GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Oxidative stress proteins as an indicator of a low quality of eucalyptus clones for the pulp and paper industry

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Genet. Mol. Res. 11 (4): 3798-3813 (2012)
Received January 18, 2011
Accepted May 14, 2012
Published August 17, 2012
DOI http://dx.doi.org/10.4238/2012.August.17.13

ABSTRACT. *Eucalyptus* is a genus widely cultivated in many tropical and subtropical regions of the world as one of the main sources of raw materials for the pulp and paper industry. Identification of clones and selection of genotypes with desirable agronomic characteristics would be useful. We assessed eucalyptus full-sibs that varied in wood quality, using a combination of two-dimensional gel electrophoresis and mass spectrometry to identify differentially expressed proteins as candidates for quality markers. Thirty-one differently expressed proteins were identified, including three proteins of clone X1, four of clone X2, and 12 each of clones X3 and X4. These proteins are involved in various biological processes, including polyphosphate biosynthesis, catalytic activity, nucleotide excision repair, cellular metabolic processes, cell redox homeostasis, response to salt stress, response to temperature,

oxidation and reduction processes, cellular water homeostasis, and protein phosphorylation. In the cambial region of each clone, the proteins ketol-acid reductoisomerase, uncharacterized protein MG428, receptor-like serine/threonine-protein kinase and a heat shock protein were found in larger quantities in clone X4 than in clone X1. These proteins are known to be related to protection against oxidative stress and biosynthesis of lignin. A high buildup of proteins involved in response to stress in the cambial region of eucalyptus would indicate clones with undesirable characteristics for use in the pulp and paper industry.

Key words: Differential proteins; 2-D PAGE; NanoLC-ESI-Q-TOF; Heat shock proteins

INTRODUCTION

Wood is globally used in multiple forest-based industries, which generate millions of work fronts and billions of dollars in income, involving pulp and paper, solid wood products, and other products. As it represents the main source of biomass on Earth, it is estimated that it also carries out an important function as an alternative raw material for renewable and environmental production of biofilms, solid wood and fiber, and serves as the largest deposit of excess atmospheric CO_2 (Plomion et al., 2001). Currently, plant fibers represent the main source of raw material for the production of cellulosic mass, most of which - approximately 95% of world production - is obtained from the trunks of trees. The genus *Eucalyptus* is the main source of wood worldwide and more widely used in industrial plantations in Brazil. The area of eucalyptus forests is booming in most Brazilian states with tradition in the forestry of this group of species, or in states considered as new borders of forestry, with cumulative growth of 23.0% between 2005 and 2010 in the country (ABRAF). The choice of this genus is determined by the type of soil and by tropical climate that favors its rapid growth (D'Almeida, 1988). In 2009, Brazil became the world's fourth largest manufacturer of cellulose, surpassing traditional producer countries such as Finland and Sweden (ABRAF).

The selection of elite clones of eucalyptus trees has prompted research on the characterization of the xylogenesis process, at which point the genes expressed and the resultant proteins will determine the chemical and physical properties of the wood. Chemically, the structural macromolecular constituents (cellulose, hemicellulose and lignin) and the extractives making up the wood (Morais et al., 2005) and their proportions in the tissue directly influence the industrial process, where they are utilized. In the production of pulp and paper, higher levels of cellulose and hemicellulose are considered essential, while the same cannot be said for the extractive and lignins, which are ideal in the furniture and fuel industries. Substances of typically lipophilic nature, called extractives, are undesirable for the production of cellulose because they reduce the yield and quality of pulp, in addition to causing great damage to the industries during the different processing stages, leading to the formation of pitch, which is waste that deposits and accumulates in the machinery and also contaminates the products (Barbosa et al., 2005). The same happens with the lignin content that directly affects the conversion of wood into cellulose kraft, because it impacts alkali consumption, pulp yield and generation of dry solids in the liquor recovery system. All this results in high costs and

the production of chemical waste that is released into the environment, increasing pollution.

There has been extensive research on genes expressed in wood, identifying key genes in the process of xylogenesis (such as the myb transcription factor, phenylalanine ammonia lyase, glycine hydroxymethyltransferase) and contributing to building an accessible database of genes involved in the genetic control of the properties of wood, including metabolic routes (Whetten et al., 2001). However, the process of wood formation is still not well characterized. Proteomic analysis has become a timely tool in the functional characterization of trees and, along with analysis of transcripts, has been increasingly used in the study of plants (Rengel et al., 2009). The analysis of protein profiling in two-dimensional (2-D) gels is important for two reasons: i) the determination of proteins relate directly to the functional mechanisms, while the determination of transcripts is by definition indirectly linked to cellular functions, and ii) recent findings have pointed out that the abundance of protein can hardly be predicted from the abundance of corresponding mRNA transcripts (Plomion et al., 2000). In this context, it is worth recalling that proteins are responsible for the final phenotype of the cell, i.e., the final product of gene expression after undergoing several posttranslational modifications, such as the removal of signal peptide, phosphorylation and glycosylation. In addition, the concept stating "one gene one protein" is already outdated; it is known that a gene may be responsible for the production of more than one protein by the process of alternative splicing (Brett et al., 2002).

