



Analysis of genes related to xylem cell wall development based on transcriptomics in *Populus alba* 'Berolinensis' tension wood

Lin Feng¹ · Youchao He¹ · Xu Li¹ · Meiqi Zhou¹ · Chao Wang¹

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Abstract *Populus alba* 'Berolinensis' is a fast-growing, high-yielding species with strong biotic and abiotic stress resistance, and widely planted for timber, shelter belts and aesthetic purposes. In this study, molecular development is explored and the important genes regulating xylem formation in *P. alba* 'Berolinensis' under artificial bending treatments was identified. Anatomical investigation indicated that tension wood (TW) was characterized by eccentric growth of xylem and was enriched in cellulose; the degree of lignification was lower than for normal wood (NW) and opposite wood (OW). RNA-Seq-based transcriptome analysis was performed using developing xylem from three wood types (TW, OW and NW). A large number of differentially expressed genes (DEGs) were screened and 4889 counted. In GO and KEGG enrichment results, genes involved in plant hormone signal transduction, phenylpropanoid biosynthesis, and cell wall and secondary cell wall biogenesis play major

roles in xylem development under artificial bending. Eight expansin (*PalEXP*) genes were identified from the RNA-seq data; four were differentially expressed during tension wood formation. Phylogenetic analysis indicated that *PalEXLB1* belongs to the EXPB subfamily and that the other *PalEXPs* are members of the EXPA subfamily. A transcriptional regulatory network construction showed 10 transcription factors located in the first and second layers upstream of EXP, including WRKY, ERF and bHLH. RT-qPCR analysis in leaves, stems and roots combined with transcriptome analysis suggests that *PalEXPA2*, *PalEXPA4* and *PalEXPA15* play significant regulatory roles in cell wall formation during tension wood development. The candidate genes involved in xylem cell wall development during tension wood formation marks an important step toward identifying the molecular regulatory mechanism of xylem development and wood property improvement in *P. alba* 'Berolinensis'.

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✉ Chao Wang
wangchao@nefu.edu.cn

¹ State Key Laboratory of Tree Genetics and Breeding, Northeast Forestry University, 150040, Harbin, People's Republic of China

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Introduction

As an important renewable resource, wood provides a large number of ecological services and biomass products. Wood formation depends on the growth of secondary xylem, which involves several processes, including cell division and expansion, secondary wall deposition, lignification, and programmed cell death (Mellerowicz et al 2001; Zhang et al 2020). Cellulose, lignin, hemicellulose and several proteins are the main components of cell walls (Rennie and Scheller 2014).

With regards to the bent or curved portions of woody dicotyledonous plant stems or branches, xylem tissue formed

on the outside due to tensile force is called tension wood (TW), making the upper side of the stems or branches of trees thicker and eccentric to a certain extent (Timell 1969). Opposite wood (OW) refers to wood on the inner side of the bent wood (Pilate et al 2004a). Wood without any irregularities is termed normal wood (NW). Due to external forces, many plants produce a gelatinous fiber layer (G layer) inside tension wood cell walls, and its main component is crystalline cellulose (Pilate et al 2004b). Compared with normal wood, its appearance replaces the S3 layer of secondary cell walls (Andersson-Gunnerås et al 2006). Its most remarkable feature is that the cellulose content increases, making the cell wall thicker, and the lignin and xylan contents less. Anatomically, it has been found that the amount and size of vessels in tension wood are fewer, and fibers and vessels are longer. Certain chemical attributes have the potential to enhance the effectiveness of wood conversion for biofuel and material manufacturing (Yu et al 2021), but their physical properties are affected due to the presence of gelatinous fibers, making them unfavorable for fiber board (Vilkovská et al 2018). Improving the understanding of the molecular and genetic regulatory mechanisms involved in tension wood formation can facilitate strategic improvements in wood fiber and energy utilization and play a key role in promoting wood engineering to produce materials and energy more effectively.

Populus is a genus of fast-growing species and identifying the molecular mechanism of wood formation is of considerable significance for the improvement of timber resources. RNA sequencing (RNA-seq) has been used to explore the differentially expressed genes during tension wood development in various poplar species such as *P. trichocarpa* Torr. & A. Gray ex Hook. (Yu et al 2021), *P. × euramericana* ‘Zhonglin46’ (Liu et al 2023), *P. davidiana* × *bolleana* ‘Shanxin’ (Zhao et al 2021), and *P. × tomentosa* Carr. (Chen et al 2015). These studies have identified key genes involved in wood formation that will directly or indirectly regulate tension wood development (Jin et al 2011; Chen et al 2015). Some of the key genes have been identified, including those involved in hormone regulation, transcription factors (TFs), lignin and cellulose biosynthesis enzymes, and other potential regulatory genes. For example, castasterone, one of several brassinosteroids (BRs), has a low expression in the xylem of opposite wood (Jin et al 2020). Two TFs, *PtrHSFB3-1* and *PtrMYB092*, have been identified as mediators of transcriptional reprogramming during tension wood formation. They interact with regulatory DNA sequences to specifically regulate the biosynthesis of secondary cell walls (Liu et al 2021). As one of the highly expressed genes in tension wood, fasciclin-like arabinogalactan (FLAs) were highly concentrated on the inner side of the G layer, possibly exercising a specific function in structural formation (Ma et al 2023). The PtFLA6 proteins, which are part of the

fasciclin-like arabinogalactan protein family, were found to be predominantly accumulated in the xylem tissues of the tension wood (Wang et al 2017). The *PtCesA* gene, which is involved in cellulose biosynthesis, may be transcriptionally regulated during tension wood development under stress (Wu et al 2000). The low expression of cinnamyl alcohol dehydrogenase (CAD) protein, a key enzyme in lignin biosynthesis, leads to inhibition of lignin monomer biosynthesis and lower lignin content in tension wood (Xiao et al 2021a). Most studies on tension wood have focused on *FLA* and *CesA* or genes involved in lignin biosynthesis, but regulation mechanisms of eccentric growth and changes in vessel size have not been explained. Research on the participation of expansin (EXP) in secondary growth is increasing (Wang et al 2011) and *EXP* gene may be involved in the regulation of vessel and fiber development during tension wood formation under bending stress. *PttEXP1* identified from hybrid aspen was specifically expressed in mature stems exhibiting secondary growth and found in the cambium and radial expansion region. In addition, *PttEXP5* is similar to *PttEXP1* and is the major expansin in tension wood development (Gray-Mitsumune et al 2004).

