Research Article

Eucalyptus urograndis stem proteome is responsive to short-term cold stress

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Abstract

Eucalyptus urograndis is a hybrid eucalyptus of major economic importance to the Brazilian pulp and paper industry. Although widely used in forest nurseries around the country, little is known about the biochemical changes imposed by environmental stress in this species. In this study, we evaluated the changes in the stem proteome after short-term stimulation by exposure to low temperature. Using two-dimensional gel electrophoresis coupled to high-resolution mass spectrometry-based protein identification, 12 proteins were found to be differentially regulated and successfully identified after stringent database searches against a protein database from a closely related species (*Eucalyptus grandis*). The identification of these proteins indicated that the *E. urograndis* stem proteome responded quickly to low temperature, mostly by down-regulating specific proteins involved in energy metabolism, protein synthesis and signaling. The results of this study represent the first step in understanding the molecular and biochemical responses of *E. urograndis* to thermal stress.

Keywords: abiotic stress, mass spectrometry, omics, proteomics.

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Introduction

Eucalyptus is one of the most important plant genera used in the pulp and paper industry. This genus contains ~700 species that originated in Australia and Indonesia, in addition to several hybrids developed to exploit different plant traits. Currently, *Eucalyptus* plants are cultivated worldwide because of their rapid adaptability to different climatic conditions and easy use in plant breeding programs. The most widely used species in forest plantations and breeding programs are *Eucalyptus grandis*, *Eucalyptus globulus*, *Eucalyptus urophylla* and *Eucalyptus camaldulensis*. In addition to these species, several intra- and interspecific hybrids that combine important traits from both parental lines have been successfully bred. *Eucalyptus urograndis* is one of the most important interspecific hybrids because it combines the rapid growth of *E. grandis* and the disease/climate tolerance of *E. urophylla* (Kullan *et al.*, 2012). This species is currently the hybrid preferred by the Brazilian pulp industry and is consequently the mostly propagated species in commercial forest nurseries in this country.

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Although *E. urograndis* has been successfully adapted to the Brazilian climate, adult trees and young plants are subject to metabolic alterations induced by fluctuations in the growth conditions. The projected increase in the worlds average temperature may result in a new global scenario in which plants may have to cope with erratic temperature patterns of unseasonable warm/cold spells; untimely deacclimation may also lead to freeze damage (Gu *et al.*, 2008; Pagter and Williams, 2013).

Large-scale gene expression profiling provides a robust, high-throughput means of detecting important genes and of guiding plant breeding programs to improve specific plant traits, such as thermotolerance. Proteomics, the large-scale study of the proteins present in a particular biological system, is a powerful multi-disciplinary approach that focuses on the characterization of biological molecules that are synthesized as the final product of gene expression. Information regarding the differential regulation of protein(s) may therefore be useful for monitoring the metabolic responses of plants to environmental disturbances and assisting in the genetic transformation of target plant species. Despite their importance for the pulp industry, few studies have examined the proteome of *Eucalyptus* species (Celedon *et al.*, 2007; Bedon *et al.*, 2011, 2012; Britto *et al.*, 2012; Valdés *et al.*, 2013), with most of them investigating the changes induced by drought stress (Bedon *et al.*, 2011, 2012; Valdés *et al.*, 2013). In this work, we examined the

response of young *E. urograndis* plants to low temperature and identified stem proteins that are potentially related to cold stress responses in this hybrid plant.

Materials and Methods

Plant material

Young *E. urograndis S. T. Blake* plants (clone PL-3335) were obtained from a local nursery and cultivated in the greenhouse of the Department of Technology at the State University of São Paulo (Jaboticabal, SP, Brazil). After one month of growth, the plants were transferred to growth chambers with a 12 h photoperiod and a temperature of 15 °C for exposure to cold and 30 °C for normal conditions.