The objective of this study was to characterize the differential proteome of the cambial region of full-sib eucalyptus clones with contrasting characteristics for wood quality applied in industrial production of pulp and paper, for the purpose of identifying marker proteins for the quality of timber for the paper industry. We analyzed the protein profile of the cambial region of clones X1, X2, X3, and X4 with 2-D polyacrylamide gel electrophoresis (PAGE) and electrospray quadrupole time-of-flight mass spectrometry (NanoLC-ESI-Q-TOF). Proteins related to responses to stress showed greater accumulation in clone X4, which has a high content of extractives and lignin, undesirable characteristics for the pulp and paper industry.

MATERIAL AND METHODS

Plant material

The plant material was obtained from trees of 8 years or older of a population of full-sib hybrid *Eucalyptus*, from the Progeny of Matrix 1106 of a commercial plantation in Teixeira de Freitas, BA, Brazil, belonging to Suzano Papel e Celulose S.A. We performed the sampling in the cambial region in one tree for each clone, thereby including not only the cambial region but also the cells of the phloem and xylem, primary and secondary, more distant from the heartwood, and consequently, still potentially differentiating. The tissue was collected using a nondestructive method, which consisted of removing a window of 20 x 15 cm from the bark of trees at diameter at breast height followed by immediate scraping of the tissue present on the surface of the trunk and on the inner surface of the bark. The collected sample was immediately frozen in liquid nitrogen to avoid oxidation and stored at -80°C until extraction of total protein.

Characterization of clones

The clones selected for proteomic and molecular analyses were chosen by Companhia

Suzano de Papel e Celulose S.A. after prior intra-laboratory assessment of trees of the commercial planting by contrasting values of different characteristics involved in the quality of wood, including basic density, total lignin, extractive and gross yield of pulp, and paternity test. For this, the trees/clones were felled, peeled and sent to chipping in the factory production line. The logs were processed into chips after the industrial grinders. The chips generated were prepared and selected for cooking, according to standard SCAN-CM 40:94, and packed in polyethylene bags to conserve moisture. Using the quartering technique, chips of each clone repetition were sampled for chemical analyses and basic density determination.

The chemical analyses were carried out on wood sawdust, classified between 40 and 60 mesh, obtained from the chips of the woods examined, using a Wiley mill. The analyses were performed according to GQAMB's standardized procedure. The cooking was performed in an M/K digester. The operational conditions of cooking were held constant, and the alkaline load was varied to obtain a kappa number of 18.0 ± 0.5 in purified pulp. Cooking, washing and purification of the pulps were according to the standardized procedures of GQAMB. Cooking was performed in duplicate for each wood sample to ensure adequate levels of repeatability and accuracy of the results, besides generating non-bleached pulps in sufficient quantity for subsequent analyses.

The basic density of the chips and chemical analyses were carried out according to TAPPI (Technical Association of the Pulp and Paper Industry) and ABTCP (Associação Brasileira Técnica de Celulose e Papel) standards. The determination of acid-soluble Klason lignin was done according to Goldschmid (1971); the determination of acid-insoluble Klason lignin was done according to Gomide and Demuner (1986), where total lignin was the sum of the two results. For the assessment of wood extractive content, the solvents and solvent mixtures used were: dichloromethane according to TAPPI standard T204 cm-97 and ethanol: toluene according to standard T264 om-82.

Protein extraction

The phenolic extraction method was used for the purification of total protein of the clones studied. Approximately 5 g of the cambial region was crushed to a fine powder in liquid nitrogen with a mortar and pestle. The powder was mixed with 10 mL extraction buffer (0.7 M sucrose, 0.5 M Tris base, pH 7.8, 0.03 M HCl, 0.1 M KCl, 0.005 M EDTA, 0.002 M DTT, and 0.002 M PMSF), according to Hurkman and Tanaka (1986), with adjustments. The mixture was homogenized for 10 min at room temperature (RT) and sonicated under refrigeration for 15 min with 5-s pulses each, 70 power, and intervals of 15 s in an ultrasonic processor (Gex 130, 130 W). Next, a volume of phenol saturated with Tris, pH 8.0, was added, and the mixture was homogenized for 10 min at RT. The phases were then separated by centrifugation at 10,000 *g* for 30 min at 4°C. The phenolic phase was recovered and re-extracted two more times with an equal volume of extraction buffer. The protein fraction was allowed to precipitate for 12 h at -20°C by the addition of 5 volumes 0.1 M ammonium acetate diluted in methanol at -20°C. The precipitated material was collected by centrifugation at 10,000 *g* for 30 min at 4°C and washed three times with 0.1 M ammonium acetate in cold methanol and once with 80% acetone at -20°C. The protein was allowed to dry at RT for 12 h and solubilized in 300 μL rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.002% bromophenol blue). The insoluble residue was removed by centrifugation, and the total protein of samples was quanti-

fied with the 2-D Quant kit (GE Healthcare), using bovine serum albumin as standard. The samples were then stored at -20°C until use.