As a cell wall protein that induces extension and expansion (Wang et al 2011), EXP was first isolated from cucumber seedlings (McQueen-Mason et al 1992), and its activity was regulated by pH (Cosgrove 2000; Artzi et al 2016). It includes four main subfamilies, α -expansin (EXPA), β -expansin (EXPB), expansin-like A (EXLA) and expansin-like B (EXLB) (Kende et al 2004). *EXPA* subfamily genes are involved in regulating acid-induced cell wall relaxation, *EXPB* subfamily genes are related to pollen tube growth, and *EXLA* and *EXLB* genes may be related to cell wall modification (Cosgrove 2015). To date, the functions of some family members have been verified. For example, *GhEXPA8a* and *GhEXP8g* promote the elongation of fiber cells in upland cotton (*Gossypium hirsutum* L.) (Lv et al 2020). *DsEXLA2* overexpressed transgenic plants exhibited higher heights, thicker stems, larger leaves, less epidermal hair numbers and smaller stomatal apertures compared to controls. Cellulose content in the stem of transgenic plants increased, and cell walls were thickened significantly (Li et al 2022). In addition, *EXP* genes can also respond to abiotic stress by changing the tightness of cell walls, a process accompanied by lignin biosynthesis (Feng et al 2019). As a key wall loosening factor involved in the growth of primary walls (Cosgrove 2005), *EXP* gene has also been found to play a role in the formation of secondary walls (Wang et al 2011). However, research on the *EXP* gene is not extensive, although molecular mechanism studies on wood traits regulated by the *EXP* gene have important value.

There are a number of new varieties of *Populus* with excellent characteristics (Clifton-Brown et al 2019). *Populus alba* ‘*Berolinensis*’ is a hybrid of *P. alba* as the female

and *P. Berolinensis* Dippel as the male and has excellent qualities for both paper-making and bioenergy (Wang et al 2008). *P. alba* 'Berolinensis' at five-years meets the requirements for paper-making in terms of pulping performance, chemical composition, fiber morphology and other indicators (Wang et al 1999). It has rapidly become the most widely used variety of *Populus* in China because of its tolerance to cold and drought. Therefore, the identification of high-quality genes related to wood formation in *P. alba* 'Berolinensis' is of considerable significance for poplar wood improvement in semi-arid northeast China. In this study, the gene expression pathways regulating the development of tension wood were analyzed using transcriptome sequencing technology; the genes involved in the development of cell walls were screened. Eight *EXP* genes (*PalEXPs*) were screened in RNA-seq data, four were differentially expressed between TW, OW and NW. The bHLH, WRKY transcription factors were identified as upstream factors of *PalEXP* genes by transcriptional regulatory network construction, which revealed the molecular regulation mechanism in the formation of tension wood and provided information and gene resources for the improvement of wood properties using molecular biological methods.

Materials and methods

Plant materials and experimental treatment

Four-year-old clonal *P. alba* 'Berolinensis' saplings were obtained from the breeding base of Improved Variety Bases in Fulaerji District, Qiqihar, Heilongjiang province. The materials were cultivated outdoors (average temperature range 17–27 °C). The stems of the treatment group were bent in a lean angle of 45° in June (the period of active cambium cells and the developing xylem). Straight stems were used as the control (Fig. S1). Five saplings were pooled into one sample with three replicates. A total 15 saplings were used for bending and another 15 were the control. After two weeks, stems of similar heights above the ground were removed from both groups. The bark was peeled and the xylem gently scraped away with a scalpel and collected. The upper side of the bent stems was removed and labelled as TW (tension wood), the lower side as OW (opposite wood), and the developing xylem from the straight stems was collected as NW (normal wood). All samples were pre-cooled in liquid nitrogen and stored at –80 °C for subsequent transcriptome sequencing and quantitative reverse transcription polymerase chain reaction (RT-qPCR) experiments. Stem samples of 5 cm were used for anatomical analysis.

Anatomical analysis

A microscope slide was prepared of 3 cm of stem and fixed in FAA fixative (10% formalin, 5% acetic acid, 50% ethanol). To observe the developing xylem, the fixed samples were sectioned with a sliding microtome (Leica 1007, Wetzlar, Germany). Paraffin sections were prepared with sequential steps of dehydration, transparency, wax dipping, embedding, block trimming, slicing, sticking and dewaxing. The samples were stained with safranin O-fast green or by the Wiesner (phloroglucinol-HCl) reaction (Pradhan Mitra et al. 2014; Chi et al 2023) and photographed under a stereo light microscope (Olympus SZX7, Tokyo, Japan) and an optical microscope (Olympus BX43, Tokyo, Japan).

RNA sequencing, de novo transcriptome assembly and assessment

The total RNA of nine samples (NW1, NW2, NW3, TW1, TW2, TW3, OW1, OW2, and OW3) was extracted by the CTAB method (Zhao et al 2012) and assessed using 1.0% agarose gel electrophoresis. The A260/280 ratio was measured by a NanoDrop 2000 UV–visible spectrophotometer (Thermo Scientific, Waltham, MA, USA). A library of standard-compliant RNA was constructed using the NEBNext® Ultra™ RNA Library Prep Kit, and the constructed library was sequenced on an Illumina® platform (New England Biolabs, Ipswich, MA, USA). Based on the filtered clean data, a full-length transcript sequence was assembled with Trinity (Trinity Release v2.4.0) (Grabherr et al 2011). The longest transcript sequence in each gene was taken as the unigene. All transcripts assembled by Trinity and unigene were evaluated by BUSCO to determine their integrity (Simão et al 2015). Based on the unigene sequence assembled by Trinity and the ORF sequence predicted by TransDecoder, the annotation information of the assembly result in the corresponding database was obtained by Tools including Blast (<http://www.ncbi.nlm.nih.gov/BLAST/>), Hmmscan (Finn et al 2011), SignalP (Petersen et al 2011), and TmHMM (Krogh et al 2001), and the comprehensive functional annotation results were then obtained through Trinotate (Trinotate Release V3.0.2). The RNA-seq data were upload to the NCBI Short Read Archive (SRA) with the accession number PRJNA954564.

Expression level analysis and DEGs identification

FPKM was used for the quantitative estimation of unigene expression values (Trapnell 2010). Differential genes with \log_2 -fold change ≥ 1 and $q < 0.05$ were selected by DEG-seq (Wang 2010) as differentially expressed genes (DEGs). The data were normalized using the negative binomial distribution statistical method, and the resulting *P* value was subjected to

multiple test correction to control false positives according to the Benjamini–Hochberg procedure.

