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ORIGINAL ARTICLE

EST analysis of functional genes associated with cell wall biosynthesis and modification in the secondary xylem of the yellow poplar (*Liriodendron tulipifera*) stem during early stage of tension wood formation

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Abstract A cDNA library was constructed from secondary xylem in the stem of a 2-year-old yellow poplar after being bent for 6 h with a 45° configuration to isolate genes related to cell wall modification during the early stages of tension wood formation. A total of 6,141 ESTs were sequenced to generate a database of 5,982 high-quality expressed sequence tags (ESTs). These sequences were clustered into 1,733 unigenes, including 822 contigs and 911 singletons. Homologs of the genes regulate many aspects of secondary xylem development, including those for primary and secondary metabolism, plant growth hormones, transcription factors, cell wall biosynthesis and modification, and stress responses. Although there were only 1,733 annotated ESTs (28.9%), the annotated ESTs obtained in this study provided sequences for a broad array of transcripts expressed in the stem upon mechanical bending, and the majority of them were the first representatives of their respective gene families in *Liriodendron tulipifera*. In the case of lignin, xylem-specific COMTs were identified and their expressions were significantly downregulated in the tension wood-forming tissues. Additionally, the majority of the auxin- and BR-related genes were downregulated significantly in response to mechanical bending treatment. Despite the small number of ESTs sequenced in this study, many genes that are relevant to cell wall biosynthesis and modification have been isolated. Expression analysis of selected genes allow us to identify the regulatory genes that may perform essential functions during the early stages of tension wood formation and associated cell wall modification.

Keywords *Liriodendron tulipifera* · Tension wood · EST · Lignin · Cell wall

H. Jin and J. Do contributed equally to the study.

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Introduction

Wood is one of the major renewable biomaterials, and consists mainly of cellulose, hemicelluloses and lignin. As wood is valuable in building construction, pulp and paper industries, it has more recently become valuable in the field of environmental-friendly biofuels, it has been receiving more attention, compensating for increasing demand for high-quality wood biomass with strong environmental resistance. However, it might be possible to use biotechnology to alleviate the pressures on native forest land. With this objective in mind, a great deal of progress has been made toward understanding the biosynthesis and

modifications of wood biomass (i.e., mainly secondary xylem), and these efforts have generally involved the extensive isolation and functional characterization of genes from economically important tree species (Allona et al. 1998; Whetten et al. 2001; Paux et al. 2004; Ranjan et al. 2004; Sterky et al. 2004; Paux et al. 2005; Andersson-Gunneråas et al. 2006). In case of *Populus trichocarpa* (black cottonwood), full genome sequencing had been recently completed (Tuskan et al. 2006) and a variety of molecular resources including EST (Sterky et al. 2004), proteomics (Plomion et al. 2006) and transcriptome analysis (Sjödin et al. 2006) as well as T-DNA insertional activation tagging mutagenesis (Busov et al. 2003) have been successfully employed.

Secondary xylem, also called wood, is heterogeneous in terms of cell types. It consists primarily of tracheary elements, such as the tracheid in gymnosperms and vessels in angiosperms, as well as a relatively small amount of parenchyma cells (typically less than 10%), the numbers and arrangement of which vary among species. For example, the secondary xylem contains fibers and often fiber tracheids that develop thick cell walls with smaller cell lumens as compared to vessel elements; this provides structural support. Although vessels and fibers are functionally specialized for different purposes, both cell types undergo a similar developmental process during the formation of secondary xylem, i.e., programmed cell death, after secondary cell assembly and lignifications (Fukuda 1997). The cell wall lignification stage enables the plants to achieve a variety of important functions for the secondary xylem; the hydrophobic lignin macromolecules prevent significant water loss during long-distance water conduction, and the deposition of lignin into lignifying cells (vessel and fiber in angiosperm) provides mechanical support, thereby enabling woody plants to stand upright through, at least in part, their covalent and non-covalent interactions with major cell wall components such as cellulose microfibrils and hemicelluloses (Kwon 2000). The biosynthesis of these three principal cell wall components of wood was shown to be affected not only by endogenous stimuli, but also by external environmental stimuli, but mostly by the combined activities of endogenous and external stimuli. For example, branches and stems displaced as the result of wind or mechanical stress in angiosperm woody species results in the formation of a specialized tissue referred to as tension wood (Cronshaw and Morey 1965; Norberg and Meier 1966). Tension wood develops on the upper sides of leaning stems and drooping branches, where tensile stress is exerted (Scurfield 1973; Hellgren et al. 2004). Tension wood is characterized by eccentric growth promotion and modified cell wall structure with altered chemical composition of the secondary cell wall. The chemical modification associated with tension wood formation includes increased cellulose content with higher cellulose crystallinity (Okuyama et al. 1990), reduced lignin content with alterations in monolignol composition (Terashima et al. 1998; Joseleau et al. 2004), and changes in hemicelluloses composition (Kwon 2000) relative to those of opposite wood and normal wood. Although the existence of tension wood has been regarded as a detrimental property in wood, particularly the structural and chemical modifications occurring during tension wood formation in angiosperms, it is also regarded as a good model system to uncover the mechanism underlying cell wall biogenesis and modification during secondary xylem development (Kwon 2008). Additionally, it is also regarded as a good genetic pool for the isolation of essential regulators controlling the cellulose and lignin biosynthesis in the secondary xylem.

Liriodendron tulipifera, L. is a member of the Magnoliaceae family in the order Magnoliales, which is one of the early branching angiosperm lineages referred to as "basal angiosperm" (Liang et al. 2007), with many common names such as tulip tree, tulip poplar, yellow poplar, white poplar and whitewood (Liang et al. 2007). As it is a quickly growing tree species with relatively high stress resistance to both abiotic and biotic stresses, it is regarded as a future renewable bio-material not only for use in the bioenergy industry, but also as a useful substitute for petroleum products (Liang et al. 2007; Kwon 2008; Cho et al. 2010). In addition to its future potential as a biomaterial, it is very important for comparative studies of biological evolutionary processes in land plants, since the yellow poplar is phylogenetically critical among the angiosperms and due to possible biotechnological applications in its genetic improvement. Additionally, the transformation system and ability to produce clonal trees with somatic embryos make this an excellent tree species model (Wilde et al. 1992; Rugh et al. 1998). However, there is currently very little available information regarding functional genes and gene structure. For example, the public NCBI database (http://www.ncbi.nlm.nih.gov/) provides mostly partial cDNA sequences with four full-length cDNAs encoding for laccases related to cell wall lignification (LaFayette et al. 1999). More recently, however, the Floral Genome Project generated a cDNA library from yellow poplar floral tissues (Albert et al. 2005) in an EST data set containing approximately 6,000 unigenes (http://pgn.cornell.edu/). Additionally, the construction of a bacterial artificial chromosomal (BAC) library resulted in 182 genomic end sequences for *Liriodendron tulipifera* (Liang et al. 2007), which includes partial sequences for lignin biosynthetic genes including cinnamyl alcohol dehydrogenase (accession number DQ223432), 4-coumarate:CoA ligase (accession number DQ223433) and phenylalanine ammonia-lyases (accession number DQ223434). However, very few essential regulators have thus far been isolated for the genetic improvement of the yellow poplar

to make it a better source of biomaterials, i.e., growth promotion with increased levels of cellulose content and downregulation of lignin biosynthesis with monolignol modification toward an increasing S/G ratio. Since the secondary xylem of angiosperm has been shown to result in the formation of such tissues under tensile stress (Scurfield 1973; Hellgren et al. 2004; Jin and Kwon 2009), the stems of 2-year-old yellow poplar were bent at 45° for 6 h to induce the expression of functional genes in the early stages of tension wood formation. The resultant tension wood-like tissues with adjacent phloem tissues were employed for the construction of the cDNA library. A total of 5,982 ESTs were sequenced and analyzed. As this is the first EST data set established from secondary xylem upon mechanical bending for 6 h in the yellow poplar stem, many genes associated with cell wall biosynthesis/modification during early stage of tension wood formation have been sequenced. Among 5,982 ESTs, a total of 1,733 annotated ESTs contain genes for cell wall biosynthesis and modification, hormone biosynthesis and signaling, and stress and defense responses. Additionally, the expression analysis of lignin biosynthetic genes and several hormone-related genes allows us to identify regulatory genes that might play essential roles during the early stage of tension wood formation.

Materials and methods

Plant materials

For EST construction, 2-year-old yellow poplars planted on 5 April 2008 and maintained in an experimental field were used in this experiment. Two months after bud breaking, the straight stem of the yellow poplar was bent to maintain an angle of approximately 45° as previously described (Jin and Kwon 2009). After 6 h of bending, the upper tissues of the bent stem were carefully harvested with a razor blade and stored in liquid nitrogen until being used for EST construction. Opposite tissues were also harvested from bent trees for 6 h and employed for quantitative RT-PCR.

RNA extraction and complementary DNA library construction

Only the upper tissues of the stems bent for 6 h were homogenized with a pestle under liquid nitrogen and total RNA was extracted using the pine tree method described by Chang et al. (2003). After checking the quality and quantity of total RNA using a spectrophotometer and electrophoresis, 50 μg of extracted crude RNA was enzymatically treated with DNase I (Takara BIO INC. Japan) to remove contaminant genomic DNA. Messenger RNA was further purified from 100 μg of total RNA using an Absolutely mRNA Purification Kit (Stratagene, USA) in accordance with the manufacturer's instructions. The quantity and quality of mRNA were evaluated by the ratio of absorbance at 260 and 280 nm.

