WOOD RESEARCH
64 (6): 2019
943-956

2D-DIGE IDENTIFICATION OF PROTEINS IN WOOD-FORMING TISSUE OF *PINUS KORAIENSIS* SEEDLING STEM AFTER ARTIFICIAL BENDING TREATMENT

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(Received January 2019)

ABSTRACT

Compression wood is an ideal model for exploring the molecular mechanism of wood formation. To supplement the proteome data in compression wood formation, two-dimensional difference gel electrophoresis (2D-DIGE) proteome technology was used to investigate the protein response to bending the stem of *Pinus koraiensis* seedlings. The results showed that most of the proteins in wood-formation tissues were distributed in pH from 4-8 and the molecular weight was around 30-97 KDa. In total 24 identified proteins were mainly functional on amino acid metabolism, cell wall synthesis, secondary metabolism, and stress response. Proteins related to methionine pathway and lignin biosynthesis were up-regulated in the formation of the compression wood. On the contrary, lipid metabolism-related proteins were down-regulated during the formation of the compression wood. In additional, some proteins involved in energy metabolism and photosystem were also changed in the tissues during the formation of the compression wood. These findings suggested that 2D-DIGE was a feasible and timesaving technology in proteome analysis of wood-forming tissue. Moreover, proteins were not involved in lignin synthesis pathways, but other metabolites were changed as a response to stem bent treatment. The identified proteins in compression wood formation insight to further investigating the molecular mechanism of wood cell wall biosynthesis.

KEYWORDS: Compression wood, wood-forming tissue, differential protein, 2D-DIGE, wood formation.

INTRODUCTION

Wood formation is the result from synergy of genetic factors and environment factors during tree growth. When living trees faces special environment factors, such as slope, winds, or snow loading, the wood will react by producing modified anatomical and chemical properties (Timell 1986). In conifers, reaction wood generally formed at the low side of leaning stems or branches, which is termed as compression wood. Because of the high content of lignin (Singh and Donaldson 1999, Villalobos et al. 2012) and larger microfiber angle in the secondary cell wall (Plomion et al. 2000); compression wood is commonly regarded as a defective material for pulping and wood drying processes (Timell 1986). However, based on such differences in anatomical and chemical characteristics from normal wood, compression wood is ideal for the study of the molecular mechanism of wood formation (Plomion et al. 2000). There are many reports focused on the genes (Allona et al. 1998, Zhang et al. 2001, Yamashita et al. 2009, Villalobos et al. 2012) and biochemical make up of cell wall lignification (Donaldson et al. 1999, Saito and Fukushima 2005, Mast et al. 2009, Kim et al. 2011, Donaldson and Knox, 2012) involved in compression wood formation. However, to date, only limited studies have investigated the proteins in compression wood formation (Plomion et al. 2000, Gion et al. 2005, Mast et al. 2010). Proteins are large macromolecules, which play an important role in plant physiology development, for instance, DNA replication, catalyzing metabolism, responding to stimuli, cell signaling and ligand binding. In other words, proteins are the chief actors carrying out the duties specified by the information encoded in genes (Lodish et al. 2004). Investigation of proteins in differentiating xylem will provide us with some essential knowledge for revealing mechanism of cell wall formation in trees. Understanding all the information from the gene level, protein level, or metabolite level involved in wood formation is a powerful strategy to uncover the controlling of chemical and mechanical properties of wood.

Two-dimensional difference gel electrophoresis (2D-DIGE) is an advanced method of proteome analysis, which can eliminate the inter-gel variation and reduce time consumption compared to the method of the traditional 2D electrophotesis (Alban et al. 2003). This proteomics technology was wildly used in Grape berry proteome (Carli et al. 2011), Arabidopsis proteome (Choudhary et al. 2016), and *Hevea brasiliensis* (Xiang et al. 2012). *P. koraiensis* is an economically and ecologically essential tree species in Northeast of China and East Asia (Piao et al. 2011). In this work, 2D-DIGE quantitative proteomics technology was employed for investigating the protein changes in compression wood formation of *P. koraiensis* seedlings. To ensure the severe compression wood formed, 4-year-old *P. koraiensis* seedlings were bent for 15 days at 45o from vertical growth direction. Wood-forming tissue was collected and proteins were extracted by the modified Ttrichloroacetic acid (TCA) system. Different proteins were qualitatively and quantitatively analyzed according to the MS/MS data and previously published literature.