Gene function annotation, GO, KEGG analysis and identification of DEGs related to cell wall formation

The genes were compared in NR (NCBI nonredundant protein sequences), NT (nucleotide sequence database), and UniProt (universal protein resource) databases, and the function and characteristics of known nucleotide sequences were recorded. All DEGs were mapped to specific terms within the gene ontology (GO) database (<http://www.geneontology.org>). Subsequently, the number of genes associated with each was determined. GO enrichment analysis was performed by the topGO R package to identify significantly enriched terms. KOBAS 3.0 software (<http://kobas.cbi.pku.edu.cn/index.php>) tested the statistical enrichment of DEGs in the Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.kegg.jp/>) pathways (Xie et al 2011). To visualize data, the Heatmap plug-in on the TBtools platform was used to draw heatmaps (Chen et al 2020).

RT–qPCR analysis

SYBR®Green Real-time PCR Master Mix (Toyobo, Co., Ltd., Osaka, Japan) were used for RT–qPCR experiments as described (Gang et al 2019). The reference gene was 18S rRNA (Li et al 2021). The primers used are listed in Table S1. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of each transcript (Schmittgen and Livak 2008). Five saplings were selected for three wood types (TW, OW, NW) and multi-sample mixing was performed to achieve biological repetitions with three technical replicates.

Bioinformatics analysis of the PalEXP family genes

Sequences of *PalEXP* family genes are listed in Supplementary file7. ExPASy website (<http://web.expasy.org/cgi-bin/protscale/>) was used to analyze the hydrophilicity of the PalEXPs (Schneider et al 2004). To predict the *PalEXP* family gene transmembrane region, TMHMM Server v. 2.0 (<https://services.healthtech.dtu.dk/services/TMHMM-2.0/>) has been used (Krogh et al 2001). The protein sequence was imported into BioEdit software 7.0.1 for multisequence alignment (Tippmann 2004) and the phylogenetic analysis of EXP proteins carried out by MEGA 6.0 software (Hall 2013).

Construction of transcriptional regulatory network of EXP upstream TFs

According to Jia et al (2022), a transcriptional regulatory network was constructed using a partial correlation

coefficient-based algorithm. The differentially expressed genes (DEGs) from all comparison groups were divided into two categories: (1) regulatory genes, including genes encoding transcription factors (TFs), and (2) structural genes. The *PalEXP* genes were utilized as the foundational genes to build the regulatory network. The relationship between TFs (derived from the regulatory genes in the DEGs) and *PalEXP* genes was determined using the partial correlation coefficient-based algorithm (Mukaka 2012).

Results

Anatomical characteristics of TW, OW and NW

Slides with paraffin sections were stained with safranin O-fast green or by the Wiesner (phloroglucinol-HCl) reaction. By observing the distance between the cambium and the annual ring of the previous year, it was found that bending produced eccentric growth in the leaning trunks, and the width of the tension wood was significantly higher than that of NW and OW. In addition, the number of vessels decreased, and the lumen was smaller in xylem (Fig. 1).

The staining results show that the tension wood cell walls were rich in green (Fig. 1A). The developing xylem cells near the cambium on the outer side of the TW, OW and NW were also green (Figs. 1A, D, B, F, C and H), indicating cellulose accumulation but lower lignin deposition. The mature xylem cells near the previous annual ring were stained red in the OW and NW (Figs. 1G and I) but were still green in TW (Fig. 1E). This indicates that the tension wood was rich in cellulose and higher than in OW and NW. The degree of lignification in OW and NW was higher than in TW. The existence of G-fibers, characteristic of TW markers, was found in mature tension wood stained green (Fig. 1E). The results of Weisner staining showed lighter red in TW and darker in OW and NW, indicating that the G-layer lacked lignin deposition and that the lignin in OW and NW was higher than in TW. (Figs. 1A', B', C', E', G' and I').

Transcriptome assembly and functional annotation of the unigenes in TW, OW and NW

Some 142,819,912 (TW), 146,012,770 (OW), and 152,134,284 (NW) raw reads were generated by RNA-seq. After filtering, 133,843,072 (TW), 133,709,274 (OW), and 141,165,714 (NW) clean reads with high quality were obtained. The Q30 values were above 90% in all samples and the data amount and base content were used for further analysis (Table S2). The proportion of clean reads for each sequence sample reflects the quality of sequencing data, and the analysis results show that the proportion of clean reads was close to 90%, indicating

