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METABOLIC PROFILES IN WOOD FORMING TISSUE DURING TENSION WOOD FORMATION

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ABSTRACT

Metabolic profiling in wood-forming tissue has become a valuable tool for reveal the mechanism of wood formation because almost all the chemical components of wood are the result of metabolism in living tree. In angiosperms, tissue that differs from normal wood develops on the upper side of an inclined stem or branch and is referred to as tension wood. Here, we aimed to determine whether such metabolic profiling were associated with different extent tension wood formation in *Populus simonii* young seedlings were artificial inclined and the Gas chromatography-Mass spectrometry (GC-MS) were employed to analysis the whole metabolites. Cross sections of newly wood were observed by Scanning Electron Microscope. Growth traits and lignin content also were investigated. Different degree tension wood was formed in stem inclination 0°-70°. Tree growth was seriously affected by stem inclination in greater angle. Varieties and abundances of metabolites were significantly affected by inclined angle. The patterns of relative abundance of D-glucose and D-fructose in accordance with cellulose content in smaller incline angle treatment. Amino acid was increased in stem inclination. L-proline was significantly increased in stem inclination 30°. Additionally, the majority of lipids were significantly increased in response to stem inclination treatment. These results indicated that metabolites may perform essential roles in tension wood cell wall biosynthesis and architecture.

KEYWORDS: Tension wood; gas chromatography-mass spectrometry (GC-MS); metabolites; wood forming tissue.

INTRODUCTION

The variation of wood structure and chemical properties is caused by abiotic and biotic stresses in tree growth. In order to back toward its vertical orientation in inclined or crooked tree stem, trees usually develop reaction wood which has special organization structure and chemical constitutes (Scurfield 1973; Timell 1986). Reaction wood in gymnosperms is referred to as tension wood (TW) and is formed on the upper side of the leaning (Du and Yamamoto 2007). In fact, TW also is formed in fast grown plantation broad-leaved tree and always regard as a serious defect in wood utility because of its difference with normal wood in cell wall structure and chemical components. Especially, TW is characterized by the gelatinous cell wall layer and changed cellulose content affecting the timbers milling properties.

For nearly hundred years, the TW formation was investigated in anatomy, constitutes, biochemistry and physiology. Since nineties last century, modern molecular biology technology has been applied to TW forming tissue on genomic, transcriptome and proteomics levels (Wu et al. 2000; Paux et al. 2005; Kaku et al. 2009; Jin et al. 2011). Some hypothesis, like growth stress, gravity response, hormone distribution and pressure expansion, were stated based on these research results. All of these show that it is an integral development and respond on different levels in tension wood formation. Yet, our knowledge is still limited about the mechanism of TW formation on overall level. Recently, metabonomics, as the important part of system biology, was been interested in field of plant science and wood science. Metabolites can be regarded as the ultimate output of gene expression under the influence of environment (Fiehn 2002). Research on variation pattern of metabolic in reaction wood formation could predict wood properties and interpret gene function in wood formation. Andersson-Gunnerås et al. (2006) bent the stem at 45° and investigated Carbon flux in tension wood forming tissues at transcriptome and metabolome level. The result suggested that decreased activity were the pathway for C flux through guanosine 5'-diphosphate sugars to biosynthesis of lignin and cell wall matrix carbohydrates (Andersson-Gunnerås et al. 2006). Comparison with the kinds and relative abundance of metabolites in developing wood tissue between juvenile wood and compression wood, which were significantly difference on intermediates which involve in lignin and cellulose synthesis (Yeh et al. 2006). Metabolites patterns were accordance with main chemicals in compression wood formation in *Pinus koraiensis* which were related to stem inclination periods and angles (Shi and Li 2012a; Shi et al. 2012). Morris et al. (2004) indicated that different wood properties genotypes could be distinctly segregate by metabolic profiles. Our previous study had indicated that both HPLC/MS and GC/MS employed to metabolite analyze wood forming tissue provided the basis for methods to separate the wood samples obtained for the two classes of wood (Shi and Li 2012b).

