene regulation of dehydrins (table 1). Comparing the frequency of cis-elements present in *EniDHNs* and in *EuglDHNs* significant differences were found between ABRE elements especially in *DHN2* where ABRE and G-box elements in *E. nitens* were higher than *E. globulus*. This can give an understanding of regulatory systems in the stress-responsive dehydrin genes expression and helping understand the difference in the level of stress tolerance of both species.

** The frequency observed is higher than expected for cis-acting element in the promoter region*

Table 2 Putative ***cis*-**acting regulatory elements involved in stress-responsive expression in the promoter regions of four *EniDHNs* in *E. nitens*

***cis* elements**	**Sequence**	**Frequency of the *cis*-element present in the *dhns* of *E. nitens***				**Function**	**Reference**
		Dhn 1	*Dhn 2*	*Dhn 3*	*Dhn 10*		
ABRE	TACGTG	3*	4*	3*	1	ABA responsiveness	Allagulova et al (2003)
Box-4	ATTAAT	2	0	1	0	Light responsiveness	Lois et al. (1989)
CAAT-Box	CAAT	2	3	8*	2*	Common in promoter and enhancer regions.	Straub et al. (1994)
CRT	GCCGAC	4*	2	0	0	Low-temperature responsiveness	Qin et al. (2004)
G-Box	CACGTG	1	7*	5*	0	Promoter regulatory element involved in dehydration responsiveness	Maruyama et al (2011)
HSE	AAAAAATTTC	0	1*	1	0	Heat stress responsiveness	Pastuglia et al. (1997)
MBS	CGGTCA	1	0	1	0	Drought responsiveness	Nash et al. (1990)
MYC	CACGTG	2*	0	4*	0	MYC recognition site found in promoters of dehydration-responsive gene.	Chinnusamy et al. (2003)
TCA-element	CCATCTTTTT	0	0	0	3*	SA responsiveness	Pastuglia et al. (1997)

Competing interests

The author declares that they have no competing interests.

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Genetic approaches to increase biomass yield in the woody perennial *Populus*

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Key words: Populus, transformation, wood, flowering genes

Background

In Germany, the phasing out of nuclear energy till 2022 was determined. A national law pretends that 30% of the power should come from renewable resources in 2020. Especially biomass-based energy provides great potential for this aim. Biomass contributes already the biggest part in the renewable energy mix [1]. Fast growing poplars can be cultivated in short rotation coppices for several years with little care and can be grown even onto soils which are not suitable for food crops. With increased biomass yield, short rotation coppices could become more profitable. For increase of the biomass, seven candidate genes were chosen for transgene approaches. Two of them, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) and *FRUITFUL* (*FUL*) are usually described as flowering genes and are involved in the transition from vegetative to reproductive stage [2]. In Arabidopsis, double mutants with this genes show a perennial phenotype including the production of wood [3]. Furthermore, it was revealed in a transcriptome analysis of an aspen hybrid that several genes are expressed in different tissues. Five genes that are similarly expressed in the developing xylem and the catkins or the roots (unpublished data, Kersten et al., Thünen Institute of Forest Genetics, Grosshansdorf, Germany) were chosen. Five of these mostly unknown genes were completing the set of candidate genes. With them both the overexpression and knock-down were executed, respectively.

Methods

Two different poplar hybrid clones were used for transformation. Both clones, Esch5 (*P. tremula* × *P. tremuloides*) and INRA 717-1B4 (*P.* × *canescens*) are genetically well characterized and reliable for transformations [4, 5]. All transformations were mediated by *Agrobacterium*, mainly according to Fladung et al. [4] and slightly modified by the authors. Binary plasmids were used, which contained kanamycin or hygromycin resistance genes for transformants' selection beside the gene of interest. All used genes were under control of the constitutive promoters 35S or Ubiquitin. Beside single transformations, also double transformations were executed. In every independent regenerate, PCRs were accomplished for both the resistance gene and the gene of interest to confirm transgene status. With Southern Blot analyses, the copy numbers were determined. Single copy plants were preferably used for expression analyses by qRT-PCR. Additionally, plants with more copies of the transgene were kept for possible quantitative effects. All lines were transferred to soil, cultivated for several vegetation periods in the greenhouse and observed for noticeable phenotypes. In winter season, every plant was measured for their height and stem diameter. The produced wood was analyzed by project partners for its compound properties.