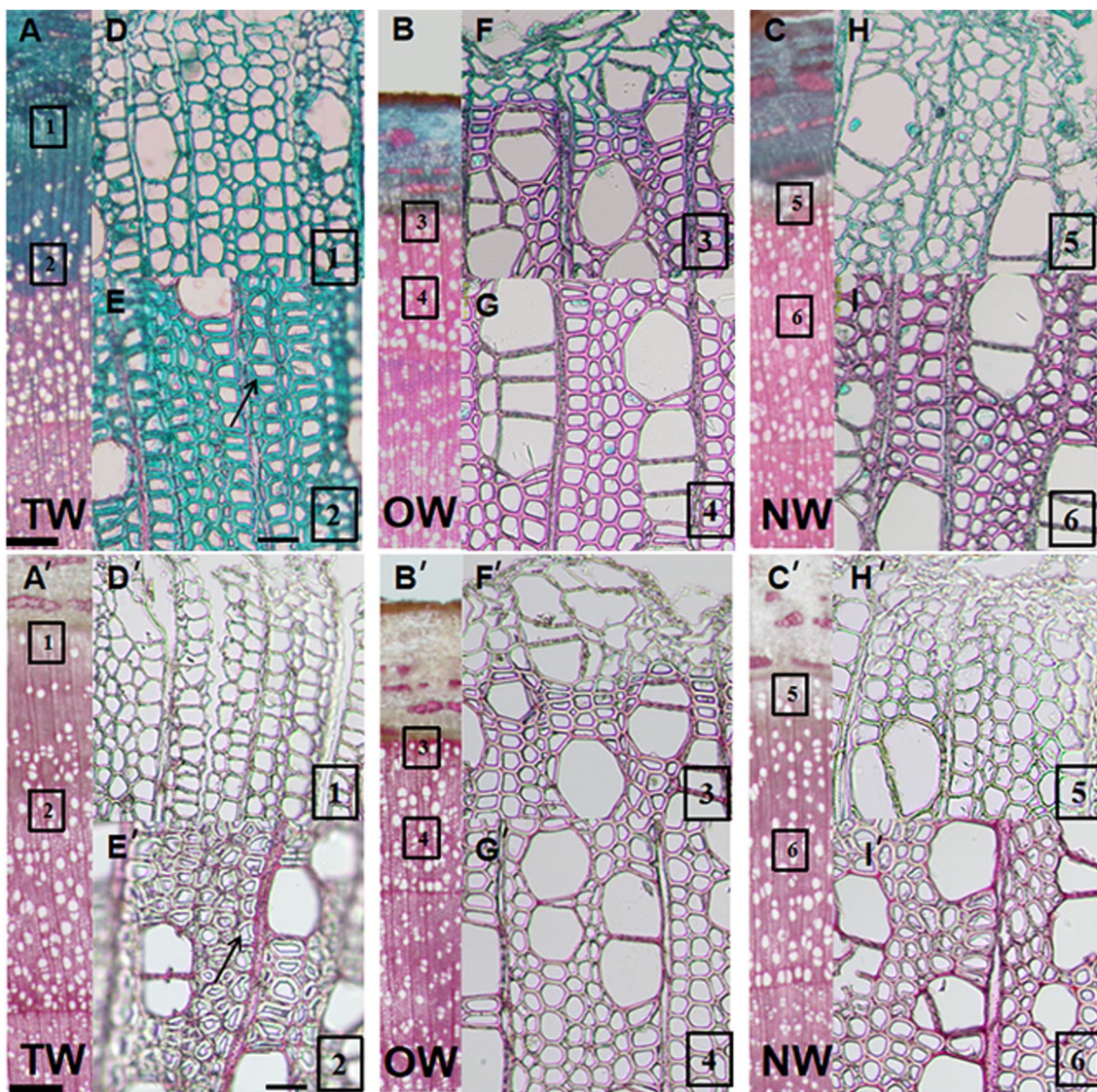


Fig. 1 Anatomical observations on TW, OW and NW in *P. alba* 'Berolinensis' A, B, C, D, E, F, G, H, I: safranin O-fast green in which cellulose-enriched cell walls are green and lignified cell walls red; A', B', C', D', E', F', G', H', I': Wiesner (phloroglucinol-HCl) reaction; lignin-enriched cell walls are red; A, B and C: sliding sec-

tions, scale bar=500 μ m; D, E, F, G, H, I: paraffin sections equivalent to the position of the number in A, B and C, scale bar=50 μ m; numbers with black boxes indicate the developing or mature parts of the xylem; the black arrow points to G-layer; the red arrow points to annual ring of the previous year

good quality data (Fig. S2). Altogether, 60,712 unigenes were obtained from the assembly results, of which the GC content was 39.9%. The length distribution of the assembled unigenes was as follows: the average length of the unigenes was 1017.09 bp, and the length of N50 was 1688 bp. Among these unigenes, 41,043 (67.6%) were > 400 bp in length, 21,706 (35.7%) were > 1000 bp, and 8666 (14.3%) were > 2000 bp (Table S3). BUSCO evaluation was conducted on all transcripts and unigenes assembled by Trinity. The number of missing BUSCOs was low, with a core protein alignment of 93.6%, annotation rate

of 75.0%, and reads utilization rate of 75.5%. Accuracy, integrity, and core protein comparison evaluations indicated good transcriptome assembly quality (Table S3).

To characterize the gene functions involved in cell wall development in *P. alba* 'Berolinensis', the unigenes were annotated against public databases, including UniProt, NR, and NT. Annotated unigenes were counted, and the annotation of each database is shown using an UpSetR plot. The results showed that 18,382 genes were common to all databases (Fig. S3).

Expression level analysis of unigenes and identification of DEGs

The fragments per kilobase per million (FPKM) method estimates gene expression levels using RNA-Seq (Trapnell 2010). In this study, the expression levels of unigenes in each sample were analyzed, and boxplots used to visualize the distribution of expression levels. The overall expression distribution was found to be similar across all samples, with only a small percentage of genes showing differential expression (Fig. S4). To investigate key genes involved in the regulation of xylem development during the formation of tension wood, differentially expressed genes (DEGs) were identified based on $q < 0.05$ and $\log_2(\text{ratio}) \geq 1$ in the transcriptome data. A total of 4489 DEGs were identified in three comparisons. In the comparison between OW and NW, there were 1563 upregulated and 373 downregulated transcripts. In the comparison between TW and NW, there were 1407 upregulated and 507 downregulated transcripts and between TW and OW, there were 1326 upregulated and 1840 downregulated transcripts (Fig. S5A).

An upset plot was constructed to intuitively reflect the common and unique DEG numbers of each comparison group of the TW, OW and NW transcriptomes. There were 423 common DEGs between the OW vs. NW and TW vs. NW groups, 1123 between the TW vs. NW and TW vs. OW groups, and 139 DEGs common to all three groups. These results suggest that these 423 DEGs shared by OW vs. NW and TW vs. NW might be responsive to bending treatment stimulation, and the 1123 DEGs common to TW versus NW and TW versus OW might be involved in the development of tension wood (Fig. S5B).

GO enrichment analyses of DEGs

To analyze the biological function of DEGs involved in tension wood development, GO enrichment analyses were carried out. The figure shows that DEGs in GO terms, including cell wall organization or biogenesis and secondary cell wall biogenesis (biological processes), xylan O-acetyltransferase activity (molecular functions), and cell wall (cellular components), were enriched among the three groups. It is noteworthy that in the TW versus NW group, the DEGs were significantly highly enriched in cell wall organization or biogenesis, plant-type secondary cell wall biogenesis and hemicellulose metabolic process, among others (Fig. 2). In conclusion, the DEGs from TW versus NW and TW versus OW were enriched more in GO terms related to cell wall and secondary wall metabolic and biosynthesis processes, which suggests that transcripts involved in plant cell wall development play significant roles during the formation of tension wood in *P. alba* 'Berolinensis' and that these DEGs are key regulatory factors.

KEGG enrichment analyses of DEGs

KEGG enrichment analysis further revealed the gene regulatory pathways in developing xylem under bending stress. The DEGs in all three comparison groups were significantly enriched in the plant hormone signal transduction and phenylpropanoid biosynthesis pathways (Fig. 3), suggesting that these pathways played important roles in *P. alba* 'Berolinensis' xylem development in response to artificial bending.