Protein extraction

After 24 h of cultivation, *E. urograndis* stems were isolated and stored at -80 °C until analysis. Protein extracts were obtained by mixing 1.5 g of tissues with 4 mL of extraction media containing 500 mM Tris, 50 mM EDTA, 700 mM sucrose and 100 mM KCl (pH 8.0). After 5 min of incubation at 4 °C, 4 mL of equilibrated phenol (Sigma-Aldrich) was added to the solution and the mixture was stirred for 15 min prior to centrifugation (4000 x *g*, 10 min, 4 °C). After centrifugation, the phenol fraction was collected, transferred to a new tube and five volumes of cold methanol containing 100 mM ammonium acetate was added. Protein precipitation was done for 12 h and the pellet was resuspended in 300 μL of sample buffer (125 mM Tris-HCl, 1% SDS, 1% dithiothreitol and 20% glycerol) after centrifugation (4000 x *g*, 15 min; 4 °C). Protein quantitation was done with the Bradford assay using bovine serum albumin as the standard.

Two-dimensional electrophoresis

For two-dimensional (2D) electrophoresis, 100 μg aliquots of proteins were precipitated from the protein extract by adding five volumes of cold acetone followed by incubation for 2 h at -20 °C. After centrifugation (4000 x *g*, 10 min, 4 °C) the protein pellets were resuspended in 150 μL of rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 1% IPG buffer, 0.002% bromophenol blue) followed by passive sample loading onto IPG-gel strips (7 cm, 4-7 pH gradient) for 12 h. Isoelectric focusing was done using an Ettan IPGPhor 3 system (GE Healthcare) for 20 h and terminated after accumulating 37 kVh. Prior to separation by SDS-PAGE, proteins were reduced (6 M urea, 75 mM Tris-HCl, 30% glycerol, 2% SDS, 0.002% bromophenol blue and 125 mM dithiothreitol) and alkylated (6 M urea, 75 mM Tris-HCl, 30% glycerol, 2% SDS, 0.002% bromophenol blue and 125 mM iodoacetamide) for 30 min at room temperature. Vertical electrophoresis was done in a discontinuous buffer system for 2 h at 100 V. After SDS-PAGE, protein spots were visualized by staining with Coo-massie colloidal G-250 for 12 h followed by gel destaining for 3 h using a 30% methanol solution.

Detection of differentially regulated proteins

The 2D gels were analyzed using the program Image Master Platinum v.7 (GE Healthcare). Gel images were acquired in transparent mode using a green color filter. Spots were detected automatically using the parameters smooth, minimum area and saliency. Spot matching was done by using only one landmark with manual correction if necessary. Differential regulation was detected based on the normalized spot volume (%V) parameter. Spot matches between control and treatment gels with a two-fold difference in the %V that was significant ($p < 0.0.5$) in pair-wise analyses using Students *t*-test were considered to be differentially stained and, consequently, selected for mass spectrometry analysis. Supplementary data from the 2D gel image analyses is provided in Table S1.

Identification of proteins by tandem mass spectrometry

Prior to the mass spectrometric analyses, proteins were digested *in gel* according to Shevchenko *et al.* (2007). Peptides were introduced in the mass spectrometer after separation by reverse phase chromatography using an EASY-nLC 1000 system (Thermo Scientific). After loading onto the analytical column, separation of the tryptic peptides was done using a C18 nano-column (15 cm, 2 μm, 100 Å) for 60 min at a flow rate of 300 nL/min. Mass spectrometric analyses were done in a Q-Exactive instrument (Thermo Scientific) using the data-dependent acquisition method for the ten most abundant peptide ions. Fragment spectra were acquired after HCD (high-energy collision dissociation) fragmentation of the isolated peptide ions under a stepped normalized collision energy equal to 35% (±5%). Spectral correlation was done using the Sequest search tool contained in the Proteome Discoverer software (Thermo Scientific) and run against the *E. grandis* protein database available for download from the Phytozome portal (Myburg *et al.*, 2014). The search parameters were adjusted for an error tolerance equal to 0.02 Da for the fragment ions and 10 ppm for the parent ions. Oxidation of methionines and carbamidomethylation of cysteines were selected as dynamic and static modifications, respectively, during database searches. Peptide-spectrum matches were considered significant if they had a cross-correlation score ≥1.5, 2.0 or 2.5 for singly, doubly or triply-charged ions, respectively.

