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Genome-wide transcriptional profiling reveals molecular signatures of secondary xylem differentiation in *Populus tomentosa*

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ABSTRACT. Wood formation occurs via cell division, primary cell wall and secondary wall formation, and programmed cell death in the vascular cambium. Transcriptional profiling of secondary xylem differentiation is essential for understanding the molecular mechanisms underlying wood formation. Differential gene expression in secondary xylem differentiation of *Populus* has been previously investigated using cDNA microarray analysis. However, little is known about the molecular mechanisms from a genome-wide perspective. In this study, the Affymetrix poplar genome chips containing 61,413 probes were used to investigate the changes in the transcriptome during secondary xylem differentiation in Chinese white poplar (*Populus tomentosa*). Two xylem tissues (newly formed and lignified) were sampled for genome-wide

transcriptional profiling. In total, 6843 genes (~11%) were identified with differential expression in the two xylem tissues. Many genes involved in cell division, primary wall modification, and cellulose synthesis were preferentially expressed in the newly formed xylem. In contrast, many genes, including *4-coumarate*:*cinnamate-4-hydroxylase* (*C4H*), *4-coumarate*:*CoA ligase* (*4CL*), *cinnamyl alcohol dehydrogenase* (*CAD*), and *caffeoyl CoA 3*-O-*methyltransferase* (*CCoAOMT*), associated with lignin biosynthesis were more transcribed in the lignified xylem. The two xylem tissues also showed differential expression of genes related to various hormones; thus, the secondary xylem differentiation could be regulated by hormone signaling. Furthermore, many transcription factor genes were preferentially expressed in the lignified xylem, suggesting that wood lignification involves extensive transcription regulation. The genome-wide transcriptional profiling of secondary xylem differentiation could provide additional insights into the molecular basis of wood formation in poplar species.

Key words: Wood formation; Affymetrix microarrays; Secondary xylem differentiation; Lignified xylem; Vascular cambium; Newly formed xylem

INTRODUCTION

Tree growth consists of elongation growth originating from the shoot apical buds (Cutter, 1965) and periclinal growth driven by the vascular cambium (Larson, 1994). The vascular cambium is a group of meristematic cells derived from procambial cells formed during primary growth. The division of cambial initials produces xylem mother cells (Larson, 1994), which then develop primary and secondary xylem (wood). Formation of wood includes a series of developmental processes, such as cell division, primary cell wall synthesis, secondary wall formation, and programmed cell death (Hertzberg et al., 2001).

The molecular basis of wood formation has been previously studied in many forest tree species by using cDNA microarray analysis (Sterky et al., 1998; Li et al., 2009b), Affymetrix DNA chips (Bao et al., 2009), and RNA sequencing (Ramsköld et al., 2012). These studies have identified many genes that are differentially expressed in various wood developmental stages and highlighted the complex molecular mechanisms involved in wood formation. Furthermore, the sequenced genomes of *Populus trichocarpa* (Tuskan et al., 2006) and *Picea abies* (Norway spruce; Nystedt et al., 2013) provide fundamental basis for furthering study on wood formation in forest tree species.

The Chinese white poplar (*Populus tomentosa* Carr.) is the most important poplar species widely planted in commercial forests in Northern China. It plays a major role in the furniture and paper industries and territorial environmental protection. Previous studies on this species have mainly focused on the genes or gene families related to various stress resistances. Only one study investigated wood formation in this species at the transcriptional level by using cDNA microarray analysis of about 3000 clones (Wang et al., 2009). While they identified many genes that are highly expressed in the secondary xylem, knowledge on the genome-wide transcriptome changes during secondary xylem differentiation in Chinese white poplar and

other poplar species remains largely limited. In this study, the Affymetrix poplar genome microarrays containing 61,413 probes were used to compare the xylem trancriptomes of Chinese white poplar during xylem differentiation. This study aimed to identify genes that are differentially expressed in the newly formed and lignified xylem from a genome-wide perspective.

MATERIAL AND METHODS

Plant materials and sampling

Five-two-year-old individuals of a *P. tomentosa* clone were selected for sampling. These trees were grown under normal conditions at Xiaotangshan, Beijing, China. Developing xylem tissues were sampled in early July (summer). A piece of bark (approximately 5 cm in length) was first removed from each tree at about 1 m high above the ground. The newly formed xylem (approximately 0.5 mm deep) was collected from the exposed surface by using a knife. The xylem was further scraped at a depth of 1-2 mm toward the inner xylem, representing lignified xylem. These samples were immediately placed in separate Falcon™ tubes filled with liquid nitrogen, and then stored in a freeze at -80°C until RNA extraction. All samples were uploaded at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.ov/geo/) with the accession number GSE46946.

RNA extraction

The two developing xylem samples collected from the five trees were pooled separately and then divided into three subsamples for RNA extraction. RNAs were extracted using the RNeasy plant mini kits (Qiagen, Inc., Valencia, CA, USA). The quality of RNAs was checked by performing agarose gel electrophoresis and reading absorbance. The 18s/28s ratios of all RNA samples ranged from 1.3 to 1.8, and the $A_{260/280}$ ratios were 1.8-2.1, indicating good quality for downstream use.

Microarray platform and array hybridization

The Affymetrix Poplar Genome GeneChip consisted of 61,413 probes representing over 56,000 transcripts. Microarray hybridization was performed at the Shanghai Bioarray Co. Ltd., according to Affymetrix© protocols. Signals of the hybridized arrays were screened using an Affymetrix scanner. Raw gene expression data were read and analyzed using the Affymetrix GeneChip® Operating Software. The quality of data was verified using a list of parameters such as probe array image inspection, B2 oligo performance, average background, noise values, poly-A controls, hybridization controls, internal control genes, percent present, scaling, and normalization factors (Bao et al., 2009).

