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Arabidopsis whole-transcriptome profiling defines the features of coordinated regulations that occur during secondary growth

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Abstract

Secondary growth in the inflorescence stems of *Arabidopsis* plants was induced by a combination of short-day and long-day treatments. The induced stems were divided into three different stem developmental stages (i.e., immature, intermediate, and mature) with regard to secondary growth. Whole transcriptome microarrays were used to examine the changes in global gene expression occurring at the different stem developmental stages. Over 70% of the *Arabidopsis* transcriptome was expressed in the stem tissues. In the mature stems with secondary growth, 567 genes were upregulated 5-fold or higher and 530 were down-regulated, when compared to immature stems (with no secondary growth) and 10-day old seedlings (with no inflorescence stem). The transcription phenotypes obtained from the stems at different developmental stages largely confirm the existing insights into the biochemical processes involved in the sequential events that lead to wood formation. The major difference found between the stems undergoing secondary growth and only primary growth was in the expression profiles of transcriptional regulation-and signal transduction-related genes. An analysis of several shoot apical meristem (SAM) activity-related gene expression patterns in the stems indicated that the genetic control of secondary meristem activity might be governed by a different mechanism from that of SAM. The current study established the expression patterns of many unknown genes and identified candidate genes that are involved in the genetic regulation of secondary growth. The findings described in this report should improve our understanding of the molecular mechanisms that regulate the growth and development of the stem.

Introduction

Plant stem plays several crucial roles in plant growth by providing a physical support for growing biomass and a conduit for the translocation of nutrients and water. As the largest storage organ in trees, it is of economic significance because most forest products are derived from the stem and help alleviate global warming by sequestering and storing carbons in long-term storage. Our current understanding of the molecular biology of stem growth and development is limited.

Thickening of stem occurs as a result of growth and differentiation of secondary tissues produced by the vascular cambium (or the secondary meristem). This secondary growth is a highly ordered developmental process, which involves patterned division of vascular cambium cells and a subsequent regulated differentiation of cambial derivatives into secondary xylem and phloem tissues. Stem diameter growth requires various molecular signals that are differentially transduced by cell-to-cell contacts, relative to cell positions, and turn on and off in response to both external and internal stimuli. Upon the receipt of such positional signals, the cell in the vascular cambium then expresses the appropriate genes in a highly coordinated manner. The differential expression of

key genes involved in the developmental pathway determines the epigenetic state of the vascular cambium, which controls the cambial activity and differentiation. For instance, the cells undergo differentiation if only the genes encoding features specific to vascular development are expressed in the cambium cells.

Significant progress has been made in the study of the gene expression and signaling mechanisms responsible for secondary wall formation, lignin and cellulose biosynthesis (Arioli *et al*., 1998; Vander Mijnsbrugge *et al*., 2000; Hertzberg *et al*., 2001; Moyle *et al*., 2002; Kirst *et al*., 2003), and xylem development (Fukuda, 1997a,b). Zhao *et al*. (2000) characterized xylem-specific proteases in *Arabidopsis*. In addition, Baima *et al*. (2001) demonstrated that the ectopic expression of a homeodomain-leucine zipper homeobox gene (*AtHB8*) increased the production of xylem tissue and promoted vascular cell differentiation in *Arabidopsis*. *Arabidopsis thaliana*, the most well studied herbaceous model plant species, undergoes secondary growth and produces secondary xylem when it is properly kept from flowering by the repeated removal of inflorescences (i.e., decapitation) (Lev-Yadun, 1994; Zhao *et al*., 2000; Little *et al*., 2002). Furthermore, it has been shown to express all of the major components of wood development during its ontogeny and has been used as a model for the study of wood and fiber production in trees (Zhao *et al*., 2000; Chaffey *et al*., 2002). We developed an experiment system for the study of the entire secondary xylem development process, which does not involve decapitation treatment. *Arabidopsis* sustains vegetative growth when grown under short-day (8 h light/16 h dark) conditions. After a brief long-day treatment (5–10 days) of these short-day grown plants, we obtained thick inflorescence stems with various stem heights. The stems produce secondary xylem tissues and the extent of secondary xylem development was correlated with stem height (Ko *et al*., 2004).

In order to better understand the molecular events that occur during secondary growth, the steadystate transcript levels of the stems in different developmental stages with regard to secondary growth were analyzed using the *Arabidopsis* whole-transcriptome GeneChip (23 K) arrays. This study provides detailed and complete information on the changes in global gene expression that define the metabolic and physiological processes characteristic of stem growth in *Arabidopsis*. In addition, the genes discovered from this study will be the focal point of further efforts to unravel the genetic mechanisms involved in secondary growth.

Materials and methods

Plant material

A. thaliana (ecotype Columbia) was grown in a growth chamber under short-day conditions (8 h light/16 h dark) at 23 °C for 7 weeks. To induce inflorescence stem, the short-day growth plants were shifted to long-day conditions (16 h light/ 8 h dark) for 5–10 days according to the experimental design as described in Ko *et al*. (2004). After the long-day treatment, plants were moved back to the short-day conditions.

Histological analysis

The stem area located immediately above the rosette was cross-sectioned by hand and stained with 2% phloroglucinol to visualize secondary cell walls. Microtome (Leica RM2025, Leica Instruments Gmbh, Germany) sectioning was utilized and tissues were stained with toluidine blue (0.05%) to observe vascular cambium. For confocal laser scanning microscopy, the mature stem of *Arabidopsis* and second stem internode of poplar (4-months old) were cross-sectioned by hand and stained with 0.1% aqueous solution of Saffranin O for 15 min., respectively. A Zeiss (Jena, Germany) PASCAL confocal laser scanning microscope, with a 488 nm excitation mirror, a 560 nm emission filter and a 505–530 nm emission filter was used to record images. Image analysis was performed using Laser scanning microscope PASCAL LSM version 3.0 SP3 software.

Gene expression analysis using affymetrix GeneChip®

For total RNA isolation, main stems sections (3 cm from the rosette level) from 20 to 30 individual *Arabidopsis* plants were harvested for each of the three stem stages: immature (5 cm height), intermediate (10–15 cm) and mature (> 25 cm). For the *Arabidopsis* seedling samples, whole seedlings (ecotype Columbia) were grown for 10 days on agar

plates (MS + 2% sucrose + 0.3% phytagel) in long-day condition. It should be noted that the samples were pooled from several batches of plants so that much of the variation in gene expression patterns caused by both subtle changes in environmental conditions and individual plant differences could be eliminated. Furthermore, all samples were harvested around 4:00 PM. For reproducibility, all experiments were duplicated. All methods for the preparation of cRNA from mRNA, as well as the subsequent steps leading to hybridization and scanning of the U95 GeneChip Arrays, were provided by the manufacturer (Affymetrix, Santa Clara, CA) (Ko *et al.*, 2004). Briefly, first-stranded cDNAs were synthesized using 1 μg of mRNA from the three samples (immature, intermediate, and mature stems) with a special oligo(dT)24 primer containing a T7 RNA polymerase promoter at its 5′ end. After second-strand synthesis, biotin-labeled cRNA was generated from the cDNA sample, by an *in vitro* transcription reaction, using a BioArray RNA Transcript Labeling Kit (Enzo Diagnostics, New York) with biotin-labeled CTP and UTP. cRNA (20 μg) was fragmented by heating it at 94 °C for 35 min in a fragmentation buffer (40 mM Tris–acetate, pH 8.1, 125 mM KOAc, and 30 mM MgOAc). An aliquot of fragmented and unfragmented cRNA was analyzed by formaldehyde/agarose gel electrophoresis to ensure appropriate size distribution (average size, 700 bp of unfragmented cRNA and 100 bp after fragmentation). The control cRNA mixture was composed of a second set of four cDNA biotinylated *in vitro* antisense transcripts encoding the *Escherichia coli* biotin synthesis genes *bioB*, *bioC*, and *bioD* and the P1 bacteriophage *cre* recombinase gene. Probes corresponding to these bacterial transcripts are also represented on all Affymetrix GeneChips (including test chips). Each synthetic transcript was quantified and represented at copy numbers of 2×10^8 to 2×10^{10}, corresponding approximately to the expected dynamic range of detection for the GeneChip. This set of control cRNAs allows for the monitoring of hybridization, washing, and staining conditions and also provides a second set of reference samples for normalizing between experiments. The cRNA hybridization mixes were hybridized on GeneChip arrays at 45 °C for 16 h in a rotisserie oven set at 60 rpm (GeneChip Hybridization Oven 640, Affymetrix). Then, the arrays were washed with SSPE, stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR) and washed again (GeneChip Fluidics Station 400, Affymetrix). Finally, the chip was scanned using a GeneArray Scanner (HP and Affymetrix). The average difference and expression call, for each of the duplicated samples, was computed using Affymetrix GeneChip Analysis Suite version 5.0 with default parameters. The resulting hybridization intensity values (i.e., signal intensity) reflect the abundance of a given mRNA relative to the total mRNA population and were used in all subsequent analyses. Normalization and K-Mean clustering was done by GeneSpring 4.2.1 software (Silicon Genetics, Redwood City, CA), in which the software divides each gene's signal intensity by the median signal intensity of the entire probes. This normalization allows the comparison of relative changes. The expression pattern clusters were predefined by K-means clustering algorithms as implemented in the GeneSpring program using the Pearson correlation distance definition.