MATERIALS AND METHODS

Chemicals

Lysine, N, N'-methylene bisacrylamide, Glycine, Agarose, Urea, Thiourea, CHAPs, DTT, Carrier Ampholytes, IPG cover oil, BPB, Coomassie Blue (G-250), H_3PO_4, Ammonium Sulfate, Iodoacetamide, 2-D-clean-up Kit (GE, Healthcare, 80-6484-51, USA), 2-D-Quant(GE, Amersham Biosciences, 80-6483-56, USA), IPG Strips (24cm, pI3-11), IPG Buffer (pH3-10), Dyes (Cy2, Cy3 and Cy5): 1-(5-carboxypentyl)-1'-propylindocarbocyanine halide N-hydroxysuccinimidyl ester(Cy3), 1-(5-carboxypentyl)-1'-methylindodicarbocyanine

halide N-hydroxysuccinimidyl ester (Cy5), and 3-(4-carboxymethyl) phenylmethyl-3;-ethyloxacarbocyanine halide N-hydroxysuccinimidyl ester (Cy2) were purchased from GE Healthcare, USA. Acrylamide, Trishydroxymethyl aminomethane (Tris), TEMED, and β-Mercaptoethanol were purchased from Amresco, USA. SDS was purchased from Sanland, USA. Trichloroacetic acid (TCA), acetone (chromatography level), methanol (chromatography level) and distillated water were purchased from a chemical company of China.

Plant material and treatment

10 four-year old *Pinus koraiensis* seedlings were planted in plastic pots filled with a mixture of black soil and compost. The seedlings were sorted into two groups and 5 seedlings were used as biological replicates for each group. The stem of group one was bent to an angle of approximately 45° and kept there as previously described (Shi and Li 2012). The seedlings underwent 15-day worth of bending during the month of July at which time vigorous cambial growth. Fig. 1 shows the bending treated and control seedling. The experimental procedure used to wood-forming tissues collection was previously described (Shi and Li 2015). After 15 days of bending, the underside wood-forming tissues of the bent seedlings stems were carefully harvested following experimental procedure. Near to the fixed point, cortex and phloem was peeled off successively and wood-forming tissues were scraped with a razor on the exposed fresh xylem surface. To vertical growth stem, wood-forming tissues were collected from similar of the stem height. As-collected wood-forming tissues were immersed in liquid nitrogen and stored at -80°C in laboratory. In addition, two cm long samples of the seedlings stems were reserved for the micro-examination.

Cross section observation

3 mm thickness cross sections were cut from samples along the vertical of the stems and the lower side of the bending stems. After washing with distilled water, the sections were air-dried and mounted on the sample stage. As prepared samples were then coated with an approximately 1 nm thick gold layer and observed with a Scanning Electron Microscope (SEM, Quanta 200, FEI, USA) at an accelerating voltage of 10.0 kV.

Protein sample preparation and labeling

The method of extracting crude protein from wood forming tissue requires 2 g of frozen tissue be ground into fine powders in liquid nitrogen, then transferred to a 1.5 ml centrifuge tube and mixed with 1ml extraction solutions (10% TCA and 0.07% β-mercaptoethanol). After adding extraction solutions and mixing, the liquid was precipitated at -20°C for 20 min, and centrifuged at 4°C 12000 rpm for 30 min. The supernatant was removed from the centrifuge tube and the resulting protein pellet were washed with prechilled acetone and centrifuged at 4°C 12000 rpm for 30 min. Then the pellet was washed twice with distilled water and centrifuged at 4°C 12000 rpm for 30 min. The precipitation was vacuum freeze dried and stored at -80°C.

