Transcriptome Profiling of Vertical Stem Segments Provides Insights into the Genetic Regulation of Secondary Growth in Hybrid Aspen Trees

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In order to better understand the genetic regulation of secondary growth in hybrid aspen (Populus tremula L.×P. alba L.), we carried out a series of cDNA-amplified fragment length polymorphism (AFLP)-based transcriptome analyses in vertical stem segments that represent a gradient of developmental stages with regard to secondary growth. This approach allowed us to screen >80% of the transcriptome expressed in six samples and identify genes differentially expressed with the progress of secondary growth, in a tissue-specific manner. Of the 76,800 transcript-derived fragments (TDFs) analyzed, 271 TDFs were selected and sequenced based on their differential expression patterns. Many of the xylem-up-regulated genes were involved in cell wall and lignin biosynthesis, while the bark-up-regulated genes had diverse functional roles. About 25% of the xylem-up-regulated TDFs analyzed were involved in the phenylpropanoid biosynthesis pathway, which produces the cell wall polymer lignin and various wood extractives. In addition, many of the TDFs showing secondary xylem-specific expression were annotated as genes not previously reported in Populus, including novel cell death proteins, cytoskeleton-interacting proteins, transporters and putative transcription factors.

Keywords: cDNA-AFLP — Populus — Secondary growth — Stem — Wood formation.

Abbreviations: AFLP, amplified fragment length polymorphism; HD-Zip, homeo-box domain leucine zipper; L, leaf sample; PCD, programmed cell death; PG, primary growth sample; SAM, S-adenosyl-L-methionine; SG, secondary growth sample; SXF, secondary xylem-forming; TDF, transcript-derived fragment; TS, transition zone sample; X, xylem sample.

The nucleotide sequences reported in this paper have been submitted to GenBank under accession numbers CF216193–CF216471.

Introduction

An important characteristic of typical trees is that they can undergo considerable secondary growth and expand their stem girth each growing season. Secondary growth (i.e. wood formation) is achieved by massive production of secondary xylem and phloem cells originating from the vascular cambium. The secondary tissues provide necessary mechanical support and a conduit for the long-distance transport of water and nutrients, allowing trees to grow tall and eventually out-compete other herbaceous vegetation for light and nutrient uptake. In addition, secondary xylem, commonly referred to as wood, is of primary importance to humans as timber for construction, wood pulp for paper manufacturing, and firewood. It is the most environmentally cost-effective renewable source of energy. The increasing global demand of forest utilization necessitates gaining a fundamental understanding of the biochemical processes involved in secondary growth.

The transition from primary to secondary growth is apparent in expanding shoots of trees, in which the apical portion is soft and green, while the lower portion becomes stiff and woody. This is the result of cell wall thickening and lignification of secondary xylem cells located centripetally to the vascular cambium. In the primary tissues, the conducting elements are clustered into vascular bundles derived from the procambium. Primary xylem cells can be characterized as protoxylem and metaxylem according to the time of differentiation (Dickison 2000). Primary phloem consists of sieve tubes that form only primary cell walls. As the stem matures, procambium gives rise to the fascicular cambium, and interfascicular cambium starts to form between the vascular bundles. Together, these cambia form a continuous cylinder that is called ‘vascular cambium’. The vascular cambium increases the stem diameter by periclinal divisions and the circumference by anticlinal divisions, resulting in the developmental continuum of secondary phloem and xylem (called ‘secondary growth’) (Chaffey 1999). In angiosperm trees including Populus species, secondary xylem consists of fibers, axial parenchyma, vessel elements, and ray cells that form thick cell walls consisting of three successive layers ‘S1’, ‘S2’ and ‘S3’, with the exception of ray cells which develop a protective layer (Mellerowicz et al. 2001, Chaffey et al. 2002). Secondary phloem consists of sieve elements and phloem parenchyma that form only primary cell wall, and phloem fibers that form thick secondary cell walls.

Currently, most of the published research concerning secondary xylem formation is based on sequencing analysis of cDNA clones derived from wood-forming tissues (Allona et al. 1998, Sterky et al. 1998, Lorenz and Dean 2002, Kirst et al.

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in the identification of the genes differentially expressed during wood formation in pine (Le Provost et al. 2003).

