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METABOLITES ANALYSIS OF WOOD FORMING TISSUE

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ABSTRACT

A comprehensive experimental result will be obtained by a multi-analytical platform compared to a single method. We describe here the use of High Performance Liquid Chromatogram Mass Spectrometry (HPLC-MS) and Gas Chromatogram Mass Spectrometry (GC-MS) through the analysis of poplar metabolites from wood forming tissue extracts. It was possible to identify and calculate the chemical composition and relative abundance of these compounds respectively. Comparison of compression wood and normal wood showed that each phenotype possesses a distinct metabolic profile. GC-MS results indicated that important substrate for cellulose synthesis is obviously lower in wood forming tissue of compression wood.

KEYWORDS: Compression wood, gas chromatogram mass spectrometry (GC-MS), high performance liquid chromatogram mass spectrometry (HPLC-MS), metabolite, *Pinus koraiensis*, wood forming tissue.

INTRODUCTION

Metabonomics is the quantitative measurement of the dynamic multi parametric metabolic responses of living systems to external stimuli or genetic modification (Goodacre 2004). Metabonomics is one significant part of function genomic and integral biology. Metabolites can be regarded as the ultimate output of gene expression under the influence of environment (Fiehn 2002). It is difficult to identify the compounds due to its wide varieties. The mass spectrum couple with chromatogram separation is useful and is widely employed on metabonomics research. HPLC can reduce the ion inhibiting effect, which is caused by altogether elution component. Plant samples don't need other chemical treatments for HPLC/MS, which has a better selectivity and great sensitivity. In contrast GC-MS requires the use of derivatization reactions and this is likely to change the samples. It limits that GC-MS just provides an analytical technique for the

thermally unstable mixture. Indeed, it seems obvious that deploying multiple methods is possible to result in a more comprehensive set of metabolic data than using each alone. The use of coupling analytical platforms will provide a better strategy for metabonomics.

Wood is an important biological resource from the secondary growth of trees. However, we know little about the genetic mechanisms of wood formation (Li et al. 2006). Metabonomics of wood forming tissue will not only explain the internal relationship between tree growth and environment but also explore profound key genetic regulation factor in wood formation and development. Furthermore, this could provide scientific proof for improvement of wood quality. Metabolite profile had been reported in *Arabidopsis* (Fiehn et al. 2000), potato (Shepherd et al. 2007), wheat (Hamzehzarghanie et al. 2005), and maize (Alvarez et al. 2008). It indicated that these compounds play a vital role in growing development and signal transduction. Nevertheless, it is rarely analyzed for metabolites of wood formation. Trees usually produce reaction wood, which has a specific atomical structure and chemical constitution in response to gravistimulus (Timell 1973a, b, c). Reaction wood in gymnosperms is referred to as compression wood and develops on the lower side of leaning stems or branches (Du 2007). Its formation "pushes" the stems or branches toward a vertical orientation (Scurfield 1973). At present, reaction wood is believed as the best experiment system for wood formation research. In this paper, we report the application of HPLC-MS and GC-MS in metabolites analysis of wood forming tissue from *Pinus koraiensis*. Both techniques were used to compare the metabolite profiles of compression wood and normal wood.

MATERIAL AND METHODS

Plant material and sample preparation

Pinus koraiensis trees which were from the same clone were planted on the Northeast Forestry University research forest, Harbin of China (45°72′ °N, 126°62′°E). Each of the compression and normal trees were picked three randomly. Compression wood is on the underside of bent stem and normal wood from the vertical tree as control. Wood forming tissue (immature xylem) was collected immediately with a fresh scalpel after bark, phloem and cambium peeled in July 20, 2010. These samples were placed in liquid nitrogen and stored at -80°C until use. Suitable frozen tissue was ground to a fine powder using liquid N_2-chilled copper/capsule. Samples were kept freezing at all times and once ground, were returned at -80°C.

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).