Results

Within the project, 40 *Agrobacterium*-mediated transformations have been accomplished. Numerous independent transgenic lines were obtained. Each line was tested on genetic level for their antibiotic resistance and presence of the gene of interest. Southern Blot analyses showed that in addition to numerous single copy lines, multi copy lines were produced with up to seven copies of the transformed T-DNA. Currently, expression analyses are ongoing by qRT-PCR.

In total, 2460 transgenic plants were transferred to soil and cultivated under greenhouse conditions. Some interesting phenotypes could be identified. One transgenic line, that contained overexpression vectors for *SOC1* and *FUL* showed an outstanding phenotype (Fig. 1). It seemed, these plants lost their tree-like phenotype. Another line struggled to produce stems and grew mainly as a callus regenerate.

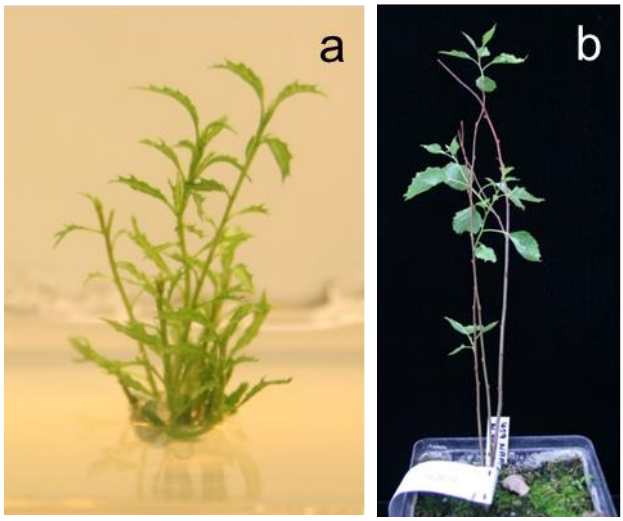

Fig. 1a, 1b: Two transgenic clones of *P. tremula* × *P. tremuloides* are revealing an outstanding phenotype

In these clones, both *SOC1* and *FUL* are over-expressed. The plants have denticulate leaves, fragile roots and weak wood formation (a: *in vitro*, four months, 2.8 cm. b: greenhouse grown plant, two years).

Older plants which have produced woody biomass were harvested and analyzed in relation to their non-transgenic control plants. Under the one year old clones, some show higher or lower growth than their control. To exclude non-genetic factors, the trees were measured once more in the second year. Currently, the data are being evaluated. In the composition of the wood, only small shifts could be observed. Their genetic background will be assured with a second analysis.

Conclusions

Overexpression of the chosen candidate genes expressed both in the flower and the developing xylem did not lead to an increase of biomass. However, we obtained evidence that such candidate genes could have an impact on the formation of biomass. *Arabidopsis* mutants with down regulation of both *SOC1* and *FUL* show a perennial phenotype [3]. According to that, it was expected that overexpression of both genes lead to an opposite phenotype. By the current state, this expectation was achieved with the described phenotype in independent transgenic lines of two different poplar clones. The complement approach to increase biomass with a knockdown of both

SOC1 and *FUL* in *Populus* seemed to be ineffective (unpublished results), what suggests that both genes are not a quantitative but a qualitative effector.
Since the analyses are ongoing, final conclusions cannot be drawn at this point. Expression analyses as well as TAIL-PCRs to determine transgene localization and to exclude position effects are scheduled.

Competing interests
The author declares that they have no competing interests.