Gene filtering and function annotation

Differentially expressed genes were selected based on a 2-fold difference of gene expression between the newly formed and lignified xylem ($\log_2$ ratios ≥ 1 or ≤ -1) with P values of ≤ 0.01 for statistical significance. The identified genes were annotated using the Affymetrix Poplar Chip annotation (against JGI release v2.2; Tuskan et al., 2006); Uniprot

(http://www.uniprot.org/uniprot); gene models of poplar (Tuskan et al., 2006); *Arabidopsis* (TAIR10; http://www.arabidopsis.org/); rice (Ouyang et al., 2007); and medicago (Li et al., 2009a), as well as Gene Ontology (GO) terms (http://www.geneontology.org/). The probes without a poplar gene model were also annotated using the GenBank accession numbers and the poplar predicted gene set v2.2 (http://www.phytozome.net/poplar; Tuskan et al., 2006). AgriGO (Du et al., 2010) was used to identify genes related to biological processes and cellular components. Genes involved in cell wall formation were identified using the Cell Wall Navigator (http://bioweb.ucr.edu/Cellwall/; Girke et al., 2004). Transcription factors were identified using the Plant TFDB (Zhang et al., 2011). Heat map of differential gene expression was generated using R (R Core Team, 2012).

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Eight genes were chosen from the differentially expressed genes identified in the microarray analysis and used for the validation by using real-time RT-PCR. These genes included phosphatase 2C (*POPTR_0013s01370*), patatin-like protein 9 (*POPTR_0005s23170*), basic chitinase (*CV277108*), an unknown protein (*POPTR_0008s14970*), thaumatin (*POPTR_0009s13510*), expansin-A4 (*POPTR_0008s05720*), expansin-A1 (*POPTR_0008s08790*), and expansin-A8 (*POPTR_0013s15080*). Primers were designed with a melting temperature of 58°C and products around 58-77 bp in length (**Table S1**). The amplification reaction was set in 25 µL consisting of 1X SYBR Premix Ex Taq™, 5 µM of each primer, and 5 ng cDNA. RT-PCR was run using an Opticon2 thermocycler real-time PCR machine (BioRad, USA). The cycling parameters were set as follows: 2 min at 50°C, 5 min at 94°C, 50 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C, and a final elongation for 10 min at 72°C. The specificity of the RT-PCR products was checked by performing a melting curve program from 70 to 95°C with holding for 10 s at each 0.5°C. Fluorescence signals were detected at 58°C during the melting curve program. *ACTIN* (GenBank accession: AY261523.1) was used as a reference gene. Each sample was run three times, and data were analyzed using the Opticon Monitor Analysis 3.1 software (BioRad).

RESULTS AND DISCUSSION

Xylem transcriptome changed from newly formed to lignified xylem

While comparing between the newly formed and lignified xylem, of the 61,413 probes in the microarrays, 3134 were found to be preferentially expressed in the newly formed xylem, and 3709 were more highly expressed in the lignified xylem. This indicated that only a small proportion of the poplar xylem transcriptome (approximately 11%) had differential expression during secondary xylem differentiation. Almost all the identified genes with differential expression (95.3% for newly formed xylem and 93.7% for lignified xylem) had homologs in the sequenced poplar genome (Tuskan et al., 2006). Of the 3134 probes preferentially expressed in the newly formed xylem, 77.5% had homologs in Uniprot, and 94.8% had homologs in the TAIR databases. On the other hand, 69.1 and 92.3% of the 3709 probes for the lignified xylem had significant matches in the two public databases, respectively.

Functional annotation and classification of differentially expressed genes

Differentially expressed genes were functionally annotated using the GO terms (Table 1). The majority of these genes (80.4% for newly formed xylem and 79.7% for lignified xylem) had a role in biological process. Genes involved in translation (GO: 0006412), cell wall organization (GO: 0071555), cellulose biosynthetic process (GO: 0030244), and cytoskeleton organization (GO: 0007010) were highly transcribed in the newly formed xylem. On the other hand, genes with a role in transcription (GO: 0006355), hormone stimulus (GO: 0009725), and protein metabolism (GO: 0019538) were preferentially expressed in the lignified xylem. In molecular function, genes functioning in the structural constituents of ribosome (GO: 0003735), hydrolase activity (GO: 0004553), cellulose synthase (UDP-forming; GO: 0016760), and actin binding (GO: 0003779) were preferentially expressed in the newly formed xylem; on the other hand, genes involved in transcription activity (GO: 0003700), zinc-ion binding (GO: 0008270), and nucleic acid binding (GO: 0003676) were relatively highly expressed in the lignified xylem. In cellular component, genes participating in ribosome (GO: 0005840), cytoskeleton (GO: 0005856), and cell junction (GO: 0030054) were more abundant in the newly formed xylem whereas the genes as components in the nucleus and peroxisomal membrane were preferentially expressed in the lignified xylem.

Table 1. Comparison of Gene Ontology (GO) terms for genes preferentially expressed in the newly formed and lignified xylem.