Extraction of polar metabolites

Metabolites from developing xylem tissue were extracted according to the protocol of Jan Lisec (Lisec et al. 2006). The frozen (-80°C) ground immature xylem tissue (50 mg ± 2 mg) was first extracted with 1 ml 100 % methanol (pre-cooled at -20°C) and 45 µl of Ribitol (2 mg.ml^{-1} stock in dH_2O) as an internal quantitative standard (IS) and vortex for 10 s. The mixture was for 15 min at 70°C in thermomixer at 950 r.p.m. and centrifuge for 10 min at 12000 g.500 µl

supernatant was transferred to a new 1.5 ml tube, then added 500 µl chloroform (pre-cooled at -20°C) and vortex for 10 s. The mixture was for 5 min at 37°C in thermomixer at 950 r.p.m. 500 µl ddH_2O (4°C) was added and vortex for 15 s centrifuge 15 min at 4000 g and transfer 200 µl from the upper phase (methanol/water, polar phase) into a new tube stored at -80°C.

HPLC-MS Analysis

The samples were analyzed on high performance liquid chromatography system 3000 (AB Sciex, USA) with a (150×4.6) mm Agilent Eclipse XDB-C18 column (5 µm particle size). All samples were eluted using a linear gradient methanol/water 0–100 % with a flow rate of 1 $ml.min^{-1}$ for 60 min. 10 µl injection was used throughout at 25°C. Mass spectrometer operated with electrospray ionization source and positive and negative scan modes. The acquisition range was from m/z 100-1000 amu. The ion source voltage and temperature were set at 4.5 kV and 300°C, respectively. Atomizer 12 psi and curtain gas 10 psi was used for analysis. Mass spectrometric data was collected using analysis software analyst 1.4.

GC-MS Analysis

200 µl sample was dried for 4 hours at -60°C in freeze vacuum concentrator and was redissolved in 50 µl of methoxyamine hydrochloride (20 $mg.ml^{-1}$ in pyridine) with shaking for 2 hours at 37°C. The samples were derivatized with 100 µl of N-methyl-N-trimethlsilyl-trifluoroacetamide (MSTFA), 30 min at 37°C in thermomixer at 260 r.p.m. In order to react sufficiently, the samples stood for overnight at room temperature.

The samples were analyzed on Varian 450GC-240MS (USA) which fitted with a VF-5 ms (30 m×0.25 mm×0.25 µm) column. The helium carrier gas (99.99 %) flow was 2 $ml.min^{-1}$. The GC oven was held at 70°C for 1 min and then ramped at 5°$C.min^{-1}$ to 300°C where it was held for 5 min and cooled to 70°C. 1 µl injection with a 50:1000 split and flow of 1 $ml.min^{-1}$ was used thoroughly. It took about 52 min for one sample. A threshold cutoff for metabolite analysis was set at 2 % of the peak area of the internal standard. Metabolites were identified with an on line 240 EI-Mass Spectra (Varian, USA) and MS Workstation software (version 6.9.3). The ion source was 300°C and Mass spectra were acquired over the range m/z 50-1000 with an electron ionization of 70 eV. The mass ions were scanned at the rate of 2 $spectum.s^{-1}$.

Qualitative and data processing

The metabolites were identified using NIST Mass Spectral Search Program (National Institute of Standard and Technology, version, USA) based on the comparison to authentic compounds with retention time and mass spectra. The data was processed using principal components analysis (SPSS, Version 19.0) and analyzed (Excel 2007). Each peak in a chromatogram was expressed relatively to the area of the ribitol internal standard peak. Furthermore, peak areas were normalized across all chromatograms by adjusting for the exact amount of tissue (mg fresh weight) used in each sample extraction.

RESULTS AND DISCUSSION

HPLC-MS positive ion results

Both positive and negative Electronic Supplementary Information (ESI) were used to High Performance Liquid Chromatogram-Mass spectrum (HPLC-MS) which performed on polar metabolites from wood forming tissue of normal and compression wood. The two ionization

methods detected different classes of molecule and therefore produced different profiles, and these profiles also elaborated different matters. The results show obvious differences between total ion current chromatograms obtained from the positive ESI HPLC-MS of compression and normal wood (Fig. 1). Subsequent, statistic analysis by principal component analysis makes clear separation between these two different wood tissues. Ions which contributed to the PCA separation were seen at m/z 432.5, 415.6 (26.012 min), 374.8 (28.044 min), 425.5 (44.725 min) and 282.6 (51.347 min), which were higher in compression wood samples whilst the ions at m/z 422.4, 405.6 (10.535 min), 274.7 (18.588 min) and 302.6 (23.751 min) which were higher in the normal wood samples (Tab. 1).