RNA extraction, northern blot analysis and RT-PCR

Immature, intermediate and mature stem samples were collected as described above. Total RNA was extracted using the Trizol reagent method (Gibco-BRL, Gaithersburg, MD). For northern blot analysis, 10 μg of total RNA of each sample was denatured and separated using a 1% agarose formaldehyde gel. RNA was transferred onto a Hybond-N+ membrane (Stratagene, La Jolla, CA) by capillary techniques. Gene specific probes were prepared by PCR and labeled with [γ^{32}P]–dCTP using a Prime-it II Random Primer Labeling kit (Stratagene, La Jolla, CA). Hybridization was carried out according to the manufacturer's instructions and then exposed to Kodak Biomax film (Sigma). Ethidium bromide-stained ribosomal RNA was used as a loading control. 5 μg of total RNA from immature and mature stems were reverse transcribed using Superscript II reverse transcriptase (Life technologies) in 20 μl reactions. PCR was carried out for 30 cycles using 1 μl of the RT reaction as a template. Genomic DNA (100 ng) was also used as a template for validation of the PCR reaction. Amplified DNA fragments were separated on 1% agarose gels and stained with ethidium bromide. The primers used for amplification of *WUS* were: WUS_F (5′-CCGTT

AACTTTGTGAACAAAAG) and WUS_R (5′-AAAGAGCTTTAATCCCGAGC); for *STM*: STM_F (5′-ATGGAGAGTGGTTCCAACAGC) and STM_R (5′-TCAAAGCATGGTGGAGGAGAT); for *CLV1*: CLV1_F (5′-CCTTGAAAGTGGACGAGAAGA) and CLV1_R (5′-ATCAAGTTCGCCACGGATTT); for *CLV3*: CLV3_F (5′-ATGGATTCGAAGAGTTTTCTG) and CLV3_R (5′-TCAAGGGAGCTGAAAGTTGTT).

Results

Changes in global gene expression in the stems

We produced inflorescence stems with various heights, which represent different stem developmental stages as described in Ko *et al.* (2004). These stems were then divided into three developmental stages (immature, intermediate and mature stage) (Figure 1). Namely, plants in the immature stage have no visible secondary xylem, while plants in the mature stage have well-developed secondary xylem. Plants in the intermediate stage are in transition from primary to secondary growth. Confocal laser scanning microscopy analysis showed that the secondary xylem (i.e., wood) formed in the mature stem was remarkably similar to that of poplar tree (Figure 2), further confirming that *Arabidopsis* can serve as a model for secondary xylem development.

The GeneChip *Arabidopsis* ATH1 Genome Array (Affymetrix Inc.) representing 22,620 genes was used to obtain the transcription phenotypes of the stems. The individual gene expression value

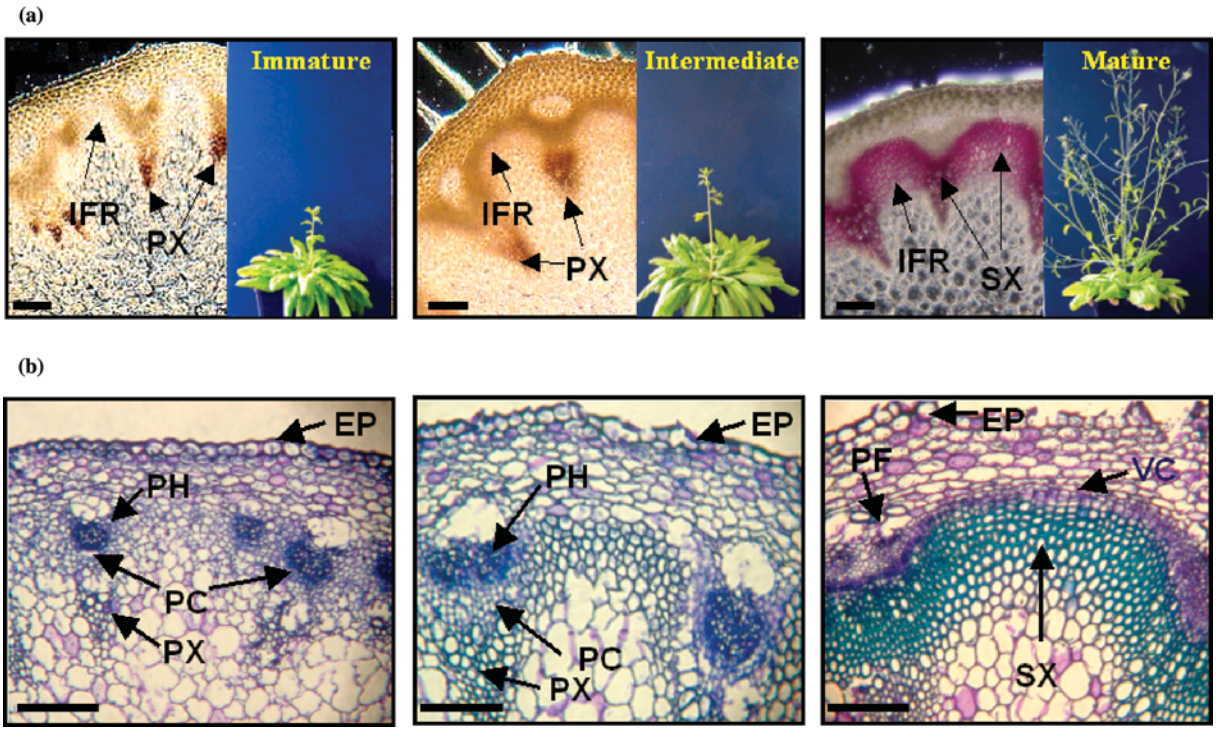

Figure 1. Developmental stages of secondary xylem tissue in *Arabidopsis* stem. (a) Secondary xylem development is related to plant stem growth. All plants (immature~mature) are the same age (8-week-old) and have similar stem thickness, but differ in the height growth of inflorescence stems. The heights of the stems are 5, 10 and 35 cm, respectively. The basal part of the each stem was cross-sectioned and stained with 2% phloroglucinol-HCl to visualize secondary xylem as red colour in the left panel. IFR, interfascicular region; PX, primary xylem; SX, secondary xylem. Bar indicates 0.2 mm of length. (b) Secondary xylem developed from the vascular cambium of mature *Arabidopsis* plant stems. From the left to right, immature, intermediate and mature stem. Microtome-sections were stained with 0.05% toluidine blue O. Lignified cells were stained with dark green color. EP, epidermis; IFR, interfascicular region; PC, procambium; PH, phloem; PX, primary xylem; VC, vascular cambium; PF, phloem fiber; SX, secondary xylem. Bar indicates 0.2 mm of length.

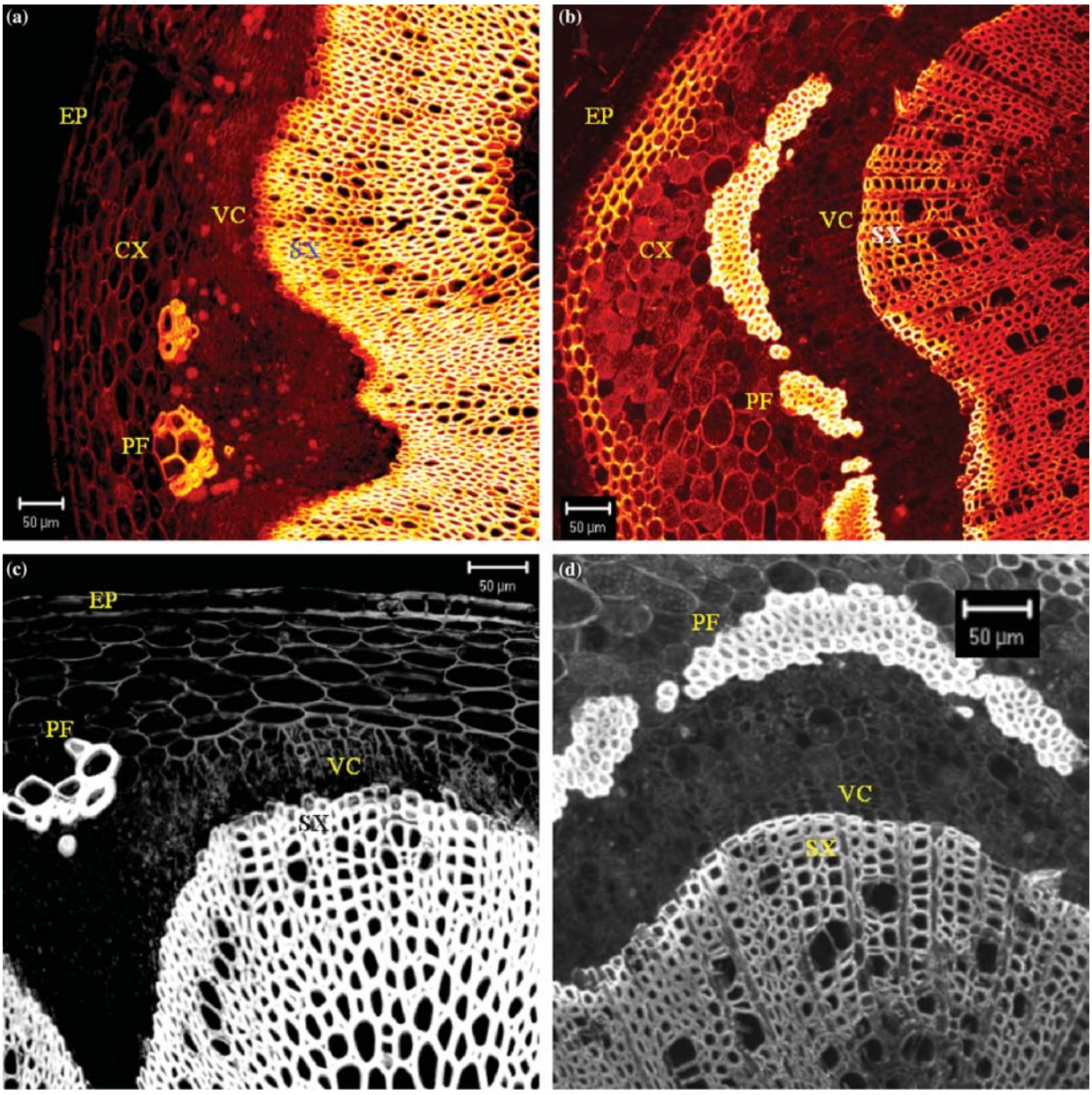

Figure 2. Comparison of wood formation in *Arabidopsis* with poplar by confocal image analysis. (a) Cross section of a mature stem of *Arabidopsis*. (b) Poplar (*Populus tremula* × *P. alba*) stem cross-section. (c and d). Magnified images of vascular cambium region in *Arabidopsis* and poplar, respectively. EP, epidermis; CX, cortex; PF, phloem fiber; VC, vascular cambium; SX, secondary xylem. Images (a and b) are modified using the 'glow scale' function in the Laser scanning microscope PASCAL LSM version 3.0 SP3 software. Images of (c) and (d) were obtained by channel 1 emission filtering (560 nm) alone.