Although there were extensive efforts in unraveling the genetic regulation of wood formation (Allona et al. 1998, Sterky et al. 1998, Hertzberg et al. 2001, Lorenz and Dean 2002), the molecular events involved in the transition from primary to secondary growth are largely unknown. Vertical segments of a tree stem provide an experimental system for such study as they represent different developmental stages such as primary growth (top), transition from primary growth to secondary growth, and secondary growth (bottom). In the current study, we used cDNA-AFLP analysis to obtain the first view of transcription phenotypes from a gradient of vertical stem segments in different developmental stages with regard to secondary growth and to identify secondary xylem- and bark-up-regulated TDFs, including several novel genes.

Results and Discussion

Vertical stem segments represent different developmental stages

To study the changes occurring in the transcriptome during secondary growth, we used three different stem segments that represent discrete developmental stages with regard to secondary growth (Fig. 1). The first sample (PG, primary growth stage) was an approximately 1 cm segment directly below the apical meristem (Fig. 1B, F); where vascular bundles are formed from procambial cells and consist of primary xylem and phloem tissues. The second sample (TS, transition stage) was from the region between the leaf plastochron index (LPI) 4 and 6, where the secondary vascular cambium has emerged and started producing secondary tissues (Fig. 1C, G). The third sample (SG, secondary growth stage) was a stem segment in which secondary xylem and phloem fibers are already heavily lignified and the amount of secondary xylem increases rapidly (Fig. 1D, H). To discriminate between bark and xylem expressed genes, we further sampled bark, xylem and mature leaves. The basal part of the stem includes bark and highly expanded secondary xylem that can easily separate from each other at the soft active cambial region. Consequently, sample four (B; bark) was the pealed bark, and sample five (X; xylem) was the remaining xylem and pith (Fig. 1E, I). The anatomy of the lower segment is largely similar to that of SG. The only difference is that more xylem cells with thicker cell walls and more lignin deposition are present in the lower stem (Fig. 1E, I). Figure 11 shows that the cambial region is attached mainly to the bark, rather than the xylem. Only the newly produced xylem mother cells that are not lignified remain on the periphery of the xylem disk. Finally, mature leaves (L) were used as an indicator for the genes that are not involved in processes of secondary growth. It should be noted that the major leaf veins belonging to classes I and II were removed as sources of genes involved in vascular development. These six samples gave us a unique opportunity to analyze the process of secondary growth in a tissue-specific manner.

Fig. 1 Sectioning of 1-month-old hybrid aspen trees for sample collection. (A) Samples were collected at four different heights named PG, TS, SG, bark and xylem. The bark and xylem samples were obtained by peeling off the bark from the stem (detail in E). The remaining part was the xylem with the pith. (B, C, D and E) Handmade transverse sections of the stem that are representative of each sample. The blue lines depict the point of sectioning. (F) The vascular bundles of the upper stem. (G) The newly formed secondary xylem consists of cells with thick and lignified cell walls but phloem remains primary walled. Phloem fibers are still developing without fully lignified cell walls. (H) Advanced and massive xylem formation. Phloem remains at the same cell number as in (G). Phloem fibers are heavily lignified with thick cell walls, but their number does not increase. (I) Detail of the newly formed xylem (left) and bark (right) samples. Observe that the cambial layer is carried on the bark sample. The pink cells in the edge of the xylem sample are differentiating secondary xylem cells. Scale bars: (B–E) 0.5 mm, (F–H) 100 µm and (I) 75 µm.

2003, Yang et al. 2003), separation of proteins isolated from wood tissues (Costa et al. 1999, Vander-Mijnsbrugge et al. 2000) and cDNA microarray analysis (Hertzberg et al. 2001, Whetten et al. 2001, Demura et al. 2002, Yang et al. 2003, Yang et al. 2004). While these approaches provided very useful information on wood formation-associated gene expression, the coverage of the transcriptome is limited by the number of cDNA clones sequenced for expressed sequence tag (EST) analysis or printed on the microarrays. Furthermore, rare transcripts are often not represented in those analyses. cDNA-AFLP (amplified fragment length polymorphism) provides an alternative for gene expression analysis with a more sensitive and broader coverage of the transcriptome in any particular tissue sample (Bachem et al. 1996, Ko et al. 2003). In contrast to most hybridization-based techniques, cDNA-AFLP allows for the discrimination of homologous genes without the need for prior sequence knowledge. This method has been successfully used in the study of the mechanisms underlying transdifferentiation of Zinnia mesophyll cells (Milioni et al. 2002) as well as
Whole transcriptome analysis during the initiation of secondary growth.