Mostly studies demonstrated that TW generally induced by stem inclination at one angle. Yamashita et al. (2008) showed that mild to severe compression wood could be formed by growing saplings at different inclinations (0 -50°). So, the objectives of this work were to identify metabolites altered by stem inclination angles in 4-year-old *Populus simonii* seedlings. Effects of inclination angle were evaluated by assessing grown traits, lignin content and cross section structure. The results help to reveal the metabolism molecular basis in tension wood cell wall formation.

MATERIAL AND METHODS

Plant materials and sample preparation

The experiments were conducted from May to July, 2011 in a plot at Flower Biological Engineering Institution of Northeast Forestry University. 4-year-old *P. simonii* seedlings (average 146.2 cm in height and 12.9 mm in diameter) were planted in plastic pots filled with a mixture of black soil and compost. The straight stems of seedlings (five saplings per treatment) were bent at 10, 30, 50 and 70° angle to the vertical on May 9, 2011, the bent method according to former study (Shi and Li 2012a). The five straight seedlings were as control. Samples collect method was performed following the procedure described by Paux et al. (2005). Shortly, the wood forming tissue was rapidly harvested from the upside of the bent stem (with sterile scalpel) after removing the bark, and control samples were from about 10 cm above the ground. Samples were kept frozen at all times at -80°C until ground. The samples were quickly ground to a fine powder using a liquid N_2-chilled mortar and pestle.

Chemicals

Ribitol (CAS No. 488-81-3), Solution A (Methoxyamine hydrochloride (CAS No. 593-56-6) soluble in Pyridine (CAS No. 110-86-1), 20 mg•ml^{-1}, Solution B (Alkanes soluble in N-Methyl-N-(trimethylsilyl) trifluoroacetamide (CAS No. 24589-78-4), MSTFA, 20 µl•ml^{-1}, were purchased from Sigma (USA); Methanol and Chloroform (Chromatography grade) were purchased from Kermel (Tianjin, China).

Growth traits and cross section

Tree height and diameter at bending piont were measured. Bare-handed cross sections about 3-mm thick from samples derived from the region (10 cm over ground) of the vertical stems or the upper side of the inclined stems. The section was air-dried after washing with distilled water and then observed with a Scanning Electron Microscope (SEM, Quanta 200, FEI, USA) at an accelerating voltage of 10.0 kV.

Lignin content

Lignin content was measured with acetyl bromide method described previous report (Iiyama and Wallis 1988; Rodrigues et al. 1999; Foster et al. 2010). 1.5-2.5 mg freeze-dried samples were placed in 1.5 ml tube and digested with 100 µl acetyl bromide/acetic acid (25 %, V/V) and 4 µl 70 % perchloric acid at 70°C, 300 rpm for 30 minutes. Terminated the reaction on ice for 10 minutes and added 200 µl 2 M sodium hydroxide. Then mixed and standing on ice for 10 minutes. The mixture was transferred to a new 5.0 ml tube and added up to 4 ml with acetic acid. The percentage of acetyl bromide soluble lignin (% ABSL) was determined with absorbance at 280 nm.

Metabolites analysis

Metabolites in wood forming tissue were extracted according to the protocol of former studies (Lisec et al. 2006; Shi and Li 2012a). Briefly, the frozen (-80°C) and ground wood forming tissue (50 ± 2 mg) was first extracted with 1 ml of 100 % methanol and 45 µl of Ribitol as an internal quantitative standard (IS) and vortex. The mixture was mixed for 15 min at 70°C in a thermomixer at 950 rpm and centrifuged. 500 µl of supernatant was transferred to a new tube, then 500 µl of chloroform was added and vortexed. The mixture was in the thermomixer for 5 min at 37°C at 950 rpm. 500 µl of dH_2O was added and vortexed, centrifuged 15 min at

4000 g, and 200 µl was transferred from the upper phase into a new tube and stored at -80°C. Before methoximation, the samples were dried in a freeze vacuum concentrator and methoximated with 50 µl of solution A at 37°C for 2 hours. Then trimethylsilylated with 100 µl solution B for 30 min at 37°C in the thermomixer at 260 rpm.