2-D gel electrophoresis

Isoelectric focusing (IEF) was performed using the Ettan DIGE system (GE Healthcare) and Immobiline DryStrip gels, 13 cm long and pH 3-10 (linear). The solution with 250 µg protein, after addition of 0.002% DTT and 0.5% ampholytes, pH 3-10, in a final volume of 250 µL, was loaded in the strip holder containing the IEF tape and covered with mineral oil (Immobiline DryStrip Cover Fluid, GE Healthcare). IEF was conducted according to the following conditions: instrument at 20°C; maximum current limit 50 µA/strip; rehydration at 50 V for 12 h; step 1, 500 V for 2 min; step 2, gradient at 8000 V for 1 min; step 3, gradient at 8000 V for 2.5 min, and step 4, at 5000 V for 10 s. After the first dimension, the proteins on the IEF tape were reduced and alkylated before electrophoresis by equilibrium at RT for 15 min in equilibrium buffer (75 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 0.002% bromophenol blue) containing 1% (w/v) DTT, followed by incubation for 15 min in equilibrium buffer containing 2.5% (w/v) iodoacetamide. The gel tapes were sealed at the top of the 12% SDS-PAGE with agarose and bromophenol blue and run at 25 mA/strip at 12°C, using Tris-glycine-SDS buffer (Laemmli, 1970). The gels were stained with Colloidal Coomassie blue G-250 (Neuhoff et al., 1988).

Image analysis and detection of spots

The gels of four clones prepared in triplicate were compared, two by two, and statistical analyses of abundance between samples were performed with the program Image Master 2-D Platinum 6.0 (GE Healthcare) according to instructions. Protein spots that were differentially expressed were marked and excised from the gels.

In-gel digestion

The gel pieces were bleached with 25 mM ammonium bicarbonate in 50% acetonitrile and dried with acetonitrile. The gel pieces were digested with 150 ng Trypsin (Promega) in 25 mM ammonium bicarbonate, pH 8.0, initially for 10 min at 4°C, and then overnight at 37°C. The samples were then transferred to the appropriate tubes. Mass spectrometry was performed using NanoLC-ESI-Q-TOF Micro™ (Micromass, Waters, Milford, MA, USA).

Identification of protein using NanoLC-ESI-Q-TOF analysis

The peptides were separated by nano-chromatography using a reverse-phase column (nanoAcquity UPLC - Waters) coupled to a mass spectrometer NanoLC-ESI-Q-TOF Micro™ (Micromass, Waters). The peptides were desalted using a Symmetry pre-column (Waters) C18 (5 µm, 180 µm in internal diameter x 20 mm in length) and an analytical column (1.7 µm BEH300 - C18 100 µm x 100 mm). The flow rate used was 0.6 µL/min with 50 min/run, where 4 mL of each sample were injected. The peptides were separated using a gradient of 1% acetonitrile for 1 min, 1 to 50% in 40 min, 50 to 85% in 5 min, holding the concentration for

another 2 min, returning the concentration to 1% in 1 min and maintaining this condition for 2 min, totaling 50 min of run time. The separated peptides were ionized in a capillary at 3000 V, fragmented in positive ion mode, with selection of the relative intensity of at least 10 counts; the three most intense ions (scans) were analyzed with collision energy ranging between 20 and 95 eV according to mass/load (mz) of peptides.

Analysis of MS spectra

The analysis of the spectra was carried out with Global Server program ProteinLynx 4.2 (Waters) configured to Tryptic digestion with a lost cleavage site, error tolerance of 30 ppm and tolerance for error of mass equal to 0.3 Da. The sequences of peptides were built by MsMs of peptide selected by quadrupole, preferably double- or triple-protonated. The MASCOT MSMS Ion Search program (www.matrixscience.com) was also used for spectrum analysis. The sequences generated from the spectra were compared against the Swiss-Prot and MSDB databases.

RESULTS

Analysis of chemical composition of clones

The data for the characteristics assessed (wood density, total lignin content, content of extractives, and gross yield) are presented in Table 1. Clone X1 had lower lignin content (25.7%), less extractives (2.41%), higher wood density (0.555 g/cm^3), and higher gross yield (52.1%). Clone X2 showed lower lignin content (25.3%), more extractives (6.08%), higher wood density (0.537 g/cm^3), and lower gross yield (47.6%). Clone X3 had higher lignin content (26.5%), less extractives (2.71%), lower wood density (0.434 g/cm^3), and higher gross yield (51.8%). Clone X4 showed higher lignin content (28.1%), more extractives (7.01%), lower wood density (0.426 g/cm^3), and lower gross yield (47.0%).

Table 1. Characterization of *Eucalyptus* clones for the different components involved in wood quality.

Clone	Density (g/cm^3)	Total lignin (%)	Extractive (%)	Gross yield (%)
X1	0.555	25.7	2.41	52.1
X2	0.537	25.3	6.08	47.6
X3	0.434	26.5	2.71	51.8
X4	0.426	28.1	7.10	47.0

Analysis of the protein pattern in 2-D of the different clones

Total protein extracted from each sample of the cambial region was separated using 2-D gels. The protein profile showed spots distributed in the range of 14.4 to 97.0 kDa and pH 3-10. A total of 347 spots were detected on the gel of clone X1, 336 spots for X2, 341 spots for X3, and 489 spots for X4. According to ImageMaster 7.0 analysis, the spots showed the same distribution for all clones. In clone X1, 75% of the spots were distributed in pH 3-7 and 25% in pH 7-8, and 41% of the spots were concentrated in pH 6-7. In clone X2, 69% of the spots were distributed in pH 3-7 and 31% in pH 6-7, and 41% of the spots were concentrated

in pH 6-7. In clone X3, 66% of the spots were distributed in pH 3-7 and 34% in pH 7-8, and 44% of the spots were concentrated in pH 6-7. In clone X4, 66% of the spots were distributed in pH 3-7 and 34% in pH 7-8, and 34% of the spots were concentrated in pH 6-7 (Figure 1).