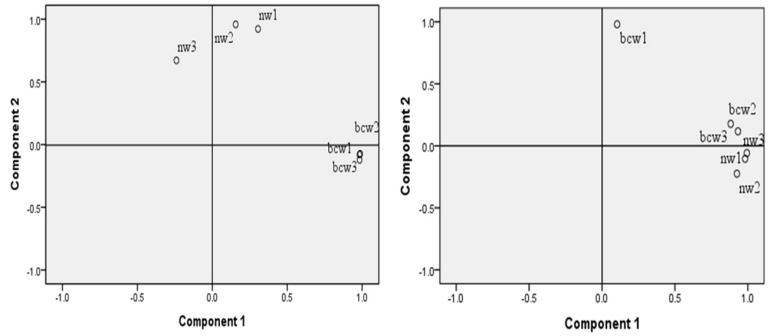

Fig. 1: PCA scores of HPLC-MS positive ion (left) and negative ion (right) analysis of matebolite obtained from compression wood (marked as bcw in the figure) and normal wood (marked as nw in the figure).

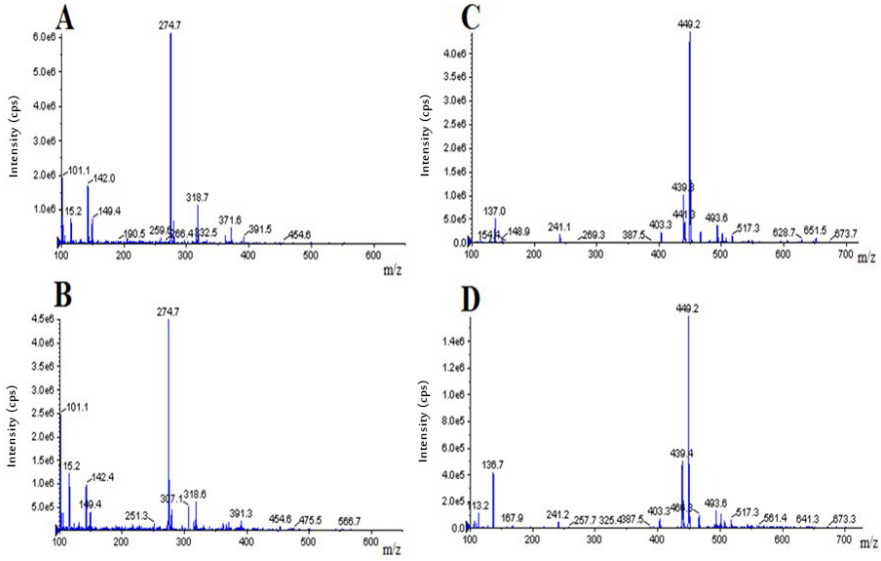

Fig. 2: High performance liquid chromatogram mass spectrum of extracted ion for m/z 274. 7(17.735-18.588) and 449.2 (8.478-8.654 min) in normal (A, C) and compression wood (B, D).

Unlike the GC-MS which has large databases, the use of HPLC-MS-based techniques for metabonomic research is still in its infancy and databases for biomolecules have not yet been constructed. So the identities of these compounds remain unknown. However, the identity of these unknowns is being actively pursued and, hopefully, these compounds will not only provide useful markers in metabolic pathway of tree physiology but will also yield novel and useful insights into the wood formation.

HPLC-MS negative ion results

As with the positive ESI data both visual inspection and PCA of the corresponding negative ESI HPLC-MS total ion chromatogram results also revealed a clear separation between the two wood tissues. The extracted ion chromatogram for one of the metabolites detected by negative ESI that was seen to vary between compression and normal wood, together with the mass spectrum obtained for this compound was shown in Fig. 2. Those ions detected by negative ESI contributed most to the differences observed between the two types included m/z 435.5, 425.3 (3.261-3.713 min), 375.5, 365.5, 253.4 (50.72-51.071 min) which were higher in the compression woods and 511.4, 465.3 (4.342-4.515 min), 435.3, 425.2, 389.3 (6.196-6.447 min), 449.2, 439.4 (8.478-8.654 min), 449.4, 439.3, 403.3 (10.360-10.535 min), 681.7, 671.7 (51.322-51.699 min), 485.6 (54.684-55.135 min) which were higher in the normal woods (Tab. 2).