Results and Discussion

In this study, we used proteomics to obtain information about the *E. urograndis* biochemical responses to short-term (24 h) cold stimulus. For this, we initially compared the total protein content between plants grown at a

low temperature (15 °C) and those grown at 30 °C. As expected, there were no major changes in protein accumulation after 24 h of exposure to low temperature. Using the extraction procedure described here, protein yields of 0.36 ± 0.09 mg/g (mean ± SD) and 0.45 ± 0.06 mg/g of fresh tissue were obtained for stems of plants grown at 30 °C and 15 °C, respectively.

To gain insight into the fine biochemical alterations that could affect growth and wood biosynthesis, the stem proteome was analyzed using 2D gel electrophoresis applied to young *E. urograndis* plants grown under both conditions. A subsequent comparative analysis detected 12 protein spots with significant densitometric variation (Figure 1, Table 1). Tandem mass spectrometric analyses followed by database searches using a closely related *Eucalyptus* species resulted in the successful identification of all proteins from the selected gel spots. The differential regulation of proteins from a wide variety of cellular functions indicates a complex regulatory network in *Eucalyptus* cells in response to cold stress. Down-regulation of the proteins fructose bisphosphate aldolase (Eucgr.K02073.1, spot 16), glyceraldehyde 3-phosphate dehydrogenase (Eucgr.I01564.1, spot 93) and NADH ubiquinone oxidoreductase (Eucgr.A02717.1, spot 98) suggested a reduction in the energy-related metabolism of *E. urograndis* stems shortly after cold stress induction.

Low temperature stress also decreased the expression of proteins involved in gene transcription, such as the protein Eucgr.K02072.1, described as a transcription factor GT-2 containing a trihelix DNA-binding/SANT domain (spot 87). InterPro and Prosite prediction analyses revealed the presence of a myb-like domain from residue 98 through residue 163 of this differentially regulated protein (Figure 2A). A novel myb transcription factor was recently reported to enhance cold tolerance in *Arabidopsis* (Su *et al.*,

2014). Up-regulation of myb-related transcripts/proteins upon cold stress has also been identified in other plants, such as *Anthurium* sp. (Tian *et al.*, 2013) and *Lilium lancifolium* (Wang *et al.*, 2014). Although not extensively described, a correlation between myb proteins and cold stress has already been reported for *Eucalyptus*. Keller *et al.* (2009) identified myb-like proteins as the most abundant transcription factors in *E. gunnii* leaves exposed to cold stress. Large-scale transcript analysis of *E. grandis*, *E. globulus* and *E. urophylla* xylem tissue indicated a differential expression of myb-like proteins across these *Eucalyptus* species (Salazar *et al.*, 2013). In the present work, the protein Eucgr.K02072.1 identified in young *E. urograndis* stems was down-regulated after short-term cold exposure.

In addition to the suggested role in plant responses to cold stress, over-expression experiments indicate that myb transcription factors may also regulate lignin genes, such as cinnamoyl-coenzyme A reductase and cinnamyl alcohol dehydrogenase (Goicoechea *et al.*, 2005; Barakat *et al.*, 2009; Legay *et al.*, 2010), thereby affecting cell wall thickness and plant growth. Multiple sequence alignments of the *E. urograndis* protein Eucgr.K02072.1 against the 287 *E. grandis* genes reported to contain myb-like motifs revealed high sequence similarity between this protein and the *E. grandis* transcribed genes containing an alcohol dehydrogenase transcription factor motif (Eucgr.H04383.1, Eucgr.A01571.1, Eucgr.J02994.1, Eucgr.A00307.1 and Eucgr.C01064.1 in Figure 2B). This result suggests that if the down-regulated protein Eucgr.K02072.1 is capable of binding to a cinnamyl alcohol dehydrogenase DNA sequence then it may directly affect lignin biosynthesis in *E. urograndis* stems or could play a role in the NADH/NAD$^+$ balance, and thus in the cell redox status, in order to counteract the imposed temperature stress (Hashida *et al.*, 2009). Currently, one of the best known plant regulatory systems active during cold sensing and acclimation is the C-repeat binding factor (CBF) pathway. In *Eucalyptus*, two *CBF* genes have recently been isolated from *Eucalyptus*
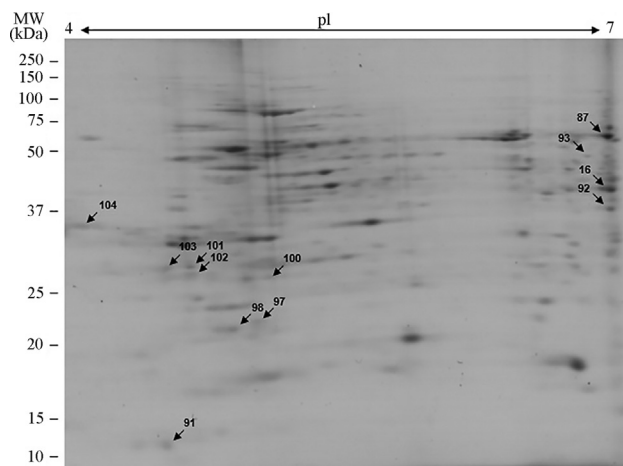