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Dynamics of repressive and active epigenetic marks during cork development

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Key words: phellem, 5-methylcytosine, DNMTs, H3K4me3

Background

Cork oak (*Quercus suber* L.) is one of the most important Mediterranean forest trees with high ecologic and economic value in Portugal due to its outer bark, the cork. After the trees reach a legally defined perimeter, cork can be harvested every nine years in a renewable and sustainable basis. Cork is a thick periderm composed by multiple layers of phellem cells produced by a secondary meristem, the cork cambium or phellogen. During the first year of the tree, phellem cells expand and differentiate through the inner deposition of suberin in their walls [1]. Subsequently new cell layers arise while the older ones undergo an irreversible developmental program ending with cell death. This process renders cork mainly composed of impermeable dead cells.
Ontogenetic development, ageing and maturation are characterized by altered patterns of cell differentiation accompanied by drastic chromatin remodelling, where DNA methylation and histone modifications play essential roles. The regulation of specific developmental processes involving epigenetic marks is well known and has been studied in *Q. suber* revealing a correlation between DNA methylation levels and tissue maturity [2]. DNA marks such as trimethylation of histone H3 at lysine 4 (H3K4me3), associated with transcriptionally active regions of chromatin, together with the transcriptional repressive methylation of the cytosines in the DNA, contribute to the flexibility of transcriptional regulation necessary for plant development. A family of DNA methyltransferases (DNMTs) comprising three specific functional classes in plants is responsible for methylating the DNA: the MET class maintains the methylation in CpG zones; the CMT (chromomethylase) class maintains the methylation in CpHpG sequences in plants and the DRM (Domain-Rearranged-Methyltransferase) class is associated with the *de novo* methylation in any context [3].
In this work, we report the DNA methylation and trimethylation of Histone H3 at lysine 4 dynamics associated with cork development supported by the transcriptional profile of three putative DNA methyltransferase genes of the CMT, DRM and MET classes.

Methods

Immunodetection of 5-methylcytosine (5-mC) and H3K4me3 was performed in herbaceous and slightly lignified twigs (one-year-old), and reproduction cork (harvested from adult trees in full activity) using specific antibodies (anti-5mC, ab10805; anti- H3K4me3, ab8580). Prior to immunodetection and cork formation studies, the samples were collected and fixed either

in formalin–acetic acid–alcohol or in 4% paraformaldehyde and embedded in GMA or paraffin according to the purpose. Tissue autofluorescence was used to identify the cork formation. Measurements of the fluorescent signal intensity were performed using the AxioVision measurement module of epifluorescence microscope Axio Imager.Z1.
To evaluate gene expression, cDNA was produced from RNA extracted from phellogen and contiguous differentiating tissue of the reproduction cork of six adult trees. Three DNMTs were selected from the cork oak unigene library [4].

Results

We took advantage of the suberin autofluorescence to evaluate cork formation. In the herbaceous twigs, as expected, no secondary meristematic activity was observed but only primary growth. In the one-year-old twigs, some phellogen divisions have already occurred giving rise to three phellem layers composed of yet living cells highly autofluorescent due to the deposition of suberin in their walls. In the reproduction cork, we observed cells belonging to the tear zone with no suberin in their walls and several compressed layers of phellem cells with thin suberized walls. It is known that the cork cells show empty lumen, however we observed living cells with cytoplasmic content in approximately seven layers beyond the tear zone.
Immunolocalization of DNA methylation revealed similar levels of 5-mC in the different tissues of the herbaceous twigs, nevertheless, in the one-year-old twig this mark is tissue dependent revealing an increase from the phellogen to the differentiating phellem. Epidermis cells that are in an accelerated process of cell death showed the highest level. Also, no differences were found in the level of H3K4me3 in phellogen and contiguous differentiating tissue, as well as in other type of tissues such as cortex, phloem and xylem in contrast with the epidermis cells.
In the reproduction cork, a fourfold increase in the DNA methylation level from the tear zone to the older phellem living cell layer was detected. This increase during phellem differentiation until complete loss of the cellular content is accompanied by a condensation of the nuclei and drastic alterations in the chromatin structure.
All QsDNMTs are transcriptionally active in the phellogen and contiguous differentiating tissue; QsDRM2, revealed to be the gene with the highest relative expression what is in accordance with the nature of this tissue.

Conclusions

Cork development is accompanied by a drastic increase in DNA methylation, associated with severe alterations in the chromatin structure and nuclear area decrease. Moreover, the DNA methylation pattern found in phellem cells is characteristic of the programmed cell death undergone by these cells.
The DNA methylation patterns observed are supported by the transcriptional profile of several DNMTs. Furthermore, the increase in the DNA methylation level as phellem cells are becoming more differentiated is strongly supported by the high expression level of QsDRM2, responsible by the *de novo* methylation. Also, the similar levels of the H3K4me3 detected in all the tissues of young twigs are in accordance with the high cellular activity of these tissues.
All these observations clearly show an association of the “epigenetic marks” in cork development.