Expression analysis and identification of DEGs related to cell wall formation

According to previous studies on genes associated with cell walls and through conducting GO and KEGG enrichment analyses, we identified DEGs associated with the formation of the cell wall in the transcriptomes of TW, OW, and NW. Most DEGs involved in lignin biosynthesis, like *POD* (peroxidase), *CCR* (cinnamoyl-CoA reductase), *PAL* (phenylalanine ammonia-lyase), *CAD* and *CYP450* (cytochrome P450), were downregulated in TW vs. OW (Fig. 4A). Transcript levels of most DEGs encoding *CesA* (cellulose synthase) and *SuSy* (sucrose synthase), which are involved in cellulose synthesis, increased in TW vs. OW (Fig. 4B). Among the *CesA* transcripts, homologue of *AtCesA8* (DN19943_c0_g3 and DN23646_c3_g1) were upregulated in TW vs. OW. Most DEGs involved in MAPK signaling pathway were upregulated in OW versus NW, downregulated in TW vs. OW. *PR* (pathogenesis-related protein) was significantly regulated among them (Fig. 4C). Based on the information provided, the expression levels of *PEs* (pectinesterases) were downregulated in the development of tension wood cell walls compared to opposite wood. *FLAs* (fasciclin-like arabinogalactan proteins) were upregulated in TW compared to NW and OW. *EXPs* were downregulated in OW compared to NW (Fig. 4D). In the process of plant hormone signal transduction, transcripts encoding *AUX* (auxin influx carriers), *LAX* and *CCND3* (cyclin D3 protein) were upregulated in TW vs. OW and TW vs. NW. Transcripts encoding *NPR3-like*, *AXR1* and *BRUI-like* were downregulated in TW compared with OW and NW (Fig. 4E). In total, 21 TFs shared among the three comparative groups were identified, including bHLH, ERF, WRKY, MYB, and NFY. These TF genes are mainly up-regulated in OW or TW compared to NW, indicating their involvement in the transcriptional regulation of xylem development in response to bending stimuli (Fig. 4F). These results indicate that the identified transcripts and TFs play significant roles in developing xylem cell walls in response to artificial bending.

To validate the transcriptome analysis results, RT-qPCR analysis was carried out to assess the expression levels of genes associated with cell wall synthesis, including *PAL* (TRINITY_DN21468), *PalCesA*

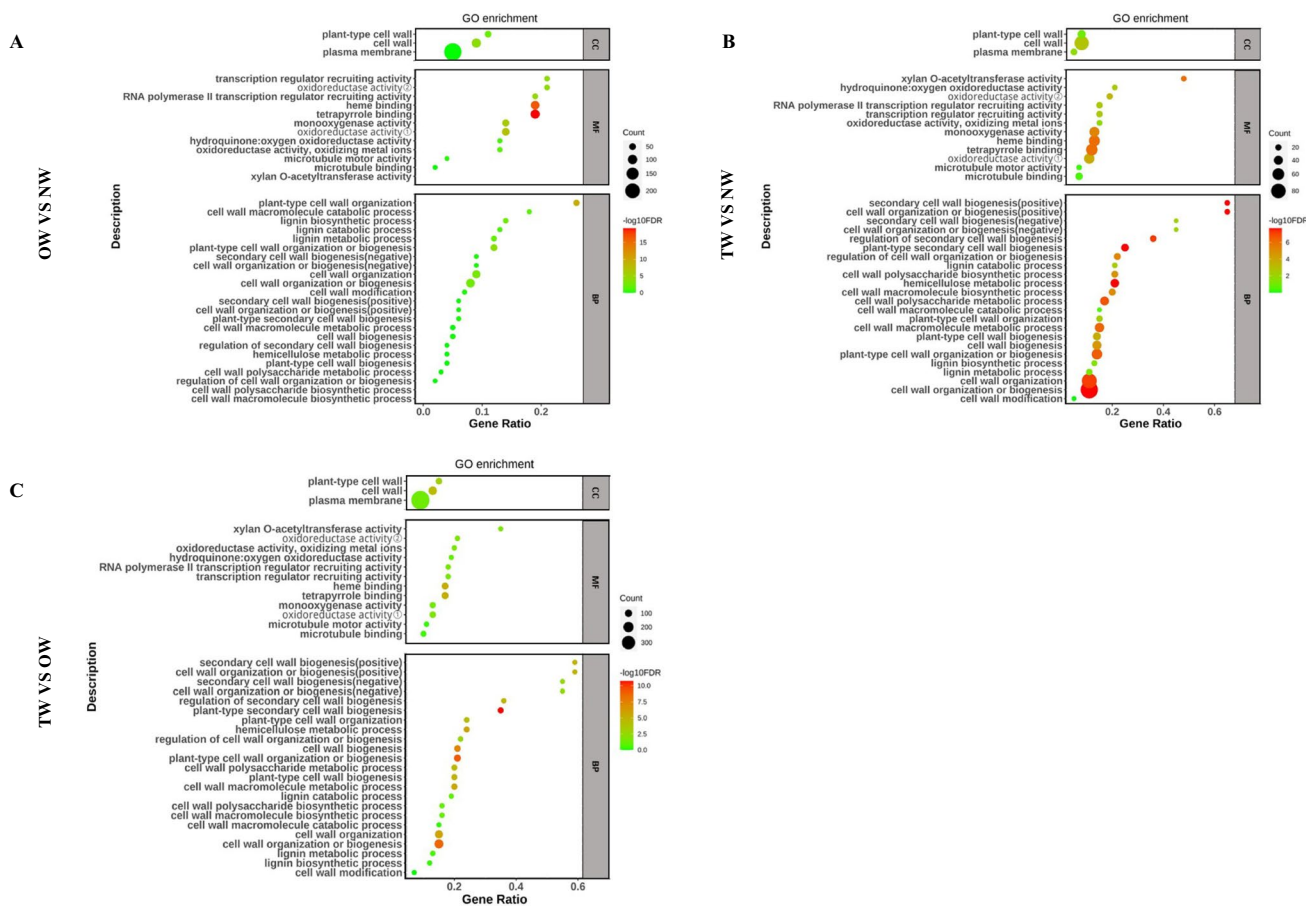


Fig. 2 GO functional enrichment analysis of DEGs in *P. alba* 'Berolinensis'. GO analysis of DEGs identified in OW vs. NW (A), TW versus NW (B), and TW versus OW (C). The x-axis represents the transcript ratios, and the y-axis the term enriched in BP (biological

process), MF (molecular function) and CC (cellular component). The size of the point represents the transcript count; the color of the point represents the *p* value