The derivatized extracts were analyzed using a Varian 450 GC-240 MS system (USA), and the metabolites were separated on a VF-5 ms 30 m×0.25 mm×0.25 µm column with a helium (99.99 %) carrier gas flow of 2 ml•min^{-1}. The front inlet temperature was 250°C and oven was held at 70°C for 1 min and then raised by 5°C• min^{-1} to 300°C where it was held for 5 min and cooled to 70°C. It took about 52 min per sample. The injection volume was one mircroliter. The helium gas flow rate was 1 ml• min^{-1} and the injector split ratio was 1:20. A threshold cutoff for metabolite analysis was set at 2 % of the peak area of the internal standard. The mass spectrometer was operated in the electron impact ionization mode at 70 eV. The ion source was 300°C and data acquisition was performed in the full scan from m/z 50 to 1000. The mass ions were scanned at the rate of 2 spectrum•s^{-1}.

The metabolites were identified using NIST Mass Spectral Search Program (National Institute of Standard and Technology, 2005, USA) based on comparison to authentic compounds with retention time and mass spectra. The relative abundance of all single compounds was displayed by peak areas of unknown compound divide the peak area of IS. Data of growth parameters, lignin and cellulose content and metabolites were expressed as the mean standard ± deviation (n=5). The data were analyzed and mapped by Origin Pro.8.0 and Microsoft Excel 2007.

RESULTS AND DISCUSSION

Growth responses

The height increment and diameter at bending piont increment were significantly changed under all stem mechanical incline treatment. The height increment decreased with increasing stem inclination angle, and there was minimal at 70° treatment. In another hand, the diameter increment was sharply increased at 10°, and then the increment was reduced at 30°, but it was hardly any changes at 50 and 70° (Fig. 1). The results of variance analysis showed that incline angle significantly affect the height and diameter growth of young seedlings ($P<0.05$). However, the changing pattern of diameter increment was inconsistent with the research on poplar clone 107 which was decreased along with increased of inclination angle (Liu 2010). These results suggested that bending stress could inhibit the growth increment at greater inclination angle. Wood formation is the results of continuously growth on height and diameter in tree. Previous studies of planted conifers suggested that mild compression wood always formed along with higher growth rate (Donaldson et al. 2004). On the contrary, other works showed that stem inclination treatment in conifer trees could inhibit its axial growth and result in growth rate reduced (Longman and Wareing 1958; Sinnott 1952). These observations indicated that mild reaction wood formed with higher diameter growth at smaller inclination and the axial growth is inhibited by stem inclination.

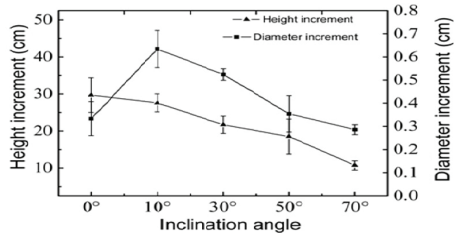

Fig. 1: Variation of growth parameters under inclination treatment. Each bar is the mean ± SE (n=5) for each treatment.

Cross section and lignin content

To confirm the TW formation in 4-year-old *Populus simonii* seedlings, stem cross section was observed using SEM. Gelatinous layer is a typical characteristic in inner cell wall of TW. From Fig. 2 different degree gelatinous layer was observed in all incline treated samples. The thickness of gelatinous layer was observably increased with increased inclination angle, and almost occupied the whole cell lumen at 70°. The stick together of cell wall in 10 and 50° maybe result from the over soften before sample slicing.