Competing interests

The author declares that they have no competing interests.

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The epigenetic landscape of *Quercus suber* L. female flower development

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Key words: Cork oak, H3K4me3, 5-Mc, Pistillate flower

Background

Montados, the *Quercus suber* L. forests are traditional Mediterranean agro-silvo-pastoral systems with environmental importance contributing to the preservation of some important habitats. Montados are usually naturally regenerated being, therefore, dependent on seed production. Studies on *Q. suber* reproductive biology are of great importance for further understanding of the reproductive success of this species (1). Unlike most angiosperms, in which fertilization occurs within few days after pollination, in almost all Fagales there is a delay between pollination and fertilization (progamic phase) (2) due to the incomplete development of the pistillate flowers at pollination. Therefore, *Q. suber,* is a monoecious tree with a long progamic phase: female flowers appear in Spring and only get fully developed a few months later, if pollinated. Usually, the pollen tube growth is arrested at the base of the style for around 6 weeks, whilst ovule differentiation occurs. After fertilization, only one of the six ovules develops into seed (3). This cascade of events involves changes in the pattern of cellular differentiation that are regulated by epigenetic mechanisms. The study of chromatin remodelling involving the patterns of epigenetic marks during pistillate flower development has never been attempted in cork oak. In this study, we analyse the dynamics of the epigenetic marks during the female flower development in this oak species.

Methods

Female flowers in different developmental stages were collected from mid-March to August and fixed in 4 % paraformaldehyde prior to paraffin inclusion. Sections of 10 µm obtained with a rotary microtome were rehydrated after paraffin removal, and stained with 0.1% toluidine blue solution to confirm the developmental stage. Immunolocalization of modified histone H3K4me3 and of 5-mC was performed with monoclonal antibodies (1:100 and 1:50 abcam, respectively) against these chromatin modifications and were detected with a secondary antibody associated with Alexa fluorochrome (1:100 upstate). The cell walls and DNA were counterstained with calcofluor and DAPI, respectively. To identify lignified structures, a saturated solution of phloroglucinol in 20% HCl was used.

Results and Conclusions

The different stages of cork oak female flower development from the early start until the beginning of the embryo development were evaluated in sections of flowers with different sizes stained with toluidine blue. In the smaller flowers the style occupies the majority of the pistillate flower with a noticeable transmission tissue (TT). The further development of the flower is accompanied by the enlargement of the locules positioned at the base of the style. These cavities where the primordia of the ovules can early be detected are surrounded by conspicuous unicellular trichomes. During flower progression the ovary develops and becomes functional, the cupule increases and large scales are detected. After fertilization TT cells become non-functional due to cell death; the thick cell walls become lignified as detected by the positive phloroglucinol reaction. In advanced stages, after fertilization, the zygote/developing embryo was located at the micropylar end, and a free nucleate endosperm is visible.

Immunolocalization of epigenetic marks related to silence (5-mC) and active transcription (H3K4me3) were detected in the same flower stages. In the first stage flowers, before pollination, the two marks were well presented in all the tissues. The TT shows high intensity of both marks whereas the rest of the style shows the highest levels of H3H4me3. Pollination takes place when the ovule primordia were detected (3). At this stage the higher level of H3K4me3 was detected in the TT, in the ovule primordia and first layers of the ovary, while the styles and the scales show stronger signals with 5-mC antibody indicating possible cell inactivity. Longitudinal and transverse sections of flowers where ovules are fully maturated show that the ovules present high levels of H3K4me3 in all the tissues with emphasis to the internal integument, the megaspore mother cell, nucellus epidermis, and subdermal layer. In this phase we detected 5-mC in ovary nuclei and high amount in the scales with the highest signal in the outer layers. At this stage new scales are being produced in the cupule, showing high levels of H3K4me3.
When embryo sacs start to develop the TT is very small and the ovary occupies a large portion of the flower. High amounts of H3K4me3 are in cells surrounding the embryo sacs, in the inner integument. On the other hand almost no H3K4me3 signal can be seen in the TT nuclei what agrees with the decay of these cells after fertilization. Trichomes inside the locules show a progressive level of DNA methylation along flower development until cell death and complete disappearance in more advanced stages.