A certain amount frozen protein was mixed with 500 μL solubilization buffer (7 mol·L^{-1} Urea, 2 mol·L^{-1} Thiourea, 4% (w/v) CHAPS, 40 mmol·L^{-1} Tris-Base) and dissolved completely at room temperature. After 5 min of sonic treatment, the mixture was centrifuged for 60 min at 4°C and 4000 rpm. The supernatant was then transferred to a new tube and purified using the 2-D Clean-up Kit (GE, Healthcare, 80-6484-51, USA). The pH value of as-purified protein was adjusted to 8.0-9.0 using 250 mmol·L^{-1} NaOH or HCl. The quantitative of protein was determined using a 2D-Quant Kit (GE, Amersham Biosciences, 80-6483-56, USA).

2D-DIGE and image capture

Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) was used to quantitatively determine the components of the proteins in wood-forming tissue. Before two-dimensional gel electrophoresis (2-DE), all proteins were labelled by different dyes (Cy2, Cy3 and Cy5). 50 μg control normal wood protein, 50 μg compression wood protein, and 50 μg internal reference protein (25 μg normal wood and 25 μg compression wood protein) were labelled with 1 μL 400 pmol·μL^{-1} Cy3, Cy5, and Cy2 dyes, respectively. After being mixed with the dye the samples were then finely mixed in an ice bath for 30 min away from light. 1 μL 10 mmol·L^{-1} lysine was added into mixtures of wood proteins and their respective dyes in order to stop the reaction. 2-DE was performed on 24 cm IPG strips (pH3-11) under 12.5%T and 2.6%C SDS-PAGE. Once electrophoresis was finished, image capture of all gels was carried out using Typhoon 9400 fluorescence scanner under emitted light wavelengths of 520 nm (Cy2), 580 nm (Cy3), and 670 nm (Cy5). Captured images were analyzed with the DeCyder software and its differential in-gel analysis (DIA) modules were used for the comparison of different gels. The exclusion filter was set to Slope >1.1, Area < 100, Volum < 100, Peak Hight < 100 or > 64000, and the difference in expression more than two folds was marked. A total of 15 gel images (5 controls, 5 treated and 5 internal standards) were used for statistical analysis. The abundance of each protein was estimated by the volumes. Student's T-test function implemented into DECYDER was used for comparing differences in spot intensity between groups ($p<0.05$) and an absolute ratio of at least 2 fold were used for protein identification.

Protein identification

The fluorescence labeling gel electrophoresis map was matched with the preparation of the gel, and the corresponding differences in the proteins were found in the Coomassie Blue stained gel. Target protein spots were removed with a sterile pipette tip and digested by trypsin in a EP tube. The digestion of the protein samples was concentrated using ZipTipC-18RP tips (Millipore, Billerica, MA, USA) and eluted by 50% acetonitrile and 0.1% trifluoroacetate. Finally, proteins samples were mixed into 1 μL 5 mg·mL^{-1} CHCA and spotted on the steel target to dry. MS was carried on 4800 plus MALDI TOF/TOF™ (AB SCIEX, USA). Peptides matching were performed by MASCOT searching (Matrix Science, Version 2.1) against NCBInr protein database. Fixed modification was set as carbamidomethyl, variable modification was set as phosphorylation (STY), max miss cleavage was set as 1, mass tolerance was set ±0.2 Da.

RESULTS AND DISCUSSION

Scanning electron microscopy (SEM)

Newly formed tracheid wall characteristics showed significant differences after fifteen days of stem bending treatment compared to vertical seedlings (Fig. 2). In general, compression wood featured rounded and thick-walled tracheid cells (Timell 1986). As shown in Fig. 1, tracheid cell walls were obviously thicker after the bending treatment than in the vertical seedlings stems.

Fig. 1: Digital photo of control (a) and bending treated (b) seedlings.