GC-MS results

Total ion chromatograms of each sample were obtained by GC/MS analysis of TMS-derivatives from wood forming tissue extracts. Chromatograms of metabolites from compression and normal wood immature xylem showed distinct difference. There are thirty-seven and twenty nine peaks which were greater than 2 % of total peak area in normal and compression wood respectively. These ions were observed to be either specificity for wood source or difference in relative abundance. Due to the complexity and diversity of metabolites, 2 $mg.ml^{-1}$ ribitol was only as internal standard for quantifying. Postulated identities were listed based on high-scoring matches (mass spectrum, retention time) from National institute of standards and technology (NIST) and the reported. Spectra of these compounds together with database matches were given in Fig. 3. These metabolites were assigned identities and classified as amino acid, carbohydrate, alcohols, organic acids, N-compounds and others. In addition, some compounds were defined as unknowns because they did not hit in the database.

About 90 % metabolites are relatively lower in the extracts obtained from compression wood compared to the normal wood (Tab. 3). The difference in carbohydrate is the most outstanding. The reduction of fructose and glucose in BCW were consistent with the lower cellulose content in BCW (34 %) compared to NW (44 %) (Yeh et al. 2005). At present, it acknowledges that UDP-glucose server as the substrate for polymerization of glucan chains which were gathered to cellulose. So the amount of UDP-glucose will affect the rate of deposition of cellulose in fibers. However, UDP-glucose can be formed by two pathways. The first pathway is from glucose to glucose-6-phosphate, glucose-1-phosphate and is followed by the conversion to UDP-glucose by pyrophosphorylase (Babb and Haigler 2001, Delmer and Haigler 2002). The second pathway is the conversion sucrose to UDP-glucose and a portion of the fructose released is recycled into additional sucrose metabolism to support subsequent cellulose synthesis. Moreover, over-expression of UDP-glucose pyrophosphorylase in hybrid poplar revealed significant increases in cellulose contents and concurrent decreased in lignin contents (Coleman et al. 2007). These effects result in the lower cellulose content in BCW compared with NW.

Tab. 1: Ions detected by positive ion HPLC-MS and comparison of normal wood and compression wood.

Retention time/min	Principal ions	Elevated in NW	Elevated in BCW
10.285-10.535	422.4, 405.6	+	-
17.735-18.588	274.7	+	-
22.751-24.833	302.6	+	-
26.012-26.364	432.5, 415.6	-	+
28.044-28.747	374.8, 330.7	-	+
44.725-45.028	425.5, 403.6	-	+
51.122-51.347	282.6	-	+

Note: + increase, - decrease

Tab. 2: Ions detected by negative ion HPLC-MS and comparison of normal wood and compression wood.

Retention time/min	Principal ions	Elevated in NW	Elevated in BCW
3.261-3.713	435.5, 425.3	-	+
4.342-4.515	511.4, 465.3	+	-
6.196-6.447	435.3,425.2,389.3	+	-
8.478-8.654	449.2, 439.4	+	-
10.360-10.535	449.4,439.3,403.3	+	-
50.720-51.071	375.5,365.5,253.4	-	+
51.322-51.699	681.7, 671.7	+	-
54.684-55.135	485.6	+	-

Note: + increase, - decrease

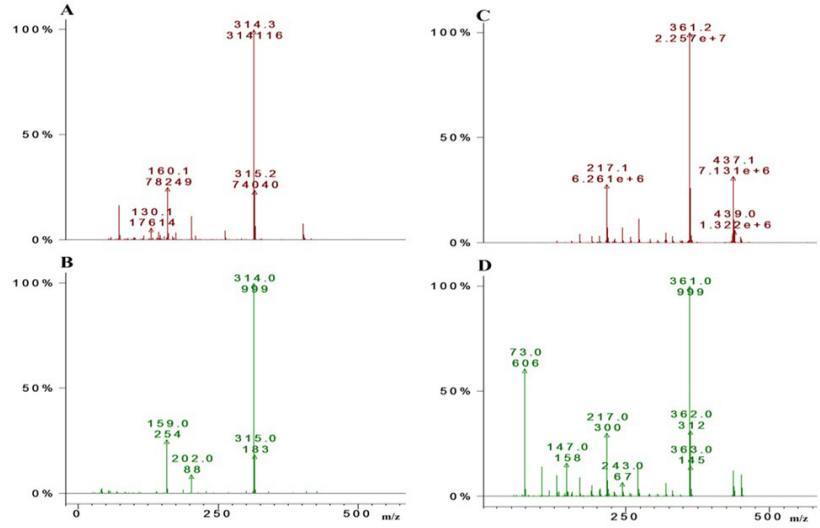

Fig. 3: Identification of perturbed metabolites from GC-EIMS analysis: A) EIMS for peak eluting at 36.537-36.656 min and B) its library match. 3-Trifluoromethylbenzylamine,N,N-diundecy, C) EIMS for peak eluting at 39.666-40.004 min and D) its library match alpha, D-Glucopyranoside 1,3,4,6-tetrakis-O- (trimethylsilyl)-, beta-D-fructof.

