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Insights into temperature modulation of the *Eucalyptus globulus* and *Eucalyptus grandis* antioxidant and lignification subproteomes

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ABSTRACT

Eucalyptus grandis and *Eucalyptus globulus* are among the most widely cultivated trees, differing in lignin composition and plantation areas, as *E. grandis* is mostly cultivated in tropical regions while *E. globulus* is preferred in temperate areas. As temperature is a key modulator in plant metabolism, a large-scale proteome analysis was carried out to investigate changes in the antioxidant system and the lignification metabolism in plantlets grown at different temperatures. Our strategy allowed the identification of 3111 stem proteins. A total of 103 antioxidant proteins were detected in the stems of both species. Hierarchical clustering revealed that alterations in the antioxidant proteins are more prominent when Eucalyptus seedlings were exposed to high temperature and that the superoxide isoforms coded by the gene Eucgr.B03930 are the most abundant antioxidant enzymes induced by thermal stimulus. Regarding the lignin biosynthesis, our proteomics approach resulted in the identification of 13 of the 17 core proteins involved in this metabolism, corroborating with gene predictions and the proposed lignin toolbox. Quantitative analyses revealed significant differences in 8 protein isoforms, including the ferulate 5-hydroxylase isoform F5H1, a key enzyme in catalyzing the synthesis of sinapyl alcohol, and the cinnamyl alcohol dehydrogenase isoform CAD2, the last enzyme in monolignol biosynthesis. Data are available via ProteomeXchange with identifier PXD005743.

1. Introduction

According to the Food and Agriculture Organization of the United Nations (FAO), planted forests correspond to a global area of approximately 264 mi hectares, most of them comprising Pinus and Eucalyptus species (Del Lungo et al., 2006). Within the economically important Eucalyptus plants, *Eucalyptus grandis* is probably one of the most planted and economically important species due to its fast growth rate and adaptability to different climate conditions. Recently, the landmark genome sequencing of a woody perennial organism was accomplished (Myburg et al., 2014) paving the way to explore the genetic information regarding the growth and the metabolic responses in *E. grandis* and in closely related species.

After water deficit, high and low temperatures are among the most influential stresses limiting plant productivity (Boyer, 1982; Cramer et al., 2011). Although not representing the first line of responses against thermal stress, reactive oxygen species (ROS) generation is a common phenomenon used against plant abiotic stresses. As the production of ROS at high levels may negatively affect the cellular machinery, it has to be rapidly degraded to counteract potential damages induced by those unstable molecular species. Common enzymatic scavengers of plant ROS are well described and include the enzymes superoxide dismutase, ascorbate peroxidase, glutathione peroxidase and catalase (Apel and Hirt, 2004; Das and Roychoudhury, 2014; Foyer, 2005).

Due to their crucial impact on plant metabolism, temperature regions are a worldwide and locally limiting factor for crop plantations, including planted forests. Eucalyptus plant distribution is based on climate zones, where the species *E. grandis* and its hybrids are mostly cultivated in tropical regions and the species *Eucalyptus globulus* is preferred for cultivation in temperate areas (Potts, 2004; Dasgupta et al., 2015). As the latter is a cold tolerant hardwood tree with major economical importance, the molecular responses induced by low temperature have been explored. Using a qRT-PCR strategy, Fernández et al. (2010) observed a differential regulation

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of the genes ELIP (early light-inducible protein), NCED (9-cis-epoxycarotenoid dioxygenase) and GS (galactinol synthase) in *E. globulus* plants exposed to cold. Gamboa et al. (2007) isolated a CBF (cold-binding factor) cDNA clone from *E. globulus* plants after exposure to low environmental temperatures, while Fernández et al. (2012) observed the upregulation of three dehydrin genes during cold acclimation. Although these targeted gene expression studies have been used to understand *E. globulus* responses to thermal stress, there are no reports on large scale technologies used to unveil this phenomenon.

In addition to the low-temperature tolerance trait, *E. globulus* plant cell walls differ from other *Eucalyptus* species as its lignin polymer presents a different composition in terms of the *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) subunits. As the ratio of S (doubly methylated, on the 3- and 5-hydroxyl moieties) to G (singly methylated, on the 3-hydroxyl group moiety) subunits dictates the degree of lignin condensation, the high S/G ratio in *E. globulus* lignin is an attractive feature for cell wall degradation during paper and pulp production (Araújo et al., 2014a, 2014b; Bhuiya and Liu, 2010; Araújo et al., 2014b). However, a deep understanding of all steps involved in Eucalyptus lignin biosynthesis is essential prior to any attempts to engineer plant cell walls in this genus. Recently, Carocha et al. (2015) carried out a deep analysis of the core genes involved in the lignin biosynthesis of the closely related species *E. grandis*, using RNA-seq high-throughput expression profiling combined with real-time quantitative PCR.

Due to its potential to provide insights into the proteins that were effectively decoded from the plant's genome, shotgun proteomics technologies were used here to study changes in the gene expression of *E. globulus* and *E. grandis* induced by cultivation in different temperatures, with a focus on profiling ROS-related proteins and lignin plant cell wall-related enzymes.