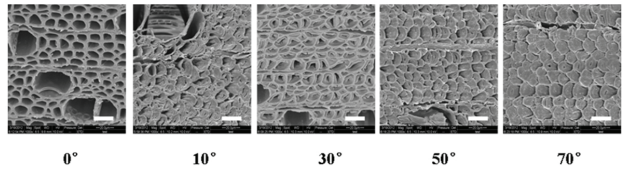

Fig. 2: SEM of cross section in different inclination angle (Bar: 20 μm).

Compared with vertical tree, higher cellulose content and lower lignin content is a characteristic in tension wood, and which is caused by gelatinous layer in wood cell wall. In this work, lignin content in wood forming tissue decreased in all inclination treatment than vertical seedlings, but the changes was not significant at $P<0.05$ (Fig. 3). These indicated that metabolism pathway in tree was changed allocated to different products. In addition, SEM observed showed that cell wall thicken by the gelatinous layer and supporting the cellulose content may increased in treatment trees (Fig. 2). Yamashita et al. (2009) showed that lignin content and the percentage of cell walls increased with stem inclinations up to 20 to 30°, but did not increase further with increased stem inclination in Japanese cypress (*Chamaecyparis obtusa*) saplings. This also suggested that the degree of development of reaction wood reaches a limit at around 30°, not in gymnosperms but in angiosperms.

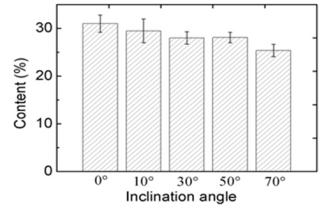

Fig. 3: Variation of lignin content under inclination treatment. Each bar is the mean SE (n=5) for each treatment.

Metabolites profiling responses to inclination

To explore metabolites profile response to different stem inclination angles treatment, the kinds and relative abundance of metabolites in tension wood forming tissue was analyzed by GC-MS. The polar metabolites from wood forming tissue of normal and inclined stem samples were identified. These identified metabolites include saccharides, amino acids, lipids, alcohols, organic acids, *N*-compounds and unknown (Tab. 1). The relative content of organic acids is the highest, with saccharides, and amino acids following. Variance analysis between bending treated and untreated samples was performed using T test. Some metabolites showed significantly difference at $P<0.05$ and $P<0.01$.

Tab. 1: Classification and proportion of metabolites in wood forming tissue of normal tree.

Classification	Proportion
Alcohols	9.09 %
Alkanes	3.03 %
Amino acids	12.12 %
Lipids	9.09 %
N-compounds	6.06 %
Organic acids	24.24 %
Saccharides	15.15 %
Unknown	21.21 %

Because of the extraction from wood forming tissue is a mixture and the components is unknown. Furthermore, no target analysis for mixture is one type of metabonomics. The metabolites which identified in all samples were selected and compared to each other in relative abundance (Fig. 4). Amino acid is an important substrate for protein synthesis and plays a critical regulation in wood cell wall formation. The change pattern of L-aspartic acid was similar to L-serine, with an increase in the 10-50° and they decreased to the level at controls (Fig. 4a). However, the abundance of L-proline was sharply increased in 0-30°, after which it did not increase further.

The relative abundance change pattern of D-fructose was similar to D-glucose with an increase in 10° inclined compared to control. At a stem inclination of 30-50°, their abundances were almost the same at vertical samplings, after which decreased slightly at 70° (Fig. 4b). The relative abundance of a, D-glucopyranoside increased with stem inclination up to 50°, after which it decreased to the level at 0°. On the other hand, D-glucose and D-turanose was decreased in every treated seedling. The abundance of D-turanose was significantly decreased about six-fold after in 70° of inclination.