The transcriptome of the six different samples was analyzed by employing the cDNA-AFLP technique, which generated an estimated 76,800 transcript-derived fragments (TDFs). Based on the analysis of publicly available full-length aspen gene sequences (see Materials and Methods) and other plant sequences (Ko et al. 2003), the enzyme pair is estimated to cover approximately 80% of the whole transcriptome. The produced gel images were visually inspected for differences in the intensity of each TDF between the six samples. Several distinct expression patterns could be observed (Fig. 2A), revealing high complexity of the transcriptome among the six different samples. Selection of bark or xylem expressed genes was carried out by comparing samples B, X and L. TDFs expressed only in the bark (B) and/or xylem (X) were selected for further evaluation (Fig. 2B, 1–11). The majority of tissue-specific TDFs were observed in the xylem (patterns 5–7 in Fig. 2B), reflecting the unique biology of this tissue. Also, many TDFs were expressed in both bark and leaves (Fig. 2B, 14–15), probably due to the fact that bark at this age is still green and has photosynthetic activity. The term ‘bark-’ or ‘xylem-specific’ expression will be used from this point on for TDFs that are abundantly or exclusively expressed in the bark or xylem, respectively. Using the TDFs that were previously selected as bark- or xylem-specific, we extended the comparison to the other three samples (PG, TS and SG) in order to determine if their expression was initiated during primary or secondary growth. For example, pattern 1 in Figure 2B shows a TDF expressed in bark tissue during secondary growth, while pattern 3 indicates that this TDF was expressed in bark during primary growth and continued to be expressed during secondary growth. Northern blot analysis was used to confirm the cDNA-AFLP expression pattern of representative TDFs (Fig. 3).

A total of 271 TDFs were isolated, of which 180 were xylem specific, 70 were bark specific and 21 were L or PG up-regulated. The last were selected as markers of green tissue metabolism (Fig. 2B, 13–15), together with some constitutively expressed TDFs (Fig. 2B, 16). Twenty-two TDFs were similar to unknown or putative proteins and six did not have any significant similarity. The average size of the ‘no-hit’ sequences was 186 bp. None of the ‘no-hit’ sequences aligned to any predicted or non-predicted open reading frame on the *P. trichocarpa* genome, but forms a hairpin structure (data not shown).

Homologs of the TDFs were also found in the PopulusDB database (http://poppel.fysbot.umu.se/), which contains 102,019 *Populus* ESTs from cDNA libraries of 18 different tissues and treatments. This comparison allowed us to monitor the...
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### Bark-upregulated genes

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**Fig. 4** Bark-upregulated genes. ID: TDF identification number; PtC: Annotated through a query with a clone from the poplar draft genome sequence; PtG: Annotated through a query with a gene from the poplar genome assembly. 1: PG sample, 2: TS sample, 3: SG sample, 4: Bark sample, 5: Xylem sample, 6: Leaf sample, Cam: Cambium, C: Leaf, F: Flower, I: Senescence, K: Apical shoot, M: Mature female flowers, N: Bark, P: Petioles, Q: Dormant bud, R: Root, S: Imbibed seed, T: Apical shoot meristem, UK: Apical shoot, UL: Cold stressed leaves, UM: Mature female flowers, UR: Root, VM: Mature male flowers, Y: Pathogen infected leaf.
**Genetic regulation of secondary growth**

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**Fig. 5** Xylem-upregulated genes. ID: TDF identification number; PtC: Annotated through a query with a clone from the poplar draft genome sequence; PtG: Annotated through a query with a gene from the poplar genome assembly. 1: PG sample, 2: TS sample, 3: SG sample, 4: Bark sample, 5: Xylem sample, 6: Leaf sample, Cam: Cambium, C: Leaf, F: Flower, I: Senescence, K: Apical shoot, M: Mature female flowers, N: Bark, P: Petioles, Q: Dormant bud, R: Root, S: Imbibed seed, T: Apical shoot meristem, UK: Apical shoot, UL: Cold stressed leaves, UM: Mature female flowers, UR: Root, V: Mature male flowers, Y: Pathogen infected leaf.
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expression of the identified genes in other tissues as well. Results from BlastN searches are presented in Fig. 4 and 5, where EST matches were divided based upon expression in cambium, wood or other libraries. Most of the xylem-specific TDFs had an EST homolog in the cambium or wood-forming libraries. A significant number of bark-specific TDFs matched to cambium ESTs, among other tissues. It is well known that EST libraries have the tendency to over-represent the more abundant genes and miss the low expressed genes. Many of the newly identified genes in this study are represented by less abundant genes and miss the low expressed genes. Many of the EST libraries have the tendency to over-represent the more abundant genes and miss the low expressed genes. Many of the newly identified genes in this study are represented by less abundant genes and miss the low expressed genes.