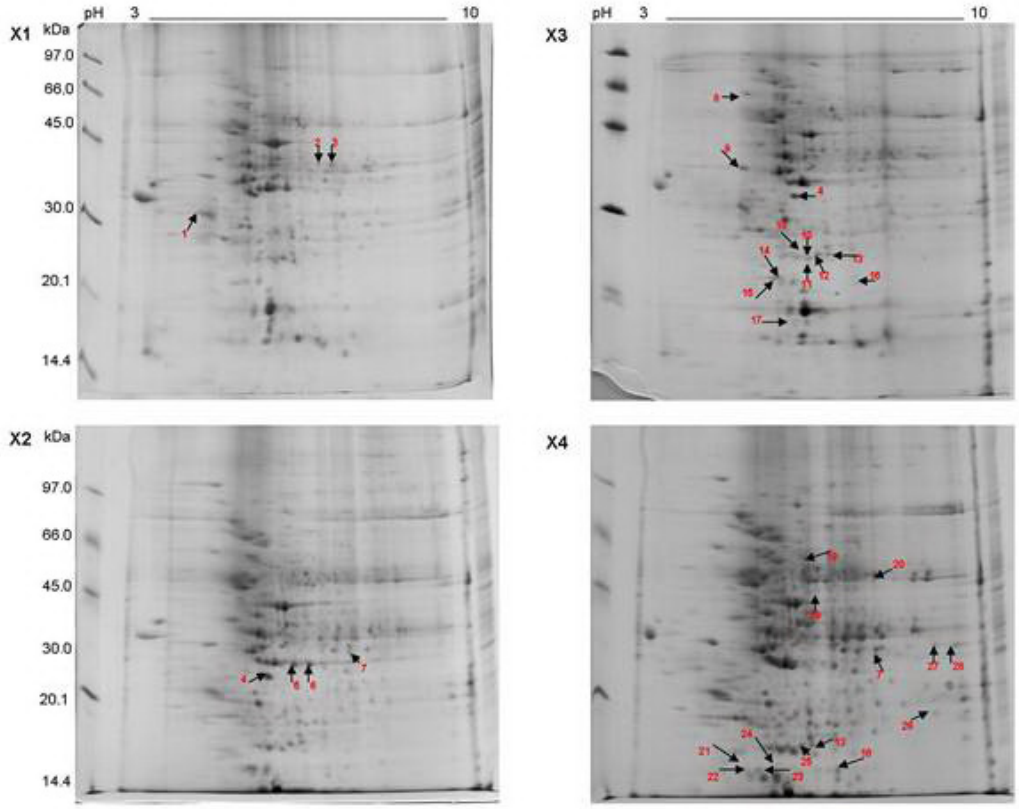

Figure 1. Protein profile on 2-D gel of the cambial region of eucalyptus clones with contrasting characteristics for quality of wood for pulp and paper industry. The clones are identified as X1, X2, X3, and X4. The gels were stained with Colloidal Coomassie blue. Molecular masses of the pattern in kDa are indicated to the left of the images for X1 and X3.

According to the two-by-two analyses performed and using clone X4 as reference, it was possible to differentiate common proteins between clones as under- and overexpressed proteins. When clones X4 and X1 were compared, 407 spots were found exclusive to clone X4, whereas 266 spots were exclusive to clone X1. From the 87 uncommon spots identified, we observed 31 overexpressed and 56 underexpressed spots for clone X1. When clones X4 and X2 were compared, 245 spots were exclusive to clone X4 and 92 spots were exclusive to clone X2, and of the 248 uncommon spots, only 10 were overexpressed for clone X2 and 238 were underexpressed. As for the comparison between clones X4 and X3, of the 708 spots compared, 51% were exclusive to clone X4 and 30% were exclusive to X3. For the 133 uncommon spots, 50% were over- and 50% were underexpressed in clone X3. In mass spectrometry

analysis, only 31 proteins differentially expressed were identified, namely 3 proteins of clone X1, 4 of clone X2, 12 of clone X3 and 12 of clone X4. The expression profile of the cambial region of the clones showed an interesting behavior. Some differences in the accumulation of some proteins are highlighted in Figure 2, including proteins detected only in one of the clones such as spots 1 and 2 of xylem X1 (Figure 2A and B), spot 17 of xylem X3 (Figure 2C) and spot 27 of xylem X4 (Figure 2D). There were also common proteins between clones and with similar expression, such as spot 4 in xylem X2 and xylem X3 (Figure 2E and F). It is interesting that spots 13 and 16 of clone X3 (lowest expression) were also detected in clones X2 and X4, showing the highest expression in the latter (Figures 2G and H). The identification of the protein for each of the spots, theoretical molecular weight, theoretical p*I*, score of combined spectra researched at the Swiss-Prot and MSDB databases, the annotation by biological process and quantification by the program are summarized in Table 2.