A directional Lambda ZAP cDNA synthesis/Gigapack III Gold Cloning Kit (Stratagene, USA) was employed for the construction of the cDNA library. Reverse transcriptase of mRNA for the production of first-strand cDNA synthesis was primed from the poly-A tail using an oligo-dT linker-primer harboring an *Xho*I cloning site. After second-strand cDNA synthesis, the RNase H (1.5 unit μl^{-1}) and DNA polymerase I (9.0 unit μl^{-1}) enzymes were added to the second-strand synthesis reaction, and synthesis proceeded for 2.5 h at 16°C. *Eco*RI linkers were ligated to the 5′-terminal regions. *Xho*I digestion released the *Eco*RI adapter and residual linker-primer from the 3′ end of the cDNA. These fragments were separated on a column containing Sepharose® CL-2B gel filtration media, thus yielding three fractions. The fractions were then independently ligated into the ZAP Express vector (pBK-CMV) and the primary library produced via in vitro packaging of the ligation product with a ZAP Express cDNA Gigapack III Gold cloning Kit (Stratagene, USA). cDNA was directionally cloned into the pBK-CMV vector, which allows for both prokaryotic and eukaryotic expression of large sequences. The in vivo excision of cloned fragments was carried out into the phagemids described by the manufacturer and employed in the infection of the *Escherichia coli* strain XLOLR prior to sequencing. The cDNAs were plated on LB-kanamycin (50 μg ml^{-1}) plates (rectangle 23.5 cm × 23.5 cm) with X-gal/IPTG for blue/white selection. White colonies were randomly manually selected and inoculated into 384-well plates (Corning, USA) containing 40 μl of TB/kanamycin and then incubated for 16 h at 37°C with a fixation culture. The cDNA library was mixed with glycerol solution (65% glycerin, 0.1 M $MgSO_4$, 0.025 M Tris–HCl, pH 8.0) combined with 40 μl of bacterial culture products and were prepared and stored at −80°C. The insert sizes ranged from 400 bp to at least 4.0 kb.

The primary library was titrated and diluted to 10^7 pfu lambda phages in SM buffer and the packing extract was stored at 4°C. The mixture of 10^8 pfu XL1-Blue MRF cell and 10^9 pfu ExAssist helper phage was packaged into the lambda phage and mass excision was conducted. The phagemids were then titrated after 15 min of incubation at 37°C in 1× NZY broth. The cultivated phagemids were spread and incubated for colonization in LB media.

Sequencing cDNA clones

Single plasmid colonies were transferred into 540 μl of Terrific Broth (TB) medium supplemented with antibiotics (kanamycin at 50 μg ml^{-1}) in 96 deep well plates and

incubated overnight at 37°C with gentle rotation (550 rpm; HT-MegaGrow shaking incubator, Bioneer, Korea). The plasmid DNA was extracted and purified via a modification of an alkaline lysis method commonly employed for the isolation of plasmid DNA from bacteria (Birnboim and Doly 1979; Kelley et al. 1999) designed to optimize DNA yield. Using the prepared PCR products as templates, sequencing reactions were conducted in accordance with the manufacturer's instructions with a BigDye Terminator chemistry (Ver. 3.1) Cycle Sequencing Kit (Applied Biosystems, CA). PCR amplification was conducted in volumes of 3 μl with 250 ng of plasmid DNA, 0.5 μl of universal primer (3 pmol), 0.87 μl of 5× sequencing buffer, 1.38 μl of distilled water and 0.25 μl of BDT v3.1, using a GeneAmp PCR System 9700 (Applied Biosystems, CA). The reaction was conducted under the following conditions: 35 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min. The PCR products were purified via ethanol precipitation and resolved on an ABI 3730XL DNA Analyzer (Applied Biosystems, CA). The universal sequences of primers employed for forward primer was T3 and the reverse primer was T7.

DNA sequence trimming and assembly

After a total of 5,982 selected 5′-end sequences were completed, all 5′-end sequences were trimmed by Phred (Ewing and Green 1998; Ewing et al. 1998) score 20 prior to assembling ESTs, and the poly-A/T, vector and adaptor sequences were removed. Then, sequences shorter than 100 bp were excluded from analysis. The remaining 52,406 5′-end sequences were clustered into clusters and singletons (12,830) using the TGICL and CAP3 programs with default parameters of 40 bp overlap, a minimum identity of 95% and a 30-bp maximum mismatched overhang (Huang and Madan 1999; Pertea et al. 2003). The sequences reported in this paper were deposited in the NCBI database under accession number LtTW0001–LtTW0531.

Bioinformatics analysis of ESTs

For the homolog search and functional categorization of ESTs using BLASTX and TBLASTX (minimum match amino acids ≥50 aa, identity ≥25% and *E* value of ≤1E −10), we employed a variety of databases: the NCBI non-redundant database nucleotide and protein databases for general annotation, and the non-redundant DrugBank database for finding drug target candidates, in October 2006. The domain structures with potential functions in gene and gene ontology (GO) were predicted using InterProScan (Data version v14.0) with an *E* value of ≤1E−5. To predict potential signal peptides, we also conducted Orfpredictor (https://fungalgenome.concordia.ca/tools/OrfPredictor.html) and SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/), using both the neural network and Hidden Markov models to identify a possible signal peptide in the predicted proteins. For prediction, we employed alpha helices and the position of the intervening loop of transmembrane TMHMM version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). To evaluate the significance of in silico gene expression, we counted each read from the library from which it derived in assembled contigs. We subsequently conducted the Fisher exact test ($P < 0.01$) at IDEG6 (http://telethon.bio.unipd.it/bioinfo/IDEG6_form/).

Quantitative RT-PCR

The upper (tension wood (TW)) and lower stems (opposite wood (OW)) bent for 6 h were individually harvested. Fresh tissues (3 g, fresh weight) from tension wood and opposite wood were individually pulverized in liquid nitrogen using a mortar and pestle. After homogenization, total RNA was purified via the method described by Chang et al. (2003). The quality and quantity of purified total RNA were confirmed via spectrophotometric analysis and gel electrophoresis. As much as 5 μg of total RNA was used to synthesize the first-strand cDNAs using Superscript II reverse transcriptase according to the manufacturer's instructions (Invitrogen, USA). In brief, total RNA was heat denatured for 10 min at 70°C and used for the reverse transcription reaction in buffer containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT, 0.5 mM each deoxyribonucleoside triphosphate (dNTP), 1 mM random hexamer (Invitrogen, USA) and 200 units of Superscript II (Invitrogen, USA) in a total volume of 20 μl for 50 min at 42°C. After incubation, the resultant first-strand cDNAs were diluted with sterile distilled H_2O. For quantitative RT-PCR analysis, diluted first-strand cDNAs were used in 20 μl of Ready-to-use hot start PCR reaction mixture containing LightCycler FastStart DNA Master SYBR Green I (Roche, Germany). For each reaction, a reaction mixture without cDNA was employed as a negative control. PCR reactions were conducted using a GenAmp PCR system 9700 Cycler (Perkin-Elmer, USA) and the amplification conditions for polymerase chain reaction (PCR) consisted of 45 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 5 s, extension at 72°C for 9 s with 95°C pre-incubation for 10 min, and additional extension at 72°C for 10 min, in accordance with the manufacturer's instructions. The sequences of the primers used are listed in the Supplementary Tables (Suppl. Tables 1–3). The fold changes of gene expression in the tension wood relative to those of opposite wood were analyzed via the Delta–Delta CT method (Livak and

Schmittgen 2001). As an internal control, primers designed based on the sequence of CL37Contig1, which is homologous to *Gossypium hirsutum* actin (ACT11) with 89.9% identity were employed. The sequences of the forward and reverse primers for actin were 5′-GCGTGATCTCACCG ACGC-3′ and 5′-TAAGAGATGGCTGGAAGAGGAC-3′, respectively.

Results

EST Sequencing and classification of functional genes

From the constructed cDNA library, 6,141 clones were sequenced and a total of 6,046 clones were selected after the base-calling process. During the vector trimming process, contaminant trimming and repeat masking processes, a total of 5,982 clones with an average insert size of approximately 1.4 kb were finally selected for sequence analysis (Table 1). Among the 5,982 ESTs submitted for the BLASTX search, 1,733 (28.9%) ESTs significantly matched the previously identified gene transcripts provided in the NCBI public database, but 4,249 (71.3%) ESTs were not. These ESTs were assembled into 822 clusters and 911 singletons using a TGICL module (Table 1). Among the 5,982 sequenced ESTs, 1,733 (28.9%) ESTs significantly matched the formerly identified gene transcripts and thus were annotated as respective gene transcripts. Among the annotated ESTs, 122 ESTs (2.0%) were homologous with gene transcripts of unknown function.

The EST sequences with strong similarities were analyzed further using the GO algorithm for classification according to biological processes, cellular localization and molecular functions (Fig. 1). A total of 2,491 sequences (41.6%) were classified as genes associated with biological processes such as primary and secondary metabolic processes, macromolecular metabolic processes, transport, stress responses, cell communications and other functions. Among the genes classified as biological processes, more than 50% of the genes were related with primary and secondary metabolic processes. Additionally, 217 sequences (3.6%) were related to various stress and defense responses, including abiotic stresses, chemical stimuli and endogenous stimuli. On the other hand, 3,053 ESTs (51.0%) were classified as cellular components; this number includes intracellular organelles with highest frequency, protein complex, vesicles, membrane part and non-membrane bounded organelles and others. Among 5,982 ESTs, 1,295 ESTs (21.6%) have been classified as molecular functions with hydrolase, ion binding, protein binding, nucleotide binding, transferase, oxidoreductase, lyase, translation factor and transcription factor activity, as well as some other activities.

Although the number of annotated ESTs was only 1,733 (28.9%), probably due to their biological characteristics as early angiosperms, the analysis of functional genes using the GO algorithm indicates that the annotated ESTs obtained in this study provide sequences for a broad array of transcripts expressed in the stem upon mechanical bending for 6 h, and thus provide a variety of useful genes expressed in the early stages of cell wall modification during tension wood formation in the stem of yellow poplar.