Category	GO term	Number of genes	
		Newly formed xylem	Lignified xylem
BP	GO:0006412|translation	94	15
BP	GO:0006886|intracellular protein transport	33	4
BP	GO:0051258|protein polymerization	29	1
BP	GO:0007018|microtubule-based movement	25	3
BP	GO:0071555|cell wall organization	10	1
BP	GO:0042545|cell wall modification	7	1
BP	GO:0030244|cellulose biosynthetic process	15	
BP	GO:0006414|translational elongation	11	
BP	GO:0007010|cytoskeleton organization	7	
BP	GO:0006355|"regulation of transcription, DNA-dependent"	36	124
BP	GO:0009725|response to hormone stimulus	3	16
BP	GO:0019538|protein metabolic process	30	68
MF	GO:0003735|structural constituent of ribosome	99	13
MF	GO:0005525|GTP binding	80	18
MF	GO:0004553|hydrolase activity	69	22
MF	GO:0003924|GTPase activity	50	5
MF	GO:0016760|cellulose synthase (UDP-forming) activity	12	
MF	GO:0004634|phosphopyruvate hydratase activity	9	
MF	GO:0003779|actin binding	8	
MF	GO:0003700|transcription factor activity	112	312
MF	GO:0008270|zinc ion binding	53	145
MF	GO:0003676|nucleic acid binding	32	86
MF	GO:0009055|electron carrier activity	28	64
MF	GO:0003723|RNA binding	19	44
CC	GO:0005840|ribosome	99	13
CC	GO:0005737|cytoplasm	40	9
CC	GO:0043234|protein complex	29	1
CC	GO:0005783|endoplasmic reticulum	21	3
CC	GO:0030131|clathrin adaptor complex	14	
CC	GO:0000139|Golgi membrane	10	
CC	GO:0000015|phosphopyruvate hydratase complex	9	
CC	GO:0005856|cytoskeleton	8	
CC	GO:0030054|cell junction	3	
CC	GO:0005634|nucleus	39	114
CC	GO:0005778|peroxisomal membrane		5

BP = biological process; MF = molecular function; CC = cellular component.

In the GO terms with biological process, genes involved in lignin metabolic process were more enriched in the lignified xylem (Figure 1). In contrast, genes involved in cytoskeleton were considerably more enriched in the newly formed xylem (Figure 2). The identified differentially expressed genes were further classified into 16 functional groups (Figure 3). Genes with hydrolase activity, kinase activity, microtubule motor activity, structural constituents of ribosomes, structural molecule activity, and transferase activity were more highly expressed in the newly formed xylem. On the other hand, genes with DNA binding, nucleic acid binding, RNA binding, transcription factor activity, and unknown functions were up-regulated in the lignified xylem. These results indicated that the two stages of xylem differentiation had significant differences in their xylem transcriptomes.

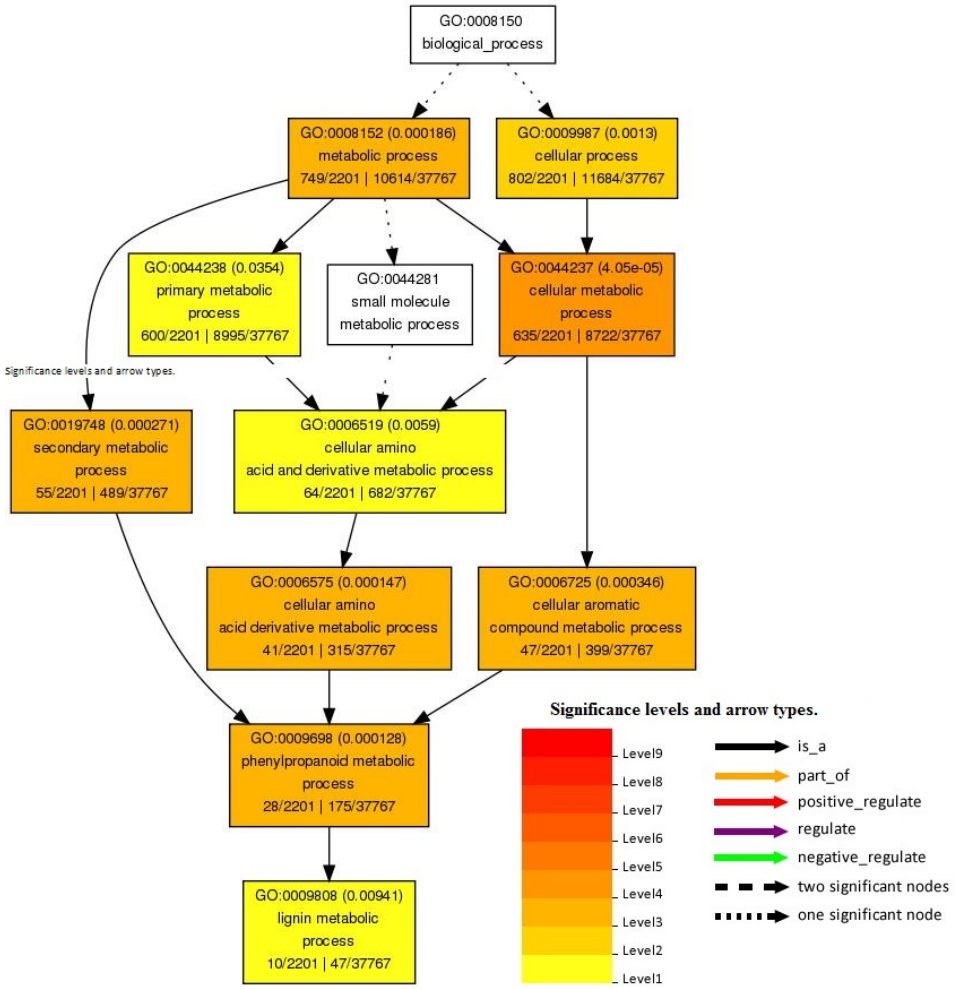

Figure 1. Gene Ontology (GO) terms of lignin metabolic process were significantly enriched in the lignified xylem. the AgriGO software was used for the analysis with P values <0.05 for statistical significances. The significant GO term node is presented by proportional color, as indicated by the scale.