In conclusion, the cork oak developing pistillate flower shows dynamic epigenetic marks during developmental suggesting that chromatin remodelling events are likely to contribute to the establishment of the fate of the cells during female gametophyte development and fertilization events.

Competing interests

The author declares that they have no competing interests.

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Functional characterization of three Eucalyptus MYB genes controlling wood formation and evaluation of their impacts on saccharification

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Keywords: Secondary cell wall, saccharification, MYB genes, Eucalyptus

Backgrown

Either used for pulping, for sawn timber or as an energy source, wood is an important raw material for mankind reported to be the fifth most important world trade product [1]. Wood, also known as secondary xylem, is formed by the thick secondary walls (SCW) from fibers and tracheary elements. These SCW are constituted mainly from cellulose, hemicelluloses and lignin, and despite their industrial uses, they are crucial for land plants, since they give support to the aerial structures and allow an efficient water transport. Xylem cells derive from vascular cambium, a lateral meristem that actively divides and produces daughter cells which follow elongation, deposition of secondary cell wall, and programmed cell death to differentiate into the different xylem cell types. Cambial activity and

xylem differentiation are tightly regulated by a hierarchical network of transcription factors, where auxin transport and auxin-related genes control meristematic activity and cell fate, whereas NAC and MYB proteins, among others, transcriptionally regulate the activity of lignin, cellulose and hemicellulose biosynthetic genes (reviewed in [2]).

Eucalyptus species and hybrids are the most planted hardwoods worldwide because their fast growth, high adaptability and valuable wood properties, thus being one of the world leading sources of wood biomass [3]. However, despite the huge economic importance of Eucalyptus, only a few regulatory genes controlling wood formation have been functionally characterized up to now. Among them, two members of the R2R3 MYB transcription factor family, EgMYB1 [4] and EgMYB2 [5], were shown to be a repressor and an activator of SCW formation, respectively. Thanks to the *E. grandis* genome sequence [3], we performed a genome-wide survey of the MYB family that highlighted EgMYB137, which has no clear ortholog in Arabidopsis, as a potential regulator of wood formation [6]. The main aim of this study was to better understand the role of these three MYB transcription factors (EgMYB137, EgMYB1 and EgMYB2) in wood formation.

Methods

We overexpressed the three genes as dominant chimeric repressors (DR) by fusing each of them to the EAR transcriptional repression domain. We overexpressed (OE) only EgMYB137 in its native form because transgenic plants overexpressing EgMYB1 and EgMYB2 had already been characterized [4, 5]. Because Eucalyptus spp are very difficult to transform and regenerate, we transformed instead both poplar and Arabidopsis plants. Phenotyping of the transgenics plants was performed at transcriptional, histochemical and biochemical (pyrolysis, Klason, thioacidolysis and saccharification measurements) levels mainly focusing on xylem and SCW characteristics.

Results and discussion

Histochemical analyses of stem sections revealed that both dominant repression and overexpression lines of EgMYB137 exhibited thinner cell walls. Moreover, lignin content was also reduced, especially in DR plants. Similarly to EgMYB137, EgMYB1-DR plants also showed thinner cell walls and reduced lignin content, displaying analogous but stronger phenotypes as compared to the corresponding over-expression plants already characterized [4]. Concerning to the lignin monomeric composition, EgMYB137-DR lines showed a higher S/G ratio. In contrast, no significant changes in S/G ratio were found in EgMYB1-DR plants, which showed, however, a higher content in H lignin units. Despite these differences in lignin composition, both EgMYB137-DR and EgMYB1-DR lines have a significantly higher yield of saccharification and a lower pentoses vs hexoses ratio, likely reflecting a higher proportion of cellulose vs hemicelluloses. However, the cellulose of EgMYB137-DR poplar plants seemed to be more

accessible to hydrolytic enzymes as suggested by a higher sugars release after saccharification.

EgMYB2-DR plants showed an opposite phenotype compared to that reported earlier for the EgMYB2-OE plants [5], showing thinner cell walls and lower lignin content. Moreover, these lines exhibited a higher saccharification yield, probably reflecting the better accessibility to cellulose as a consequence of the decrease in lignin content.