Table 1 Statistics of *Liriodendron tulipifera* stem ESTs

Categories	No. of clones (%)
Total clones sequenced	6,141
ESTs submitted for blast search	5,982
No. of EST clusters (contig)	822
No. of EST singletons	911
Annotated ESTs	1,733 (28.9)
EST with homology to other organism (contig)	756 (12.6)
EST with homology to other organism (singletons)	855 (14.3)
EST with unknown function (contig)	66 (1.1)
EST with unknown function (singletons)	56 (0.9)

Genes for cell wall biosynthesis and modification

Tension wood is usually characterized with eccentric growth promotion, where tensile strength is exerted and changes of cell types occur, i.e., increased fiber cell types with decreased vessel types in cells (Zobel and van Buijtenen 1989). Additionally, fiber cell walls in the tension wood are much thicker, with increased levels of carbohydrates in the cell wall. Previous chemical analyses of tension wood in the stems of 2-year-old yellow poplar with mechanical bending demonstrated that the cellulose contents were significantly higher in tension wood than in opposite wood at 7 and 14 days (Moon et al. 2011). In addition, hemicellulose contents were also modified in the tension wood as compared to the opposite wood at 7 and 14 days of bent samples (Moon et al. 2011). In this study, however, stems were bent for only 6 h at an angle of 45° to isolate early regulatory genes relevant to cell wall modification during tension wood formation, since a previous study for the isolation of regulatory genes from *Eucalyptus* tension wood isolated these successfully from samples bent for 6 h (Paux et al. 2005). Despite the small number of ESTs (5,982) sequenced thus far, many genes associated with cellulose and hemicelluloses biosynthesis have been isolated (Table 2). The sequences of CL157Contig1 and CL786Contig1 were homologous to the *Eucalyptus grandis*

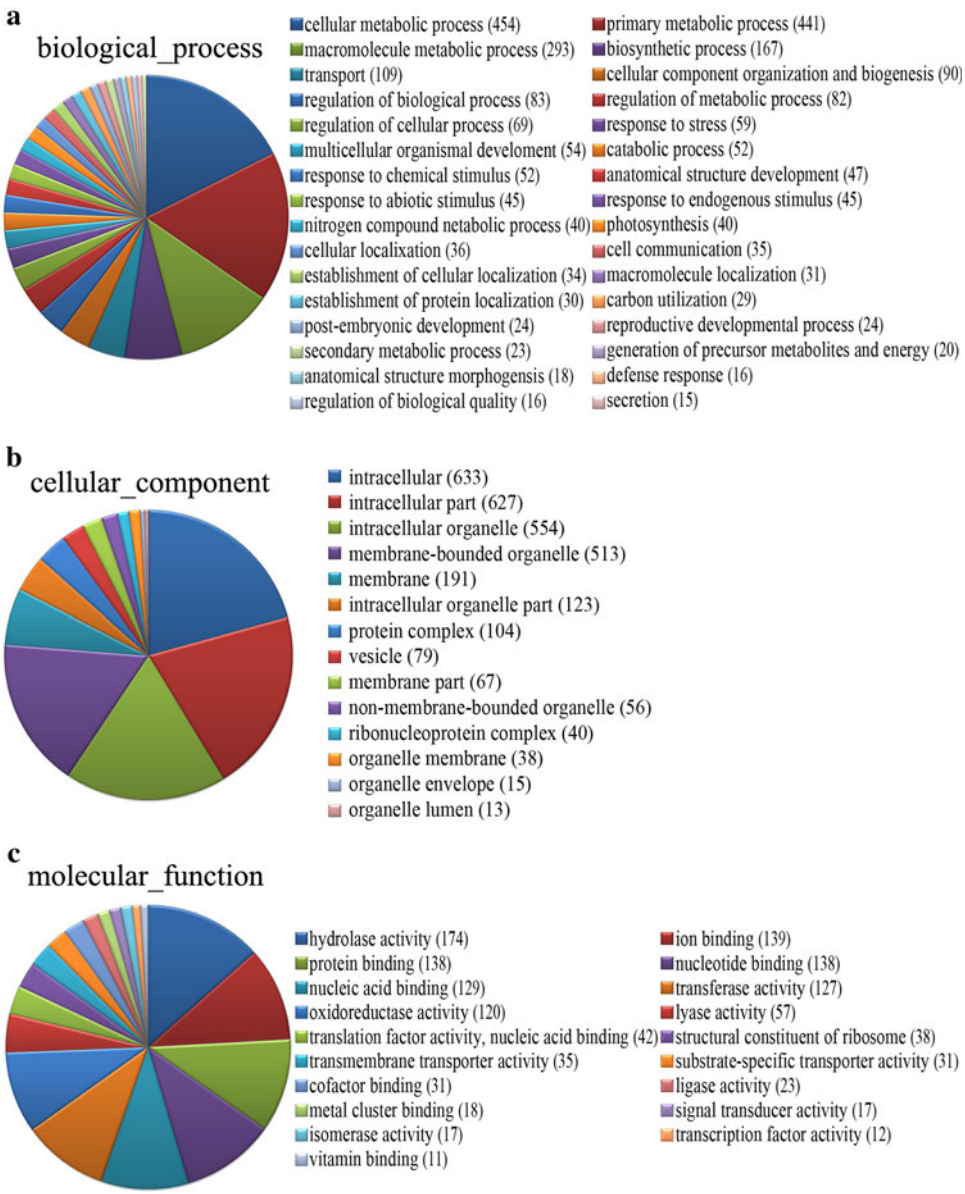

Fig. 1 Result of gene ontology (GO) annotations based on biological process (**a**), cellular components (**b**) and molecular function (**c**)

cellulose synthase 3 (EgCesA3) and cellulose synthase 6 (EgCesA6), respectively. In the case of EgCesA3, its expression was shown to be highly specific to xylem in the secondary xylem (Ranik and Myburg 2006). Meanwhile, the singleton TW3-4a-T3_G01 was highly homologous to *Populus tremuloides* CesA (PtrCesA), the transcripts of which were expressed predominantly in the primary wall forming expanding cells of aspen organs including the leaves, petioles, stems and roots, as revealed via in situ hybridization (Kalluri and Joshi 2004). The CL336Contig1 sequence was homologous to *Medicago truncatula* glycosyl transferase family 48, which might be a component of 1,3-beta-glucan synthase.

Interestingly, many enzymes associated with the degradation of cellulose and hemicelluloses have been isolated from the secondary xylem upon mechanical bending in the yellow poplar stem. This includes TW3-4a-T3_M15 (*Acidothermus cellulolyticus* cellulose-binding family II protein), CL722Contig1 (*Gossypium hirsutum* endo-1, 4-beta-glucanase), CL130contig1 (*Aspergillus fumigates* Af293 endo-1,3(4)-beta-glucanase), CL9Contig1 (*Schizosaccharomyces pombe* α-glucosidase), CL118Contig1 (*Oryza sativa* β-glucosidase), TW16-1a-T3_E05 (*Streptomyces* sp. SPB74 β-glucosidase), CL489Contig1 (*Gossypium barbadense* Glycosyl hydrolase family 19 protein), TW2-3a-T3_F07 (*Arabidopsis thaliana* Glycosyl hydrolase family protein 17), TW4-1a-T3_O23 (*Populus tremula* × *Populus tremuloides* endo-1,4-β-xylanase), CL263Contig1 (*Pyrus communis* alpha-L-arabinofuranosidase) and CL583Contig1 (*Gossypium hirsutum* xyloglucan endotransglucosylase).