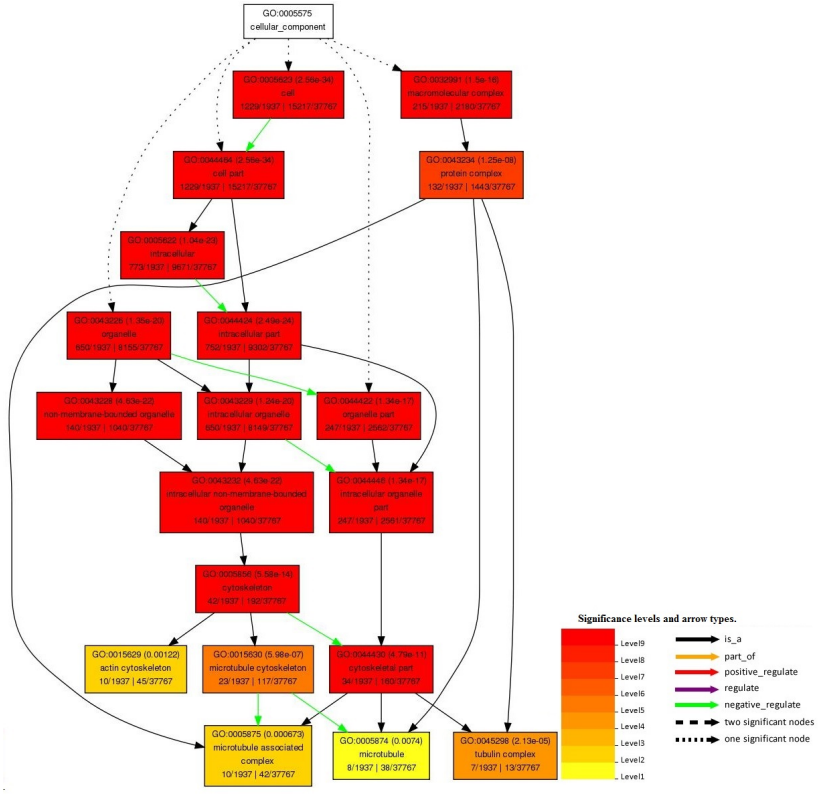

Figure 2. Gene Ontology (GO) terms of cytoskeleton in "Cellular Component" were significantly enriched in the newly formed xylem. The AgriGO software was used for the analysis with P values <0.05 for statistical significances. The significant GO term node is presented by proportional color, as indicated by the scale.

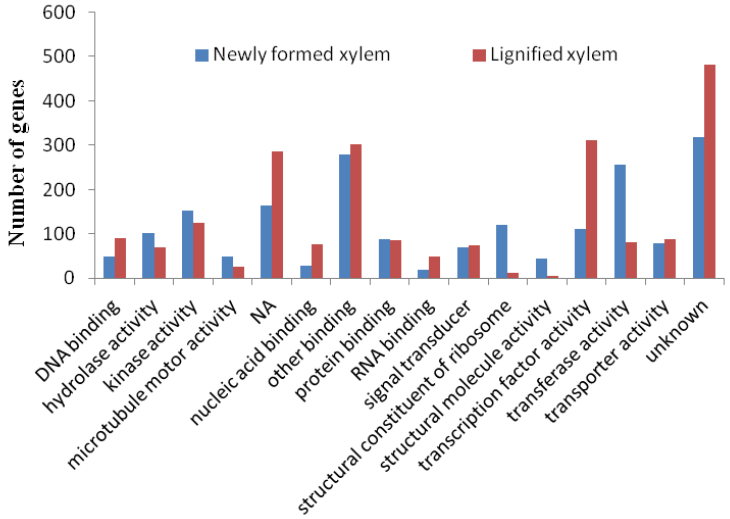

Figure 3. Functional categories of genes differentially expressed in the newly formed and lignified xylem.

Differential expression of cell wall-related genes

Many differentially expressed genes were related to cytoskeleton, cell wall structural proteins (Table 2), and cell wall biosynthesis (Table 3 and Figure 4). Red and green colors indicate higher and lower transcript levels, respectively. The JGI gene models are shown on the right. A and B represent replicates 1 and 2 of the newly formed xylem, respectively; G and H indicate replicates 1 and 2 of the lignified xylem, respectively. Genes involved in cytoskeleton development were exclusively up-regulated in the newly formed xylem, such as genes encoding actins, tubulins, and tubulin-folding cofactor. Actins and tubulins are important units of actin microfilaments and microtubules, respectively (Oakley et al., 2007). Li et al. (2009b) found that the actin genes were highly abundant in the radiate pine xylem expressed sequence tags, indicating their possible function in wood formation. Microtubules play an important role in cellulose microfibril arrangement and deposition, and *tubulins* were highly expressed in the xylem (Li et al., 2009b).

Table 2. Cytoskeleton-related genes and genes encoding cell wall structural proteins were preferentially expressed in newly formed and lignified xylem.