Figure 1 - Representative 2D protein gel obtained for *E. urograndis* stems. Protein spots selected for mass spectrometry-based identification are marked with an arrow and number, as in Table 1. Molecular mass markers are indicated to the left of the gel.
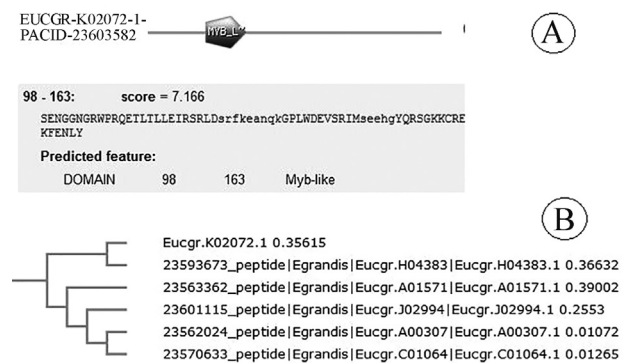

Figure 2 - Structure and molecular phylogeny analysis for the *E. urograndis* protein Eucgr.K02072.1 (A) ScanProsite output. Predicted myb-like domain profile extends from amino acid residue 98 through residue 163. (B) Neighbor-joining tree branch containing the *E. urograndis* protein Eucgr.K02072.1 and the closely-related *E. grandis* sequences.

Table 1 - List of differentially regulated proteins identified in the stems of young *E. urograndis* plants after growth at low temperature for 24 h.

Spot[a]	Identifier[b]	Description[c]	Score[d]	Coverage[e]	Peptides[f]	Expression profile[g]
16	Eucgr.K02073.1	Fructose-bisphosphate aldolase	43.23	34.92	GTVELAGTNGETTQGLDGLAQR-IGPTEPSQLAINENANGLAR-GILAADESTGTIGKR-LSSINVENVEENRR-GILAADESTGTIGK-VAPEVIAEYTVR-LSSINVENVEENR-	
87	Eucgr.K02072.1	Transcription factor GT-2 and related proteins, contains trihelix DNA-binding/SANT domain	31.80	27.61	TKVVGVDTSGDIVK-AEEDGVAcVEFIAGK-	
91	Eucgr.C04037.1	Membrane-associated progesterone receptor component-related/cytochrome b5-like heme/steroid binding domain	2.40	14.71	SFYGPGGAYAAFSGK-	
92	Eucgr.B00856.1	Receptor for activated protein kinase (RACK1)	6.56	12.54	AHTDmVTAIAVPIDNADmIVTSSR-FSPNTLQPTIVSASWDR-	
93	Eucgr.I01564.1	Glyceraldehyde 3-phosphate dehydrogenase	3.12	3.73	AAALNIVPTSTGAAK-	
97	Eucgr.D02028.2	Pentapeptide repeats (8 copies)	5.15	10.37	mVLNEANLTNAVLAR-ESDFSGSTFNGAYLEK-	
98	Eucgr.A02717.1	NADH-ubiquinone oxidoreductase-mitochondrial inner membrane	7.55	8.88	QTTGIVGLDVVPNAR-	
100	Eucgr.H03609.3	Endo-1, 3-1, 4-β-D-glucanase/dienelactone hydrolase	3.49	9.44	LAGTEDIQAAVILHPGR-	
101	Eucgr.F02130.2	14-3-3 protein	23.79	31.25	AAQDIAQADLASTHPIR-LVVGWTPAAELTVEER-LGLALNFSVFYYEILNQSDK-	

Table 1 (cont.)