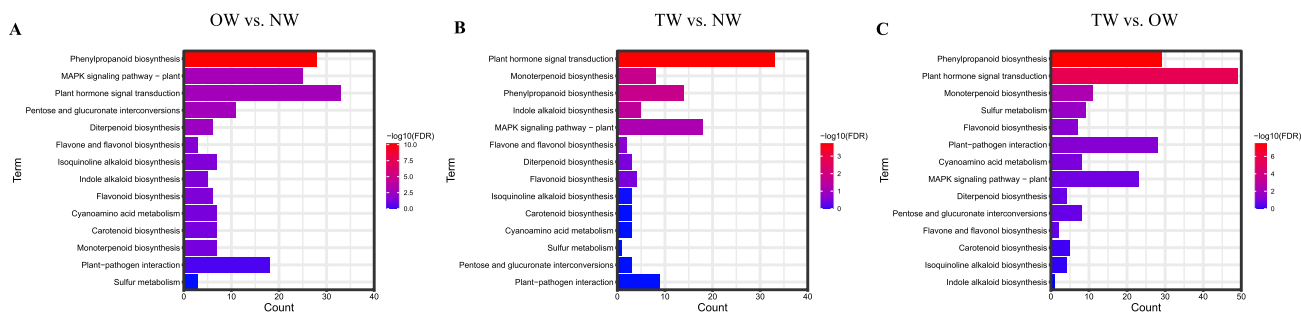


Fig. 3 KEGG pathway involved in the DEGs of *P. alba* 'Berolinensis'. KEGG analysis of DEGs identified in OW versus. NW (A), TW versus NW (B), and TW versus OW (C)

(TRINITY_DN23646), *FLA* (TRINITY_DN20133), *EXPA2* (TRINITY_DN7305_c0_g1), *EXPA4* (TRINITY_DN16617_c0_g3), *EXPA15* (TRINITY_DN15609_c1_g2), and *EXLB1* (TRINITY_DN8270_c0_g1). These genes exhibited differential expression between TW, OW, and NW transcriptomes. The results demonstrate a

consistent expression pattern for *PAL*, *CesA*, *FLA*, and *EXP* genes in TW, OW, and NW when analyzed by both RT-qPCR and RNA-seq methods (Fig. 5).

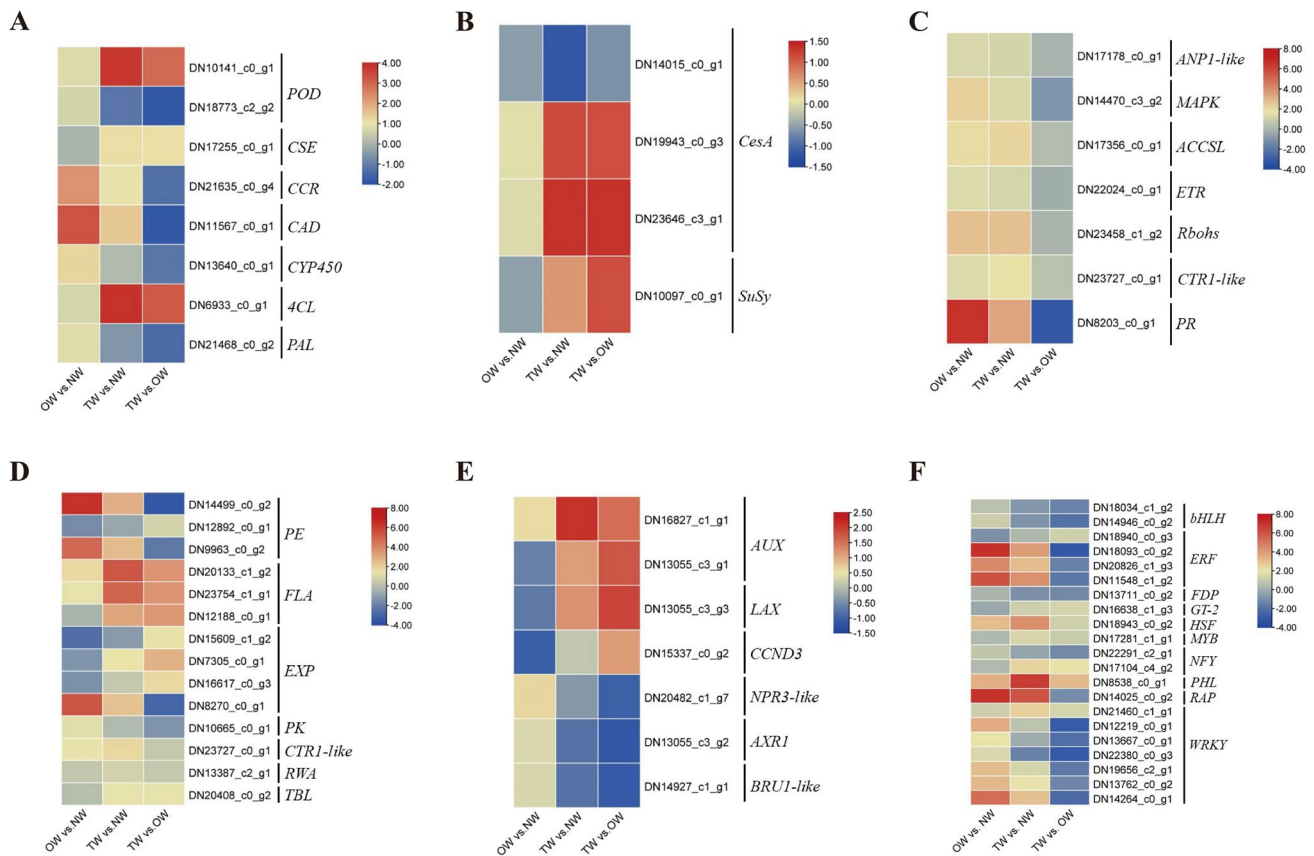


Fig. 4 Candidate transcripts in response to artificial bending in *P. alba* 'Berolinensis'. Heatmaps (A–F) show the expression of the DEGs identified in lignin biosynthesis (A), cellulose biosynthesis (B), MAPK signaling pathway (C), development of cell wall (D) plant hormone signal transduction (E) and transcription regulation (F) in response to artificial bending. Rows represent different tran-

scripts and columns three kinds of groups (OW vs. NW, TW vs. NW, and TW vs. OW). The expression levels of transcripts are represented by squares, with red indicating up-regulation and blue indicating down-regulation. Differentially expressed genes (DEGs) were identified based on $q < 0.05$ and $\log_2 \text{ratio} \geq 1$, and then aligned with databases using the BLASTx algorithm at an E-value cutoff of 10^{-5}

Identification and characterization of PalEXP family members using transcriptome data

Eight EXP family genes of *P. alba* 'Berolinensis' were screened from the transcriptome data. The protein lengths of PalEXPs ranged from 249 to 274 aa, with molecular weights from 26.59 to 30.12 kDa. The isoelectric points varied between 6.71 and 9.62, indicating that most of these proteins were hydrophilic (Table S4). By comparing and analyzing the amino acid sequences of the eight PalEXPs, it was observed that there were obvious conserved structural features between the sequences and that the C-terminal domains are relatively conserved (Fig. S6).