The tracheid walls on the cross sections were approximately round or oval shape in bending treatment stems (Fig. 2b), but rectangular or polygon shape in vertical seedlings stems (Fig. 2a). Furthermore, many helical cracks, another feature in compression wood (Yamashita et al. 2009), appeared in the internal surface of tracheid wall in bending treatment stems. These features confirmed that the compression wood was formed within the 15 days of the bending treatment of the stems.

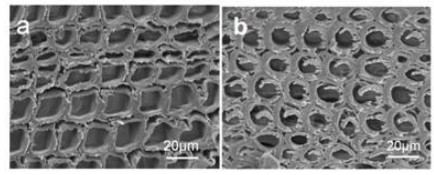

Fig. 2: SEM pictures of cross section of normal wood (a) and compression wood (b).

Stem bending responsive proteins detected by 2-D DIGE

The fluorescence dye labelled proteins in the wood-forming tissue are presented in Fig. 3a.

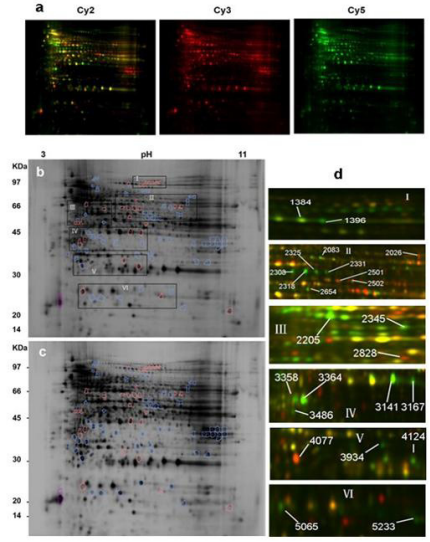

Fig. 3: 2DE-DIGE map of wood-forming tissue: a) Images of dye labelled protein from wood-forming tissue. Cy2 (overlay, internal control, Cy3 (red, normal wood), Cy5) green, compression wood). b) Bending after 15 days. c) Control. d) Fluorography from internal control and highlights of the differential spots presented in boxed areas indicated in (b).

Proteins in the compression wood-forming tissues were labelled by Cy5 dye and scanned under a wavelength of 670 nm. The normal wood-forming tissues were labelled by Cy3 dye and scanned under a wavelength of 580 nm. The mixture of compression wood and normal wood proteins served as internal standard, which was labelled by Cy2 dye and scanned under a wavelength of 520 nm. Based on the preliminary 2DE-PAGE separations of wood-forming tissue extracts within a pH 3 to 11, it was noticed that most of the protein spots were distributed in a pH between 4-8 and molecular weight ranking from 30-97 KDa. Sixty-seven spots, representing 3.5% of the total number of spots, saw significantly change over 2.0-folds. It can be clearly found differential proteins in the compression wood and normal wood-forming tissue (Fig. 3a). Differential proteins labelled by dyes and scanned under different radiation were benefiting to dig the differential proteins spots on the SDS-PAGE.

Fig. 3 shows the overall appearance of the 2-DE separation of wood-forming tissue extracts image were divided into two parts; the first one corresponding to the full image of a 2DE-PAGE (the highlighted regions of variable spots between normal and bending samples in boxes with dotted lines) (Fig. 3b,c), and the second present a magnification of the regions in the 2DE-DIGE gel (Fig. 3d).The image was divided into two parts, one , and another part presenting a magnification of those regions in a 24 out of the 67 protein spots were sequenced and putatively identified with matching MASCOT database. After undergoing sequencing these spots had their spot number, protein name, fold change and other data identified (Tab. 1). Of the identified spots seven belonged to trees, and seven were *Arabidopsis* proteins, and10 were similar to sequences from other plants. Several proteins were detected in more than one spot, suggesting that a percentage of the identified spots corresponded to post-translational modifications of proteins or were members of multigenic protein families. As indicated by the calculated fold-change, 19 of the identified spots were differentially up-regulated due to bending treatment and only 5 spots were down-regulated due to bending treatment. According to the assumed function, identified proteins from wood-forming tissue of *P. koraiensis* were classified into either amino acid metabolism (34%), cell wall synthesis (17%), secondary metabolism (8%), and stress response (8%) (Fig. 4).