Tab. 3: Gas chromatogram mass spectra of significant metabolites and comparison of normal wood and compression wood.
Abbreviation: TMS=trimethylsilylate derivative

Retention time (min)	Principal ions m/z (relative intensity)	Description (from best library fit)	Relative abundance	
			NW	BCW
7.078-7.153	191.0 (36), 147.1 (100), 73.1 (56)	Lactic acid TMS	+	-
9.156-9.261	147.1 (100), 133.1 (32), 73.1 (49)	Ethanedioic acid TMS	+	-
11.114-11.204	144.1 (100), 73.1 (34)	Valine,N-(trimethylsilyl)-,TMS	+	*
12.558-12.633	300.0 (26), 299.1 (100)	Silanol,trimethyl-,phosphate(3:1)	+	-
12.647-12.737	158.1 (100), 73.1 (40)	N,O-Bis(trimethylsilyl)leucine	+	-
13.188-13.278	158.1 (100), 73.1 (31)	Isoleucine,N-(Trimethylsilyl)-TMS	+	*
13.324-13.428	142.1	d-proline,N-ethoxycarbonyl-, pentadeayl ester	+	*
14.949-15.038	218.0 (39), 204.1 (100), 73.1 (56)	Serine tritms	+	*
15.626-15.647	291.1 (26), 218.1 (73), 101.1 (31), 73.1 (100)	N,O,O,-Tris(Trimethylsilyl)-L-threonine	+	*
18.265-18.273	233.0 (25), 147.1 (100)	Malic acid, O-trimethylsilyl-, bis(trimethylsilyl)ester	-	+
19.072-19.205	156.0 (100), 73.0 (38)	L-proline,5-oxo-1(Trimethylsilyl)-, TMS	+	*
19.235-19.294	174.1 (49), 73.1 (100)	Butanoic acid TMS	+	-
19.509-19.599	263.2	Trimethyl(2,6 ditert,-butyphenoxy) Silane		
22.563-22.667	231.1 (67), 147.1 (30), 73.1 (100)	Asparagine, N,N2-bis(trimethylsilyl)-, TMS	+	*
24.417-24.507	217.0 (100), 73.1 (42)	Arabinofuranose,1,2,3,5-tetrakis-O-(trimethylsilyl)-	-	+
24.834-24.917	156.1 (100), 73.1 (55)	I-Glutamine, tris(trimethylsilyl) derive.	+	*
25.527-25.631	204 (100), 147.1 (27), 73.1 (45)	1-Cyclohexene-1-carboxylic acid TMS	+	-
25.691-25.897	433.1 (36), 343.1 (40), 318.1 (71), 260.1 (100), 217.0 (73), 207.0 (44), 191.0 (59), 133.1 (43),129.0 (28), 73.1 (96)	unknown	-	-
26.335-26.439	346.1 (28), 345.2 (100), 255.2 (45), 147.1 (31),73.1 (48)	2-Thiobarbituric acid TMS	-	-
26.575-26.678	307.0 (46), 217.0 (80), 147.1 (35), 73.1 (100)	D-Fructose,1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime	+	-
26.769-26.887	307.0 (52), 217.0 (98), 147.1 (48), 103.0 (33),73.1 (100)	D-Fructose,1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime	+	-
27.474-27.562	319.1 (66), 205.0 (35), 147.1 (65), 73.1 (100)	Glucose,2,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime	+	-