was obtained as 'signal intensity' and ranged from approximately 40–90 000 (with the average value of 2,015). About 71% of the probe sets (16,035 genes) on the GeneChip were expressed (''presence'' calling) in the stem tissues. The results of the GeneChip analysis were confirmed by northern hybridization analysis using several differentially expressed genes (Ko *et al*., 2004; data not shown). Hierarchical clustering of the 16,035 genes expressed in the stems identified six groups of differentially expressed genes from the three developmental stages: Groups I and II are composed of the genes up regulated in the immature stage, Groups III and IV in the intermediate stage,

and Groups V and VI in the mature stage (Figure 3a and b). The putative functions of the genes were assigned and categorized according to the MIPS (http://mips.gsf.de/proj/thal/db/tables/tables_func_frame.html). The distribution of the genes in each expression group appears to reflect the physiological changes that might occur in the different stages (Figure 3c). For instance, the number of genes involved in protein synthesis and cell cycle control was higher in Groups I and II (i.e., up regulated in immature stage) and, then, sharply decreased in Groups V and VI (i.e., upregulated in mature stage). This seems to be consistent with the fact that the stems of plants in the immature stage are more juvenile and therefore require more vigorous protein synthesis and cell division activities. On the other hand, primary metabolism-related genes were similarly represented in all of the six groups. It is notable that the representation of signal transduction and transcriptional regulation-related genes was increased in Groups V and VI.

Of the 16, 035 genes expressed in the stem tissues, 12,417 genes (77.4%) were present in all three stages (Figure 4a). The number of genes with overlapping expression was significantly lower between the immature and mature stages (152), when compared to those between the immature and intermediate stages (820) and intermediate and mature stages (924). Mature stems had the highest number of stage-specific genes (847), followed by the intermediate (574) and immature stage (301). In order to identify the genes upregulated (≥ 5-fold) in the mature stems, we compared the gene expression profiles between the immature and mature stems. Those identified genes were then subtracted by the genes upregulated (≥ 3-fold) in 10-day old whole seedlings (compared to the immature stem). The subtraction increases the odds for finding stem developmental stage-specific genes by removing the commonly upregulated genes in both seedling and the mature stem (e.g., Group XI in Figure S1). Results from hierarchical clustering analysis of the gene expression profiles from three stem developmental stages and seedling are presented in Supplement Figure S1 and supplement Table S4. After removal of the commonly upregulated genes, about 6.8% of the 16,035 genes expressed in the stems were differentially regulated during the process of stem maturation. A total of 567 genes were upregulated in the mature stem, while 530 genes were downregulated when compared to the immature stem (for the gene list and expression level changes, see Supplement Table S1 and S2). Functional classification of the stem developmental stage-specific genes revealed that transcriptional regulation and signal transduction-related genes were most abundant in the mature stage, suggesting that complex metabolic and regulatory events occur at that stage (Figure 4). The functional categories that are more abundantly represented in the immature stage, when compared to mature stage, include: cell cycle, cellular organization, cellular transport, protein fate, hormonal regulation, primary metabolism, and protein synthesis. Conversely, the genes involved in transcriptional regulation, secondary metabolism, signal transduction, and transport facilitation were more represented among the mature stem upregulated genes (Figure 4c).

Expression of meristem activity- and cell division-related genes in the stem tissues

All aboveground primary tissues including stems, leaves, and flowers are derived from SAM. The SAM initiation is a complex process requiring the action of several well-defined genes including *STM* (*SHOOT MERISTEMLESS*), *WUS* (*WUSCHEL*), *ZLL*/*PND* (*ZWILLE*/*PINHEAD*), *CUC*1 (*CUP SHAPED COTYLEDON*) and *CUC*2 (Barton and Poethig, 1993; Laux *et al*., 1996; Aida *et al*., 1997; Mayer *et al*., 1998; Lynn *et al*., 1999). STM and WUS are required to maintain the population of undifferentiated cells (Laux *et al*., 1996), while the *CLAVATA* loci play a role in regulating the balance of these cells between proliferation and differentiation (Clark *et al*., 1993, 1995; Kayes and Clark, 1998). On the other hand, secondary tissues are not produced directly by the SAM, but rather by the cambium meristem. Contrary to SAM, our understanding of cambium meristem initiation and maintenance is very limited.

Although our stem samples do not include SAM, expression levels of several SAM activity-related genes were increased in the stems undergoing secondary growth (Figure 5a). These upregulated genes include *CLV1* (*CLAVATA1*, At1g75820), *CLE12* (*CLAVATA3*/*ESR-Related 12*, At1g68795), *AtHB8* (At4g32880) and *KNAT7* (At1g62990),

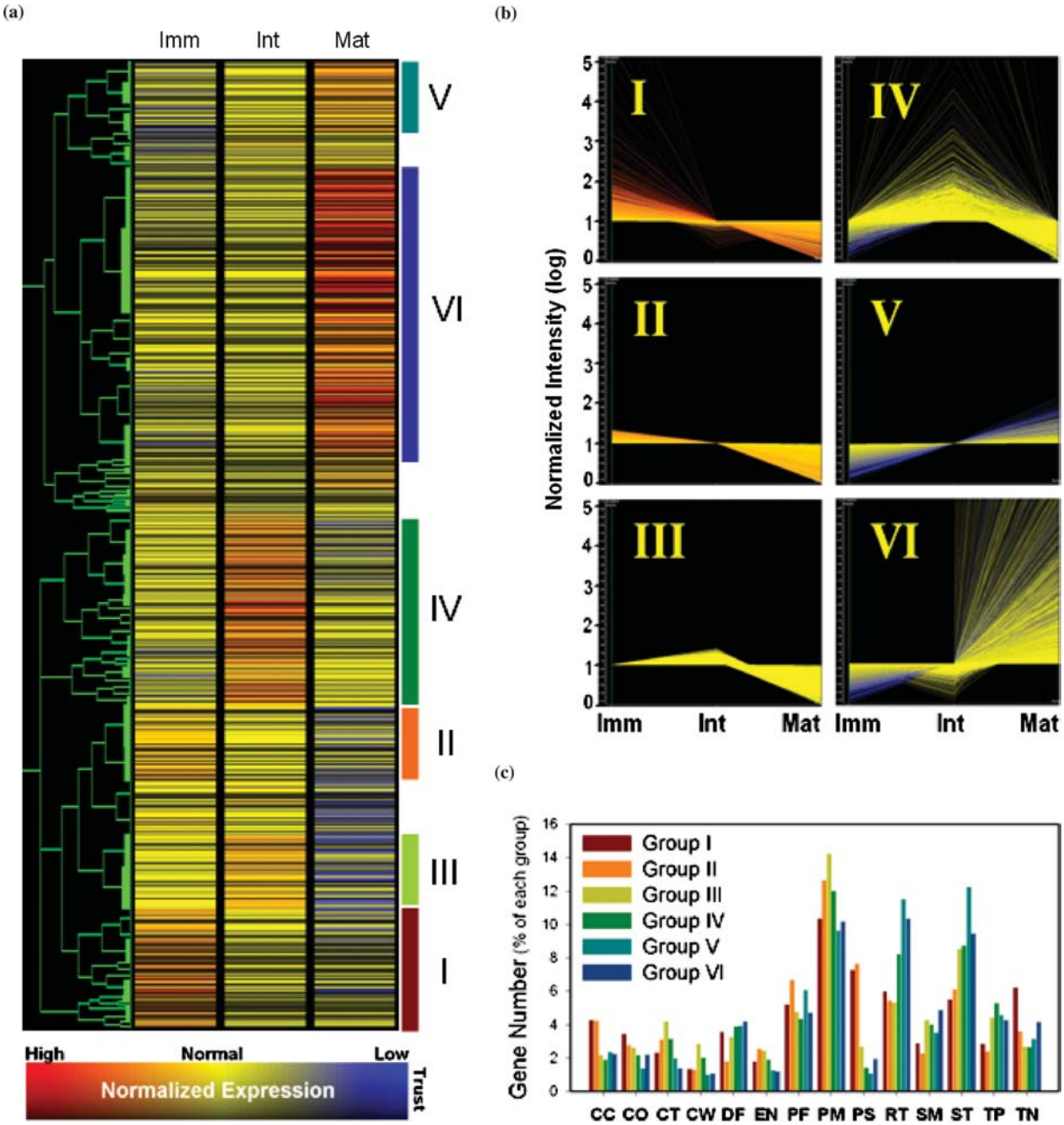

Figure 3. Whole transcriptome changes during the wood formation (from immature to mature stem). (a) Hierarchical clustering analysis. 16,035 selected genes with differential expression in the immature (imm), intermediate (int) and mature (mat) stem. Similarity was measured by the Pearson correlation with a 0.5 separation ratio and a 0.001 minimum distance, using GeneSpring 4.2.1 (Silicon Genetics) software. Colour scale bar shows normalized expression level. Trust level increased according to the colour brightness. (b) Groups of genes having different expression patterns with normalized intensity (*y*-axis). I, 1993 genes; II, 1249 genes; III, 1221 genes; IV, 3192 genes; V, 1132 genes; VI, 4086 genes. (c) Functional classification of the 6 groups in (b). Note the trends found in each of the functional categories from groups I–VI. Classification was based on the Munich Information Center for Protein Sequences (http://mips.gsf.de/proj/thal/db/tables/tables_func _frame.html); CC (cell cycle), CF (cell fate), CO (cellular organization), CT (cellular transport), CW (cell wall), DF (defense), EN (energy metabolism), HR (hormonal regulation), PD (plant development), PF (protein fate), PM (primary metabolism), PS (protein synthesis), RT (regulation of transcription), SM (secondary metabolism), ST (signal transduction), TP (transport facilitation), TN (transcription), and UN (unknown or unclassifed).