Spot[a]	Identifier[b]	Description[c]	Score[d]	Coverage[e]	Peptides[f]	Expression profile[g]
102	Eucgr.J00743.1	RNA recognition motif (RPM, RBD or RNP domain)	2.70	6.07	SAITGGAASSSLAPR-	
103	Eucgr.F02130.2	14-3-3 protein	20.93	28.37	AAQDIAQADLASTHPIR-LLDTNLVPSAAASESK-IVSSIEQKEEGR-VENELSDVcASILR-	
104	Eucgr.I01037.1	Serine protease family S10 serine carboxypeptidase	62.42	19.87	LPFTLETGYIGVNEIDDVQLFYFIESQR-mELQGYLLGNPVTDDFIDTNSR-NILEGIEAGVRPK-VNLISDEYYEDAK-	

[a]Spot number as shown in Figure 1. [b]Description or transcript name in the Phytozome portal. [c]Functional annotation associated with the identifier. [d]Sequest protein score. [e]Protein coverage (%). [f]Identified peptides that support the protein identification. [g]Expressed as % of the spot volume (left column: 15 °C; right column: 30 °C treatment; columns represent the mean ± SD (n = 3)).

gunnii, with both containing myb-like recognition motifs and ABA-responsive elements (Navarro *et al.*, 2009).

Apart from the differential regulation of a myb-like transcription factor, a protein identified as a receptor for activated C kinase (RACK) was also down-regulated in *E. urograndis* stems during cold stress (spot 92). In *Arabidopsis*, RACK1 genes seem to negatively regulate ABA responses by direct interaction with the eukaryotic initiation factor 6, a key regulator of the 80S ribosome assembly (Guo *et al.*, 2009a,b, 2011). Although the mechanisms that mediate ABA/RACK1 stress responses are still unclear, Speth *et al.* (2013) suggested that RACK1 could interfere in ABA related responses by repressing or stimulating the synthesis of microRNAs. A direct correlation between RACK1 and temperature adaptation has been proposed by Ullah *et al.* (2008) who reported that *Arabidopsis* plants exposed to elevated temperature showed a reduction in RACK1 protein expression. In addition, the status of sumoylation, a post-translational protein modification (PTM) of RACK1, could play an important role in the heat stress responses (Ullah *et al.*, 2008). As shown here, exposure to low temperature for only 24 h reduced the expression of RACK1 in *E. urograndis* stems, thereby corroborating a possible role for this protein in temperature-sensing signaling pathways.

Another *E. urograndis* stem protein that was down-regulated upon short-term cold stress was Eucgr.F02130.2, described as a 14-3-3 protein. Using a data mining approach, Furtado *et al.* (2007) identified four 14-3-3-like genes in the *E. grandis* genome. Large-scale transcript analysis detected 46 14-3-3 transcripts in juvenile xylem tissues of *E. grandis* (Carvalho *et al.*, 2008) and, using a 2D electrophoresis approach, Celedon *et al.* (2007) detected the up-regulation of five 14-3-3 protein spots in juvenile xylem in relation to three- and six-year old tissues. Interestingly, in *E. urograndis* stems, this protein was detected as down-regulated with the same expression profile in two distinct spots (101 and 103). The identification of the same protein in different spots may be explained by the existence of proteolytic cleavage products in the protein extract or by the presence of PTMs in the identified protein.