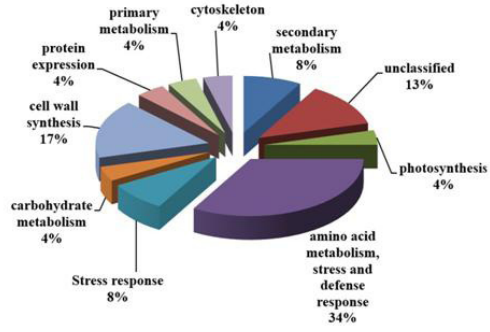

Fig. 4: Classification of identified proteins in compression wood-forming tissue.

Of the identified proteins the non-classified proteins (13%) were those peptides that had no identity in the query database; where the proteins that could not be sequenced using MS/MS were list as non-identified. Due to the limited to understanding mechanism on proteomics level of compression wood formation, identified proteins were discussed in terms of their assumed function as follows.

Tab. 1: List of identified proteins in compression wood-forming tissue. (a) Number of protein spot on the 2-D gel; (b): Theoretical PI and Mw (in Da) refer to Mascot database. CW: compression wood; NW: normal wood.

Protein spot ID[a]	Protein name	Number of matching peptides	Protein score	Protein PI / MWb	Accession No.	Reference Plant	Change folds	3D image	
								CW	NW
1384	5- methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	17	91	6.09/84645.6	METE_ARATH	*Arabidopsis thaliana*	2.24		
1396	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	17	98	6.09/84645.6	METE_ARATH	*Arabidopsis thaliana*	5.26		
2026	Pentatricopeptide repeat-containing protein At2g17670	7	36	8.47/52002.2	PP161_ARATH	*Arabidopsis thaliana*	3.10		
2083	Adenosyl homocysteinase	20	253	5.51/53640.2	SAHH_TOBAC	*Nicotiana tabacum*	2.26		
2205	Tubulin alpha-1 chain	17	122	5.02/50381.7	TBA1_ANEPH	*Anemia phyllitidis*	4.01		
2308	S-adenosylmethionine synthase 2	19	251	5.55/43497.8	METK2_PINCO	*Pinus contorta*	2.89		
2318	S-adenosylmethionine synthase 1	9	345	5.42/43525.7	METK1_ELAUM	*Elaeagnus umbellata*	7.00		
2325	S-adenosylmethionine synthase 2	20	173	5.55/43497.8	METK2_PINCO	*Pinus contorta*	3.30		
2331	ATP synthase subunit b', chloroplastic	11	34	6/24444.2	ATPX_SPIOL	*Spinacia oleracea*	2.17		
2345	S-adenosylmethionine synthase 2	22	411	5.55/43497.8	METK2_PINCO	*Pinus contorta*	2.76		
2501	Putative callose synthase 6	34	41	8.76/223998.4	CALS6_ARATH	*Arabidopsis thaliana*	2.11		
2502	RPM1-interacting protein 4	5	29	9.24/23528.1	RIN4_ARATH	*Arabidopsis thaliana*	2.88		
2654	Probable cinnamyl alcohol dehydrogenase	12	98	5.8/39468.6	CADH_PINRA	*Pinus radiata*	2.14		
2828	Photosystem I assembly protein Ycf4	6	24	9.82/21747.5	YCF4_CUCSA	*Cucumis sativus*	3.99		
3141	Bate-amyrin synthase	3	27	5.95/88748.5	BAS_BRUGY	*Bruguiera gymnorhiza*	4.20		
3167	Monothiol glutaredoxin-S4, mitochondrial	5	28	6.41/20248.2	GRXS4_ORYSJ	*Oryza sativa*	3.87		
3358	Caffeoyl-CoA O-methyltransferase	10	240	5.29/27359	CAMT_SOLTU	Solanum tuberosum	2.65		
3364	Caffeoyl-CoA O-methyltransferase	13	314	5.43/29184.2	CAMT_PINTA	Pinus taeda	4.86		
3486	14-3-3-like protein 2 (Fragments)	5	51	4.28/7715.8	14332_PSEMZ	Pseudotsuga menziesii	2.06		
3934	Glutathione S-transferase L3	5	36	5.07/27186.9	GSTL3_ARATH	Arabidopsis thaliana	6.58		
4077	Linoleate 9S-lipoxygenase 1	19	33	6.34/97666.3	LOX1_ORYSJ	Oryza sativa	3.84		
4124	ATP-dependent Clp protease proteolytic subunit	11	34	6.54/25757.1	CLPP_MESVI	Mesostigma viride	2.36		
5065	Glucose-6-phosphate 1-dehydrogenase, chloroplastic	11	29	6.11/65823.3	G6PDC_SPIOL	Spinacia oleracea	2.24		
5233	Glutathione S-transferase U13	8	26	5.65/25286.1	PP161_ARATH	Arabidopsis thaliana	10.65		