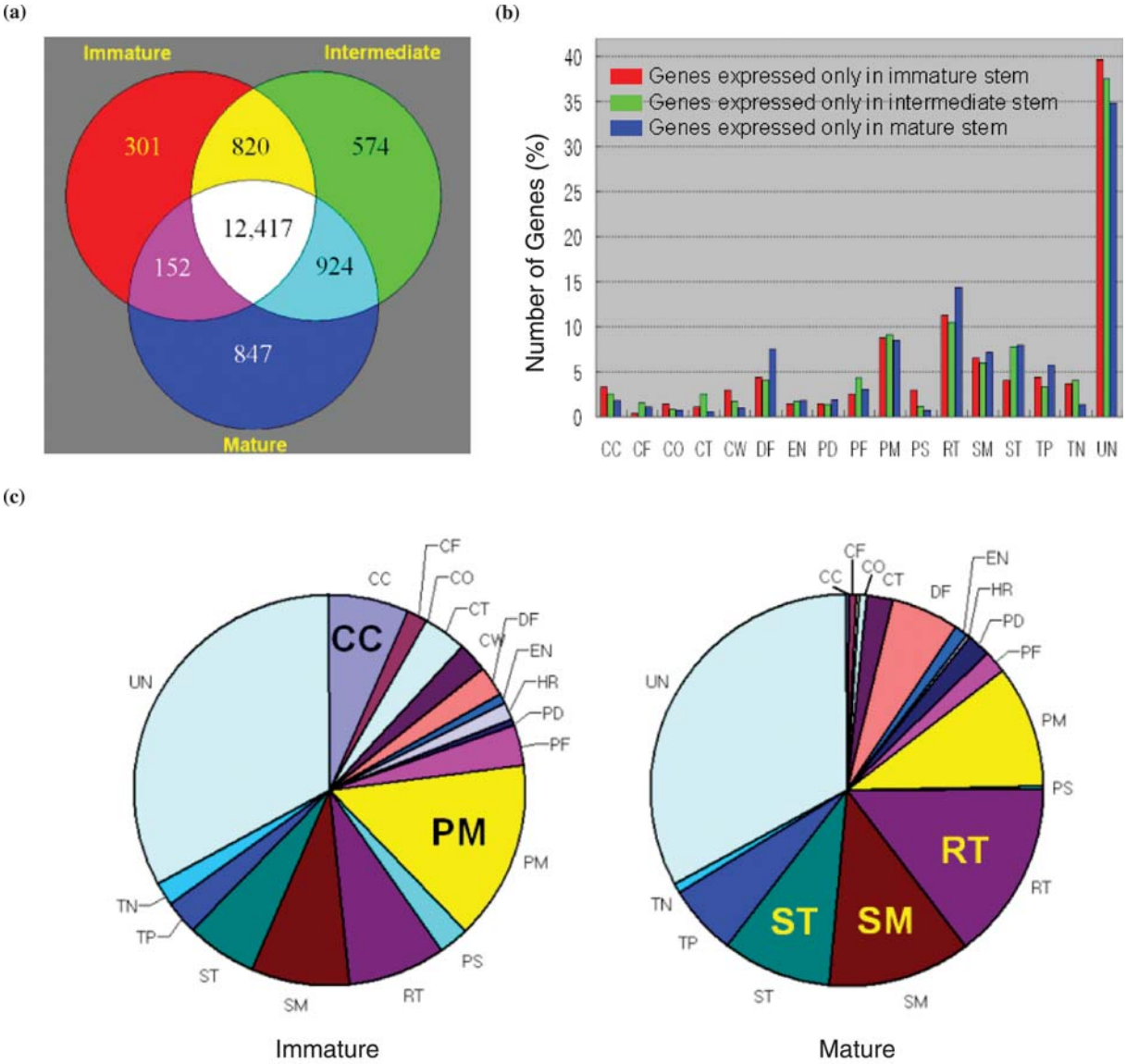

Figure 4. Characteristics of gene expression profiles in the three different stages. (a) Venn-diagram for the number of differentially expressed genes in the three sample pairs. The numbers in the overlapping areas indicate the number of shared genes in either two or three sample pairs. (b) Functional classification of the stage-specific genes obtained from (a). (c) Functional classification of only the highly expressed (> 5X) genes in the immature and mature stem. Refer Figure 3c for the classification.

all of which showed a dramatic increase in their expression levels as the stem matures. However, certain SAM activity regulators such as *WUSCHEL* (*WUS*; At2g17950), *CLV3* (*CLAVATA3*; At2g27250), *CUC1* (*CUP SHAPED COTYLEDON*; At3g15170) and *CUC2* (At5g53950), *LAS* (At1g55580), *LEAFY* (At5g61850) were not expressed in the stems ("absence" calling). Both *WUS* and *STM* (*SHOOT MERISTEMLESS*) play a critical role in the maintenance of meristematic activity in the SAM (Gallois *et al*., 2002; Lenhard *et al*., 2002). The fact that *WUS* is not expressed in the stems suggests that the maintenance and differentiation of secondary meristem (i.e., vascular cambium) may be regulated by different mechanisms from that of SAM. Since the current GeneChip does not have a probe for *STM*, we carried out RT-PCR analysis in order to investigate whether *STM* is expressed in the stem. Figure 5b shows that *STM* is expressed in the stem. The RT-PCR analysis also shows that *WUS* and *CLV3* are not expressed in the stem, confirming the GeneChip results. North-

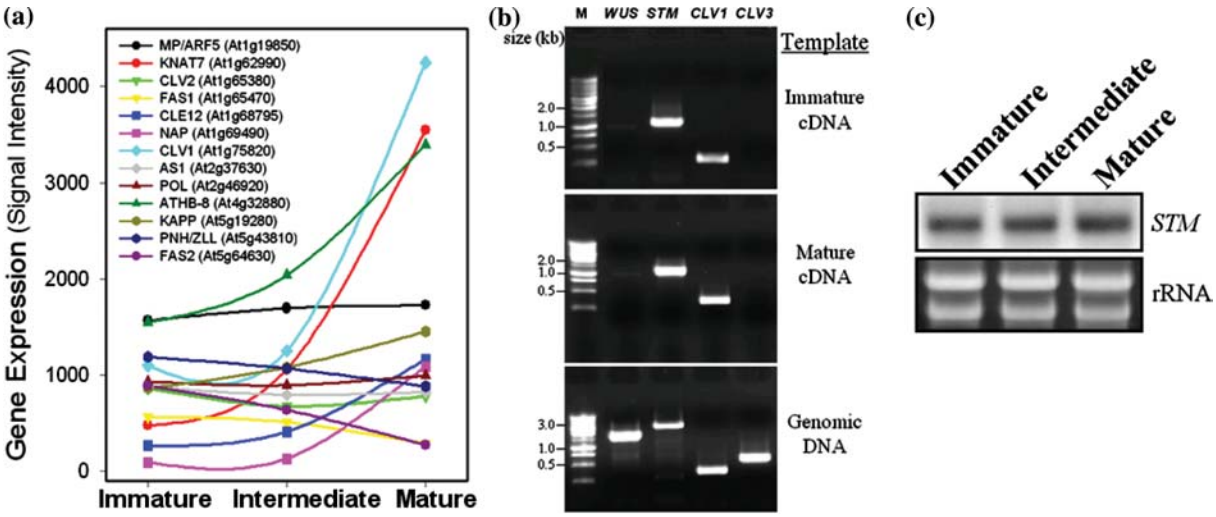

Figure 5. The expressions of shoot apical meristem regulating genes during wood formation. (a) Gene expression (Y-axis) designates the mean value of signal intensity of each gene. (b) RT (reverse transcriptase)-PCR for confirmation of GeneChip results of (a). Genomic DNA PCR was used for validation of the PCR reaction. The faint band in the 'WUS' lane was identified as an artifact based on its size. Gene specific primers (C-terminal region) used for CLV1. (c) Expression of *Arabidopsis STM*. RNA gel blot analysis was performed with 10 μg of total RNA extracted from each of the samples (immature, intermediate and mature) and hybridized with ^{32}P-labeled *STM* cDNA probe. Ethidium bromide-stained ribosomal RNA served as a loading control.

ern blot analysis shows that the *STM* gene is expressed in all three stages of stem development (Figure 5c).

Cell division from vascular cambium is the first step in secondary growth. Cell cycle control in general is governed by universally conserved molecular mechanisms, in which cyclin-dependent kinases (CDKs) play a key role. So far, two major classes of CDKs (A-type and B-type) have been studied in plants. A-type CDKs regulate both G1-to-S and G2-to-M transitions, whereas B-type CDKs control G2-to-M checkpoint (Hemerly *et al*., 1995; Magyar *et al*., 1997; Porceddu *et al*., 1999). Eleven CDKs (including 1 A-type and 3 B-type) were expressed in the stems. B-type CDKs were strongly down regulated in the mature stem while the expression levels of other types of CDK (A, C, D and E-type CDKs) were not significantly changed (Supplement Figure S2). Since monomeric CDKs lack kinase activity, CDKs must interact with a group of regulatory proteins called 'cyclins' in order to function in cell cycle control. Their protein levels fluctuate during the cell cycle (Vandepoele *et al*., 2002). Therefore, cyclins are key determinants for CDK activation. The expression levels of all cyclin genes (A-, B- and D-type) were high in the immature and intermediate stages and dropped dramatically in the mature stage (Supplement Figure S2). This suggests that active cell division may be critical during the early processes of stem development but not in later stages. CDK subunit (CKS) proteins act as docking factors and mediate the interaction of CDKs with putative substrates and other regulatory proteins. The expression of *CKS* genes was the highest in the immature stem but decreased in the mature stem. On the other hand, the expression of CDK inhibitor (*KRPs*) genes was increased in the mature stem (Supplement Figure S2). *Rb* (At3g12280) protein is a key regulator for the start of DNA replication (S-phase). Only one Rb gene was identified in the *Arabidopsis* genome. *Rb* gene expression was decreased during xylogenesis (Supplement Figure S2). Overall gene expression patterns of cell cycle regulation components clearly show that cell division activity is high in the immature/intermediate stems and low in the mature stem (Figure 6).