Some lipids were identified in wood forming tissue. The change pattern of octadecanoic acid was similar to hexadecanoic acid and monostearin, with increase in stem inclination up to 70° (Fig. 4c). Alcohols also are an important substrate and intermediate products in cycle of metabolism. The abundance of inositol was increased with stem inclination up to 50°, after which it decreased abruptly (Fig. 4d). In addition, glucitol and glycerol was nosignificant changes at different inclination angles. The organic acid variety identified in wood forming tissue was more than others. The abundance of lactic acid increased with inclination angle up to 70°. The pattern of malic acid was similar to ethanedioic acid with increased at stem inclination of 10-50°. Interestingly, the abundance of citric acid was decreased in inclined samples, which induced about 4-fold after 70° of inclination (Fig. 4e).

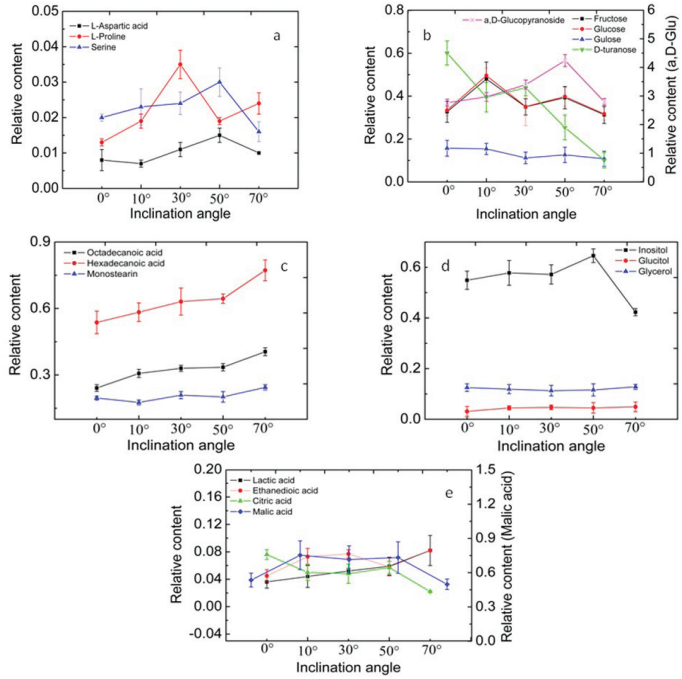

Fig. 4: Changes of partly amino acids a), saccharides b), lipids c), alcohols d) and organic acids e) under inclination treatment. Each bar is the mean ± SE (n=5) for each treatment.

Changes in the relative abundance of metabolites during various inclination angles of tension wood formation have provided new insights into the developmental metabolites that underlie the modifications in cell wall structure and composition. In this report the relative abundance of D-fructose and D-glucose increased by nearly doubled in stem inclination 10° than control samples, respectively (Fig. 4b). However, the abundance of D-fructose and D-glucose increased slightly in 30° than control seedlings. According to current studies, UDP-glucose is regarded as the immediate substrate for cellulose synthesis (Kqczkowshi 2003; Coleman et al. 2007), but UDP-glucose can be formed by two metabolic pathways. First, it changes glucose to glucose-6-phosphate, glucose-1-phosphate and at last via the conversion to UDP-glucose by pyrophosphorylase. Secondly, sucrose can be converted into UDP-glucose and fructose by sucrose synthase (Babb and Haigler 2001; Delmer and Haigler 2002). The increase of glucose in inclined stems could result in increase of UDP-glucose and the increase of fructose could increase the conversion of sucrose to UDP-glucose. On the other hand, the UDP-glucose levels increased prior to the maximum rate of secondary wall cellulose synthesis in developing cotton fibers (Carpita and Delmer 1981). All of these effects caused the higher cellulose content in the inclination stems.