Table 2 Selected enzymes for cell wall and carbohydrate metabolism

Contig ID[a]	Annotation	Access. No.	Total length	Query length	Matched aa	Percent alignment	*E* value
Cellulose/hemicellulose							
CL157Contig1	Cellulose synthase 3 (CesA3) (*Eucalyptus grandis*)	AAY60845.1	1,040	289	265	91	9.10E−157
CL786Contig1	Cellulose synthase 6 (CesA6) (*Eucalyptus grandis*)	AAY60848.1	1,097	246	128	82	2.20E−70
TW3-4a-T3_G01	Cellulose synthase (*Populus tremuloides*)	AAO25536.1	1,083	285	269	94	1.90E−159
CL130Contig1	Endo-1,3(4)-beta-glucanase (*Aspergillus fumigatus* Af293)	XP_750278.1	356	129	19	29	8.04E+00
CL722Contig1	Endo-1,4-beta-glucanase (*Gossypium hirsutum*)	AAQ08018.1	619	288	220	86	3.30E−127
CL247Contig1	Polygalacturonase (*Lycopersicon esculentum*)	AAF61444.1	452	290	98	64	2.00E−50
TW4-1a-T3_O23	Endo-1,4-beta-xylanase (*Populus tremula* × *Populus tremuloides*)	AAX33301.1	915	259	144	60	1.20E−77
CL9Contig1	Alpha-glucosidase (*Schizosaccharomyces pombe*)	BAB43946.1	969	243	8	39	2.30E+00
CL118Contig1	Beta-glucosidase (*Oryza sativa*)	BAD87322.1	513	286	78	45	1.90E−37
TW16-1a-T3_E05	Beta-glucosidase (*Streptomyces* sp. SPB74)	YP_002188841.1	844	111	14	58	2.06E−01
CL683Contig1	Beta-mannosidase 4 (*Oncidium Gower Ramsey*)	ABC55715.1	498	214	154	71	1.00E−93
TW11-3a-T3_D01	Endo-1,4-beta-mannosidase protein 2 (*Prunus persica*)	ABV32548.1	433	263	191	78	1.20E−109
CL263Contig1	Alpha-arabinofuranosidase (*Pyrus communis*)	BAF42035.1	675	285	218	76	8.20E−126
CL272Contig1	UDP-glycosyltransferase (*Arabidopsis thaliana*)	NP_179151.2	484	248	106	61	2.30E−51
CL336Contig1	Glycosyl transferase, family 48 (*Medicago truncatula*)	ABN09771.1	1,245	290	165	79	1.50E−90
CL489Contig1	Fiber glycosyl hydrolase family 19 protein (*Gossypium barbadense*)	AAQ84319.1	238	271	59	76	6.50E−29
TW2-3a-T3_F07	Glycosyl hydrolase family protein 17 (*Arabidopsis thaliana*)	NP_564059.1	184	299	92	60	2.90E−44
CL606Contig1	(1-4)-beta-mannan endohydrolase (*Arabidopsis thaliana*)	NP_195813.1	448	288	169	74	2.30E−99
CL514Contig1	Starch branching enzyme II-1 (*Malus* × *domestica*)	ABO31358.1	845	267	72	80	1.80E−39
CL559Contig1	Starch branching enzyme I (*Populus trichocarpa*)	ABN05321.1	838	254	114	61	2.00E−61
CL425Contig1	Xyloglucan galactosyltransferase (*Zea mays*)	ACG27661.1	603	241	76	70	1.70E−40
CL583Contig1	Xyloglucan endotransglucosylase (*Gossypium hirsutum*)	ABU41234.1	289	285	217	80	1.90E−135
TW3-1a-T3_I17	Alpha-1,4-glucan-protein synthase (UDP-forming) (*Medicago truncatula*)	ABD28560.1	347	285	120	70	4.30E−66
TW3-4a- T3_M15	Cellulose-binding family II protein (*Acidothermus cellulolyticus 11B*)	YP_872377.1	1,298	288	34	34	1.78E−03
CL579Contig1	Hydroxyproline-rich glycoprotein family protein (*Arabidopsis thaliana*)	NP_566709.1	532	294	144	52	7.60E−74
Extensin/expansin							
CL22Contig1	Extensin 4 (*Arabidopsis thaliana*)	BAB20085.1	246	205	43	56	3.80E−18
TW13-2a-T3_I14	Extensin (*Catharanthus roseus*)	BAA13175.1	225	186	57	35	2.60E−14
TW2-4a-T3_F12	Alpha-expansin 1 (*Populus tremula* × *Populus tremuloides*)	AAR09168.1	262	262	219	93	3.80E−132

Table 2 continued

Contig ID[a]	Annotation	Access. No.	Total length	Query length	Matched aa	Percent alignment	*E* value
Pectin							
CL123Contig1	Pectin methylesterase (*Eucalyptus globules* subsp. *globulus*)	ABD47737.1	98	375	65	70	1.10E−31
CL129Contig1	Pectin methylesterase (*Sesbania rostrata*)	CAD29733.1	554	146	57	45	7.70E−21
CL339Contig1	Pectin methylesterase (*Arabidopsis thaliana*)	NP_175787.1	586	273	131	46	2.70E−59
CL788Contig1	Pectinesterase (*Fragaria* × *ananassa*)	AAQ21127.1	211	238	131	82	1.70E−77
TW5-1a-T3_M05	Pectinesterase family protein (*Arabidopsis thaliana*)	NP_197586.1	512	266	66	44	1.90E−22

[a] Contig (CL) or singleton (NEF) number

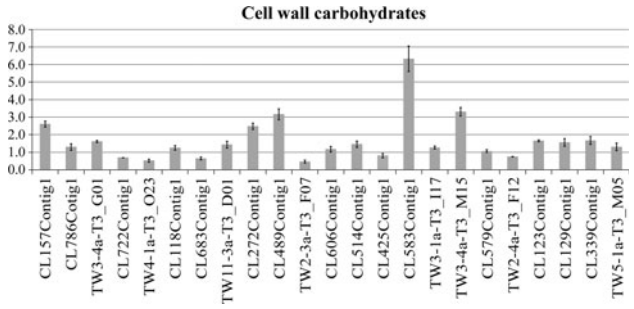

Fig. 2 Relative expression of selected genes related to cell wall carbohydrate biosynthesis and modification in the tension wood compared to opposite wood in 6 h bent sample. The expressions of CL722Contig1, TW4-1a-T3_O23, CL683Contig1, TW2-3a-T3_F07 and TW2-4a-T3_F12 were significantly lower in tension wood than in opposite wood, whereas the expression of CL583Contig1 was six times higher in tension wood than in opposite wood. Actin (CL37Contig1) was used as an internal control. All experiments were repeated three times with five duplicates

Among the sequences for cellulases and hemicellulases, CL130contig1 was highly homologous to the fungal endo-1,4-β-glucanases isolated from *Aspergillus fumigatus* Af293 (Table 2). On the other hand, the singleton TW3-4a-T3_M15 was highly homologous to the cellulolytic actinobacterial cellulose-binding family II protein. Additionally, the sequence of CL583Contig1 was highly homologous to *Gossypium hirsutum* xyloglucan endo-transglucosylase (Table 2), which has been previously reported to be significantly upregulated during the elongation stage of cotton fiber development (Michailidis et al. 2009). Interestingly, the expression of CL583Contig1 was significantly higher in tension wood-forming tissues than in opposite wood, whereas most of them are not decreased or only slightly decreased (Fig. 2). As previous research using transgenic poplar expressing fungal xyloglucanase has shown that constitutive degradation of cell wall xyloglucan contributed to the loosening of the cell wall, thus resulting in growth enhancement and cellulose accumulation (Micheli 2001; Park et al. 2004), the upregulation of CL583Contig1 in the tension wood may perform a critical role in cell wall modification and growth promotion in the tension wood-like tissues; this is one of the best characterized features of tension wood in angiosperms.

Not only cellulases and hemicellulases, but also other types of cell wall modification enzymes have been isolated from the stem of yellow poplar upon mechanical bending. This includes extension and expansion sequences. CL22 Contig1 was homologous to *Arabidopsis thaliana* Extensin 4, TW13-2a-T3_I14 to *Catharanthus roseus* extensin, and TW2-4a-T3_F12 to α-expansin1 of the hybrid poplar (*Populus tremula* × *Populus tremuloides*) as listed in Table 2. The α-expansin has been implicated in cellulose crystallinity, because the down regulation of the *Petunia*

hybrid α-expansin gene via a transgenic antisense approach resulted in a decline in crystalline cellulose in the cell wall of the petal limb (Zenoni et al. 2004; Wang et al. 2011). The expression of α-expansin was significantly lower in the tension wood-forming tissues than in opposite wood (Fig. 2), implying a role of α-expansin in the regulation of cellulose crystallinity in the early stage of cell wall biosynthesis and modification in the secondary xylem during tension wood formation.

Additionally, four contigs and one singleton were homologous to pectin methylesterase (Table 2), which has been previously theorized to inhibit symplastic and intrusive cell growth in the developing wood cells of *Populus* (Siedlecka et al. 2008). The overexpression of pectin methylesterase inhibitor has been consistently reported to increase the biomass of dicots via the promotion of cell expansion (Lionetti et al. 2010). However, in the stem of yellow poplar bent at 45° for 6 h, the transcript level of isolated pectin methylesterase (CL123Contig1, CL129Contig1, CL339Contig1) was not upregulated significantly in response to mechanical bending (Fig. 2), thereby implying that pectin modification plays a minor role in the early stages of tension wood formation upon mechanical bending treatment.

Genes for lignin biosynthesis

A previous study of tension wood formation induced by mechanical bending treatment of the yellow poplar stem showed that lignin content was downregulated significantly in the tension wood relative to that of the opposite wood (Jin and Kwon 2009; Moon et al. 2011). Additionally, the monomeric composition was altered upon mechanical bending treatment of the yellow poplar stem (Moon et al. 2011). Although previous histochemical and chemical analyses have demonstrated that mechanical bending causes modifications of lignin biosynthesis at the cellular level, our understanding of lignin modification at the molecular level is quite limited at present, as the majority of the lignin biosynthetic genes have not been isolated from the yellow poplar. In the NCBI database, for example, two partial sequences for phenylalanine ammonia lyase were available as an ozone-related gene (EU190449) and a gene associated with flower development (DQ223434), respectively. Our EST provides a total of 16 sequences that matched monolignol biosynthetic enzymes in the phenylpropanoid pathway (Table 3). These include sequences homologous to PAL (TW4-3a-T3_D21), C4H (CL243Contig1), C3H (CL458Contig1), HCT (CL1Contig6), F5H (CL170Contig1), CCR (CL492Contig1), 6 COMTs (CL83Contig1, CL83Contig2, CL145Contig1, CL570Contig1, TW3-1a-T3_G01, TW12-4a-T3_H18) and 4 CADs (CL10Contig1, CL102Contig1, CL361Contig1, TW4-1a-T3_A23). The singleton TW4-3a-T3_D21 (designated as LtPAL1) was homologous to the xylem-specific phenylalanine ammonia-lyase 5 (EU603320.1) of *Populus trichocarpa* (Shi et al. 2010), with an identity of 78% and its expression was slightly higher in the stem than in leaf (Fig. 3). The sequence of CL243Contig1 (LtC4H1) was highly homologous to *Populus tremuloides* trans-cinnamate 4-hydroxylase (C4H2-1) (DQ522294.1), with an identity of 89% (Table 4). The transcript of LtC4H1 was expressed, slightly more in both leaf and young stem than in mature stem (Fig. 3), probably because of its various functions in the general phenylpropanoid pathway, consistent with its *Arabidopsis* orthologs (Bell-Lelong et al. 1997; Mizutani et al. 1997). The sequence of CL458Contig1 (designated as LtC3H1) was highly homologous to *Populus trichocarpa* C3H3, with an identity of 72% (Table 3) and an expression that was ubiquitous but significantly higher in the leaf (Fig. 3), which also implies the diverse roles of LtC3H1 in the general phenylpropanoid pathway.