Conclusions

The three MYB genes hereby studied have a strong impact in SCW formation. One of them, EgMYB137, has never been characterized before, neither its potential ortholog in any other plants, and is therefore a new important actor of the complex hierarchical network of SCW regulation. EgMYB137 and EgMYB1 genes act as repressors of SCW formation, with DR plants showing a more dramatic phenotype when compared to the corresponding over-expressors. EgMYB2 is an activator, but the addition of the dominant repression motif had turned it into a repressor. All transgenic lines obtained showed decreased lignin content and increased saccharification yield, making them promising target candidates for biofuel production and pulp industry.

Competing interests

The author declares that they have no competing interests.

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Genome Engineering Improvement for Useful plants of a Sustainable agriculture

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Key words: genetically modified crops; site-specific nucleases; gene targeting

World agriculture needs to guarantee food security, replace fossil resources, decrease its environmental impact and adapt to a changing global climate. Whereas France and other European countries presently choose to meet the genetic aspect of these challenges by the sole use of conventional breeding, an increasing number of agriculturally important countries enlarge the available gene pool via transgenesis. Despite certain political concerns transgenesis is already an indispensible technology for French seed companies and public scientists to remain competitive at an international level.

Recent scientific advances in the field of transgenesis now provide answers to certain reserves of citizens and blur the border between breeding and transgenesis. In particular the advent of nuclease technology opens the way to extremely precise modifications of plant genomes at pre-determined sites. In this context it is strategic to ascertain top-level know-how in transgenesis in France, to actively participate in the debate of these new technologies and to demonstrate their applicability in a wide range of crop species.

If successful, the project **GENIUS (*Genome ENgineering Improvement for Useful plants of a Sustainable agriculture***) will provide French researchers and plant breeders with state of the art know-how, the necessary biological material and connected intellectual property rights for precise genome modifications in a variety of crop and horticultural species (wheat, maize, rice, oilseed rape, tomato, potato, poplar, apple, rose), laying the basis for high throughput functional genomics and efficient plant breeding. Proof of concept will concern disease resistance, salt tolerance, plant architecture and quality traits. Studies on the regulatory, economical and philosophical context will complement the experimental work.

To reach these goals, in an unprecedented effort, GENIUS has assembled a consortium of 15 public and private partners – 10 public research units in biological or social sciences with 5 biotechnology and seed companies. This consortium will create synergy between field- or species-oriented entities into a technology-oriented community.

The project started on September 1st, 2012 and will be developed over a period of 7 years and 4 months.

Competing interests

The authors declare that they have no competing interests.

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Strategic biosafety communication: a new approach

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Key words: biosafety, information, public participation,GMOs

The persistent controversy that surrounds the release of Genetically Modified Organisms (GMOs), confirms that the communication approaches adopted to-date have not achieved the expected goals. It is increasingly being recognized that the success of projects for the safe application of modern biotechnology largely relies on how successfully communication is used as a facilitating tool for public participation and informed decision-making. Experience over the last decades demonstrates that technically well-designed projects can fail or achieve poor results if decision-makers and the civil society are not properly informed, consulted, involved, integrated, and mobilized.
This chapter analyses a number of treaties and other international policy instruments, including official declarations adopted by international conferences, which establish a set of agreed principles connected with the safe application of modern biotechnology. In summary, treaties and official declarations state that (i) the benefits of biotechnology application must be shared, (ii) the risks of GMO release must be evaluated and managed by honest and transparent procedures, and (iii) the right to democratic participation in the GMO-related decision-making process must be enforced.
These principles call for active policies to promote the involvement of the civil society in decision-making about the release and commercialization of GMOs. Public participation should be ensured along the entire decision-making chain, ensuring that all the stakeholders and the public at large have the right to influence all of the steps, from the formulation of national biotechnology policies, to the establishment of national regulatory frameworks, and to the approval of each GMO release.
In this perspective, communication plays a crucial role, even if communication *per se* does not guarantee active public participation. The promotion of public participation in decision-making processes exceeds the function of the communication of risks inherent to GMO release, which is nevertheless an essential component of the risk analysis process, but assumes a broader meaning that can be captured by the proposed term 'strategic biosafety communication'. This term is preferred to the term 'risk communication' also because communication should consider all of the aspects connected with the release of GMOs, including the benefits, and not be narrowed down to the risks. Strategic biosafety communication has a two-pronged objective: to promote consensus on the safe use of GMOs and to contribute to the evaluation and management of the risks related to GMO release and commercialization.
In light of this conceptual and theoretical framework, this work critically reviews different unidirectional or asymmetric communication approaches applied in biosafety programmes and projects and suggests adopting a participatory approach. This approach recognizes knowledge symmetry for all the actors, and adopts an interactive, multidirectional communication model to encourage participation. Open access to all the relevant information is an obvious necessary, but not sufficient, prerequisite for public participation, which can only be achieved if based on reciprocal trust. The information made available should therefore honestly consider the pros and cons and transparently include ambiguities and knowledge gaps. A general challenge is represented by the necessity of reaching all the actors and not only the organized groups, such as NGOs and CSOs. An additional challenge is present in many developing countries, where connectivity and access to media are not widespread. The disclosure of information must be accompanied by the translation of texts written in scientific jargon into packages that are understood by laymen. The design of a