Of the 70 TDFs that were specifically expressed in the bark, 62 had similarity to a known protein, five were similar to a hypothetical protein and three had no significant similarity (Fig. 4). Even though fewer bark-specific TDFs were recovered in comparison with xylem, they exhibited more diverse expression patterns. This may be due to the fact that bark contains various cell types such as phloem fibers, sieve elements, companion cells, epidermal cells, epidermal cortex and cambium cells.

Gene ontological view of the tissue-specific TDFs

One of the most important cellular processes leading to secondary growth is the secondary cell wall biosynthesis. In the present study, we identified seven TDFs belonging to three known Populus cellulose synthase genes PtCesA1, PtCesA2 and PtCesA3 (Fig. 5) (Wu et al. 2000, Samuga and Joshi 2002, Joshi 2003). All of the TDFs were specifically expressed in secondary xylem-forming (SXF) tissues (namely TS, SG and X; Fig. 5). These genes are orthologous to the three Arabidopsis cellulose synthases AtCesA8, AtCesA7 and AtCesA4, which are believed to form a hexagonal rosette complex involved in secondary wall biosynthesis (Gardiner et al. 2003, Taylor et al. 2003). The P. trichocarpa genome contains two copies of PtCesA1 and PtCesA2 with few amino acid changes. Each of the four identified TDFs (Pt1104, Pt1063, Pt1009 and Pt1004) aligned to one of the four predicted P. trichocarpa genes (supplementary Fig. 5). Sucrose synthase is also believed to be part of the cellulose synthesis complex catalyzing sucrose into UDP-glucose and fructose, the first of which is further used for synthesis of cellulose (Haigler et al. 2001, Doblin et al. 2002). One sucrose synthase gene (Pt1162) showed SXF tissue-specific expression and may participate in cellulose synthesis.

Several genes that belong to the glycosyltransferase class of carbohydrate active enzymes were identified as xylem specific (Fig. 5). Pt1040 is an ortholog of the PARVUS gene and Pt1064 is a homolog of the QUASIMODO1 gene, both of which belong to the family 8 of glycosyltransferases. Mutations in both Arabidopsis genes cause defects in pectin biosynthesis (Bouton et al. 2002, Lao et al. 2003). The TDF Pt1082 belongs to a family 2 glycosyltransferase that is similar to a bacterial cellulose synthase, but shares no homology to other plant cellulose synthases. The SXF tissue-specific expression of this TDF, however, resembles that of cellulose synthases and does not exclude a possible interaction with the cellulose synthase complex. Three glycoconjugate glycosyltransferase-like TDFs (Pt1079, Pt1110 and Pt1003) showed a gradual activation during shoot growth, with Pt1079 being expressed early in PG and Pt1003 being expressed late in X (Fig. 5). Other genes involved in hemicellulose or pectin biosynthesis included a UDP-glucose dehydrogenase (Pt1125) that was expressed in the SXF tissues and in the bark, a UDP-glucuronate decarboxylase gene (Pt1305) that was present in SXF tissues, and a pectin methyl esterase (Pt1076) that was expressed only in sample X.

Arabinogalactan proteins (AGPs) are a large family of highly glycosylated cell wall-localized proteins (Gaspar et al. 2001), with various roles in plant development such as vascular development, fertilization, somatic embryogenesis, cell division, cell adhesion, cell expansion and programmed cell death (PCD) (Majewska-Sawka and Nothnagel 2000, Johnson...
et al. 2003, Zhang et al. 2003, Motose et al. 2004). We identified five TDFs (Pt1048, Pt1062, Pt1080, Pt1082 and Pt1093) that were similar to cell wall AGPs and were present in SXF tissues. However, none of the TDFs share a similar expression pattern, suggesting a role for each of these genes in different stages of cell wall formation.