Gene	Poplar gene model	TAIR annotation	Log_2 ratios (newly formed xylem/lignified xylem)
Actin 7	POPTR_0001s31700	AT5G09810	-1.38
Actin-11	POPTR_0006s20710	AT3G12110	-3.52
Tubulin beta-5	POPTR_0002s02340	AT1G20010	-4.09
Tubulin alpha-2	POPTR_0009s08850	AT1G50010	-1.28
Tubulin beta-7	POPTR_1455s00210	AT2G29550	-3.44
Tubulin beta 6	POPTR_0001s09330	AT5G12250	-2.44
Tubulin beta 8	POPTR_0006s09610	AT5G23860	-2.74
Tubulin beta 3	POPTR_0001s27960	AT5G62700	-1.54
AGP 18	POPTR_0005s18840	AT4G37450	-3.21
AGP 20	POPTR_0014s09050	AT3G61640	-3.10
AGP 30	POPTR_0011s05340	AT2G33790	-2.68
FLA 11	POPTR_0012s02220	AT5G03170	1.51
FLA 12	POPTR_0013s14790	AT5G60490	2.17
FLA 1	POPTR_0001s37650	AT5G55730	-2.35
FLA 4	POPTR_0006s18920	AT3G46550	-1.08
FLA 6	POPTR_0013s12490	AT2G20520	-1.51
FLA 7	POPTR_0014s16100	AT2G04780	-3.89
FLA 10	POPTR_0014s06740	AT3G60900	-3.12
FLA 17	POPTR_0008s01310	AT5G06390	-1.65
Proline-rich protein	POPTR_0008s06680	AT2G40070	-1.21
Glycine-rich protein	POPTR_0008s19880	AT1G27090	-1.02
Glycine-rich protein	POPTR_0011s04480	AT4G21620	-2.38
Glycine-rich protein	POPTR_0004s13090	AT5G39570	-2.05
Glycine-rich protein	POPTR_0011s03530	AT1G11440	4.05
Glycine-rich protein	POPTR_0004s03670	AT1G61255	3.52
Glycine-rich protein	POPTR_0006s12580	AT2G43630	1.24
HRGP	POPTR_0012s12920	AT5G52430	2.24
HRGP	POPTR_0002s10890	AT5G65660	2.94
HRGP	POPTR_0006s25030	AT2G25930	1.48
HRGP	POPTR_0017s09000	AT2G33490	1.04
HRGP	POPTR_0008s13830	AT1G14710	3.08
HRGP	POPTR_0005s28120	AT1G76660	-2.13
HRGP	POPTR_0017s12970	AT3G02120	-3.94
HRGP	POPTR_0001s46460	AT3G25690	-2.49
HRGP	POPTR_0004s22000	AT3G45230	-2.40
HRGP	POPTR_0009s01910	AT3G45230	-3.25
HRGP	POPTR_0006s24500	AT5G11890	-1.53

AGP = arabinogalactan protein; FLA = FASCICLIN-like arabinogalactan; HRGP = hydroxyproline-rich glycoprotein family. Log_2 ratios represent relative expression of a gene between the lignified xylem and the newly formed xylem. Positive values indicate genes preferentially expressed in the lignified xylem, whereas negative values indicate genes preferentially expressed in the newly formed xylem.

Table 3. Some cell wall-related genes preferentially expressed in the newly formed and lignified xylem.

Gene	Poplar gene model	TAIR annotation	Log_2 ratios (newly formed xylem/lignified xylem)
Expansin A1	POPTR_0010s17440	AT1G69530	-3.84
Expansin A4	POPTR_0008s05720	AT2G39700	-3.16
Expansin-like A2	POPTR_0004s18840	AT4G38400	-3.64
Pectin lyase-like	POPTR_0014s10770	AT3G62110	-3.64
Pectin lyase-like	POPTR_0003s17450	AT4G13710	-3.62
Pectin lyase-like	POPTR_0015s09930	AT5G63180	-4.33
PME 3	POPTR_0003s07040	AT3G14310	-3.31
PME 44	POPTR_0006s13670	AT4G33220	-5.24
PAE	POPTR_0005s00330	AT3G05910	-3.92
PAE	POPTR_0003s03720	AT4G19410	-4.26
PAE	POPTR_0012s13090	AT5G23870	-3.31
Plant invertase/PME	POPTR_0006s13680	AT2G26440	-3.87
Plant invertase/PME	POPTR_0012s14560	AT5G62350	-4.26
XET 33	POPTR_0014s11030	AT1G10550	-5.00
XTH 5	POPTR_0003s15800	AT5G13870	-4.94
XTH 9	POPTR_0019s14590	AT4G03210	-3.61
XTH 28	POPTR_0008s13780	AT1G14720	-3.04
CesA 1	POPTR_0018s01540	AT4G32410	-1.49
CesA 3	POPTR_0016s05520	AT5G05170	-3.49
CesA 6	POPTR_0007s07120	AT5G64740	-1.32
CesA 7	POPTR_0006s19580	AT5G17420	-1.75
CesA 8	POPTR_0011s07040	AT4G18780	-2.62
CesA A4	POPTR_0002s25970	AT5G44030	-1.39
CesA-like C6	POPTR_0002s24980	AT3G07330	-3.68
CesA-like D5	POPTR_0002s20130	AT1G02730	-2.97
GSL12	POPTR_0003s21690	AT5G13000	-2.76
Laccase 2	POPTR_0009s15860	AT2G29130	-4.58
Laccase 17	POPTR_0006s08740	AT5G60020	-4.26
Peroxidase 31	POPTR_0001s34660	AT3G28200	-4.37
PAL 2	POPTR_0010s23100	AT3G53260	2.05
PAL 1	POPTR_0016s09230	AT2G37040	2.95
SAMS	POPTR_0013s00550	AT3G17390	1.05
4CL 2	POPTR_0006s18490	AT3G21240	2.47
CCoAOMT	POPTR_0008s13600	AT1G67980	1.67
C4H	POPTR_0019s15110	AT2G30490	1.41
CAD 9	POPTR_0001s31470	AT4G39330	3.13
CAD	POPTR_0011s15120	AT1G72680	1.09

PME = pectin methylesterase; PAE = pectin acetylesterase; XET: xyloglucan:xyloglucosyl transferase; XTH = xyloglucan endotransglucosylase/hydrolase; CesA = cellulose synthase; GSL12 = glucan synthase-like 12; PAL = phenylalanine ammonia-lyase; SAMS = S-adenosylmethionine synthetase; 4CL = 4-coumarate:CoA ligase; CCoAOMT = caffeoyl CoA 3-O-methyltransferase; C4H = cinnamate-4-hydroxylase; CAD = cinnamyl alcohol dehydrogenase.