Phylogenetic analysis of EXP proteins

To explore the phylogenetic relationship of PalEXPs genes, a phylogenetic tree was constructed using EXP proteins from three species, *P. alba* 'Berolinensis', *P. trichocarpa*, and *A. thaliana* (Fig. 6). The results show that all the EXP proteins

were clustered into four subfamilies (EXPA, EXPB, EXLA and EXLB) and named as PalEXPA2, PalEXPA3, PalEXPA4, PalEXPA6, PalEXPA13, PalEXPA15, PalEXPA16 and PalEXLB1 with *Arabidopsis* EXP proteins as reference according to the phylogenetic tree. Only PalEXLB1 was clustered into the EXLB subfamily, and the remaining PalEXPs proteins belonged to the EXPA subfamily.

Construction of transcriptional regulatory network of EXP upstream transcription factors

Eight PalEXP genes were identified, and four had different expressions between TW, OW and NW according to the comparative transcriptome analysis (Fig. 5). By identifying DEGs encoding TFs in each group, the transcriptional regulatory factors that control the expression of the PalEXPs can be identified (Fig. 4F). In total, 21 TFs are shared among the three comparative groups and a transcriptional regulatory network was constructed with four differentially expressed PalEXPs using partial correlation coefficient algorithm. In

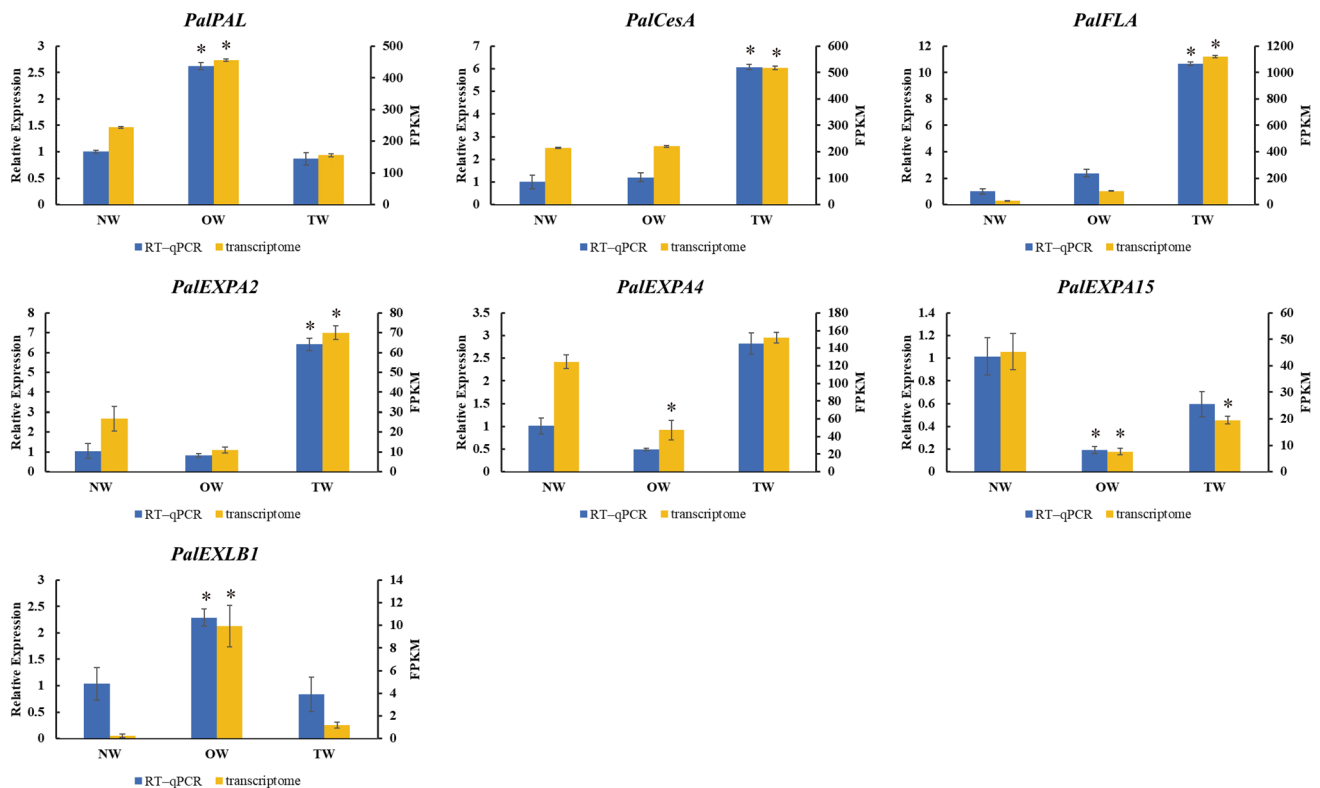


Fig. 5 Expression pattern validation of the RNA-seq DEG results by RT-qPCR of *PalPAL*, *PalCesA*, *PalFLA*, *PalEXPA2*, *PalEXPA4*, *PalEXLB1* and *PalEXPA15*. The expression was normalized to the *tubulin* gene. Relative expression levels of candidate transcripts were

calculated by the $2^{-\Delta\Delta Ct}$ method. Data are mean values \pm standard error. Asterisks indicate statistically significant differences compared to the ratio obtained by RT-qPCR alone (t-test; $p < 0.05$)

this network, there were 10 TFs involved in the regulation of *PalEXPs*, and most belonged to WRKY, ERF, bHLH families (Fig. 7). Five of these are located in the first layer of the regulatory network, and they indirectly regulate *EXPs* by regulating five TFs in the second layer. These results show that these TFs are involved in the transcription regulation of *PalEXPs* when the xylem received external bending signals. The transcriptional regulation relationship needs further verification.

Tissue-specific expression analysis of *PalEXP* genes

The results of the RT-qPCR analysis revealed distinct expression patterns of the *PalEXP* genes in different tissues of *P. alba* 'Berolinensis'. *PalEXPA2*, *PalEXPA4*, and *PalEXPA15* exhibited significantly higher expression levels in leaves compared to roots and stems. The expression levels were the lowest in roots. *PalEXPA6* and *PalEXPA3* showed high expression levels in stems and leaves, but relatively lower levels in roots. *PalEXPA16* and *PalEXPA13* exhibited slightly higher expression levels in stems compared to leaves. The highest expression levels of all four genes were

shown in stems. *PalEXLB1* was mainly expressed in roots (Fig. 8).