Twenty-five percent (45) of the 180 xylem-specific TDFs were linked to the phenylpropanoid biosynthesis pathway, which leads to the production of the cell wall polymer lignin as well as many wood extractives such as flavonoids, isoflavonoids and hydroxycinnamic acid derivatives. Fig. 6 summarizes the expression of the TDFs in the phenylpropanoid pathway. Cinnamoyl-CoA reductase (CCR; Pt1186, Pt1018), a late enzyme in the pathway, was expressed in all tissues except L (Fig. 6). This finding suggests that the same enzyme is involved in other pathways as well. Cinnamyl alcohol dehydrogenase (CAD), sinapyl alcohol dehydrogenase (SAD) and p-coumarate-3-hydroxylase (C3H) were not detected in this screening due to either absence of the restriction sites, low signal intensity or failure to fulfill the selection criteria on tissue-specific expression.

S-adenosyl methionine (SAM) is the methyl donor for caffeic-acid-O-methyltransferase (COMT) and caffeoyl-CoA-3-O-methyltransferase (CCoAOMT) in the phenylpropanoid pathway (Meng and Campbell 1998). Fig. 7 shows the SAM biosynthesis pathway (Vander-Mijnsbrugge et al. 2000) and the expression patterns of the genes involved. Interestingly, the expression patterns of those genes seem to be categorized into two groups, the first one being genes that are expressed at the same levels in both primary and secondary xylem tissues and in sample X (Fig. 7a), and the second, genes that are expressed at low levels in primary tissues (PG), highly up-regulated in TS and reaching a steady state in SG and X (Fig. 7b).

Lignin polymerization is catalyzed by peroxidases, laccases, phenol oxidases and coniferyl alcohol oxidase (Boerjan et al. 2003). In this study, we have identified two peroxidase genes (Pt1294 and Pt1042) and one laccase (Pt1024) gene that were expressed in SXF tissues (Fig. 6). The function of peroxidases depends on the presence of hydrogen peroxide, which is produced by the action of oxidases such as oxalate oxidases (Boerjan et al. 2003). A germin-like oxalate oxidase gene (Pt1037) was found to have high expression in SXF tissues that coincides with at least one of the peroxidases (Fig. 6).

Lignan biosynthesis is an alternative pathway to lignin biosynthesis that also makes use of monilignols produced by the phenylpropanoid pathway. Three TDFs were similar to previously identified lignan biosynthesis genes, two of which encode a phenylcoumaran benzyl ether reductase (Pt1101 and Pt1181) and one encoding an isoflavone reductase (Pt1144). The expression patterns of those three TDFs were similar to that of phenylpropanoid biosynthesis-related TDFs (Fig. 6).

Several transcription factor genes were differentially expressed, including MYB-, LIM-and HD-Zip domain-containing proteins. Some of the MYB transcription factors and LIM-domain containing proteins are known to regulate phenylpropanoid pathway genes in Arabidopsis and tobacco (Tamagnone et al. 1998, Borevitz et al. 2000, Kawaoka et al. 2000). Three MYB-like genes were expressed specifically in secondary xylem, two (Pt1027 and Pt1008) of which were similar in sequence to MIB48 and MYB39 of Arabidopsis, where they are expressed in woody stems (Oh et al. 2003). Blast search against the P. trichocarpa genome assembly suggests that these two TDFs may belong to the same gene. Two other TDFs similar to LIM domain-containing proteins had very low levels of expression in PG but high levels in SG, X, which is in agreement with their role in regulation of phenylpropanoid pathway genes (Kawaoka et al. 2000). Furthermore, two HD-Zip-like TDFs were present in PG and SXF samples but not in B and L. These two TDFs were similar to AtHIB15 of Arabidopsis, which is involved in procambial and xylem cell development (Ohashimoto and Fukuda 2003). Other genes that were specifically expressed in SXF tissues resembled one basic helix–loop–helix (Pt1333, bHLH) and an AP2 domain-containing protein (Pt1172; Okamura et al. 1997), both of which are responsive to ethylene. Ethylene has been implicated in secondary xylem development at the level of ethylene biosynthetic genes.
Peptides