Arabinogalactan proteins (AGPs) are one of the most important cell wall structural proteins (Showalter, 2001). AGPs are highly glycosylated hydroxyproline-rich glycoproteins (HRGPs) that are expressed mostly in the plant cell wall and plasma membrane. Thus, they are believed to have functions in plant growth and development (Showalter, 2001). Our data showed that *AGPs* were up-regulated in the newly formed xylem, suggesting their function in the earlier stage of xylem development. Fasciclin-like arabinogalactan proteins (FLAs) are a subclass of AGPs, which have glycosylated regions and cell adhesion domains (Gaspar et al., 2001). Our results showed that *FLA1*, *4*, *6*, *7*, *10*, and *17* were up-regulated in the newly formed xylem; on the other hand, *FLA12* showed preferential expression in the lignified xylem. Glycine-rich proteins (GRPs) and proline-rich proteins (PRPs) are other two important cell wall structural proteins. In this study, *PRPs* were exclusively up-regulated in the newly formed xylem. In contrast, different *GRPs* were preferentially expressed in either newly formed or lignified xylem. *HRGPs* also showed a similar expression pattern as that of *GRPs*.

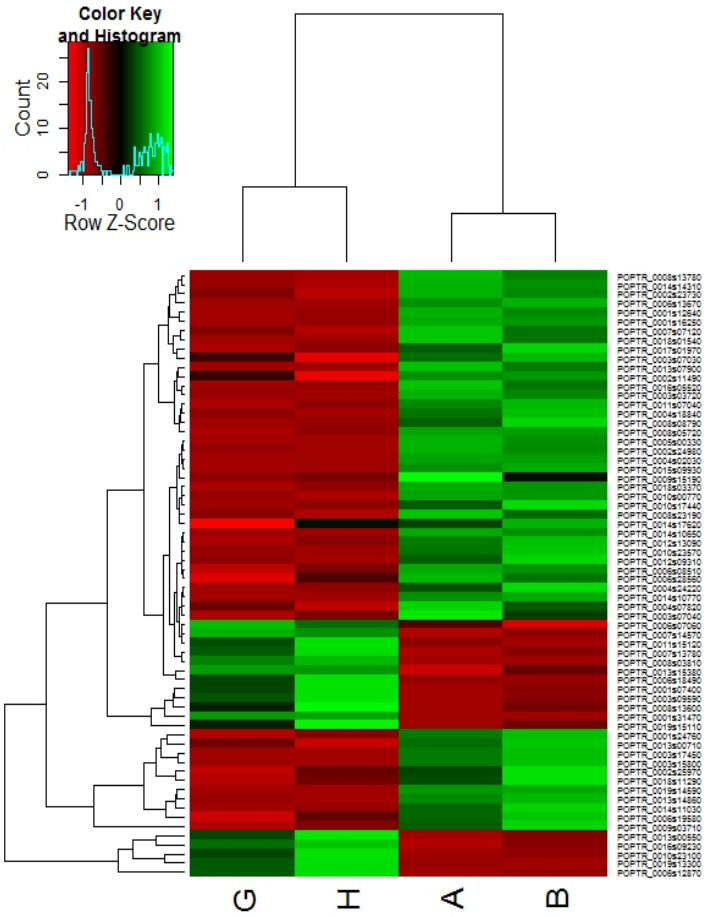

Figure 4. Heat map of differentially expressed genes involved in cell wall biosynthesis.

Many genes involved in primary wall formation were up-regulated in the newly formed xylem (Table 3). Expansin actively expressed in the cambial region had functions in the expansion of the primary cell walls of hybrid aspen (Gray-Mitsumune et al., 2004). Expansin superfamily contains expansin A, B, and expansin-like A and B. Our results showed that expansin A and B were preferentially expressed in the newly formed and lignified xylem, respectively (Table 3), suggesting their divergent roles in secondary xylem differentiation. Genes involved in cellulose biosynthesis (*cellulose synthase*, *cellulose synthase-like,* and *glucan synthase-like 12*) were up-regulated in the newly formed xylem. Some of these *cellulose synthase*s are secondary cell wall genes; thus, the newly formed xylem underwent active cellulose synthesis during both primary and secondary wall formation. Overall, the newly formed xylem sampled in summer (July) could include various secondary xylem cells at the stages of cell division, cell expansion, primary wall synthesis, and cellulose synthesis of secondary walls. In fact, the newly formed xylem sampled in this study contained some vascular cambium cells that had undergone cell division.

Lignin is a complex aromatic heteropolymer that is mainly deposited in the thickened secondary cell walls, and it is a major determinant of stem stiffness and pathogen resistance. Lignification is the most important symbol of secondary cell wall deposition in forest trees. Many genes involved in lignin biosynthesis were up-regulated in the lignified xylem tissues, such as *C4H*, *4CL*, *CAD*, *PAL*, *CCoAOMT1*, and *SAMS*. These results suggested that the lignified xylem was at the developmental stage of lignification and deposition of secondary walls.

Differential expression of transcription factors

The transcription factor (TF) database (PlantTFDB; Zhang et al., 2011) revealed a total of 44 TF families with differential expression in the newly formed and lignified xylem, accounting for 75.9% of the total TFs identified in the *P. trichocarpa* genome. These TF families included 112 and 312 genes differentially expressed in the newly formed and lignified xylem, respectively (Table 4). Most of the identified TF families were up-regulated in the lignified xylem, including *TALE* (7.1%), *bZIP* (6.7%), *ERF* (6.1%), *MYB_related* (6.1%), *ARF* (5.1%), *C3H* (4.2%), *HD-ZIP* (4.2%), *Trihelix* (4.2%), *G2-like* (3.2%), *FAR1* (2.9%), and *EIL* (2.2%). On the other hand, only a fewer TF families were more highly expressed in the newly formed xylem, such as *WOX* (3.6%).