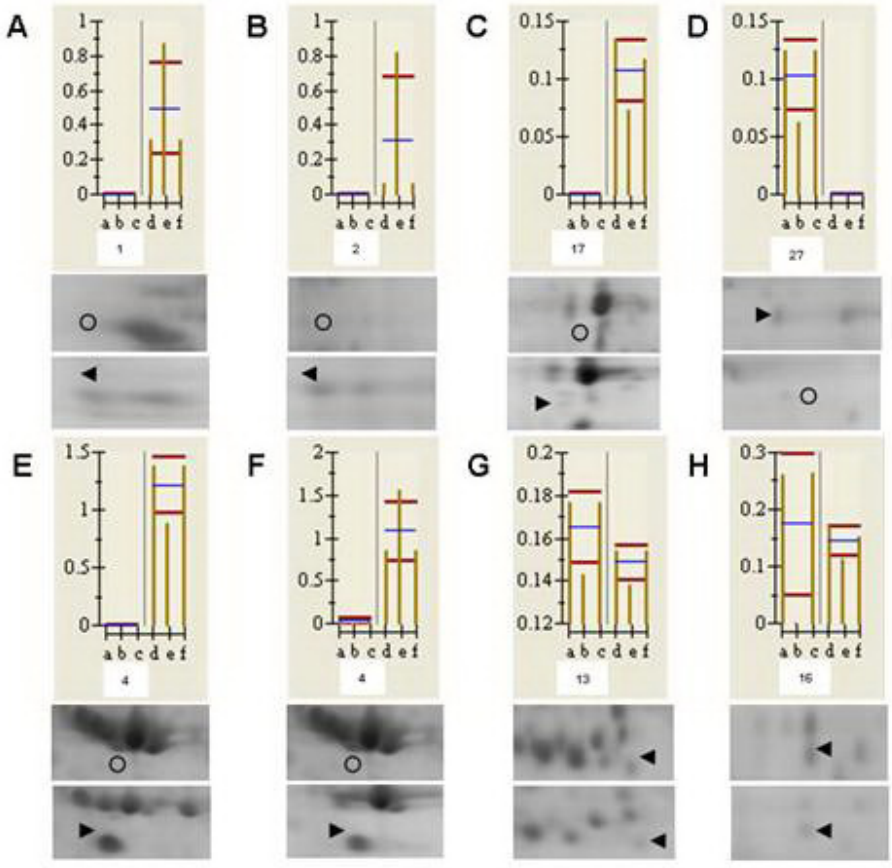

Figure 2. Spots identified in comparative analyses of protein profile of xylem of eucalyptus clones in study (X1, X2, X3, and X4). The lower case letters "a", "b" and "c", of all histograms, represent the gels of clone X4; letters "d", "e" and "f" represent clone X1 in **A**, **B** and **D**, clone X3 in **C**, **F**, **G**, and **H**, and clone X2 in **E**. Circles represent the spots that were not visualized in the region corresponding of the gel in another clone. Clones are identified as X1, X2, X3, and X4.

Table 2. Xylem proteins identified by NanoLC-ESI-Q-TOF.

Clone	ID spot	Protein description	MW (Da)/pI (pH)	PLGS score[a]	Coverage (%)	Accession	Biological process	Relation in the accumulation of proteins[b]		
								X1/ X4	X2/ X4	X3/X4
X1	1	14-3-3-like protein E	30,551/4.79	115.552	147.059	O49997	Response to stress	0.499759/-		
	2	S-adenosylmethionine synthase 2	43,227/5.61	115.546	115.552	P17562	Cellular response to iron	0.315673/-		
	3	S-adenosylmethionine synthase 1	25,636/8.19	115.546	81.197	P31155	One carbon metabolic process	0.806368/-		
X2	4	Polyphosphate kinase	46,053/5.68	149.827	17.115	Q197Y2	Polyphosphate biosynthesis		1.21276/-	1.08469/
	5	Isoflavone reductase	35,433/5.06	115.553	37.736	P52575	Oxidoreductase process		0.14232/ 0.291227	0.0336436
	6	Excinuclease ABC, B subunit	74,152/6.13	149.828	10.903	Q2FPC5	DNA damage excision		0.8875/ 0.0804537	
	7	NAD-dependent epimerase/dehydratase	36,428/5.23	149.828	42.424	Q12VM5	Cellular metabolic process	-/0.851567	0.169123/ 0.331561	0.39112/ 0.0851627
X3	8	Protein disulfide-isomerase	55,526/4.76	89.223	24.096	Q43116	Regulation of redox homeostasis			0.162958/ 0.336191
	9	Salt tolerance protein	38,426/4.91	149.828	63.953	Q5PXN9	Desiccation tolerance			0.698179/ 0.692337
	10	Putative quinone reductase	17,611/5.49	137.013	96.386	Q6YCG4	Polyphosphate biosynthesis			0.298092/ 0.520739
	11	Class II aldolase/adducin-like	27,586/5.91	-19.388	42.636	Q397V8	Oxidation-reduction process			0.126952/-
	12	Potassium efflux system protein KefA, putative	12,229/8.83	143.313	0.9074	Q88CW3	Sequestering of metal ion			0.183606/ 0.159456
	13	Proteasome subunit beta type-2-A	22,526/5.94	115.552	107.843	O23714	Transmembrane transport			0.148742/ 0.165217
	14	Polyphosphate kinase	46,053/5.68	149.828	17.115	Q197Y2	Peptidolysis involved in cellular protein catabolism			0.138605/-
	15	Small heat shock protein, chloroplastic	20,522/5.34	149.828	44.199	P09887	Polyphosphate biosynthesis			0.134562/ 0.448581
	16	Hypothetical protein	71,213/7.08	149.828	10.736	Q1E0U3	Response to stress			0.144651/ 0.174575
	17	Putative cytochrome c oxidase subunit II PS17	17,070/10.9	149.828	50.0	P84733	Sequestering of zinc ion			0.107739/-
	18	Polyphosphate kinase	46,053/5.68	149.828	17.115	Q197Y2	Oxidation-reduction process			0.105008/ 0.184504

Continued on next page

Table 2. Continued.