Protein kinases play a very significant role in signal transduction and protein activation/deactivation. Our screening revealed that two receptor kinase-coding genes (Pt1153 and Pt1176) were up-regulated in SXF tissues. Two other TDFs were annotated as mitogen-activated protein (MAP) kinases, one (Pt1069) of which was specifically expressed in SXF tissues while the other (Pt1075) was expressed in both B and X. The Arabidopsis homolog of these genes belongs to the MAPKKK family that is involved in the relay of defense responses (Zhang and Klessig 2001, Jonak et al. 2002). Also, a gene (Pt1039) homologous to a calcium-binding serine/threonine protein kinase was highly expressed in SXF tissues. Interestingly, similar protein kinases were also up-regulated during Zinnia mesophyll cell transdifferentiation to xylem cells (Demura et al. 2002).

Brassinosteroids have been shown to affect the expression of vascular development regulatory genes such as ZehB13, a Zinnia homolog of the Arabidopsis AtHB15, and other HD-ZipIII family genes (Ohashi-Ito et al. 2002, Ohashi-Ito and Fukuda 2003). A cytochrome p450–90A1-like TDF (Pt1070) was specifically expressed in the SXF tissues. In Arabidopsis, p450–90A1 is involved in brassinosteroid biosynthesis (Bancos et al. 2002).

Serine carboxypeptidases are proteins that have been implicated in the metabolism of defense-related compounds through the phenylpropanoid pathway (Shirley et al. 2001) and also in PCD during the formation of the vascular system (Dominguez et al. 2002). Six TDFs encoding serine carboxypeptidases exhibited secondary xylem-specific expression patterns (Fig. 5). Their lack of similarity to the serine carboxypeptidase-like (SCPL) acyltransferases favors a role in protein hydrolysis (Milkowski and Strack 2004). Prolyl-carboxypeptidases, in contrast to other proteases, recognize and cleave small peptides not more than 30 residues in size (Polgár 2002). One TDF (Pt1119) showed sequence homology to a prolyl-carboxypeptidase gene from Arabidopsis, which is a member of the serine carboxypeptidase clan (Rawlings and Barnett 1994). This gene was expressed throughout the stem, but at notably high levels in the xylem and low levels in PG. Three subtilisin-like serine protease TDFs were present in SXF tissues, and at especially high levels in X. Several other xylem-up-regulated TDFs were similar to defense-related genes such as Early Response to Dehydration3 (ERD3) which carries a methyltransferase domain, NtPRp27, anthranilate N-hydroxycinnamoyl-benzoyltransferase, berberine bridge enzyme, thiamatin-like, a chitinase gene and a cytochrome P450 72A1 (Fig. 5).

Inter- and intracellular transport of molecules is important for cell maintenance as well as signaling purposes. Three genes (Pt1002, Pt1073 and Pt1111) expressed in SXF tissues were similar to proton-dependent oligopeptide transporters (POTs) from Arabidopsis. These transporters recognize and transport di- and tripeptides through membranes by the use of proton motive force (Stacey et al. 2002). It is not yet determined what specific role each transporter represents, but it has been suggested that they may be involved in the transport of amino acids, peptides and nitrate (Stacey et al. 2002). It will be interesting to see if the POTs serve as transporters of oligopeptides produced by the numerous proteases mentioned above and if these peptides serve as signaling molecules for the development of the vascular system or as templates for further catabolism. Another SXF tissue-specific gene, represented by three TDFs (Pt1067, Pt1329 and Pt1179), showed similarity to a membrane-localized sugar transporter from Arabidopsis that has a signal peptide for secretory pathway localization (ARAMEMNON database; http://aramemnon.botanik.uni-koeln.de/index.html). Sugar transport is necessary for the supply of carbon backbones to organelles of the secretory pathway, involved in the synthesis of cell wall components.