The sequences of CL1Contig6 designated as LtHCT1 was homologous to PtHCT6 and its expression was higher in the stem than in the leaf (Fig. 3), thereby implying a role for this gene in the lignin/lignan biosynthetic pathway, instead of the general phenylpropanoid pathway. For F5H, CL170Contig1 was found to be homologous to *Camptotheca acuminate* F5H (Table 3), and its expression was also significantly higher in the stem than in the leaf (Fig. 3). In the case of CCR, CL492Contig1 (designated as LtCCR1) was homologous to *Populus tomentosa* CCR, with an identity of 86%. Its expression was also higher in the stem than in the leaf (Fig. 3). Therefore, CL1Contig6, CL170Contig1 and CL492Contig1 seem to encode genes for the biosynthesis of monolignols leading to the lignin and/or lignan biosynthesis in the stem. Our EST of the yellow poplar stem harbors sequences for six COMT and four CAD (Table 3). COMT catalyzes the methylation of caffeate and 5-hydroxyferulate during monolignol biosynthesis, with a greater preference for 5-hydroxyferulate than caffeate (Anterola and Lewis 2002). Two highly homologous contigs, CL83Contig1 and CL83Contig2, are also homologous to Populus COMT, CL145Contig1, CL570Contig1 and singleton TW12-4a-T3_H18 to COMT of *Rosa chinensis* var. *spontanea*, *Coffea canephora* and *Populus trichocarpa*, with relatively low identities (Table 3). All of the six COMTs were significantly highly expressed in the stem than in the leaf (Fig. 3). Interestingly, the expressions of CL83Contig1 (LtCOMT1) and CL83Contig2 (LtCOMT2) were extremely high in the mature stem than in the young stem, whereas those of CL145Contig1 (LtCOMT3), CL570Contig1 (LtCOMT4), two singletons TW3-1a-T3_G01 (LtCOMT5) and TW12-4a-T3_H18 (LtCOMT6) were slightly higher in the young stem (Fig. 3), implying a spatial and temporal regulation of their functions in the stem of yellow poplar. Additionally, the expressions of

Table 3 Selected sequences for lignin biosynthesis

Contig ID[a]	Annotation	Access. No.	Total length	Query length	Matched aa	Percent alignment	*E* value
Monolignol biosynthesis							
TW4-3a-T3_D21	PAL (*Populus trichocarpa*)	ACC63889.1	711	288	205	81	4.80E−118
CL243Contig1	C4H (*Populus tremuloides*)	ABF69102.1	505	321	221	89	4.60E−123
CL458Contig1	C3H (*Populus trichocarpa*)	ACC63870.1	508	539	364	72	2.00E−216
CL1Contig6	HCT (*Populus trichocarpa*)	EU603314.1	574	279	139	81	1.70E−76
CL170Contig1	F5H (*Camptotheca acuminata*)	AY621153.1	1,722	304	191	66	9.80E−108
CL492Contig1	CCR (*Populus tomentosa*)	ACE95172.1	338	273	217	86	2.40E−124
CL83Contig1	COMT (*Populus tomentosa*)	AAF63200.1	360	277	154	61	1.30E−85
CL83Contig2	COMT (*Populus deltoides*)	ABU53653.1	364	448	132	61	1.80E−71
CL145Contig1	COMT (*Rosa chinensis var. spontanea*)	BAC78827.1	365	291	227	79	3.20E−133
CL570Contig1	COMT (*Coffea canephora*)	AAN03726.1	350	272	187	73	1.70E−106
TW3-1a-T3_G01	COMT (*Vitis vinifera*)	AAF44672.1	386	108	60	66	6.90E−29
TW12-4a-T3_H18	COMT (*Populus trichocarpa*)	ACC63885.1	351	272	107	43	3.80E−45
CL10Contig1	CAD (*Arabidopsis thaliana*)	NP_197445.1	326	346	143	73	2.10E−79
CL102Contig1	CAD (*Liriodendron tulipifera*)	DQ223432.1	584	302	24	47	2.72E−01
CL361Contig1	CAD (*Stylosanthes humilis*)	L36823.1	980	320	37	52	1.00E−06
TW4-1a-T3_A23	CAD (*Zea mays*)	ACG45271.1	367	275	212	79	1.10E−124
Monolignol polymerization and modification							
TW1-4a-T3_F06	LAC (*Rosa hybrid cultivar*)	ACC78283.1	573	164	75	61	3.50E−39
CL474Contig1	LAC (*Liriodendron tulipifera*)	AAB17192.1	585	473	187	91	2.4E−106
TW3-3a-T3_I18	LAC (*Liriodendron tulipifera*)	AAB17193.1	586	296	280	99	2.00E−162
TW9-2a-T3_O06	Peroxidase (*Capsicum chinense*)	CAI48072.1	220	268	19	55	8.00E−08
TW5-2a-T3_I12	PPO (*Ananas comosus*)	AAK29782.1	604	252	109	49	2.50E−53
CL2Contig1	PCBER (*Nicotiana tabacum*)	BAG84267.1	308	310	224	76	8.00E−131
CL2Contig2	PCBER (*Forsythia* × *intermedia*)	AF242491.1	361	285	204	74	9.00E−113

PAL phenylalanine ammonia lyase, *C4H* cinnamate-4-hydroxylase, *C3H3* coumaroyl 3-hydroxylase, *HCT* hydroxycinnamoyl transferase, *F5H* ferulate-5-hydroxylase, *CCR* cinnamoyl CoA reductase, *COMT* caffeic acid 3-*O*-methyltransferase, *CAD* cinnamyl alcohol dehydrogenase, *LAC* laccase, *PPO* polyphenol oxidase, *PCBER* phenylcoumaran benzylic ether reductase

[a] Contig (CL) or singleton (NEF) number

CL83Contig2 and CL570Contig1 were significantly lower in tension wood than in opposite wood (Fig. 4), thereby implying a role for lignin alteration under mechanical bending treatment. In the case of CAD, CL10Contig1 (LtCAD1), CL102Contig1 (LtCAD2) and CL361Contig1 (LtCAD3) were expressed in all the tissues tested (Fig. 3); however, the expression of TW4-1a-T3_A23 (LtCAD4) was significantly lower in the leaf than in other tissues.

Additionally, several sequences related to monolignol polymerization and modification were isolated in this study. These include three new laccases, 1 peroxidase, 1 polyphenol oxidase and 2 phenylcoumaran benzylic ether reductases (Table 3). The TW1-4a-T3_F06 (LtLac2-5) was highly expressed in the leaf than in the stem, whereas the expression of CL474Contig1 (LtLac2-6) was higher in the stem than in the leaf (Fig. 3). The expression of peroxidase (LtPO1) was significantly higher in the leaf than in the stem, and the expression of LtPPO1 was higher in the young stem than in other tissues (Fig. 3). Interestingly, LtPCBER1 was ubiquitously expressed in all the tissues tested, but the expression of LtPCBER2 was slightly higher in the young stem than in leaf and mature stem (Fig. 3).

To isolate lignin-related genes that might be related with lignin downregulation and associated modifications in the secondary xylem during early stage of tension wood formation, quantitative RT-PCR was conducted. As shown in Fig. 4, the majority of the lignin biosynthetic genes including LtC4H1, LtCCR1, LtCOMT2, LtCOMT4, LtCAD1 and LtCAD4 were downregulated more significantly in tension wood than in opposite wood in response to mechanical bending. Additionally, several genes for the monolignol coupling and post-monolignol coupling enzymes were also downregulated more significantly in the tension wood than in the opposite wood (Fig. 4), with the most significant downregulation of TW9-2a-T3_O06, which is annotated as peroxidase. Therefore, EST construction and