Clone	ID spot	Protein description	MW (Da)/pI (pH)	PLGS score[a]	Coverage (%)	Accession	Biological process	Relation in the accumulation of proteins[b]		
								X1/X4	X2/X4	X3/X4
X4	19	Ketol-acid reductoisomerase, chloroplastic	63,714/6.56	115.552	40.336	Q01292	Polyphosphate biosynthesis	-/0.729245	-/0.0729245	-/0.729296
	20	Catalase	56,885/6.99	115.552	97.561	O24339	Oxidoreductase process	0.0116442/ 0.338558	0.102009/ 0.338558	-/0.338578
	21	Probable amino acid ABC transporter extracellular - arginine-binding extracellular protein ArtP	28,294/5.09	115.552	35.294	P54535	Oxidoreductase process	0.0396962/ 0.115374	0.110103/ 0.115374	0.200708/ 0.115381
	22	Chloroplast small heat shock protein fragment	20,522/5.34	115.553	88.398	P09887	Cellular metabolic process	0.0922926/ 0.185268	0.0697134/ 0.185268	-/0.185278
	23	Chloroplast small heat shock protein fragment	26,779/8.88	115.553	33.195	P030222	Amino acid transport	0.297014/ 0.448549	0.147824/ 0.448549	0.134562/ 0.448581
	24	Chloroplast small heat shock protein fragment	25,327/8.79	115.553	8.37	P31170	Response to stress	0.0842665/ 0.506744	0.391192/ 0.506744	0.22025/ 0.506771
	25	Superoxide dismutase [Fe], chloroplastic	23,027/5.43	115.552	54.455	P22302	Response to stress	-/0.159447	-/0.159447	0.183606/ 0.159456
	26	Uncharacterized protein MG428	20,246/10.01	115.552	40.936	P47667	Response to stress	0.0462524/ 0.120913	-/0.120913	0.192249/ 0.120919
	27	Annexin	4,675/4.59	149.659	170.732	Q56WE7	Oxidoreductase process	-/0.103358	-/0.103358	-/0.103364
	28	Receptor-like serine/threonine-protein kinase At2g45590	75,506/8.86	149.828	13.177	O64639	Regulation of cellular transcription, DNA-dependent	-/0.152507	-/0.152507	0.131109/ 0.152517
	29	Alcohol dehydrogenase 1	40,889/6.28	115.552	47.493	P14219	Response to abiotic stress	0.0826916/ 0.149805	0.11801/ 0.149805	0.358118/ 0.149813

The numbering of the spots coincides with the numbering shown in Figure 2. [a]PLGS score is calculated by the Protein Lynx Global Server (PLGS 2.2.5) software using a Monte Carlo algorithm to analyze all available mass spectrometry data and is a statistical measure of accuracy of assignation. A higher score implies greater confidence of protein identity (Wright et al., 2009). [b]Ratio of the accumulation of proteins quantified by NanoLC-ESI-Q-TOF performed with ImagemMaster 7.0/GE, using clone X4 as reference.

Characterization of proteins differentially accumulated

The classification by Gene Ontology was performed for a general view of the distribution of proteins differentially expressed identified in this study within the different categories of biological processes (Figure 3). Most of the proteins differentially expressed, 45.2%, were grouped in the stress response and redox processes.

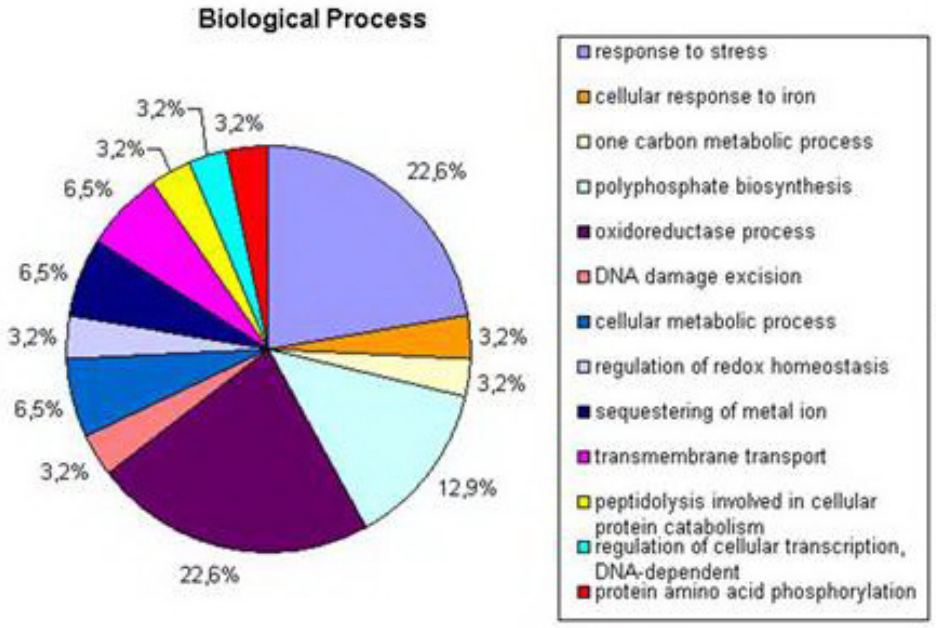

Figure 3. Classification of proteins identified by Gene Ontology using biological process.