Table 4. Transcription factor families differentially expressed in the newly formed and lignified xylem.

TF family	Number of genes	
	Newly formed xylem	Lignified xylem
WOX	4	
WRKY	14	8
NAC	10	8
TALE	2	22
bZIP	6	21
bHLH	13	21
ERF	2	19
MYB_related	1	19
C2H2	9	18
ARF	3	16
MYB	11	15
HD-ZIP	3	13
C3H	2	13
Trihelix	1	13
GRAS	5	11
G2-like		10
FAR1		9
LBD	1	7
EIL		7
HSF	2	6

TF = transcription factor.

Several TF families have been shown to have roles in wood formation, such as *C2H2*, *C3H*, *bHLH*, *bZIP*, *NAC*, *MYB-related*, *MYB*, *WRKY*, *Thrihelix*, and *ELI* (Scarpella and Meijer, 2004; Li et al., 2009b). Transgenic study and quantitative trait loci analysis revealed that *MYBs* play a role in both lignin biosynthesis and wood formation (Goicoechea et al., 2005). In the present study, MYBs were differentially expressed in the newly formed and lignified xylem, respectively, suggesting their diverse roles in the different stages of secondary xylem

differentiation. Different members of *NAC* were also differentially expressed in the newly formed and lignified xylem. *NAC* (*NAM/ATAF*/*CUC*) is highly expressed in developing xylem and differentiating tracheids (Demura et al., 2002; Kubo et al., 2005). Some other NAC genes are known to be important regulators of wood formation and show higher expression in vascular tissues (Kubo et al., 2005; Mitsuda et al., 2007). Furthermore, *NACs* (*SND1*, *NST1*, *NST2*, and *NST3*) are involved in the regulation of fiber cell differentiation and secondary wall formation (Zhong et al., 2006; Mitsuda et al., 2007; Mitsuda and Ohme-Takagi, 2008).

Differential expression of hormone-related genes

Many *auxin-related* genes were up-regulated in the newly formed xylem (Table 5), such as *AUX/IAA transcriptional regulator*, *auxin efflux carrier*, *auxin-like 1 protein*, *auxin-responsive protein*, and *SAUR-like auxin-responsive protein*. However, genes encoding *auxin response factor* (*ARF2*, *9*, *10*, *16*, *17*) were preferentially expressed in the lignified xylem. Thus, different auxin signaling genes might have diverse functions in the regulation of secondary xylem differentiation. The diverse functions might be important for maintaining auxin balance and responding to auxin stimulus (Golisz et al., 2008; Péret et al., 2012). Genes that were more transcribed in the lignified xylem might have a function in calcium ion transport and response to auxin stimulus (Hagen and Guilfoyle, 2002), whereas auxin-related genes that were preferentially expressed in the newly formed xylem were likely to have a role in cell wall modification in response to auxin (Tamaoki et al., 2008).

Table 5. Hormone-related genes differentially expressed in the newly formed and lignified xylem.

Gene	Poplar gene model	TAIR annotation	Log_2 ratios (newly formed xylem/lignified xylem)
AUX/IAA transcriptional regulator	POPTR_0013s03860	AT5G43700	-2.14
Auxin efflux carrier protein	POPTR_0011s14860	AT1G76520	-3.92
Auxin efflux carrier protein	POPTR_0016s03450	AT1G73590	-3.14
Auxin response factor 1	POPTR_0004s23770	AT1G59750	-1.13
Auxin-like 1 protein	POPTR_0002s03520	AT1G75310	-1.60
Auxin-responsive protein	POPTR_0002s25080	AT3G25290	-1.26
Auxin-responsive protein	POPTR_0014s15980	AT2G04850	-2.01
Auxin-responsive protein	POPTR_0002s25070	AT3G07390	-4.58
Auxin-responsive GH3 protein	POPTR_0009s09590	AT2G14960	-3.37
SAUR-like auxin-responsive protein	POPTR_0005s09920	AT3G12830	-1.13
SAUR-like auxin-responsive protein	POPTR_0006s13940	AT5G20810	-2.12
SAUR-like auxin-responsive protein	POPTR_0001s16460	AT1G72430	-1.99
SAUR-like auxin-responsive protein	POPTR_0009s12880	AT1G75590	-1.51
Indole-3-acetic acid inducible 14	POPTR_0010s08880	AT4G14550	-3.24
Indole-3-acetic acid inducible 29	POPTR_0006s27130	AT4G32280	-2.94
Indole acetic acid-induced protein 16	POPTR_0005s05560	AT3G04730	1.16
Auxin response factor 2	POPTR_0015s11660	AT5G62000	1.43
Auxin response factor 9	POPTR_0003s14200	AT4G23980	1.59
Auxin response factor 10	POPTR_0009s02020	AT2G28350	2.19
Auxin response factor 11	POPTR_0002s17350	AT2G46530	2.93
Auxin response factor 16	POPTR_0006s12930	AT4G30080	2.95
Auxin response factor 17	POPTR_0002s09050	AT1G77850	2.36
Auxin-responsive protein	POPTR_0014s16010	AT5G47530	1.75
Auxin-responsive GH3 protein	POPTR_0014s09120	AT2G46370	2.06
SAUR-like auxin-responsive protein	POPTR_0002s17700	AT2G46690	2.37
Ethylene-forming enzyme	POPTR_0014s15710	AT1G05010	-3.01
Ethylene insensitive 3 protein	POPTR_0008s01200	AT3G20770	1.47
Ethylene response sensor 1	POPTR_0002s20260	AT2G40940	1.60
ERE binding factor 1	POPTR_0003s15030	AT4G17500	3.82
ERE binding factor 4	POPTR_0001s40770	AT3G15210	1.06
Ethylene insensitive-like 3	POPTR_0003s22090	AT1G73730	1.46

A gene encoding ethylene-forming enzyme was up-regulated in the newly formed xylem, whereas genes encoding ethylene signaling enzymes were more transcribed in the lignified xylem (Table 5). These results indicated that ethylene signals could be involved in secondary xylem differentiation. *Ethylene insensitive 3* and *ethylene insensitive-like 3* are also transcription factors that play an essential role in higher plant growth and development and stress resistant, as well as programmed cell death (Chao et al., 1997).