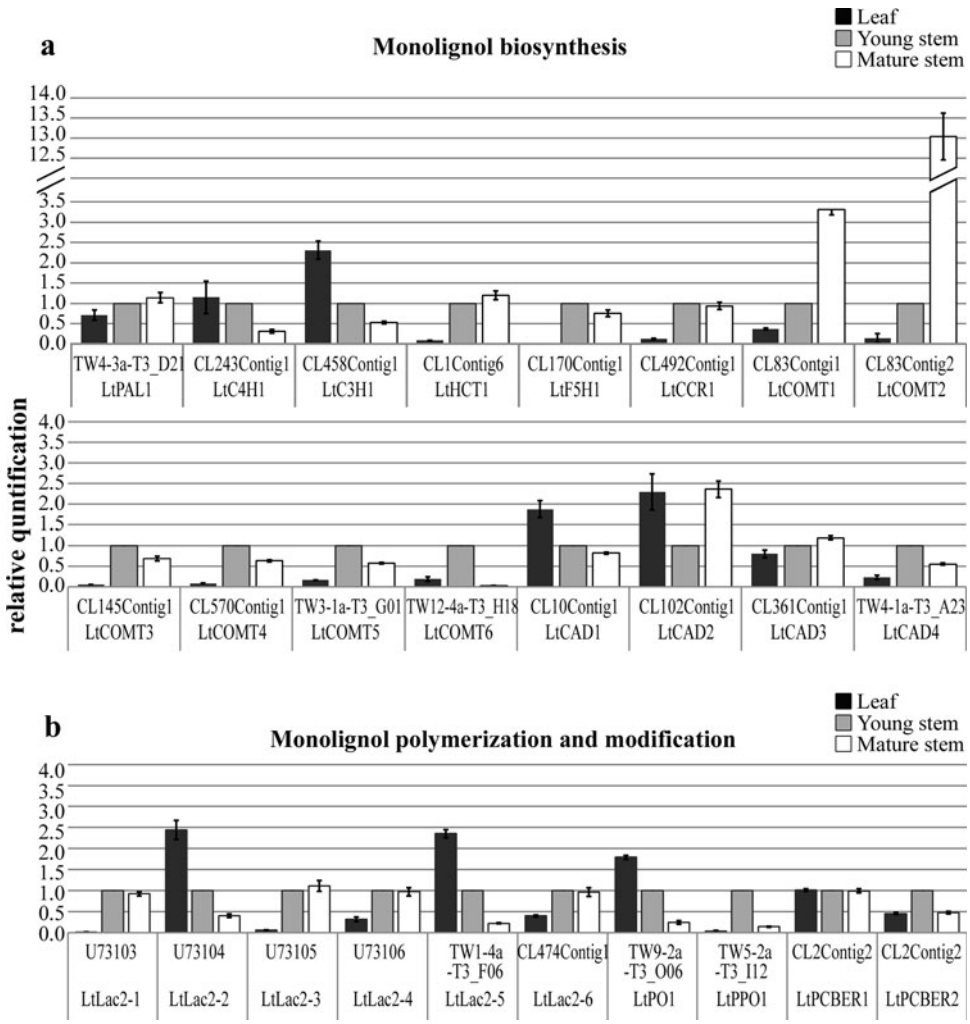

Fig. 3 Quantitative real-time RT-PCR for spatial analysis of genes related to monolignol biosynthesis and modification in the leaf, young stem and mature stem in a 2-year-old yellow poplar. Actin (CL37Contig1) was used as an internal control and the relative quantification was obtained based on the expression of each gene in the young stem as standard

quantitative real-time RT-PCR analysis allow us to isolate lignin biosynthetic genes that might be useful in uncovering the mechanism of lignin alterations during early stage of tension wood formation in the yellow poplar.

Growth hormone-related genes

Many plant growth hormones have been suggested to be essential regulators of tension wood formation; however, the molecular mechanisms underlying tension wood formation in response to tensile stress remain poorly understood. Recent research conducted by Funada et al. (2008) have shown that gibberellins exogenously applied to the vertical stem of *Fraxinus mandshurica* and *Quercus mongolica* resulted in tension wood formation at the application site (Funada et al. 2008). However, the involvement of other growth hormones is currently quite ambiguous. In the case of auxin, tension wood has been reported to be formed regardless of the amount of auxin present in the leaning stem and branches (Wardrop 1964; Cronshaw and Morey 1965). Additionally, exogenous treatment with an auxin inhibitor induced tension wood in the vertical stem (Cronshaw and Morey 1965; Morey and Cronshaw 1968); auxin has been proposed as a key regulator in tension wood formation over the long term. However, a recent study conducted by Hellgren et al. (2004) demonstrated that the amount of IAA between tension wood and opposite wood induced by gravitational stimuli did not differ significantly. Additionally, growth stimulation in the cambium of tension wood was not caused by an obvious increase in IAA (Hellgren et al. 2004). However, several Aux/IAA gene family members have been reported to be differently expressed between the tension wood and opposite wood in the hybrid aspen (Moyle et al. 2002). In this study, our EST generated at least 22 auxin-related genes, including 7 auxin response factors homologous to ARF from *Oryza sativa* (CL635Contig1, CL750Contig1), *Lycopersicon esculentum* (CL235Contig1) and *Brassica napus* (TW3-2a-T3_M17), to ARF3 from *Cucumis sativus* (TW1-2a-T3_G24) and *Arabidopsis thaliana* (TW3-4a-T3_G11), and to Arabidopsis ARF6(TW16-3a-T3_H23) as well as 2 Aux/IAA genes (CL51Contig1, CL318Contig1) and others (Table 4).

Table 4 Hormone biosynthesis and signaling

Contig ID[a]	Annotation	Access. No.	Total length	Query length	Matched aa	Percent alignment	*E* value
Auxin							
CL235Contig1	ARF 2 (*Lycopersicon esculentum*)	ABC69711.1	846	287	45	75	2.20E−17
CL635Contig1	ARF 7a (*Oryza sativa*)	BAD54030.1	991	278	230	82	7.50E−129
CL750Contig1	ARF 1 (*Oryza sativa*)	CAC83756.1	836	283	63	61	1.90E−26
TW1-2a- T3_G24	ARF 3 (*Cucumis sativus*)	BAD19063.1	916	289	260	90	3.00E−152
TW3-2a-T3_M17	ARF (*Brassica napus*)	CAG30068.1	848	296	143	73	6.50E−73
TW3-4a-T3_G11	ARF 3 (*Arabidopsis thaliana*)	AAB62404.1	608	285	167	70	2.40E−93
TW16-3a-T3_H23	ARF 6 (*Arabidopsis thaliana*)	NP_174323.1	933	298	185	63	1.80E−91
CL540Contig1	ARF-like protein (*Mangifera indica*)	AAP06759.1	840	165	50	41	5.70E−16
CL818Contig1	Auxin-responsive family protein (*Arabidopsis thaliana*)	NP_566763.1	393	258	68	46	8.00E−26
CL51Contig1	Aux/IAA protein (*Vitis vinifera*)	AAL92850.1	359	508	186	50	3.10E−83
CL318Contig1	Aux/IAA protein (*Medicago truncatula*)	ABO80473.1	670	579	271	71	1.00E−149
TW12-1a-T3_A15	IAA6-auxin-responsive Aux/IAA family member (*Zea mays*)	ACG37425.1	357	268	68	42	9.40E−25
CL391Contig1	Auxin-induced protein (*Vigna radiata*)	AAC98029.1	309	267	143	79	1.80E−76
TW7-4a-T3_B08	Auxin-inducible protein (*Zea mays*)	ACG39940.1	105	295	42	61	1.70E−17
CL57Contig1	Auxin-repressed protein (*Sesbania drummondii*)	ABQ44282.1	115	513	88	73	6.60E−41
TW16-3a-T3_B19	Auxin-repressed protein (*Prunus armeniaca*)	AAB88876.1	133	218	81	60	8.10E−33
CL594Contig1	Auxin influx protein (*Populus tomentosa*)	AAW57318.1	477	489	128	92	2.00E−61
TW6-1a-T3_K23	Auxin influx transport protein (*Casuarina glauca*)	ABN81349.1	480	251	113	86	1.20E−60
CL143Contig1	Auxin growth promoter protein (*Solanum lycopersicum*)	AAK84479.1	478	276	9	40	6.80E−02
CL519Contig1	Auxin growth promoter protein (*Solanum lycopersicum*)	AAK84479.1	478	288	7	32	1.60E−01
TW11-1a-T3_O19	Auxin-independent growth promoter (*Nicotiana tabacum*)	AAD55602.1	399	244	140	70	6.60E−80
TW3-1a-T3_A15	AIR3 (auxin induced in root cultures 3)	NP_565309.2	772	311	172	57	2.00E−85
Gibberellic acid							
CL166Contig1	GAI-like protein 1 (*Liriodendron chinense*)	ACJ65523.1	429	296	184	100	7.60E−98
TW3-4a-T3_G13	Gibberellic acid receptor-b (*Gossypium hirsutum*)	ABQ96123.1	344	268	129	75	2.40E−68
CL763Contig1	Gibberellin receptor GID1L2 (*Zea mays*)	ACG34658.1	327	333	157	52	6.30E−86
TW8-3a-T3_N23	Gibberellin receptor GID1L2 (*Zea mays*)	ACG34658.1	327	260	131	50	2.40E−70
TW3-2a-T3_I17	F-box protein GID2 (*Oryza sativa* subsp. japonica)	Q7XAK4.1	212	162	41	42	4.00E−20
TW2-4a-T3_N10	Gibberellin-regulated family protein (*Arabidopsis thaliana*)	NP_196996.1	275	267	44	68	2.00E−22
Brassinosteroid/sterol							
CL421Contig1	LRR receptor kinase precursor (*Zea mays*)	ACG45260.1	604	287	177	62	4.00E−84
TW8-1a-T3_I15	LRR receptor kinase (*Oryza sativa*)	BAD34326.1	1,214	285	132	46	5.20E−64