To examine the possible covalent attachment of protein Eucgr.F02130.2 to a phosphate group, we remined the tandem mass spectra acquired for spots 101 and 103 and compared them against the same protein database with a phosphorylation mass shift (+79.966 Da) set as a dynamic modification. Using this strategy, the peptide AAQDIAQADLASTHPIR was detected in both protein spots and possibly phosphorylated in spot 101 while not phosphorylated in spot 103 (Figure 3). Although the detection of this unique phosphopeptide could not explain the isoelectric mass shift observed between these spots (Figure 1), it was still strong evidence that Eucgr.F02130.2, described as a 14-3-3 protein, is a potential phosphorylation substrate in young *E. urograndis* stems exposed to cold
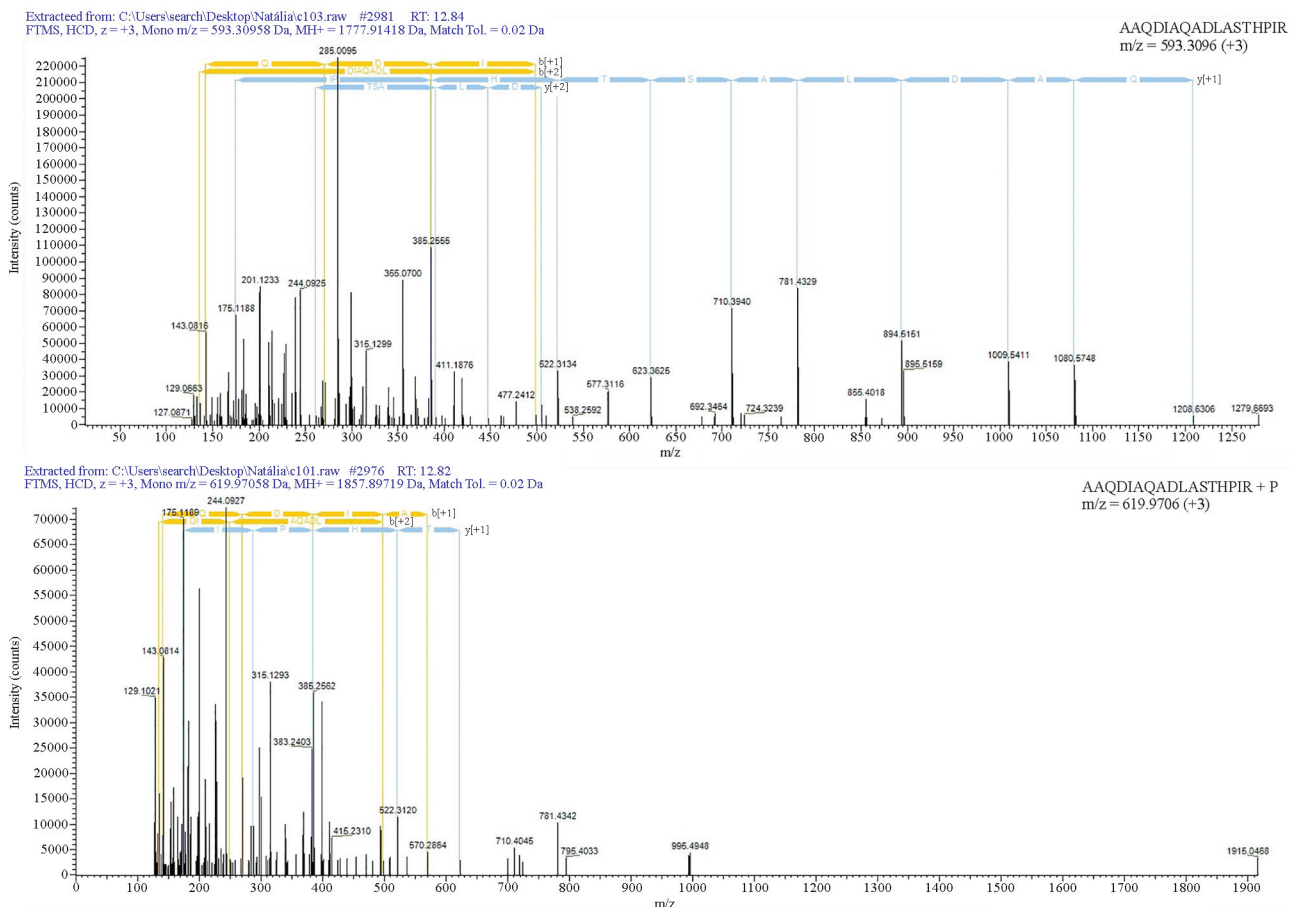
Figure 3 - Tandem mass spectra of the peptide AAQDIAQADLASTHPIR. The upper panel shows the nearly complete fragmentation pattern (with the acquisition of almost all y-ion series) of the non-phosphorylated precursor ion with m/z equal to 593.3096 (+3). The lower panel shows the fragmentation pattern of the same precursor ion with m/z equal to 619.9706 (+3).

stress. Despite the existence of transcript and protein evidence for the involvement of 14-3-3 proteins in xylem development, there have been no reports on the involvement of 14-3-3 proteins, or of their differential PTM status, in the *Eucalyptus* cold response.