biosafety communication strategy should be based on the analysis through qualitative and quantitative tools of the knowledge, attitudes and practices of different actors involved in the communication process. The participatory approach foresees active measures to solicit and discuss information inputs and opinions, including facts, views, perceptions, fears and believes from all the stakeholders and the wider public. The inputs are subsequently used for making shared decisions. This work discusses the major limits and the most important advantages of the participatory approach for strategic biosafety communication, also in light of direct experience gained in field projects. In synthesis, the approach allows the user to acquire all of the different perspectives, and to improve the risk analysis from one side, and to mediate between contrasting opinions and interests, and to gain as much ownership and consensus on biotechnology application projects as possible. In conclusion, strategic biosafety communication, understood as collective learning and social dialogue, is necessary to close the cycle from information to knowledge construction, from decision-making to social action. It should be noted that public participation in the GMO-related decision-making process can empower communities, user groups, local governments and minority groups and ultimately promote wider participation in sustainable development processes.

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GIS-based models for environmental risk assessment of genetically modified plants: poplar case study in forest ecosystems in Mediterranean environment

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Key words: Risk assessment, GMP, GIS, Populus

Background

Environmental risk assessment (ERA) and ecosystem monitoring associated with genetically modified (GM) organisms is a topic of broad interest. An essential step in the development of products based on genetically modified plants (GMPs) is an assessment of safety, including an evaluation of the potential impact of the crop and practices related to its cultivation on the environment and human or animal health. The literature describes several ways to complete a risk analysis, nevertheless, researches are still needed to implement the EU Directive within a standardized methodological reference framework. Geographical Information Systems (GIS) are a powerful tool for spatial analysis and modeling used to assist the decision-making process. GIS have been considered for ERA and monitoring of GMPs. However, the potential of GIS to assist the ERA as indicated in EFSA is an aspect to be further developed.

The aim of this work was the development of a method to assess the environmental risk generated by transgenic Bt-poplar plants. To this end, four areas of concern are considered: persistence and invasiveness of GMPs, interaction of GM plants with microorganisms, interaction of GM plants with target and non target organisms. In addition, we describe how GIS-based models might be used by risk assessors to support and complement the ERA of GMPs from local scale to landscape level taking into consideration a specific area of concern indicated in EFSA: persistence and invasiveness of the GMPs or its compatible relatives.

Methods

The proposed method integrates the quantitative approach developed by de Jesus et al. [1] with the guidelines on ERA provided by the EFSA [2]. It is performed in two steps: 1) complete a preformatted worksheet to compile the evidence of risks, 2) plot the outcome on the Matrix of Assessment. Potential hazard are grouped according to their source of exposure, along with at least one criterion for assessment of each one.