Table 4 continued

Contig ID[a]	Annotation	Access. No.	Total length	Query length	Matched aa	Percent alignment	*E* value
CL380Contig1	BR INSENSITIVE 1-associated receptor kinase 1 precursor (*Zea mays*)	ACG32036.1	217	298	145	70	1.70E−84
CL269Contig1	BRI1-KD-interacting protein 109 (*Oryza sativa*)	BAD11337.1	224	286	183	82	5.00E−89
CL423Contig1	BRL2 (*Arabidopsis thaliana*)	NP_178304.1	1,143	336	121	37	4.00E−49
CL165Contig1	BSU1 (*Medicago truncatula*)	ABN08040.1	664	291	106	50	4.00E−34
CL152Contig1	BES1/BZR1 homolog protein 4 (*Arabidopsis thaliana*)	Q9ZV88.1	325	286	96	81	7.00E−44
TW3-4a-T3_K05	BIM2 (*Arabidopsis thaliana*)	NP_177064.1	311	268	103	46	1.50E−43
CL744Contig1	BRH1 (*Arabidopsis thaliana*)	NP_191705.1	170	273	48	35	2.00E−16
TW8-4a-T3_B18	BRH1 (*Arabidopsis thaliana*)	NP_191705.1	170	292	39	45	1.80E−14
CL472Contig1	BRS1 (*Arabidopsis thaliana*)	NP_194790.1	465	297	140	47	4.00E−82
CL523Contig1	BRS1 (*Arabidopsis thaliana*)	NP_194790.1	465	274	50	38	4.00E−24
TW2-1a- T3_M23	Sterol-C5(6)-desaturase (*Nicotiana tabacum*)	AAD20458.1	271	280	197	76	2.00E−121
CL228Contig1	Membrane steroid-binding protein 1 (*Zea mays*)	ACG35045.1	232	324	145	66	8.60E−77
CL148Contig1	Steroid-binding domain-containing protein (*Oryza sativa*)	Q2QVN9	150	285	54	47	7.00E−30
CL243Contig1	Steroid 17alpha-monooxygenase (*Physcomitrella patens* subsp. *patens*)	XP_001781937.1	497	321	140	62	1.00E−75
CL299Contig1	Sterol 3-*O*-glucosyltransferase (*Panax ginseng*)	BAC22616.1	609	276	173	64	1.80E−90
CL395Contig1	3-beta hydroxysteroid dehydrogenase/isomerase (*Zea mays*)	ACG43161.1	283	294	162	63	7.10E−88
TW1-2a-T3_E04	Armadillo/beta-catenin repeat family protein (*Oryza sativa*)	ABA97086.1	806	292	204	70	6.10E−108
Ethylene							
CL1Contig5	CTR1 (*Arabidopsis thaliana*)	AAA32780.1	821	247	18	53	6.80E−01
CL138Contig1	ERS(*Phalaenopsis equestris*)	CAD91247.1	633	298	205	87	1.10E−115
CL437Contig1	Ethylene transcription factor (*Fagus sylvatica*)	CAE54591.1	378	261	152	56	1.40E−65
CL499Contig1	Ethylene-responsive transcription factor 4 (*Zea mays*)	ACG36718.1	240	340	70	38	4.60E−15
TW10-1a-T3_G19	Ethylene-responsive transcription factor RAP2-4 (*Arabidopsis thaliana*)	Q8H1E4.1	334	298	135	47	1.60E−50
TW3-2a-T3_A03	Ethylene-responsive factor (*Oryza sativa*)	AAB72193.1	636	224	200	89	7.70E−106
TW9-4a-T3_H18	Ethylene-response factor 2 (*Lycopersicon esculentum*)	AY275554.1	966	282	73	50	6.10E−29
TW8-3a-T3_F05	Ethylene-responsive protein (*Arabidopsis thaliana*)	NP_172445.2	171	194	49	47	1.60E−18
CL35Contig1	EIN3-binding F-box protein 2 (*Lycopersicon esculentum*)	ABC24972.1	665	288	123	52	2.60E−55
TW4-1a-T3_O13	Ethylene-induced calmodulin-binding protein (*Arabidopsis thaliana*)	AY510026.1	1,017	262	170	64	9.30E−65
Jasmonic acid							
CL451Contig1	JAR4 (*Nicotiana attenuata*)	ABC87760.1	577	249	89	77	9.30E−45
CL815Contig1	JAR1 (*Nicotiana attenuata*)	ABC87761.1	580	234	155	75	1.90E−86

Table 4 continued

Contig ID[a]	Annotation	Access. No.	Total length	Query length	Matched aa	Percent alignment	*E* value
ABA							
CL131Contig1	Abscisic stress ripening-like protein (*Glycine max*)	AAR26524.1	238	285	127	49	1.20E−49
TW3-1a-T3_E05	ABA DEFICIENT 2 (*Arabidopsis thaliana*)	NP_175644.1	285	205	88	73	6.20E−45
CL96Contig1	Abscisic acid glucosyltransferase (*Arabidopsis thaliana*)	NP_567953.1	481	266	91	36	2.00E−46
CL272Contig1	Abscisic acid glucosyltransferase (*Arabidopsis thaliana*)	NP_567955.1	488	248	94	55	3.00E−48
CL430Contig1	Abscisic acid glucosyltransferase (*Arabidopsis thaliana*)	NP_567955.1	488	250	58	43	1.00E−28
CL687Contig1	Abscisic acid glucosyltransferase (*Arabidopsis thaliana*)	NP_171715.1	203	273	153	77	2.00E−91

[a] Contig (CL) or singleton (NEF) number

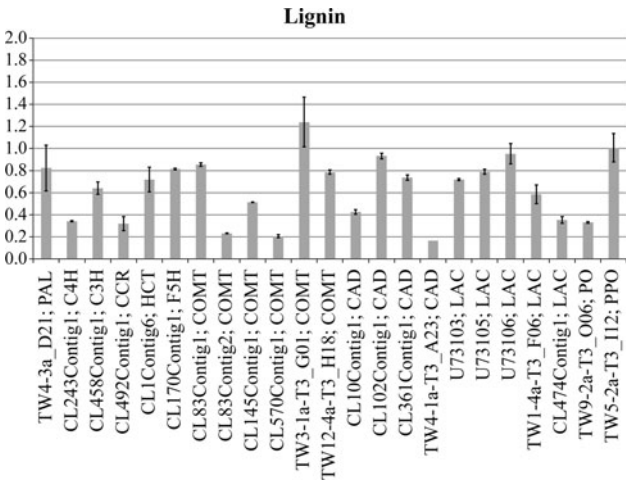

Fig. 4 Quantitative real-time RT-PCR for lignin biosynthetic genes upon mechanical bending treatment of the yellow poplar stem. Among 16 lignin biosynthetic genes, only genes that have been specifically amplified based on the amplification and melting curve were analyzed via quantitative real-time RT-PCR. Actin (CL37Contig1) was used as an internal control. All experiments were repeated three times with five duplicates

Interestingly, when the expression of auxin-related genes was compared between tension wood and opposite wood, the majority of them, except for CL750Contig1 and TW6-1a-T3_K23, was downregulated significantly in the tension wood as compared to the opposite wood (Fig. 5), implying a complex activity of auxin in the early stages of tension wood formation induced by mechanical bending.

Interestingly, our EST constructed from tension wood-like tissues induced by 6 h of bending treatment harbored up to 12 genes associated with the brassinosteroid signaling pathway (Table 4). This includes BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 precursor (CL380Contig1), LRR receptor Kinase (TW8-1a-T3_I15), LRR receptor Kinase precursor (CL421Contig1), BRI1-KD interacting protein (CL269Contig1), BRL2 (CL423Contig1), BSU1 (CL165Contig1), BES1/BZR1 homolog protein 4 (CL152Contig1), BIM2 (TW3-4a-T3_K05), BRH1 (CL744Contig1, TW8-4a-T3_B18) and BRS1 (CL472 Contig1, CL523Contig1). To determine whether the expressions of BR-related genes are influenced by mechanical bending, quantitative RT-PCR was carried out to compare the levels of expression between tension wood and opposite wood. The expression of LRR receptor kinase (TW8-1a-T3_I15) was slightly higher in tension wood-forming tissues, whereas the levels of LRR receptor kinase precursors (CL421Contig1) were significantly higher in the tension wood than in the opposite wood (Fig. 5). However, most of the well-characterized, positive regulators of the BR signaling pathway, including BRI1-LIKE 2 (CL423 Contig1), BES1/BZR1 homolog protein 3 (CL152Contig1) and BIM2 (TW3-4a-T3_K05) were significantly lower in

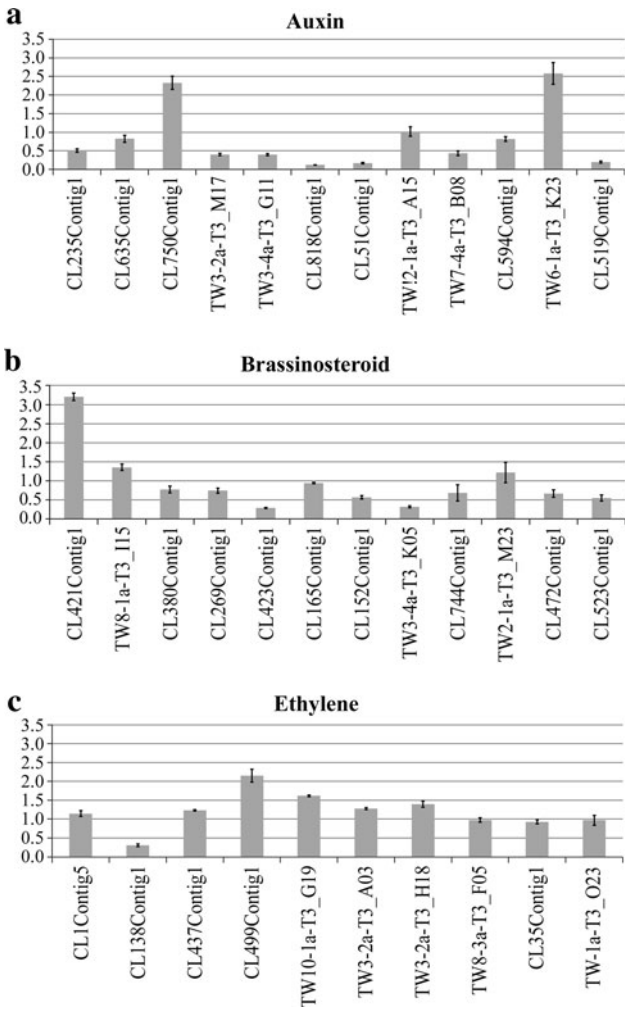

Fig. 5 Expression levels of auxin (**a**), BR (**b**) and ethylene (**c**) related genes of interest in tension wood compared to those of opposite wood. Actin (CL37Contig1) was employed as an internal control. All experiments were repeated three times with five duplicates

tension wood-forming tissues than in opposite wood (Fig. 5).