Amino acid metabolism, stress and defense response

Caused by the vigorous tree growth period, the largest percentage of identified proteins related to the amino acid, metabolism, stress and defense response, which is the same finding was reported in the previously studies (Gion et al. 2005, Mast et al. 2010). In plant vivo, some proteins were responsible for regulating the content of the downstream amino acids. Two glutathione S-transferases (GST), Glutathione S-transferase U13 (spot 5233) and Glutathione S-transferase L3 (spot 3934) proteins were identified and exhibited upregulation at10.65-fold and 6.58-fold in the stems that underwent bending treatment, respectively. In *Pinus tabuliformis*, GSTU13 and GSTL3 were present in all tissues, such as leaf, phloem of the stem and roots, and radicle (Lan et al. 2013). GSTs played an important role in biotic and abiotic stress response as well as cellular detoxification in the tissue (Wisser et al. 2011, Lan et al. 2013). Since phenylalanine is a synthetic starter of lignin biosynthesis (Pascual et al. 2016), then the upregulation of GSTL3, was associated with the aromatic amino acid metabolic process. This association suggested that GSTL3 could be related to the higher lignin content in the compression wood. S-adenosylmethionine synthase 1 (spot 2318) and S-adenosylmethionine synthase 2 (spot 2308, spot 2325, spot 2345) are two isoforms of the same enzyme involved in the methylation during lignin biosynthesis (Ye et al. 1994). It was observed that spots 2318, 2308, 2325, and 2345 were up-regulated when the bent stems samples were compared to the vertical stem samples by 7.00, 2.89, 3.3, and 2.76 folds, respectively, which aligns with was also observed by Plomion et al (2000). As we known, S-adenosylmethionine synthases (SAMS) plays a vital role in plant metabolic cycles because S-adenosylmethionine biosynthesis was regulated by SAMS through the reaction with methionine and ATP. It was observed in other studies that SAMS were preferentially expressed in xylem tissue in plant (Vander Mijnsbrugge et al. 2000, Chen et al. 2005). According to the biosynthesis of the lignin in wood cell walls, more methyl is consumed in the polymerization of lignin monomers, which means SAMS were a methyl donor and plays an important role in the xylogenesis progress of wood formation (Vander Mijnsbrugge et al. 2000). This finding indicated that the over expression of SAMS in bent stem samples was consistent with the rich lignin content in the compression wood of the conifers. Adenosyl homocysteinase (spot 2083) was one of the enzymes involved the methionine cycle and was up-regulated 2.26 folds in the bent stems samples. Adenosyl homocysteinase was also detected in Arabidopsis and was considered being nitrosylated in plants (Lindermayr et al. 2005).