Formation and concomitant repression of lateral shoot meristems is important for tree architecture. The stem segment samples used in this experiment included both nodes and internodes, which favored the retrieval of genes related to lateral organ formation. Five TDFs expressed in SXF tissues were similar to proteins that are involved in lateral organ formation. Pt1116, Pt1159 and Pt1171 were similar to three genes (AIR3 and two AIR12-like) involved in auxin-induced lateral root formation (Neuteboom et al. 1999a). AIR3 is a serine protease that promotes lateral root formation (Neuteboom et al. 1999b), but a role for AIR12 has not been determined. Pt1072 is an ortholog of MAX4 from Arabidopsis and RAMOSUS1 from pea, both of which negatively regulate shoot branching through long-distance signaling from the root to the shoot (Sorefan et al. 2003, Foo et al. 2005). The SXF tissue-specific expression of this gene suggests a tight control of shoot branching at the onset of secondary growth. Finally, Pt1140 is an ortholog of the Arabidopsis gene LATERAL ORGAN BOUNDARIES (LOB)-domain 37 (LBD37) with unknown function, and was also expressed in SXF tissues. LOB, a homolog of LBD37, is specifically expressed at the base of lateral organs (Shuai et al. 2002).

The cytoskeleton contributes significantly to the formation of the cell wall through both the microtubule and actin networks. Two genes (Pt1120 and Pt1130) homologous to WAVE-DAMPEND2 (WVD2)-LIKE of Arabidopsis exhibited SXF tissue-specific expression. WVD2 mutants show defects in expansion and rotational polarity of plant organs probably due to changes in microtubule orientation, which may further lead to changes in cellulose microfibril orientation (Yuen et al. 2003). Another gene (Pt1132), similar to the Arabidopsis ARP3-like gene that is a member of the ARP2/3 complex, was expressed in SXF tissues and PG. ARP2/3 complex is responsible for actin polymerization, and mutations in ARP2/3 genes lead to the alteration of cell shape and reduction in Golgi streaming in Arabidopsis plants (Mathur et al. 2003, El-Assal et al. 2004).
Of the TDFs similar to hypothetical proteins, Pt1102 and Pt1043 were most interesting, due to their high up-regulation in SXF tissues (Fig. 5). Even though Pt1043 is present in the *P. trichocarpa* genome, it has no homology to *Arabidopsis* or any other sequenced genes. Hypothetical proteins identified in this study, which exhibit clear SXF tissue-specific expression, need further analysis for identification of their role in secondary growth.

In conclusion, the cDNA-AFLP analysis was successfully used to document the flux in poplar stem transcriptome which occurs during the transition from primary to secondary growth and the maturation of secondary tissues. While the results largely confirm the existing insights, the current study provides several novel observations: (i) it represents one of the most extensive transcriptome analyses (>76,000 TDFs) with regard to secondary growth; (ii) we obtained transcription phenotypes from a gradient of vertical stem segments, which identify stem developmental stage- and tissue-specific genes; (iii) the majority of tissue-specific TDFs were obtained from secondary xylem, reflecting the unique biology of this understudied tissue; secondary xylem-specific TDFs includes genes encoding peptide transporters, cytoskeleton-interacting proteins, regulators of lateral organ formation, and a multitude of cell death- and defense-related proteins; and (iv) the results provide insights into several biological processes that have not been studied previously in terms of secondary growth (e.g. cellular transport, ethylene-responsive genes and lateral organ formation). The functional characterization of the genes identified in the present study will contribute to our understanding of the molecular mechanisms underlying secondary growth.

**Materials and Methods**

**Plant material**

The plants that were used for the cDNA-AFLP belong to the aspen *P. tremula* L.*×* *P. alba* L. INRA clone 717 hybrid. These plants were clonally propagated through tissue culture as described in Han et al. (2000). Plants were then potted in commercial soil and grown under aspen. The plants were then potted in commercial soil and grown under a Nikon Diaphot, inverse microscope. Pictures were taken with a blue, bright blue or white, and most primary (non-lignified) walls stain pectin/pectic substances pink to purple. Lignified walls stain greenish-blue, bright blue or white, and most primary (non-lignified) walls stain pinkish-purple (e.g. parenchyma). Stained specimens were observed under a Nikon Diaphot, inverse microscope. Pictures were taken with a Sony MAVICA digital camera.