Ethylene was also considered to perform a function in tension wood formation, since the expression of poplar ACC oxidases evidences an asymmetrical expression pattern between tension wood and opposite wood (Andersson-Gunneråas et al. 2003). In this study, we isolated a total of five contigs for ethylene-response sensor 1 (CL138 Contig1), constitutive triple response 1 (CL1Contig5), EIN3-binding F-box protein 2 (CL35Contig1), ethylene transcription factor (CL437Contig1), ethylene-responsive transcription factor 4 (CL499Contig1), and five singletons for ethylene-response transcription factor RAP2-4 (TW10-1a-T3_G19), ethylene-response factor (TW3-2a-T3_A03, TW9-4a-T3_H18), ethylene-responsive protein(TW8-3a-T3_F05) and ethylene-induced calmodulin-binding protein (TW8-3a-T3_O13) (Table 4). Unlike auxin and BR, the expression of most ethylene-related genes except for three ethylene-response proteins (CL499Contig1, TW10-1a-T3_G19, TW9-4a-T3_H18) did not differ significantly between tension wood and opposite wood (Fig. 5). On the other hand, the ethylene receptor ERS1, which is a negative regulator of ethylene signaling (Liu et al. 2010), was downregulated significantly in the tension wood-forming tissues (Fig. 5), thereby implying the role of ethylene in the early stage of tension wood formation in response to mechanical bending.

Quantitative real-time RT-PCR of growth hormone-related genes helped us to identify the important genes that might be involved in the early stages of tension wood formation induced by mechanical bending; however, their exact roles for tension wood formation in response to mechanical bending have yet to be discovered. Further molecular characterization of the mechanical bending-responsive genes isolated herein will be necessary to better understand the roles of auxin, brassinosteroids and ethylene on tension wood formation and its associated cell wall modification at the molecular level.

Discussion

In this study, ESTs were constructed from specialized secondary xylem developed in the stems of 2-year-old yellow poplar via 6 h of mechanical bending treatment to isolate the functional genes associated with cell wall biosynthesis and modification during early stage of tension wood formation, i.e., growth promotion, upregulation of cellulose biosynthesis and downregulation of lignin biosynthesis with modifications of lignin monomeric composition. Since the yellow poplar is an evolutionally early angiosperm, only 1,733 ESTs were annotated (28.9%), and many useful genes involved in cell wall biosynthesis and xylem formation were obtained. GO classifications based on biological processes, cellular components and molecular functions demonstrate that a broad variety of genes were observed in the yellow poplar stem library, even though only 5,982 ESTs were sequenced and analyzed. The majority of the genes detected herein are the first representatives of their respective gene family for *Liriodendron tulipifera*. Prior to this EST project, the Floral Genome Project and BAC library constructed by Liang et al. (2007) generated a cDNA library from a floral tissues data set with approximately 6,000 unigenes and 182 genomic end sequences for *Liriodendron tulipifera*, respectively. However, very few nucleotide sequence entries have been deposited in the GenBank database for the genes involved in cell wall biosynthesis and the modification of the yellow poplar. With regard to the lignin-related gene, for example, only four full-length cDNA sequences encoding for

laccases and partial sequences for PAL, 4CL and CAD were available in the NCBI database. This EST study provides a total of 23 sequences associated with lignin biosynthetic genes including PAL, C4H, C3H, HCT, F5H, CCR, COMT, CAD, laccase and peroxidase; several of them appear to be xylem specific. Aside from lignin, many genes encoding for proteins associated with cell wall carbohydrate biosynthesis were also obtained, i.e., cellulose synthase, endo-1,3(4)- β-glucanase, endo-1,4-β-xylanase, polygalacturonase, α-glucosidase, β-glucosidase, endo-1,4-β-mannosidase protein, α-arabinofuranosidase, UDP-glycosyltransferase, fiber glycosyl hydrolase, (1-4)-β-mannan endohydrolase, starch branching enzymes, xyloglucan galactosyltransferase, xyloglucan endotransglucosylase, α-1,4-glucan-protein synthase, pectin methylesterase and pectinesterase. The sequences of these have not been previously provided in any public database prior to this project. Additionally, many genes for plant hormones such as auxin, gibberellic acid, sterol/brassinosteroid, ethylene, jasmonic acid, and ABA were also identified. Surprisingly, the numbers of genes in the signaling pathway of auxin and BR/Sterol were as high as 22 for each, while other types of hormones, including gibberellic acid (6), ethylene (10), jasmonic acid (2) and ABA (6), were <10. Many genes for secondary metabolites as well as transcription factors were also identified in our EST (data not shown), largely as full-length cDNAs. Therefore, our approach to the construction of ESTs from the stem of the yellow poplar upon mechanical bending was proved to be a powerful tool for the identification of genes not only for normal growth and development of the secondary xylem, but also for stress responses induced by mechanical bending treatment in the stem of the yellow poplar.

Because the objective of our research was to isolate genes that are involved in the structural and chemical modifications of cell wall associated with tension wood formation, i.e., growth promotion toward tension wood, increased frequency of fiber cell types, increased amount of cellulose with increased crystallinity, modification of hemicelluloses, and reduced lignin content with the alteration of monomeric composition, the expression levels of selected genes for cell wall carbohydrate, lignin and growth hormones was compared between the tension wood and opposite wood in the stem bent for 6 h at a 45° configuration. Even though the changes in gene expression upon mechanical bending cannot be explained in this study, quantitative real-time RT-PCR helps us to identify genes relevant to responses to mechanical bending treatment. Among the cell wall carbohydrate-related genes, xyloglucan endotransglucosylase (CL583Contig1), which has been shown to be upregulated significantly during cotton fiber elongation stage (Michailidis et al. 2009), was six times higher in tension wood-forming tissues than in opposite wood. Previous studies using transgenic poplar expressing fungal xyloglucanase have demonstrated that the constitutive degradation of cell wall xyloglucan contributed to the loosening of the cell wall, ultimately resulting in enhancements of growth and cellulose accumulation (Micheli 2001; Park et al. 2004). Therefore, xyloglucan endotransglucosylase may perform an important role in cell wall loosening prior to growth promotion at the early stage of tension wood formation. It is worth noting that the genes for the many cellulase and hemicellulases isolated from tension wood-forming tissues were homologous to those of cell wall-degrading microorganisms, thereby implying a role in the re-architecture of the cell wall structure during tension wood formation in response to mechanical bending. Therefore, the molecular function of isolated cell wall-modifying enzymes should be characterized further with regard to their role in many of the anatomical characteristics of tension wood, i.e., growth promotion with increases in the numbers of fiber cells under tensile stress conditions.

Our ESTs helped us to identify many of the genes in lignin biosynthetic pathways. Tissue-specific expressions of lignin biosynthetic genes permit us to identify genes that might be important for xylem development, as their transcripts were mainly detected from lignified stems. These include CL1 Contig6 (LtHCT1), CL170Contig1 (LtF5H1), CL492Contig1 (LtCCR1) and COMTs (CL83Contig1, CL83Contig2, CL145Contig1, CL570Contig1, TW3-1a-T3_G01 and TW12-4a-T3_H18). Interestingly, the expression of two COMTs (CL83Contig2, CL570Contig1) were reduced more significantly in the tension wood-like tissues than in the opposite wood after 6 h of mechanical bending treatment (Fig. 4), thereby implying a specific role for the downregulation of lignin biosynthesis with alterations of monomeric composition upon mechanical bending. Therefore, the functional characterization of these genes may provide us with some insights into the alteration of lignin biosynthesis during tension wood formation induced by mechanical bending at the molecular level.

High frequencies of auxin- and BR-related genes imply a role in tension wood formation and associated cell wall modification upon mechanical bending conditions. However, the majority of the auxin-related genes were downregulated in the tension wood as compared to the opposite wood, although the expressions of ARF1 (CL750Contig1) and auxin influx transport protein (TW6-1a-T3_K23) were twofold higher in the tension wood than in the opposite wood. Similarly, most of the BR-related genes, except for the LRR receptor kinase precursors, were downregulated in the tension wood as compared to the opposite wood. For example, positive regulators in the BR signaling pathway, i.e., BRL2, BES1/BZR1 and BIM2, were also downregulated significantly in the tension wood as compared to the opposite wood. Previously, Caño-Delgado identified three

members of the BRI1 family—BRL1, BRL2 and BRL3—as plasma-membrane localized steroid receptors in *Arabidopsis*, and proposed that only BRL1 and BRL3 may function specifically in provascular differentiation in *Arabidopsis* (Caño-Delgado et al. 2004), as a loss-of-function mutation in *brl1* induces abnormal phloem:xylem differentiation ratios and enhances the vascular defects of a weak *bri1* mutant. Additionally, a *bri1 brl1 brl3* triple mutant exhibits abnormal vascular differentiation with enhanced *bri1* dwarfism (Caño-Delgado et al. 2004). More recently, Ceserani et al. (2009) has proposed that BRL2 may also function in vascular development in the leaf, based on the mutant phenotype of BRL2/VH1. Additionally, the insertion mutant in VIT (VH1-interacting TPR-containing protein) and VIK (VH1-interacting kinase), two BRL2 interacting proteins, evidences altered sensitivity not only to brassinosteroids but also to auxin (Ceserani et al. 2009). As the size of the cells in the tension wood is much smaller than in the opposite wood in general (Jin and Kwon 2009), the downregulation of auxin- and BR-related genes may be correlated with the reduced size of fiber cells in the tension wood, thereby implying the possible involvement of auxin and BR in tension wood development. Whether or not the significant downregulation of auxin and BR is correlated with complex structural modification during tension wood formation, i.e., increased fiber cell frequency with reduced fiber cell size, than the eccentric growth promotion in tension wood as compared to the opposite wood should be dissected at the molecular level.

Despite the small number of ESTs sequenced herein, however, many of the useful genes for cell wall biosynthesis and modification, cellulose/hemicelluloses and lignin biosynthetic genes were identified. Although the exact functions of genes that were expressed differently between the tension wood and opposite wood cannot be correlated directly with the aspects observed in the tension wood only by comparing their expression levels, and quantitative RT-PCR allows us to identify genes that might function as essential regulators in the early stage of cell wall modification during tension wood formation. The further precise characterization of these genes is expected to provide a tool for understanding the underlying mechanism of tension wood formation and associated cell wall modification.

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