Similar to Adenosyl homocysteinase, 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (spots 1384 and 1396) is an enzyme belongs to the family of transferases which aids in the catalyzation reaction used to synthesize methionine, and were up-regulated for 2.24 folds and 5.26 folds in the bent stem samples, respectively. These findings suggested that the methionine metabolism pathway in wood formation was tightly modified during the compression wood formation. One fragment sequence of 14-3-3-like protein 2 (spot 3486) was also up-regulated (2.06 folds) in the bending stem. Several proteomics studies showed that the overexpression of 14-3-3 proteins could affect the activity of many proteins, such as glutamine synthetase, glutamate dehydrogenase, nitrate reductase, and AtDHAPS2 (aromatic amino acid pathway), which suggest that 14-3-3 proteins were key enzymes in both carbohydrate metabolism and amino acid synthesis (Bachmann et al. 1996; Diaz et al. 2011). Another protein, named as ATP-dependent Clp protease proteolytic subunit (spot 4124), involved in stress response, was upregulated 2.36 folds.

Cell wall synthesis and others

Compression wood formation was induced by the exogenous force and featured with an eccentric radial growth promotion of the stem (Timell 1986, Plomion et al. 2000). This also

means that the nutrients accumulated more at the same site in the tissue while compression wood is formed. As a result, some proteins associated with wood cell wall synthesis and other metabolisms were identified more prominently in certain locations. Caffeoyl-CoA O-methyltransferase (spot 3358 and 3364) and probable cinnamyl alcohol dehydrogenase (spot 2654), are enzymes that are a part of the general lignification pathway (Whetten and Sederoff 1995, Meyermans et al. 2000), both of which were enriched during the stem bending treatment; which is similar to what was observed in compression wood formation in *P. pinaster* (Plomion et al. 2000) and *P. radiate* (Mast et al. 2010). The changes of these proteins were consistent with the high lignin content in compression wood, suggesting that lignin biosynthesis was affected by compression wood formation (Plomion et al. 2000). The higher expression of proteins related to methionine metabolism and Caffeoyl-CoA O-methyltransferase during the stem bending treatment might be caused by the higher demand of methyl group in biosynthesis of lignin. This increase in cell wall thickness that occurred in the wood-forming tissues as they underwent bent stem treatment would then be a result of increase of lignin biosynthesis, tissue of bending stem. Besides involved in lignin biosynthesis, Caffeoyl-CoA O-methyltransferase also catalyzed C3 to methoxy or methylenedioxy, which is a key step in biosynthesis of aromatic secondary metabolites.

Besides the thickness cell wall and higher lignin content, eccentric growth result in wider grow rings is another typical character of compression wood. Owing to the more biosynthesis of wood cell wall in compression wood, one protein of carbohydrate metabolism (Glucose-6-phosphate 1-dehydrogenase, chloroplastic, spot 5065, 2.24-fold), proteins of the primary and secondary metabolism (ATP synthase subunit b', Chloroplastic, spot 2331, 2.17-fold and Bate-amyrin synthase, spot 3141, 4.2-fold), were up-regulated in the bent stem samples. Glucose-6-phosphate 1-dehydrogenase (G6PD) an enzyme that is a part of the pentose-phosphate pathway where it aids in reducing power (NADPH) and pentose phosphates for fatty acid as well as nucleic acid synthesis, which are important for membrane synthesis and cell division. ATP synthase is an enzyme required for generating an electrochemical potential and production of ATP (Kerner et al. 2011) and increased amounts of it suggests accumulation of metabolism and physiology in compression wood formation. Known as a key enzyme for catalyzing the cyclization of oxidosqualene into β-amyrin, bate-amyrin synthase was the precursor synthesis pathway of triterpenes in plants (Kushiro et al. 1998). These also suggested that more abundant of triterpenes secondary metabolites could be produced during compression wood formation.