Serine carboxylase-like proteins have traditionally been associated with protein turnover, although recent studies have shown that serine carboxylases share extensive sequence similarity with acyltransferases (Vogt 2010; Sasaki *et al.*, 2013). The *E. urograndis* down-regulated protein described as a serine carboxypeptidase1 (accession Eucgr.I01037.1) shared 49% similarity with the protein glucose acyltransferase from *Solanum pennellii* and *S. berthaultii* and 47% with a putative acyl-glucose-dependent anthocyanin acyltransferase from *Delphinium grandiflorum*. Acyl-glucose dependent anthocyanin acyltransferase catalyzes the acylation of anthocyanin pigments, one of the final steps in anthocyanin biosynthesis (Matsuba *et al.*, 2008). Acylation of anthocyanin is thought to assist in the stabilization of this pigment (Cheynier *et al.*, 2006) and play a major role in tonoplast transportation (Gomez *et al.*, 2009). The potential down-regulation of an

anthocyanin acyltransferase suggests a reduction in anthocyanin vacuole intake and is likely related to a decrease in the cytoplasmic biosynthesis of this secondary metabolite. This hypothesis agrees well with the proposed reduction in *E. urograndis* energy-related metabolism, as also indicated by the down-regulation of fructose bisphosphate aldolase (spot 16), glyceraldehyde 3-phosphate dehydrogenase (spot 93) and NADH-ubiquinone oxidoreductase (spot 98) noted above, and the down-regulation of the myb-related protein spot (spot 87). In some plant species, myb transcription factors have been implicated in anthocyanin biosynthesis and storage (Cutanda-Perez *et al.*, 2009; Li *et al.*, 2012; Singh *et al.*, 2014).

Eucalyptus urograndis stems also showed inhibition of the expression of proteins related to cell division and elongation. Gel spot 91, which contained the protein identified as cytochrome b5-like heme/steroid binding domain protein (Eucgr.C04037.1), showed down-regulation upon short-term cold treatment. In *Arabidopsis thaliana*, over-expression of the membrane steroid binding protein 1 resulted in shortened hypocotyls, suggesting a close relationship between cell elongation and steroid binding proteins

(Yang *et al.*, 2005). In addition, the *Arabidopsis rlf-1* (reduced lateral root formation) mutant showed reduced cotyledon growth and cell division inhibition, indicating that the *RLF* gene, which encodes a protein with a cytochrome b5-like heme/steroid binding domain (At5g09680), positively modulates cell division (Ikeyama *et al.*, 2010). In accordance with these studies, the protein endo-1,3;1,4-β-D-glucanase, reported to be involved in cell elongation (Zhu *et al.*, 2006; Guillaumie *et al.*, 2008; Komatsu and Yanagawa, 2013), was found to be down-regulated in *E. urograndis* stems after short-term exposure to low temperature stress; this finding corroborated the hypothesis of a negative relationship between cold stress and cell elongation in *E. urograndis* stems.

Conclusions

In this work, we used a proteomics approach to identify proteins potentially involved in the *E. urograndis* cold stress response. Despite using a low-throughput/low sensitivity proteomics strategy it was possible to detect the differential regulation of 12 proteins potentially involved in a quick metabolic response of *E. urograndis* stems after short-term exposure to low temperature. The proteins identified here could be useful markers for monitoring cold stress in this species or as potential targets in molecular-based programs aimed at expanding the use of this species in forest plantations.

Acknowledgments

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Internet Resources

Phytozome joint project at http://www.phytozome.net (February, 2014).

Supplementary Material

The following online material is available for this article:
Table S1 - Experimental data for *E. urograndis* stem protein isolation and 2D gel analysis.
This material is available as part of the online article from http://www.scielo.br/gmb.

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