Coordinated regulation of cell expansion during stem development

Cell expansion plays a crucial role in shaping the form and size of plants. In the present study, we examined the expression patterns of the genes encoding cell wall loosening/hydrolytic enzymes such as expansin, xyloglucan endotransglycosylase

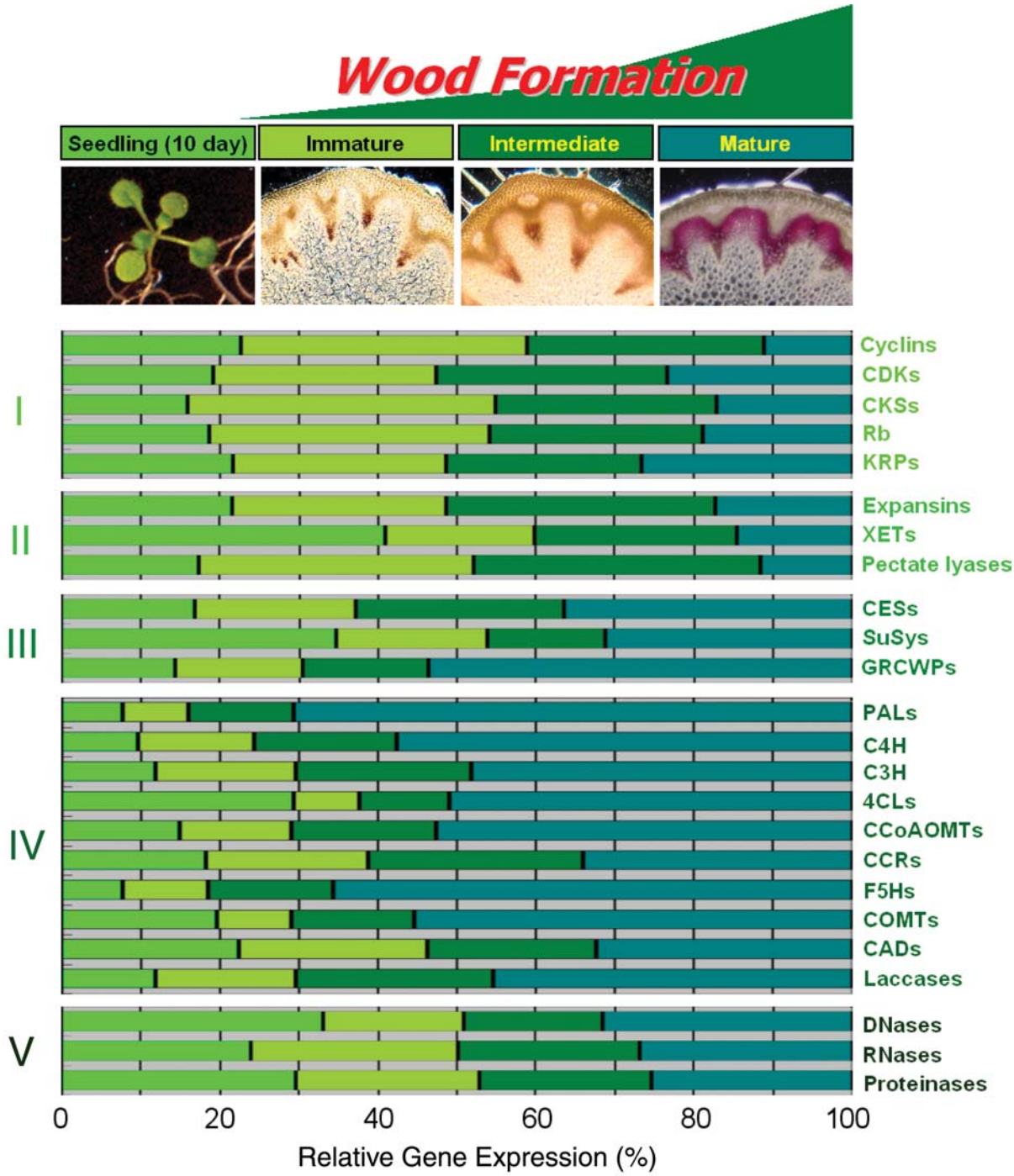

Figure 6. Summarized gene expression profile of wood formation–associated genes. I, Cell cycle related genes; II, cell expansion related genes, III, secondary cell wall biosynthesis related genes; IV, Lignin biosynthesis related genes; V, Programmed cell death related genes. Responsible gene signal intensity sums are designated by the relative gene expression percent values. 'Relative Gene Expression (%)' represents the proportion of signal intensities in each respective stage in reference to the total intensity of genes in all stages. A gene list is provided in the supplement Table S3.

(XET; recently re-named as XTH, Rose *et al*., 2002), and pectate lyase. Overall, these genes had the highest expression at the intermediate stage, where cell elongation is thought to be most active.

Of the 33-expansin genes on the GeneChip, 14 genes were not expressed in the stem tissues and 15 were highly expressed in the immature and intermediate stem. However, four expansin genes (*At-EXP15*, *At-EXPR*, *At-EXPL1*, and *At-EXPL3*) were highly up regulated in the mature stem (Supplement Figure S3). XTHs cut and rejoin xyloglucan chains. For cell expansion, cellulose microfibrils must be able to move past one another. Such cell wall loosening is achieved through the modification of xyloglucan, the major hemicellulose in plant cell walls, and the tethering of adjacent microfibrils. Of the 27 XTH genes on the GeneChip, only 15 XTHs were expressed in the stem tissues. Twelve of them including *TCH*4 (Xu *et al*., 1995) were highly expressed in the intermediate stem, suggesting their role in cell wall elongation during the transition from immature to mature stem development. However, three XTH genes (*At4g30270*, *At1g14720*, and *At3g44990*) were induced only in the mature stems (Supplement Figure S3). Pectate lyases have been extensively studied in pathogenic bacteria, which use these enzymes to macerate the tissues of the host plants by depolymerizing the pectins present in the middle lamella and primary cell wall (Henrissat and Romeu, 1995). A total of 17 pectate lyase or pectate lyase-like proteins were expressed in the stems, most of which were highly expressed in the immature and intermediate stems and minimally expressed in the mature stem (data not shown). In addition, we found that three endo-beta-1,4-glucanase genes (*At1g64390*, *At4g02290*, *At1g75680*) were up regulated in the intermediate stems. Four tonoplastic aquaporin genes (*At3g16240*, *At2g36830*, *At2g16850*, *At1g01620*) were also expressed at high levels in the immature and intermediate stage but at very low levels in the mature stage (Supplement Figure S3). Combined together, these results may support the hypothesis that there is a coordinated regulation of cell expansion during secondary growth.

Differential regulation of cell wall biosynthesis genes in the stems with secondary growth

After the cell expansion, the young secondary xylem cells start to form secondary cell walls. This process involves the biosynthesis and assembly of major cell wall compounds such as cellulose, hemicelluloses, cell wall proteins, lignins, and pectins. Figure 7 summarizes the expression patterns of carbohydrate metabolic pathway genes. The *Arabidopsis* genome contains a superfamily of more than 40 *CesA* (cellulose synthase A) and cellulose synthase-like genes. Twelve of these are structurally similar to each other (> 50% identity) and likely encode AtCesA isoenzymes, whereas the rest of the homologues showing identity below 50% are cellulose synthase-like genes (*AtCsl* genes) (http://cellwall.stanford.edu/cellwall/index.shtml). The overall expression of *AtCesA* genes was higher in the mature stage stems. The expressions of *AtCesA08* (At4g18780, *IRX1*), *AtCesA07* (At5g17420, *IRX3*), and *AtCesA04* (At5g44030, *IRX5*) were up regulated in the mature stems, indicating their potential role in secondary cell wall biosynthesis. On the other hand, the expressions of four *AtCesA* genes (At1g02730, At5g16190, At2g21770, and At4g24010) were increased in the intermediate stems and decreased in the mature stems, suggesting that they may be involved in primary cell wall biosynthesis. The other *AtCesA* genes showed no change in their expression level among the three different stem stages. Of all the *AtCesA* genes, the *AtCesA03* (At5g05170) had the highest expression level in all three stem developmental stages.

In higher plants, UDP-D-Glc (the substrate for CesA) is provided by sucrose synthase (SuSy). Of the five *SuSys* genes on the GeneChip, one *SuSy* gene (At4g02280) was dramatically up-regulated (> 40-fold) in the mature stem, although the absolute signal intensity was low (ca. 575, Supplemental Table S1). While carbohydrates largely constitute the cell wall materials, structural proteins also form a network that contributes to the cell wall architecture. Plant cell wall-associated proteins have been classified into four main groups: Gly-rich proteins, Pro-rich proteins, arabinogalactan proteins (AGPs), and Hyp-rich glycoproteins (or extensins). These proteins are cross-linked into the cell wall and probably have structural functions. Twenty-three AGP family genes were expressed in the stem tissues. Among them, *AtAGP5* (At1g35230) and *AtAGP10* (At4g09030) were highly expressed at the mature stage. Six out of nine glycin-rich cell wall proteins (GRCWP) in *Arabidopsis* were expressed in the