DISCUSSION

Proteomic characterization of eucalyptus clones

The desirable features of wood for application in the pulp and paper industry are associated with higher wood density and yield, with lower lignin content and extractives; clone X1 is the one that best meets these characteristics. Of the four clones analyzed in this study, clone X4 shows more characteristics contrasting with clone X1, making it undesirable for the pulp and paper industry. Clone X3, despite having critical features for density and lignin, has low extractive content, which makes the gross yield good for pulp. On the other hand, clone X2 is the opposite of the previous clone, where it has good wood density and low lignin content but high extractive content, which compromises the gross yield of cellulose. In this study we performed the proteomic analysis of these four clones, highlighting the contrasting clones X1 and X4, aimed at the identification of proteins that may be related to the desirable or undesirable characteristics of wood to assist in the selection of elite clones for the pulp and paper industry. In this context, it is important to note the analyses of protein profiles on 2-D

gels of the four xylems showed that several proteins were involved in response to stress, and that these were more abundant in clone X4, with less accumulation in clones X3 and X2. The majority of proteins identified in X4 were absent in clone X1.

Analysis of 2-D gels of the cambial region of clone X1 showed that the most expressed proteins were 14-3-3-like protein (spot 1) and by S-adenosylmethionine synthase (SAMS; spots 2 and 3) (Figure 2), which were detectable only in this sample. The 14-3-3-like protein, already identified in the eucalyptus genome, is known for modulating a variety of cellular processes, among them interactions between components involved in signal transduction, cell cycle control and control of growth during the life cycle of the plant, apoptosis and response to different stresses, which earned it much attention in recent years. The exact function of 14-3-3-like protein is unknown, but this protein can also act as a molecular adapter stimulating protein-protein interactions, regulate subcellular localization of proteins and activate or inhibit enzymes, in addition to regulating the function of proteins targeted for various mechanisms (Darling et al., 2005). Due to its ability to regulate the activities of several proteins involved in signal transduction, there are several levels at which this protein may play a role in response to environmental, nutritional and metabolic stress, as well as in defense against wounds and pathogen attack (Roberts et al., 2002).

It is possible that the spots of the SAMS protein of clone X1 (Figure 2) belong to a multigenic family, such as rice SAMS characterized by Breusegem et al. (1994), because the presence of seven genes was found in the databank of *Eucalyptus grandis* deposited at Phytozome. SAMS has been identified in the xylem of *Pinus pinaster* (Plomion et al., 2000) and characterized in rice (Breusegem et al., 1994), *Populus* (Vander Mijnsbrugge et al., 2000) and other organisms. This enzyme is a universal donor of the methyl group in several transmethylation reactions, which involve many types of acceptor molecules (Plomion et al., 2000). SAMS initiates the methylation of the precursor of monolignols during the biosynthesis of lignin (Ye et al., 1994), where it has also been identified in the response to salt stress in tomato plants. Several sequences corresponding to this protein were also found in expression libraries of xylem in the development of poplars and pine. The presence of various SAMS highlights the importance of methyl transfer reactions and the complexity of regulating genes for donation of methyl groups during xylogenesis (Plomion et al., 2001).

Also involved in stress response processes are the proteins identified in xylems of clones X2, X3 and X4. The proteins isoflavone reductase (spot 5, X2) and receptor-like serine/threonine-protein kinase (spot 28, X4) play an important role in the response to pathogen attack (Afzal et al., 2008). Excinuclease ABC (spot 6, X2) has a central function in nucleotide excision repair of DNA damage (Hsu et al., 1995), and catalase (spot 20, X4) is the main enzyme involved in the elimination of hydrogen peroxide in all aerobic organisms (Yang and Poovaiah, 2002), as well as superoxide dismutase (spot 25, X4), involved in the elimination of toxic oxygen species. The receptor-like serine/threonine-protein kinase, seen more in clone X4, but present in X3 and absent from clones X1 and X2, was also characterized in *Populus* and *Arabidopsis* with key roles in the process of differentiation of vascular cambium by mediating a series of signaling pathways during secondary vascular differentiation. Catalase is an enzyme that converts H_2O_2 to O_2 and H_2O, preventing primarily the potential damage caused by changes in the homeostasis of H_2O_2 (Breusegem et al., 2001). Superoxide dismutase is the first line defense of the plant against O_2 reactive species, removing O_2^- from cellular compartments where this radical is formed (Alscher et al., 2002). A protein that also contributes in