Differential expression of cell cycle-related genes

Cell proliferation is regulated by several proteins such as cyclins, cyclin-dependent kinase (CDKs), and cyclin-dependent kinase inhibitors (CKIs; Hong et al., 1998; Hattori et al., 2000; Meijer and Murray, 2001). In the present study, *CDKs* and *cyclins* were up-regulated in the newly formed xylem, whereas *CKI*, an inhibitor of *cyclin*, was preferentially expressed in the lignified xylem (Table 6). The results suggested that cell division process is more active in the newly formed xylem, and it was inhibited in the lignified xylem by the expression of *CKI* genes.

Table 6. Cell cycle-related genes differentially expressed in the newly formed and lignified xylem.

Gene	Poplar gene model	TAIR annotation	Log_2 ratios	
			Newly formed xylem	Lignified xylem
cyclin A1; *1*	POPTR_0005s20380	AT1G44110	-2.69	
cyclin D3; *1*	POPTR_0014s02310	AT4G34160	-3.61	
cyclin D3; *2*	POPTR_0005s18550	AT5G67260	-3.37	
cyclin	POPTR_0005s03620	AT3G05330	-1.90	
cyclin p3; *2*	POPTR_0002s14440	AT3G60550	-1.12	
cyclin p4; *1*	POPTR_0014s04930	AT2G44740	-2.77	
CDK B1; *2*	POPTR_0006s11390	AT2G38620	-2.64	
CDK B2; *1*	POPTR_0005s27890	AT1G76540	-2.92	
Inhibitor with CDK	POPTR_0001s32120	AT5G48820		1.61
Cycling DOF factor 2	POPTR_0017s12080	AT5G39660		1.48

CDK = cyclin-dependent kinase.

Validation of microarray gene expression by using real-time RT-PCR

Eight differentially expressed genes were selected for the validation by using real-time RT-PCR. The RT-PCR results are reported as log_2 ratios of gene expression in the newly formed xylem compared to those in the lignified xylem. Differential gene transcription (y-axis, log_2 ratio) was shown for each selected gene (x-axis; Figure 5. x-axis: eight genes involved in the validation, including phosphatase 2C (*POPTR_0013s01370*), patatin-like protein 9 (*POPTR_0005s23170*), basic chitinase (*CV277108*), unknown protein (*POPTR_0008s14970*), thaumatin (*POPTR_0009s13510*), expansin-A4 (*POPTR_0008s05720*), expansin-A1 (*POPTR_0008s08790*), and expansin-A8 (*POPTR_0013s15080*); y-axis: log_2 ratio of gene expression in the newly formed xylem compared to the lignified xylem.). No significant differences were observed between the RT-PCR and microarray gene expression results. Therefore, differentially expressed genes identified by microarray experiments in this study were relatively reliable for the transcriptome comparison during secondary xylem differentiation.

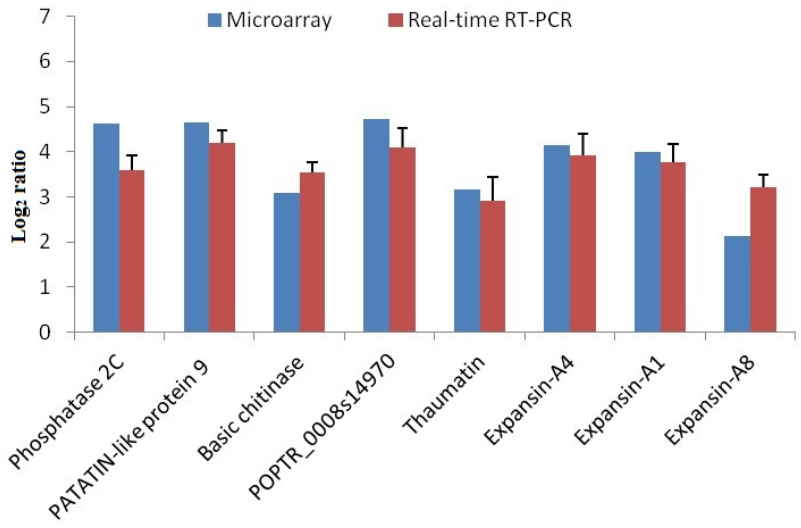

Figure 5. Validation of microarray gene expression by real-time reverse transcriptase-polymerase chain reaction.

CONCLUSION

The Affymetrix Poplar Genome Chips identified 6843 genes (approximately 11% of the total probes) with differential expression in the newly formed and lignified xylem in *P. tomentosa*. Many genes that were up-regulated in the newly formed xylem had roles in cell division, primary wall formation, and cellulose synthesis of secondary walls, such as *cyclins*, *CDKs*, *expansin*, *XHT*, *XETs*, *pectin acetylesterase*, *pectin methylesterase*, and several *cellulose synthases*. In contrast, many genes, including *C4H*, *4CL*, *CAD*, *PAL*, *CCoAOMT1*, and *SAMS*, involved in lignin biosynthesis were highly transcribed in the lignified xylem. Many transcription factors were up-regulated in the lignified xylem, suggesting that extensive transcription regulation occurs in this tissue. Furthermore, hormone-related genes were differentially expressed in the two developing xylem tissues; thus, hormone signaling genes play important roles during secondary xylem differentiation.

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Supplementary material

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