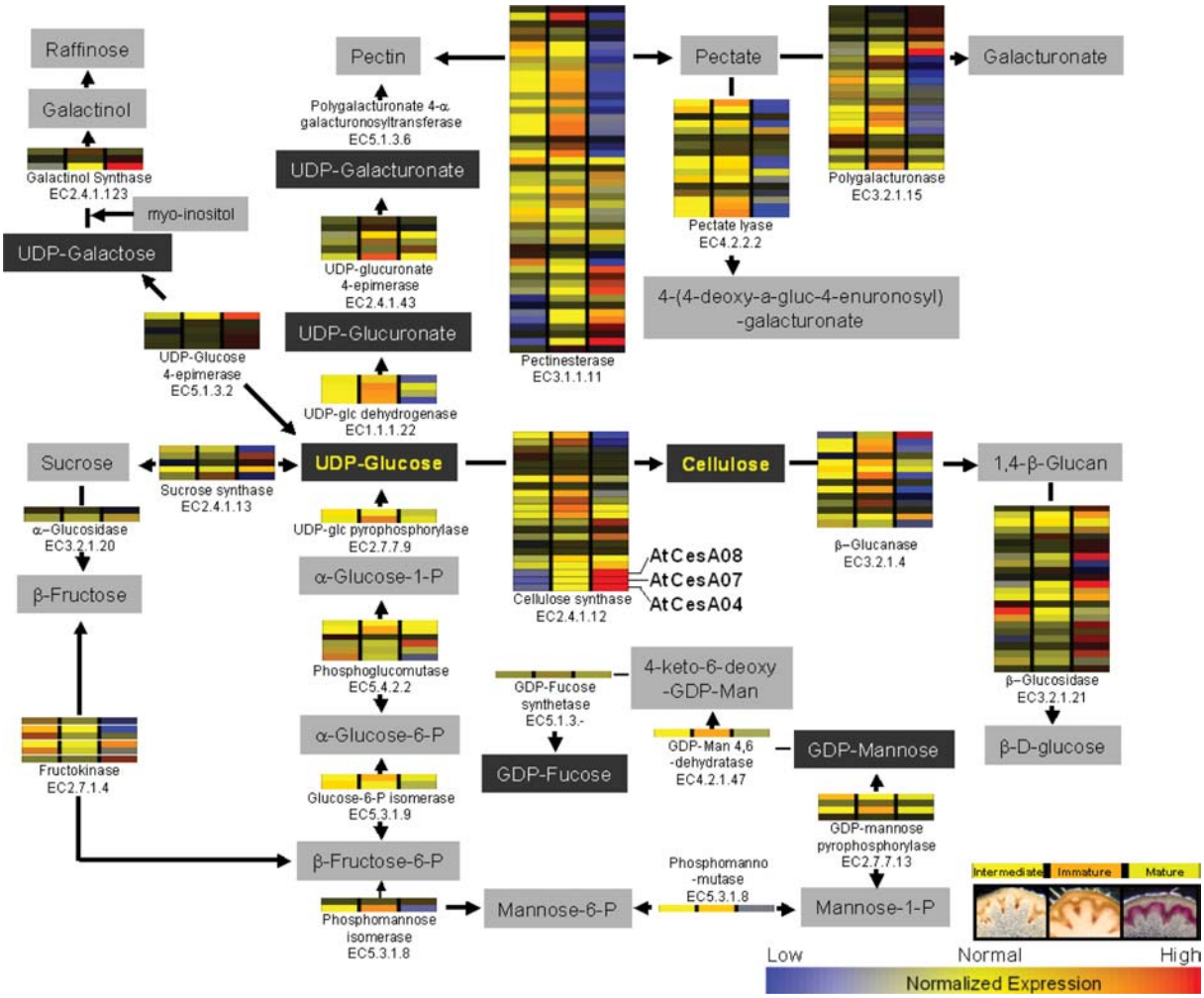

Figure 7. Selected steps in the carbohydrate metabolic pathway for the formation of cell-wall components in *Arabidopsis*. White letters on the dark gray box highlight nucleotide sugars as donor substrates of glycosyltransferases in the synthesis of glycans (Gibeaut, 2000). Colors in each bar for the three stages were drawn by GeneSpring software (Silicone Genetics) and indicate relative gene expression level. The reference bar is shown in the lower right hand corner. Brightness of the color is proportional to the signal intensity.

stems. The expressions of two GRCWP genes (At4g30450 and At4g30460) were dramatically increased (9- and 31-fold, respectively) with high signal intensities (30,726 and 29,997, respectively) in the mature stems (Table S1).

Lignins are complex phenolic polymers of plant cell walls that are linked to wood fibers. Clearly, most of the genes involved in lignin biosynthesis had an increased expression in the mature stems (Figures 6 and 8). Phenylalanine-ammonia lyase (PAL) catalyzes the first step in the phenylpropanoid pathway leading to lignin biosynthesis (Hahlbrock and Scheel, 1989). All four PAL genes were upregulated in the mature stems. Especially, the expression of *PAL4* (At3g10340) was increased about 57-fold in the mature stems. Cinnamate 4-hydroxylase (C4H), a cytochrome P450-dependent monooxygenase, catalyzes the *p*-hydroxylation of *trans*-cinnamic acid to produce p-coumaric acid, which is first step in the phenylpropanoid pathway. C4H is encoded by a single copy gene (At2g30490) in *Arabidopsis* and expressed in various *Arabidopsis* tissues, particularly in roots and cells undergoing lignification (Bell-Lelong *et al.*, 1997). During secondary xylem formation, its expression was increased about 4-fold with very high signal intensity (46,951). 4-Coumarate:CoA ligases (4CL) catalyses the formation of

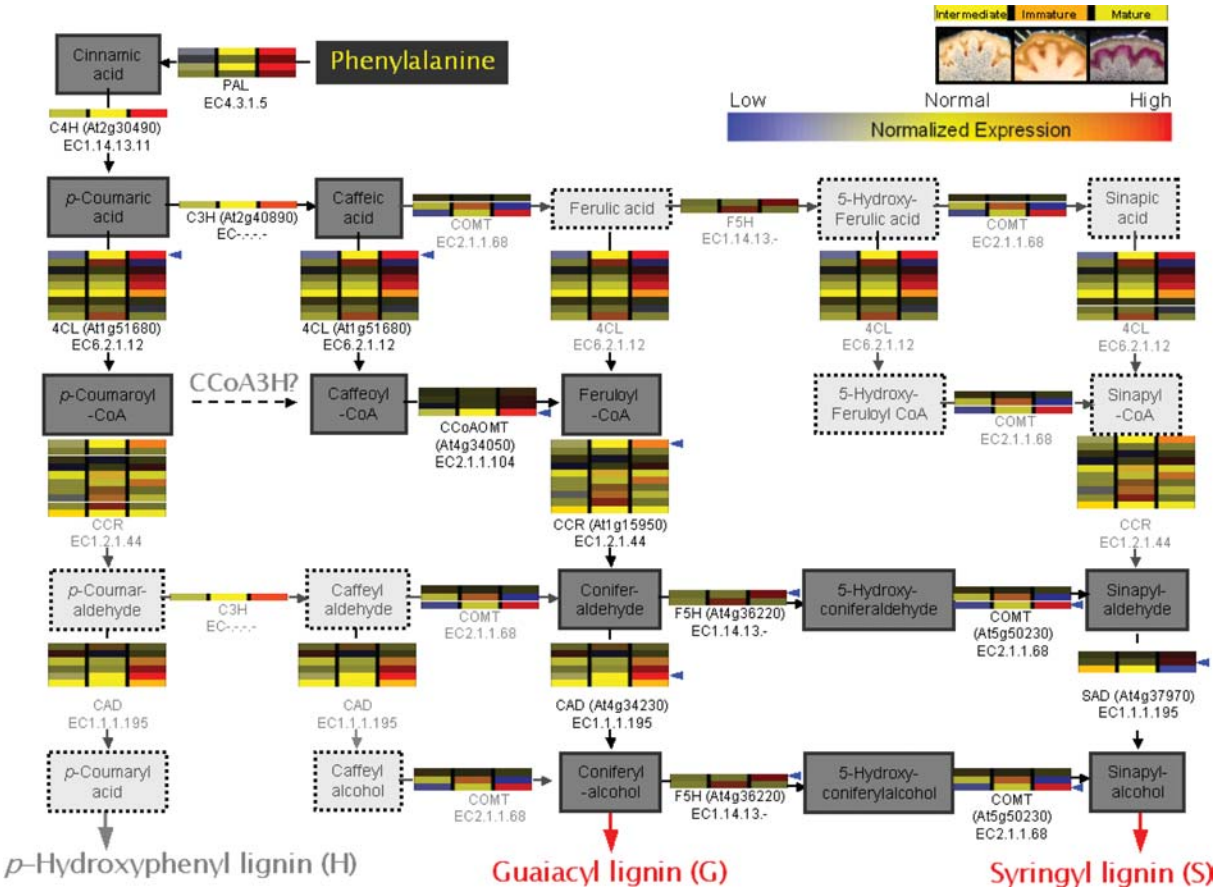

Figure 8. Metabolic pathway for lignin biosynthesis in *Arabidopsis*. Key *Arabidopsis* lignin biosynthesis reactions are highlighted by a dark gray box. AGI number in each reaction step designates the probable key gene and the blue arrowheads indicate the responsible genes. 4CL, 4-coumarate CoA ligase; C3H, p-coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; CCoAOMT, caffeoyl CoA O-methyltransferase; CCR, cinnamoyl CoA reductase; COMT, caffeic acid O-methyltransferase; F5H, ferulate 5-hydroxylase; PAL, phenylalanine ammonia-lyase; CCoA3H, p-coumaryl CoA 3-hydroxylase; SAD, sinapyl alcohol dehydrogenase. Colors in each bar for the 3 stages were drawn by GeneSpring software (Silicone Genetics) and indicate relative gene expression level. Refer the supplemental Table S3 for the each gene list.

CoA thioesters of hydroxycinnamic acids. The 23 K GeneChip contains 10 *4CL* genes, one (At1g62940) of which was not expressed in the stems. Three *4CL* genes (At1g51680, At3g21230, and At3g21240) showed increased expression in the mature stem, while two (At1g65060 and At5g38120) were down regulated. The At1g51680 gene was up regulated over 20-fold with a very high signal intensity (45,153) in the mature stem, suggesting this *4CL* gene may play a key role in lignin biosynthesis in the mature stems. Cinnamoyl-CoA reductase (CCR) catalyses the reduction of the hydroxycinnmoyl-CoA esters to their corresponding aldehydes. The *Arabidopsis* genome has a total of 11 *CCR* genes. With the exception of one gene (At2g23910), all of the CCR genes were expressed in the stems. Three *CCR* genes (At1g80820, At1g15950, and At2g33590) were up-regulated in the mature stems. Despite the fact that the At1g80820 gene was more than 36-fold up regulated, it is unlikely that this gene plays any significant role at this stage because the signal intensity was too low (about 606). However, the other upregulated gene (At1g15950), known as *irx*4 (Jones *et al*., 2001), had a very high signal intensity (19 527), suggesting its potential role in the maturation of secondary xylem cells. Caffeoyl-CoA *O*-methyltransferase (CCoOMT) preferentially catalyses the methylation of caffeoyl-CoA over 5-hydroxylferuloyl-CoA (Li *et al*., 1999) and

has a role in the biosynthesis of both S- and G-type lignin (Meyermans *et al*., 2000; Zhong *et al*., 2000). All of the four *CCoOMT* genes were up regulated during xylogenesis. One *CCoOMT* (At4g34050) was 3.6-fold up regulated in the mature stem and highly expressed with a signal intensity of 62,738 (31 times greater than the average signal intensity).