These items are predetermined on the worksheet to allow for an accurate evaluation of related risks. An index of risk and an index of significance were computed for each potential hazard. The index of risk is calculated as the product of: damage x exposure x precedent; the damage indicates the level or intensity of the impact that the GMP could have on the system, if the proposed adverse effect actually occurs; the exposure is related to the level that some components are exposed to the damage and precedent considers the previous occurrence of the adverse effect, as a consequence of the event. The Index of Significance

takes into account the location where the GMP will be cultivated, the identification and evaluation of potential adverse effects, and the evaluation of the current environmental situation. The indexes were combined using a matrix in order to assess the risk for the environment and the measures required to prevent adverse effects of GMPs.
The study was carried out within the Regional Park of Migliarino, San Rossore, Massaciuccoli (Italy). A GIS was used to collect spatial data for the study area. Topographic maps and thematic layers (soil map, land use map, forest type map, and crop map) were acquired to characterize the environment of the study area and its biodiversity at the landscape level. Aerial remote sensing data and GIS tools were used to assist field plot selection and field works planning to get data on local biodiversity (both plants and animals) in agricultural and forest ecosystems. A Global Positioning System was used to determine the geographic coordinates of the selected plots and of other instruments installed in the field (meteorological stations and pollen traps).
The spatial dataset was used to develop GIS-based models for ERA of GMPs. GM poplar cultivations were simulated in the study area and the environmental risk due to breeding between GM trees and poplar trees in the surrounding forest ecosystems was assessed. To do this, a spatially explicit analysis was performed taking into consideration the simulated distribution of GMPs (poplar), the potential distribution of wild relative species (poplar trees), and gene transfer due to pollen flow. The distribution of wild relative species at the landscape level was obtained extending local data from field plots. Gene transfer was modeled using data from pollen traps and literature data.

Results and Conclusions
We describe one case study on possible effects of *Bacillus thuringiensis* (Bt) poplars in the environment, taking into account data reported in literature and derived from the studied area. The potential hazards characterized in our case-study were related to four areas of interest and were coded with different letters.
The assigned values for the factors of moderation were based on literature data. Moreover, we observed a breeding event in the studied area (potential hazard c) in AREA1) between two poplars: P3 (*P. canescens*, naturally originated poplar trees) x F1.1 (*P. euramericana*). This event has to be taken in account because it could be considered a consequence of gene flow and, in case of genetically modified plants the incorporation of transgenes could result in greater invasiveness or loss of biodiversity with related taxa, depending upon the amount of gene flow from generation to generation and the transgenic trait(s).
Considering the distribution of the “letters” inside the Matrix of Assessment, different risk management could be suggested. The most part of potential hazards analysed does not pose significant risk, so it does not require additional actions. Whereas, potential hazard coded as “n” denotes the effect on target organisms and the potential hazard coded as “c” points out the possible consequence of gene flow, which required some restrictions to monitor the risk.
A map was produced depicting the potential areas where a risk of breeding between GMPs and wild relative species could exist in agricultural and forest ecosystems.
In poplar scenario 1 (Area 1) the gene flow between GM trees and spontaneous poplar in the surrounding ecosystems was investigated as potential source for exposure, considering that the breeding between GMPs and non GMPs represents a potential hazard reducing the genetic diversity of wild population.
In poplar scenario 2 (Area 2) the environmental risk due root exudates released by transgenic poplar was investigated as potential source for exposure (Area 2), considering as potential hazard that root exudates might affect the rhizosphere and soil community. To map such a risk, which is a consequence of risk investigated in poplar scenario 1, we

supposed that gene transfer from GMPs to soil micro-organisms occur in those areas where a risk of breeding between GMPs and its wild relatives exist.
In poplar scenario 3 (Area 3) the interactions of the GM poplar cultivations with TOs (larvae of Diptera and Lepidopteran) were examined. The level of mitigation of this issue requires some management measure. These would be developed together with the measures required to mitigate the risk of breeding considering that these two potential hazards have the same distribution.
In poplar scenario 4 (Area 4) the interactions of the GM poplar cultivations with NTOs (micro-arthropods in the soil) were addressed. The assessment of this issue for the study area indicate that no restrictions are required to mitigate the potential adverse effects of GM poplar cultivations on NTOs
Our results show how GIS might be used to assess the spatial interaction between GM plants and the receiving environment at the landscape level using data collected at the local scale. Additionally, our results show how geographic data might be used to select checkpoints for monitoring of GM crops. These results are important to further develop existing guidance on the environmental risk assessment and monitoring of GMPs according to the EU Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms.

Competing interests
The author declares that they have no competing interests.

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