**RNA extraction**

A shorter protocol of that of Wang et al. (2000) was used. Tissue was ground to a fine powder and 5 vols of extraction buffer were added followed by vortexing. The mixture was frozen at −80°C for 2 h and then placed at 37°C until just thawed, followed by centrifugation at 5,000 × g at 4°C. Supernatant was filtered through a layer of KimWipes and then 1/30 vol. of 3 M NaoAc pH 5.2 and ethanol to a final concentration of 10% (v/v). The mixture was placed on ice for 10 min and centrifuged as before. The supernatant was obtained and mixed with 1/9 vol. of 3 M NaoAc pH 5.2 and isopropanol to a final concentration of 33% (v/v). The mixture was placed at −20°C for 2 h and then centrifuged as before. The pellet was resuspended in TE pH 8.0 and placed on ice for 30 min followed by centrifugation as before. The supernatant was mixed with 1/4 vol. of 10 M LiCl and RNA was precipitated at 4°C overnight. Then it was centrifuged at 10,000 × g for 30 min followed by washing of the pellet with 70% ethanol. Pellets were dried and resuspended in diethylpyrocarbonate (DEPC)-treated water. Total RNA concentration was measured at OD260/280 using a SmartSpec spectrophotometer (BioRad, CA, USA).

**cDNA-AFLP procedure**

The cDNA-AFLP protocol was adapted from Ko et al. (2003) with slight modifications. In order to select the restriction enzyme pair on which the analysis would be based, we screened 57 complete coding sequences of aspen that were available in the GenBank database. Eighty-six percent of the sequences had target sites for the restriction enzyme pair *Apol/Msel*, which can produce restriction fragment sizes (ranging from 50 to 1,000 bp) suitable for separation within a single run of PAGE. Based on this estimation, we assumed that TDFs generated by using the *Apol/Msel* primer pair could cover at least 80% of the transcriptome. The gels were marked with fluorescent tags and exposed on a Kodak X-Omat film for 20–24 h. After developing the film, we counted the number of fragments in randomly selected lanes for different primer combinations, which resulted in an average of 100 fragments per lane. Multiplication with 128 primer combinations and six samples results in approximately 76,800 TDFs that were screened for differential expression.

**Sequence analysis**

The gel was aligned to the film and fragments were excised by cutting both film and gel using a scalpel (No. 11). The fragments were soaked in 50 µl of distilled water overnight at 37°C to elute the DNA. A 5 µl aliquot was used for re-amplification of the fragments in a 50 µl reaction using the same primers as the pre-amplification. The following program was used: 30 s at 94°C, 30 s at 60°C and 1 min at 72°C for 40 cycles. Products were analyzed on a 1.3% agarose gel and the double or smeared bands were excluded from further processing. The remaining bands were purified by ethanol precipitation with 10% 3 M NaoAc pH 5.2, overnight at −20°C and resuspended in distilled water. The *Apol* pre-primer was used for sequencing the bands in an ABI 7700 sequencer (Genomics Technology Support Facility, Michigan State University, East Lansing MI, USA). Fragments that did not yield high quality sequences were cloned in the pGEM-T Easy vector system (Promega, WI, USA) and sequenced using the M13F universal primer.

A total of 271 TDFs were selected and sequenced. The resulting sequences (with average TDF size of 214 bp) were aligned against the GenBank databases using the BLASTN and BLASTX algorithms. All the TDFs were also aligned to the Joint Genome Institute (JGI) *Populus trichocarpa* Genome Assembly v1.0 (7.5-fold draft coverage as of December 2003) using the BLASTN search engine provided on the JGI web site (http://genome.jgi-psf.org/cgi-bin/runAlignment?db=Poptr1). A cut-off E-value >10−30 and identity >90% was used as the selection criteria. Predicted *P. trichocarpa* genes that corresponded to the best
hit genomic region were then used to perform a BLASTP search in the NCBI database. The sequences that were still annotated as unknown proteins or did not have any significant match were aligned to the JGI *P. trichocarpa* genomic clone database (6-fold draft coverage as of August 2003) using the BLASTN search engine provided on the JGI web site (http://genome.jgi-psf.org/poplarb/poplar0.home.html). For each TDF, two to three genomic sequences with the highest similarity were selected (cut-off E-value >10^-25). For sequences smaller than 100 bp, an E-value cut-off of 10^-10 was used, since even at this stringency level the first hits were almost identical. The selected genome sequences were then used to search the GenBank protein database using the BlastX algorithm.

**Northern blot analysis**

Total RNA was extracted as above. A 20 μg aliquot of total RNA for each sample was analyzed on a 1% agarose gel containing formaldehyde and then transferred on a nylon membrane (Hybond N+, Amersham). The membranes were then washed (according to the manufacturer) and exposed to an X-ray film (Kodak, CT, USA).

**Supplementary material**

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oupjournals.org.

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