In vitro studies have revealed that ferulic acid 5-hydroxylase (F5H/CAld5H) prefers to catalyze the hydroxylation of coniferaldehyde over ferulic acid (Humphreys *et al*., 1999). This enzyme has been shown to be a major regulatory step in the determination of lignin monomer composition (Franke *et al*., 2000). *Arabidopsis* has two *F5Hs* (At4g36220 and At5g04330) but only one (At4g36220) was up regulated in the mature stem. Recently, Li *et al*. (2000) showed that caffeate *O*-methyltransferase (COMT/AldOMT) preferentially uses 5-hydroxyconiferaldehyde as substrate *in vitro*. Consistent with the substrate specificity, the lignin composition of the COMT-down regulated poplars is characterized by a drastic decrease in the amount of S units (Jouanin *et al*., 2000). Of the seven *COMT*s on the 23 K GeneChip, only three (At1g77530, At3g53140 and At5g20230) were expressed in the stems. Among them, only one gene (At5g20230) was upregulated in the mature stem. Cinnamyl alcohol dehydrogenase (CAD) catalyses the last step in the monolignol biosynthesis pathway, which is the reduction of cinnamaldehydes to cinnamyl alcohols. The *Arabidopsis* genome encodes 10 CADs. While three of them (At1g09500, At1g09510, At1g66800) were not expressed in the stem tissue, two (At4g34230 and At3g19450) showed significant up-regulation in the mature stem. Among the up-regulated genes, the expression of At4g34230 increased 8-fold with a very high signal intensity of 24,697 (12 times above the average signal) in the mature stem. However, *CAD1* gene (At4g39330) was down regulated. Recently, Li *et al*. (2001) reported a novel gene encoding sinapyl alcohol dehydrogenase (*SAD*) in poplar. They successfully demonstrated that the SAD is required for the biosynthesis of syringyl (S) lignin in angiosperms. Using the amino acid sequence of poplar SAD, we found four *Arabidopsis* homologues. Two of them (At2g21890 and At4g37970) were expressed more than 5-fold in the mature stem, while the other two genes (At4g37980 and At4g37990) were not expressed in the stem. The gene, At4g37970, had an especially high signal intensity of 2,598. This suggests that it may play a key role in the biosynthesis of S-lignin, which is accumulated in the phloem fiber of mature stems (data not shown).

Programmed cell death in the mature stems

The final step of secondary xylem formation involves a programmed cell death (PCD), through which the cells get rid of the cellular contents and become a part of water-conducting tissues. Specific hydrolases (cysteine and serine proteases, nucleases, and RNase) are recruited to carry out this cell-autonomous, active, and ordered suicide (Roberts and McCann, 2000). Proteinases and nucleases participate in PCD during xylogenesis (Mittler and Lam, 1995; Fukuda, 1997a,b). Of the 20 cystein protease or cystein protease-like genes, four genes were significantly up-regulated in the mature stems: At5g50260 (55-fold), At4g16190 (5-fold), At4g01610 (3-fold) and At5g60360 (2-fold). Other proteases that had the mature stem-specific expression pattern include Ser protease (At1g20160), Asp protease (At4g 04460), Asp protease (At1g62290), and Cys protease (At5g45890) (Supplement Figure S4). Both cysteine and serine proteases have been implicated in tracheid element (TE) formation in the *Zinnia* system (Minami and Fukuda, 1995; Beers and Freeman, 1997). Two cDNAs (*p48h-17* and *ZCP4*) encoding cysteine proteases have been isolated from trans-differentiating *Zinnia* cells and were associated with differentiating xylem in stems. However, *Arabidopsis* xylem-specific proteases such as *XCP1* (At4g35350), *XCP2* (At1g20850), and *XSP1* (At4g00230) (Zhao *et al*., 2000) had slightly decreased or no significant changes in their expression levels, which is consistent with an observation made in a similar study (Oh *et al*., 2003). Contrary to our expectation, the expression level of nuclease genes, including the bi-functional nuclease (*BFN1*) gene that was reported to be induced during leaf and stem senescence in *Arabidopsis* (Pérez-Amador *et al*., 2000), did not change among the three stem developmental stages. This suggests that the nucleases involved in xylogenesis-associated PCD may not be regulated at the transcriptional level.

Discussion

Whole-transcriptome profile of Arabidopsis *stem*

Recently, we described an experimental system for induction of secondary growth in the inflorescence stem (not root-hypocotyl junction) of *Arabidopsis* (Ko *et al.*, 2004). Using the system, we produced synchronized (same-age) inflorescence stems having different degrees of secondary growth. Those stems were grouped into three stem developmental stages (i.e., immature, intermediate and mature) with regard to secondary growth and used in the current study to investigate the changes in global gene expression during the three stages. The use of whole-transcriptome microarray provided a unique opportunity to obtain novel transcription phenotypes in the stems at different developmental stages. Our results show that over 70% of the genes in the *Arabidopsis* genome were expressed in the stem tissue. Plant stems play several crucial roles in the growth and development of plants. First of all, this organ provides a physical support for the growing biomass and acts as a communication center for the transmission of signals (e.g., plant growth regulators) and for the translocation of nutrients and water. In addition, stems represent the largest storage organ and play an important role in the seasonal cycle of nutrients in tree species. Considering the significance of stem on plant growth, it is not surprising that such a large proportion of the genome is expressed in the stem.

Hierarchical clustering of the expressed genes allowed us to track changes in the transcriptome as secondary xylem tissues were induced in the stem. As expected, the expression of cell division and protein synthesis-related genes were increased in young, rapidly growing stems and decreased in mature stems. There was no significant change in the expression of primary metabolism-related genes in any of the three stem developmental stages. This indicates that the mature stem was not yet undergoing senescence. However, signal transduction and transcriptional regulation-related genes were highly up regulated in the mature stems. This suggests that the cellular changes in the mature stems undergoing secondary growth involve different kinds of metabolic and regulatory demands, compared to those of the immature stems with only primary growth. Likewise, Hertzberg *et al.* (2001) previously reported that a number of transcription factors and other potential regulators of xylogenesis, as well as the genes involved in the primary metabolism, lignin and cellulose biosynthesis are under strict developmental stage-specific transcriptional regulation.

Oh *et al.* (2003) described the global gene expression changes in the bark and xylem tissues of *Arabidopsis* root-hypocotyl junction region, in which secondary growth was induced by repeated decapitation (Lev-Yadun, 1994; Zhao *et al.*, 2000). The expression patterns of most of the xylogenesis-related genes, including transcription factors *AtMYB59*, *AtMYB48*, and *AtHB-6*, were similar to the results in the current study. However, we observed that the expression patterns of some of the lignin and cell wall biosynthesis genes were not consistent with the current results. For example, Oh *et al.* (2003) found that several genes including putative *cesA* (At4g18780), *CCoAMT* (At4g34050), and *PAL* (At3g53260) were downregulated in the wood formation treated stems compared to the control stems, while these genes were upregulated in the mature stems compared to the immature stems. The discrepancy may be due to the differences in the experimental systems (repeated decapitation vs. no decapitation; long-day vs. short-and long-day combination), tissue sources (root-hypocotyl junction vs. inflorescence stem), and probe sets (8.3 k vs. 23 k). This is further supported by the observation that several pathogenesis-related genes (e.g., At2g19970, At4g36430, and At2g19990) were highly upregulated (4.4–56-fold) in the wood formation treated stems (Oh *et al.*, 2003), while they were not expressed in the mature stems of the current study.

Is the regulation of secondary growth under different mechanism from that of SAM?

Most aerial parts of plants are the results of SAM differentiation. A few cells located in the central zone of the meristem act as pluripotent stem cells. In *Arabidopsis*, the formation and maintenance of these stem cells requires the involvement of WUS and STM, which serve distinct yet complementary functions at the SAM (Clark, 2001; for review, Carles and Fletcher, 2003). STM works to prevent meristem cells from differentiating prematurely, whereas WUS specifies a subset of cells as stem cells at the apex of the meristem. In addition, other

genes involved in the regulation of SAM development include *CLAVATA* (*CLV1, CLV2*, and *CLV3*), *CUC* (1 and 2), *FAS*1, *FAS2*/*NFB1*/*MUB3, KAPP, PNH*/*ZLL*, and *POL*. The growth and differentiation of secondary meristem (i.e., vascular cambium) lead to the formation of secondary xylem (i.e., wood) and phloem. However, genetic regulation of the formation, maintenance, and differentiation of secondary meristem (i.e., vascular cambium) has not been intensively studied. Since cambium is a meristem that originated from SAM, the control of cambial cell activity could be governed by the same mechanism that specifies SAM activity. In order to address whether a common genetic mechanism governs the regulation of cell activities in both SAM and cambium meristem, we investigated the expression of four key SAM regulatory genes (*WUS*, *STM*, *CLV1*, and *CLV3*) found in the stems. Both *WUS* and *CLV3* were not expressed in the stems, suggesting that the genetic control of secondary meristem differentiation may differ from that of the primary meristem. However, *STM* and *CLV1* were expressed in both immature and mature stems. At SAM, CLV1 works as a signal receptor kinase (Clark *et al*., 1993). Interestingly, this gene was dramatically upregulated in the mature stem. It was previously determined that STM is required for both the maintenance of indeterminate cell fate and the prevention of cell differentiation at SAM, where it acts independently of CLV3 and WUS (Gallois *et al*., 2002; Lenhard *et al*., 2002). Therefore, STM may also have a fundamental role in the regulation of cambial cell activity without the WUS and CLV3 pathway. In addition, we found the expression of CLE12 (a CLV3-like gene; Sharma *et al*., 2003) was upregulated in wood-forming stems. Whether this gene functions in place of CLV3 remains to be elucidated. In a separate but related study, we analysed 24,432 publicly available ESTs derived from cambium or stem tissues of *Populus* species. While poplar homologues for both *STM* and *CLV1* were present in the cambium or stem ESTs databases, no homologue for *WUS* and *CLV3* was represented in the database (Ko and Han, unpublished). These observations lead to the hypothesis that the genetic regulation of secondary meristem maintenance and differentiation may be achieved through a different mechanism from that of primary meristem.