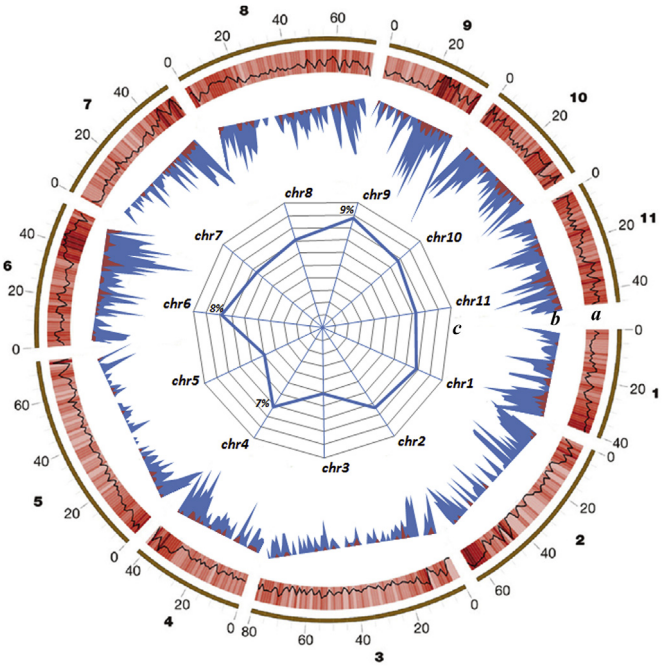

Fig. 1. Correlation between proteomics data and the *Eucalyptus grandis* genome structure as described by Myburg et al. (2014). Chromosome identities are indicated by the outermost numbers. **a**, gene density per 1 megabase (Mb) range; **b**, expressed gene density (number of identified proteins, blue peaks) and expressed gene variants (number of identified isoforms per locus, red peaks) per 1 Mb range; **c**, proteome-based genomic coverage for each proposed chromosome. Figure adapted from Myburg et al. (2014). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Results and discussion

2.1. Proteome data correlates with Eucalyptus grandis genomic architecture

Advances in large-scale nucleotide sequencing have tremendously increased the acquisition and the exploitation of plant proteome information. Although peptide *de novo* sequencing is a powerful and database-independent approach, fragment spectral mining against reference databases is still the most widely adopted strategy in the proteomics field. *Eucalyptus* genome sequencing initiatives were first launched in the early 2000s and set a milestone for forest tree biotechnology: the *E. grandis* draft genome data, a 640-megabase sequence, was assembled into 11 main chromosome linkage groups (Myburg et al., 2014). This high-quality reference is a valuable genetic resource for studies involving gene expression. As proteomics stands out from other large-scale approaches due to its capacity to target the final products of the genome information path, we firstly attempted to correlate protein abundance profile, obtained from gene encoded proteins identified herein (Tables 1S–30S), with the chromosome gene density pattern proposed by Myburg et al. (2014). The gene expression profile, in terms of the number of identified proteins, corroborated the gene density pattern for each of the 11 Eucalyptus chromosomes (Fig. 1).

The highest number of translated proteins, within a 1 megabase (Mb) genomic range, was detected in chromosomes 6 and 9, corroborating the high gene density suggested for upstream positions of those chromosomes. Additionally, the 1 Mb expression profile ranges from proteins encoded by chromosomes 3 and 5 also reflected the gene distribution suggested for those large but gene-poor chromosomes. Genomic positions of the genes coding for the protein isoforms are also in accordance with gene density patterns for all suggested chromosome linkage groups, where high gene density regions tend to express the highest number of genomic variants. Genomic coverage, in terms of identified gene products, reflected the gene distribution across *Eucalyptus* chromosomes. As a consequence of the physicochemical properties of proteins and the analytical strategies we used, the highest sequence coverage was achieved for chromosomes harboring the highest number of gene sequences (chromosomes 1, 9 and 10), while chromosomes 3 and 5 contained the lowest number of identified genes. Overall, proteomics-based gene expression analysis corroborated with the genomic architecture and the gene spatial distribution suggested by Myburg et al. (2014), providing proper support for comparative proteome analyses between the Eucalyptus species described here.

2.2. Eucalyptus antioxidant sub-proteome is regulated by thermal stimulus

Reactive oxygen species (ROS) are key elements in plant stress responses. Ascorbate peroxidase (APX), glyoxalase (GLX), glutathione peroxidase (GPX), peroxiredoxin (PRX), superoxide dismutase (SOD), thioredoxin (TRX) and catalase (CAT) are the most common antioxidant enzymes in plant cells (Foyer, 2005; Hasanuzzaman et al., 2012; Choudhury et al., 2013). In Eucalyptus species, *E. dunnii* plants grown at 4 °C showed an increase of 25% in CAT activity after 3 h of stress exposure (Liu et al., 2014). An increase in the expression of CAT was confirmed through a large-scale transcriptome analysis where the authors detected a two-fold increase in CAT-related transcripts after 24 h of treatment. Using shotgun proteomics coupled with stringent database mining against *E. grandis* genome coded proteins, we identified 103 ROS-

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