Both proteins Photosystem I assembly protein Ycf4 (spot 2828) and Linoleate 9S-lipoxygenase 1 (spot 4077), were down-regulated 3.99-fold and 3.84-fold in the bent stem samples, respectively. As an enzyme required for accumulation of the photosystem I complex, Ycf4 was not an intrinsic membrane protein but associated with the membranes (Boudreau et al. 1997). The down-regulated expression of Ycf4 indicated the deceleration of photosystem I complex in compression wood-forming tissue. Generally, 9S-lipoxygenase catalyzes the oxygenation of linoleic acid into (S)-configured fatty acid hydroperoxides (Bannenberg et al. 2009). Previous studies indicated that the oxylipins produced from 9S-lipoxygenase pathways play important roles in plant defense reactions (Bell et al. 1995). Stem bending treatment exogenously stimulates and disequilibrates the normal physiology of tree growth. As a response to these changes, compression wood is formed and can be regard as the natural defenses from trees themselves. However, the down-regulation of Linoleate 9S-lipoxygenase 1 in compression wood formation is possible due to the different regulation patterns of enzyme.

The cytoskeletal protein Tubulin alpha-1 chain, (spot 2205, 4.01-fold) is a major component for microfilaments or microtubules formation. Microtubules guide cellulose synthase distribution and behavior as they synthesize microfibrils in the cell walls (Paredez et al. 2006). Microtubule stability is promoted to a large degree by tubulin alpha/beta 1 chain (subunits) protein, which bind

to the surface of the microtubule. Microtubules are heterodimeric polymers of the alpha/beta-tubulins that are encoded by multigene families in plants (Snustad et al. 1992). Some Tubulin alpha genes were expressed preferentially in latewood of trees (Yang and Loopstra 2005). The orientation of newly deposited cellulose microfibrils was determined by cortical microtubules that are crosslinked to the cytoplasmic face of the plasma membrane. Thus, these tubulin genes could be candidate genes for S2 layer microfibril angle (MFA) in the secondary cell wall of tracheid cells. In Eucalyptus, the expression of β-tubulin protein gene was inversely to MFA (Spokevicius et al. 2007). The results indicated that the up-regulation of tubulin alpha 1 chain protein could be responsible for the increased MFA in compression wood. Some of the uncategorized proteins, such as Pentatricopeptide repeat-containing protein At2g17670 (spot 2026, -3.10-fold), RPM1-interacting protein 4 (spot 2502, -2.88-fold), and Monothiol glutaredoxin-S4 (mitochondrial, spot 3167, 3.87-fold) were observed in the wood-forming tissue. Belkhadir et al (2004) inferred that the RPM1-interacting protein 4 negatively regulated Nucleotide-Binding Site-Leucine-Rich Repeat (NBS-LRR) proteins, which plays role in improving plant disease resistance. The relationship and regulate patterns of these unclassified proteins and compression wood formation need to be further investigated.

CONCLUSIONS

The 2D-DIGE proteome technology was successfully used for investigating the responses of proteins to the bending stem treatment on *Pinus koraiensis* seedlings. Compression wood was formed as a result of bending stem treatment (at 45o from vertical growth direction) for 15 days. Most of the as-extracted proteins in the wood-formation tissue were distributed in pH from 4-8 and molecular weight ranging from 30-97 KDa. 24 spots were identified in the tissue and these proteins primarily functioned for amino acid metabolism, cell wall synthesis, secondary metabolism, and stress response. Proteins related to methionine pathway and lignin biosynthesis were over expressed in the compression wood-forming tissues. Instead, the protein related to lipid metabolism was down-regulated in compression wood. Moreover, some proteins involved in energy metabolism and photosystem that have never been reported in wood-forming tissue of compression wood were also observed. The findings help to better elaborate the proteome that occurs in within compression wood formation.

ACKNOWLEDGEMENT

This work was supported by National Natural Science Foundation of China (31600454), the Key Laboratory of Bio-based Material Science and Technology (Northeast Forestry University) Ministry of Education (SWZCL2016-09), and PAPD. Thanks to Lee Miller Smith at the University of North Texas for help in editing the language.

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