Cell elongation and cell wall biosynthesis during secondary growth

Prior to secondary wall formation, procambium cells first expand primarily along the longitudinal axis by intrusive growth (Roberts and Uhnak, 1998). Cell elongation is dependent on cell wall relaxation and expansion driven by water influx into the vacuole compartments (McCann and Roberts, 1994). The former is controlled by a series of cell wall-loosening/hydrolytic enzymes (Cosgrove, 2001), and the latter is controlled by the water channel protein aquaporin (Maurel and Chrispeels, 2001). Expansins do not follow the conventional theory of wall loosening by polysaccharide hydrolysis; rather, they act by disrupting the hydrogen bonding between cellulose and load-bearing cross- link glycans (Cosgrove, 1999). Expansin transcripts were confined to developing primary xylem cells, predominantly adjacent to the vessel members, and in the cells located at future interfasicular cambium positions (Im *et al*., 2000). However, expansins belong to a large protein family (Cosgrove, 1999) and may serve various functions throughout development. For example, a ripening-related expansin in tomato fruit may enhance accessibility of non-covalently bound polymers to endogenous enzyme action during ripening (Rose *et al*., 1997). It has also been proposed that α-expansins might be involved in leaf organogenesis (Reinhardt *et al*., 1998) and vascular cell differentiation (Cho and Kende, 1997). However, little is known about the specific roles of individual expansins. In the current study, four expansin genes (*At-EXP15*, *At-EXPR*, *At-EXPL1*, and *At-EXPL3*) were specifically up regulated in the mature stem (Supplement Figure S3). Functional characterization of these genes will improve our understanding of their roles in secondary growth. Similarly, three XTHs (At4g30270, At1g14720, and At3g44990) were specifically upregulated in the mature stem (Supplement Figure S3). The extent to which they have additional roles, other than elongation is not known. Several other possible functions for XTH that have been suggested include the incorporation of newly secreted xyloglucan into the wall and the rearrangement of the xyloglucan-cellulose network during wall assembly (Nishitani, 1997). Using poplar stems, Bourquin *et al*. (2002) have recently demonstrated XTH activity in xylem and phloem

fibers at the stage of secondary wall formation. Immunolocalization of fucosylated xylogucan with CCRC-M1 antibodies showed that its level was increased at the border between the primary and secondary wall layers when secondary wall deposition occurred. These data strongly suggest that XTH has a previously unreported role in the restructuring of primary cell walls, when secondary wall layers are deposited, probably creating and reinforcing the connections between the primary and secondary wall layers.

Hemicelluloses are crosslinked with cellulose microfibrils in the cell wall of most plant cells, forming a cellulose/hemicellulose framework that functions as the mechanical underpinning of the cell wall. UDP-glucuronic acid, which provides about half of the cell wall biomass in *Arabidopsis*, is the dominant nucleotide sugar for hemicelluloses and pectin. UDP-glucose dehydrogenase, which converts UDP-glucose to UDP-glucuronic acid, is a key enzyme that regulates the flux of UDP-glucose into several other nucleotide-sugars that are substrates for cross-linking glycans and pectin precursors synthesized in the Golgi apparatus (Seitz *et al*., 2000). *Arabidopsis* has at least five UDP-glucose dehydrogenase genes, four of which showed the highest expression in the intermediate stage. This indicates that the pathway toward UDP glucuronic acid and pectin synthesis may be largely formed directly from UDP-glucose (Figure 6).

Pectinesterases catalyse is the demethylesterification of cell wall polygalacturonans. In dicot plants, these ubiquitous cell wall enzymes are involved in important developmental processes including cellular adhesion and stem elongation. *Arabidopsis* has more than 89 pectinesterases (EC 3.1.1.11) or pectinesterase-like genes. Of those genes, 39 were expressed in the stems but showed differential expression patterns during xylogenesis (Figure 7). Most of them were highly expressed in the immature and intermediate stages but sharply decreased at the mature stage. This is consistent with their potential role in cell elongation. However, three genes (At3g59010, At2g43050, and At2g43050) were 4-fold or more up regulated in the mature stage. Previous studies have shown that pectin methylesterase activity is inversely correlated with the growth rate of expanding tissues, suggesting its possible involvement in wall rigidification (McQueen-Mason and Cosgrove, 1995). Therefore, it is prudent to suggest that genes up regulated in the mature stems may have a role in cell wall rigidification of secondary xylem cells. Pectin metabolism appears to be important in xylogenesis. In *Zinnia*, a pectate lyase (*ZePel*) transcript was found in vascular tissue by *in situ* hybridization and the gene was up regulated in the xylogenic cell culture at the early stage of induction, before the formation of secondary cell walls (Domingo *et al*., 1998). Similar expression patterns were observed with seventeen genes that encode pectate lyases or pectate lyase-like proteins in *Arabidopsis* stems (i.e., upregulated in the intermediate stems then decreased in the mature stems).

Differential expression patterns of the *AtCesA* genes observed in this study provided us with valuable insights on the role each cellulose synthase plays in cell wall biosynthesis. For instance, the upregulation of At4g18780 (AtCesA08, *IRX1*), At5g17420 (AtCesA07, *IRX3*), and At5g44030 (AtCesA04, *IRX5*) in the mature stems indicates their potential role in secondary cell wall biosynthesis, which is consistent with previous reports (Turner and Somerville, 1997; Turner *et al*., 2001; Taylor *et al*., 2003). Likewise, several *CesA* genes (At1g02730, At5g16190, At2g21770 and At4g 24010) were up regulated in the immature stems, suggesting that these cellulose synthases may have roles in primary cell wall biosynthesis. On the other hand, the AtCes03 gene (At5g05170) had very high signal intensity, but showed no significant change throughout the stem maturation, implying that this gene may be important in cellulose synthesis regardless of stem developmental stage.

Some insights into lignin biosynthesis

Lignin synthesis is one of the most important plant metabolic pathways. The contribution of lignin to the architecture and functions of secondary cell walls remains to be fully elucidated. Furthermore, the individual steps involved in lignin biosynthesis are relatively well understood. However, little or no information is available on how the transcription of all genes involved in the pathway is coordinately regulated or how levels of individual metabolic intermediates may control flux through the pathway. There are several isoforms of the enzymes involved in the lignin biosynthesis

pathway. The use of whole transcriptome GeneChip analysis allows us to obtain a transcription phenotype of the pathway. For instance, among the 10 genes encoding 4CL, the gene At1g51680 is likely to encode an important isozyme for secondary xylem formation as it is highly up regulated, with high signal intensity, compared to other *4CL* genes. Other such examples include At1g15950 for *CCR*, At4g34050 for *CCoOMT*, At5g20230 for *COMT*, and At4g34230 for *CAD*.

PAL plays a key role in linking primary metabolism to phenylpropanoid metabolism by converting L-phenylalanine to *trans*-cinnamic acid. While the expression of all four PAL genes was up regulated in mature stems, *PAL4* (At3g10340, not characterized yet) had the highest increase in its expression (about 57-fold) with high signal intensity (22,889), suggesting that *PAL4* may play an important role in the phenylpropanoid pathway during secondary xylem formation. Franke *et al*. (2002) recently isolated and characterized the *Arabidopsis* gene (*REF8*) that encodes *p*-coumarate 3-hydroxylase (C3H). Although many previous reports have suggested that C3H is a phenolase, their study with *REF8* gene clearly demonstrated that the enzyme is actually a cytochrome P450-dependent monooxygenase. This gene (At2g40890) was expressed about three-fold higher in the mature stem, compared to the immature stem, with a signal intensity of 17,635. There are two homologous genes (At1g74540 and At1g74550) that are currently annotated as putative cytochrome P450. While At1g74540 was not expressed in the stem, it had very low signal intensity and underwent no significant change in expression level during xylogenesis. These results imply that *Arabidopsis* has only one *C3H* gene for lignin biosynthesis in secondary growth.

Peroxidases are widely believed to be responsible for the final condensation of cinnamyl alcohols to form lignin. However, the high redundancies in genes and their functions (low substrate specificity) are the main obstacles for the identification of a peroxidase isoform that is specifically involved in lignification (Christensen *et al*., 2001). Of the 87 peroxidase genes on the GeneChip, 48 were expressed in the stems. Among them, 10 genes were up regulated more than 3-fold in the mature stem. Pomar *et al*. (2002) reported that class III basic peroxidases were involved in lignin synthesis. Interestingly, one gene (At4g33420) classified as class III peroxidase had about a 25-fold expression increase in the mature stem (Supplement Table S1). Laccases are thought to polymerize monolignols. However, the precise role played by these enzymes is unclear. The lignin content of transgenic poplars down-regulated for laccase remained unchanged, but the quantity of soluble phenolics increased (Ranocha *et al*., 2000). This observation suggests that laccase may play a role in cell wall cross-linking through the oxidation of simple phenolics. Ten out of the 15 laccase or -like genes were expressed in the stems, three (At5g60020, At5g05390 and At2g29130) of which were upregulated in the mature stem (Supplemental Table S1).

Summary

Secondary growth is characterized by the massive production of secondary xylem and phloem cells from the vascular cambium. Its product, wood, is of primary importance to humans as timber for construction and wood-pulp for paper manufacturing. It also represents the most environmentally cost-effective renewable source of energy. Despite its scientific and economic significance, the molecular biology of secondary growth (i.e., wood formation) in tree species is surprisingly understudied. Here, we have examined the expression patterns of the genes involved in the sequential events of secondary growth (i.e., cell division, cell expansion, cell wall biosynthesis, lignification, and programmed cell death) and identified several key candidate genes for the genetic regulation of secondary growth. The transcriptome profiling revealed the transcription phenotypes that are characteristic to different stem developmental stages and confirmed various existing insights. Furthermore, we established the expression patterns of many previously unknown genes. The findings described in this report should add new information on the genetic regulation of secondary growth.

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