detoxification processes of the plant is the ABC transporter (spot 21, X4). However, there is evidence of its participation in the flow of ions, and thus, it may also be involved in processes of growth and development in the plant (Martinoia et al., 2002). The protein disulfide isomerase (spot 8, X3), important in redox signaling, can act as a chaperone, with a role in folding secretory proteins (d'Aloisio et al., 2010). The molecular chaperones, known as small heat shock proteins (Hsp) (spot 15 in X3 and spots 22, 23, 24 in X4), belong to a multigenic family in plants (Klemenz et al., 1991) and are stress-induced proteins. In stress conditions, Hsp facilitates the folding of proteins and helps stabilize polypeptides and membranes. These proteins involved in redox processes in the plant, as well as the extractives, among them polyphenols, tannins and resins, which are characterized as harmful components in the pulp and paper industry, are induced as a defense response to attack by pathogens, but also to abiotic stress (Breusegem et al., 2001). The Hsp are abundant in the xylem of *P. pinaster* (Le Provost et al., 2003) and *Eucalyptus* (Gonçalves et al., 2011), and are believed to have a specific function in the formation of wood, also important for the development of the secondary wood.

Other proteins of the cambial region of clone X4 that showed relevance were the uncharacterized protein MG428 (spot 26), ketol-acid reductoisomerase (spot 19) and annexin (spot 27). Uncharacterized protein MG428 shows amino acid sequence similarity with the conserved domain of sigma factor, which is a transcription initiation factor that increases the binding specificity of RNA polymerase to the promoter of the gene (Lonetto et al., 1992). Ketol-acid reductoisomerase is a bi-functional enzyme that performs two distinct reactions, i.e., isomerase and reductase, one of which is involved in the biosynthesis of the amino acids valine, leucine and isoleucine (Durner et al., 1993). The absence of these amino acids in the plant inhibits development and DNA synthesis, causing death, according to tests carried out using corn herbicide.

Immunolocalization tests have shown that annexin is highly concentrated in the xylem of maize and pea seedlings (Blackbourn and Battey, 1993). This protein in plants is normally latent and becomes active when the concentration of intracellular Ca^{2+} rises (Delmer and Amor, 1995). Also, stress responses in plants triggered by damage mechanisms, environmental stress or pathogen attack, conditions under which Ca^{2+} levels are high, promote callose deposition. Annexin may also play a role as does peroxidase in oxidative stress in plant.

Plants respond to attack by pathogens by activating a wide variety of defense reactions, including transcriptional activation of defense-related genes (receptor-like serine/threonine-protein kinase), accumulation of antimicrobial phytoalexins (synthesized by isoflavone reductase), oxidative burst leading to the generation of reactive oxygen species (superoxide dismutase and catalase controlled), and the deposition of lignin and other phenolic compounds in the cell wall (Hwang et al., 2003), which, together with other chemical compounds of wood, influence the properties and performance of pulp, paper and coal (Gion et al., 2011). In studies with eucalyptus, a strong positive correlation between the pulp yield and cellulose content and negative correlation with lignin content have been observed. Pereira (1988) observed variability in chemical composition, mainly for pulp content, between trees of *Eucalyptus globulos* in the same location and different geographic locations, a fact that corroborates the differences in the profile of proteins found among clones of this study.

Based on these studies, it is possible to say that the presence of proteins involved in oxidative stress in the cambial region of eucalyptus can be a strong indicator of clones unfit for the pulp and paper industry. Accordingly, the use of certain proteins identified in xylem of

clone X4 can assist in the selection of elite clones for the pulp and paper industry.

The selection of candidate genes and validation study by gene expression in genetic materials with the quality of wood also already known is the next step in the selection of new genetic tags for the identification of elite clones of eucalyptus for paper and pulp production.

In search for varieties with specific features for each production sector, pulp and paper, energy and furniture, the study of properties of wood has become mandatory in improvement programs. These characteristics classified into categories, including physical characteristics such as wood density, and chemical composition-related features, such as cellulose and lignin content (Gion et al., 2011) have been the targets of several studies on the identification of genetic markers for later use in marker-assisted selection (Grattapaglia, 2007). Many investigators have had a keen interest in the search for information about the properties of wood, phenotypic and genetic correlations, and heritability, as well as linkage maps and QTLs (quantitative trait loci). Studies on genetic variation and heritability in *E. globulos*, for example, have demonstrated a strong correlation between extractives and lignin content. In another study, the high yield of pulp and cellulose content was associated with the low content of extractives and total lignin (Poke et al., 2006). For *Eucalyptus nitens*, Kube et al. (2001) found a high negative genetic correlation between extractive content and cellulose, which was the same correlation found by Thumma et al. (2010) in the study of wood quality with QTLs for the same species. Together, these studies suggest that selection to increase the yield of pulp or cellulose content would probably result in the reduction of lignin and extractive content, which is a favorable for the production of wood for the pulp industry (Poke et al., 2006). Thus, this pioneer study identified unique and differential proteins, by proteomic analysis, of *Eucalyptus* clones contrasting in wood quality for the pulp and paper industry.

ACKNOWLEDGMENTS

Research supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil), Fundação de Amparo à Pesquisa da Bahia (FAPESB, Brazil), and Financiadora de Estudos e Projetos (FINEP, Brazil). D.S. Britto was supported by FAPESB and Suzano Papel e Celulose S.A.

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