28.197-28.286	318.1 (27), 305.1 (48), 217.1 (67), 133.1 (30), 73.1 (100)	Neo-inositol,1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-	+	-
30.311-30.429	328.1 (26), 313.2 (55), 129.0 (42), 117.0 (100)	Hexadecanoic acid TMS	+	-
30.795-30.914	432.1 (27), 318.1 (60), 305.1 (91), 217.0 (83), 191.0 (40), 147.0 (94), 73.0 (100)	myo-inositol,1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-	+	-
31.142-31.216	434.0 (43), 433.0 (85), 343.1 (55), 204.1 (27), 147.1 (88), 73.1 (100)	unknown	+	-
33.307-33.426	314.3	Androst-2-en-17-amine,4,4-dimethyl-N-(2-phenylethyl)-,(5,alpha.)	+	-
33.870-33.974	341.2 (46), 129.0 (40), 117.0 (100)	Octadecanoic acid TMS	+	-
36.537-36.656	348.2 (25), 342.3 (100)	3-Trifluoromethylbenzylamine, N,N-diundecy	+	-
37.555-37.660	358.2 (100), 319.2 (3), 73.1 (26)	4-Methylthio-N-pheny-1,2-carbazoledicarboximide	+	-
39.337-39.456	372.2 (27), 371.2 (100), 147.1 (26)	Silane,dimethylpentyloxyhexadecyloxy-	+	-
39.666-40.004	437.0 (34), 361.1 (100), 217.0 (35)	alpha,D-Glucopyranoside,1,3,4,6-tetrakis-O-(trimethylsilyl)-,beta.-D-fructof.	+	-
42.180-42.269	487.3 (27), 399.2 (100), 203.0 (46), 147.0 (61), 73.1 (65)	Bis(trimethylsilyl)Monostearin	+	-
46.137-46.299	369.1 (100), 361.1 (41), 147.0 (32), 73.0 (87)	unknown	-	+
47.361-47.581	207.0 (87), 177.0 (31), 119.0 (100), 73.1 (62)	unknown	+	-

Note: + increased, - decreased, * Inexistence

The process of wood formation is transformed and stored carbohydrate which is the major product by photosynthesis of trees as another form like cellulose. *N*-compounds are important intermediate in the metabolism of amino acid which acts as regulator in wood formation. It can describe metabolic pathway and synthesis of cellulose via change in relative abundance of glucose and fructose. Significant increase in the pools of shikimic acid, *p*-glucocoumaryl alcohol and coniferin are consistent with an increase in lignin production in response to compression wood formation. (Yeh et al. 2006) But these compounds were not found in this paper. Inositol participates in signaling and over expression resist disease in plant (Nelson et al. 1998). Malic acid is an important intermediate product in tricarboxylic acid cycle and participates in metabolism and energy conversion in high plant. Database searching is significant approach for identification of mass spectra (Schauer et al. 2005). However, it is still limited by so many compounds related to biological process and it is not yet to be found in the available libraries. It is necessary to investigate the unknown compounds which may be valuable regulator in wood formation.

Wood formation is one of the most important biological processes to human society and the earth's environment. However, the genetic mechanisms regulating wood formation is not well known. Wood is the major product of tree growth which converts light energy, principally water, carbon dioxide and inorganic nutrients into biopolymers via photosynthesis. These carbohydrates in living tree are transformed to a variety of organics by complex biochemical

reactions for responding to environmental change and developmental of diverse tissue and organ. Furthermore, organics produce secondary metabolite through secondary metabolism. Major secondary metabolites don't take part in metabolic activity and are believed as the end product of gene expression regulation.

Wood formation is a continuous process of secondary xylem differentiation derived from vascular cambium meristematic cells and involves a series of sequential biological events, including cell division, cell function specification, cell enlarging, massive secondary wall thickening, lignifications, programmed cell death and heart wood formation. Recently, many genes have been identified for their association with xylem differentiation and wood formation (Egertsdotter et al. 2004, Bedon et al. 2007, Nairn et al. 2008). Major of these genes are involved in biosynthesis of cellulose, lignin and hemicelluloses (Pot et al. 2006, Koutaniemi et al. 2007, Decou et al. 2009).

Detection of how gene expression is responsive to environmental conditions in the course of wood formation should provide insightful clues about genetic regulation of wood properties. Moreover, some of the differentially regulated genes showed expressive patterns in association with the changes of secondary cell wall structure and composition.

CONCLUSIONS

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. It showed the difference of either category or relative abundance between compression wood and normal wood. GC-MS results indicated that important substrate for cellulose synthesis is obviously lower in wood forming tissue of compression than normal wood. Metabolite profiling is a valuable additional tool in the plant functional genomics repertoire and is worthy of wide application within wood formation. However, there was some limited overlap in single or combination techniques. No doubt on-going technical and equipment development will cause even more comprehensive metabolite profiles.

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