The relative abundance of inositol, a carbohydrate-related metabolite, decreased in tension wood formation in *Populus* (Andersson-Gunneras et al. 2006), but increased in our experiment. Inositol is widely distributing in animal and plant, but it is not well known its metabolic pathways and physiological function. Hence, the abundance of inositol decreased abruptly in heavy inclination stem results from severity restrain of tree normal growth. The changes of lactic acid,

malic acid and ethanedioic acid suggested that plant respiration enhanced and the growth rate increased. The relative abundance of citric acid which is the first product in tricarboxylic acid cycle was decreased in stem inclination< 50° (Fig. 4e) and accord with the results of Andersson-Gunnerås et al. (2006). However, the molecular basis of this pattern is not clearly.

Former study suggested that increased activity lipid biosynthesis in tension wood forming tissue of *Populus tremula* (L.) (Andersson-Gunnerås et al. 2006). In our work, the relative abundance of lipids increased in all stem inclination compare to normal samples, and main saccharides involve in cellulose synthesis was increased. At this point, it could be inferred that the carbon allocation and metabolic pathways was changed. So, as the second ingredient, the relative content of lignin will reduce. This is consistent with the acetyl bromide lignin content in inclination seedlings. Previous studies showed that the transcript relative abundance of EgCesA, an important enzyme in cellulose synthesis, increased 6.5 times in early stage of bending stress than that of the control. The transcript relative abundance of 4CL (4-Coumarate CoA ligase), CCR (Cinnamoyl CoA reductase) and CAD (Cinnamyl alcohol dehydrogenase) which are key enzymes in lignin synthesis, was sharp but transient decrease in earliest stage, which was followed by an increase up to 24 hours in stem 45° bending stress (Paux et al. 2005). Paux and others also suggested that cellulose becomes the major carbon sink in the tension zone and lignin biosynthesis would be attenuated in the same zone during tension wood formation in *Eucalyptus*, because of the shift in carbon flux towards cellulose biosynthesis. All of these data suggest increased activity for cellulose and lipids biosynthesis, and decreased activity for carboned flux through guanosine 5'-diphosphate sugars to lignin biosynthesis.

We observed increased L-proline relative abundance after stem inclination. The findings that compression wood cell walls had higher lignin content and that PtaPRP1 was highly expressed in compression wood than in vertical stem might reflect a relationship between some proline-rich proteins and lignin formation (Zhang et al. 2000). Other studies have shown that proline accumulation in roots and sap of maize increases under water stress (Alvarez et al. 2008). These all suggest that proline may play a role in response to abiotic stress.

The kinds and relative abundance of metabolites was changed in TW formation. Further research is required to confirm the relationships between these compounds and wood properties. Moreover the combination of metabonomics with genomics and proteomics, give a clearly biological overview for tension wood cell wall development.

Tilting of stems at different angles to the vertical can be induced various degrees of reaction wood. The intensity of the stem response is dependent on the angle of inclination, with the maximum effect observed at a tilt of 45° (Herrera et al. 2010). We prepared tension wood samples with various degrees of development and examined the relative abundance of metabolites among the samples. The results showed that some metabolites changed their abundance with increasing development of TW. As for fructose and glucose, these metabolites increased in abundance abruptly at the TW formation began and decreased gradually with inclination angle.

CONCLUSIONS

Tree height and diameter was affected significantly by inclination angles during the different extent tension wood formation. The height increment decreased with increasing treated angle and diameter increment sharply increased at smaller treated angle. Lignin content was reduced with treated angle but showed no significant effects by it. Metabolites in wood forming tissue displayed significantly difference in inclination treated stems compared to control samples. The relative

abundance of main saccharides was increased obviously at smaller treated angle and decreased at greater angles. The abundance of lipids was increased with inclination angle up to 70°. Three amino acids showed similar change pattern with treated angle and the abundance of L-proline changed dramatically. Also, other organic acids and alcohols, which play crucial role in energy and substance metabolism, changed their abundance with inclination angle. Tension wood tissue can be induced by inclined stem at different angles. Trees showed system response on whole levels during TW formation. The change of metabolites incorporate to cell wall structure and chemicals results from inclined wood forming tissue suggest that wood cell wall related-monomers was changed in metabolic pathway.

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