Discussion

Tension wood system to screen important regulatory genes for cell wall development

As a specialized xylem tissue, the formation of tension wood depends on gravitational stimulus or mechanical stresses (Yu et al. 2021), and this system is also a helpful tool to gain insights into the molecular mechanism of xylem formation and screen key factors regulating xylem cell wall development (Andersson-Gunnerås et al. 2006; Chen et al. 2015; Liu et al. 2021). A total of 214, 1280, and 32 differentially expressed genes (DEGs) were identified in TW vs. NW, OW vs. NW, and TW vs. OW in the rubber tree (*Hevea brasiliensis*), respectively and identified candidate genes and TFs likely involved in phenylpropanoid biosynthesis (Meng et al. 2021). In *Populus tomentosa*, 361 genes were differentially transcribed between TW and OW, 2658 between OW and NW, and 2417 between TW and NW, indicating that normal

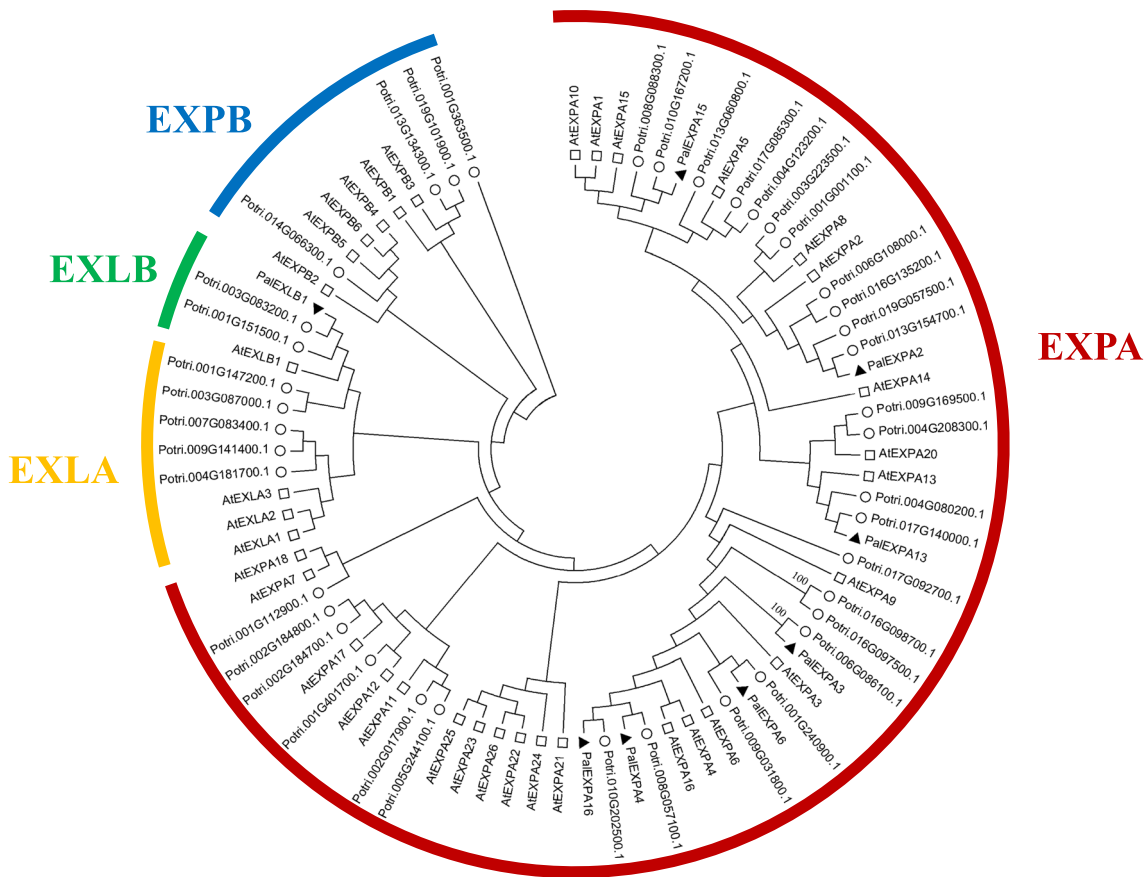
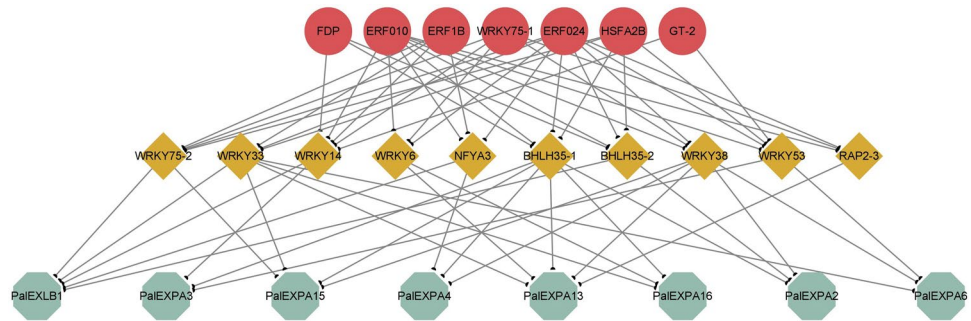


Fig. 6 The phylogenetic tree of EXP proteins from *P. alba* ‘Berolinensis’, *P. trichocarpa* and *A. thaliana*. “□” indicates *A. thaliana* EXP proteins, “○” *P. trichocarpa* EXP proteins, and “▲” *P. alba* ‘Berolinensis’ EXP proteins

Fig. 7 Transcriptional regulatory network of EXP upstream transcription factors; TFs are in red and yellow, EXPs in green



wood differs significantly from branch wood (Chen et al. 2015). In this study, the anatomical analysis of developing xylem under artificial bending in *P. alba* ‘Berolinensis’ showed eccentric growth across the section of treated stem, and higher cellulose content and less lignification in TW vs. OW and TW vs. NW. In addition, the number and size of vessels in TW were reduced, and a G layer formed in fiber cells (Fig. 1). The results of anatomical characteristics analysis of tension wood in *P. alba* ‘Berolinensis’ were similar to several studies of poplar (De Zio et al. 2020; Sousa-Baena

et al. 2022). Therefore, changes in these biological characteristics can be used as evidence for the formation of tension wood, supporting exploring gene regulatory patterns of xylem development under artificial bending and screening the important regulatory genes in xylem cell wall development based on transcriptomic analysis using TW, OW and NW. A total of 4489 DEGs were identified from the three comparison groups, with different numbers of upregulated and downregulated transcripts in each comparison. In TW vs. NW, more transcripts were up-regulated compared with

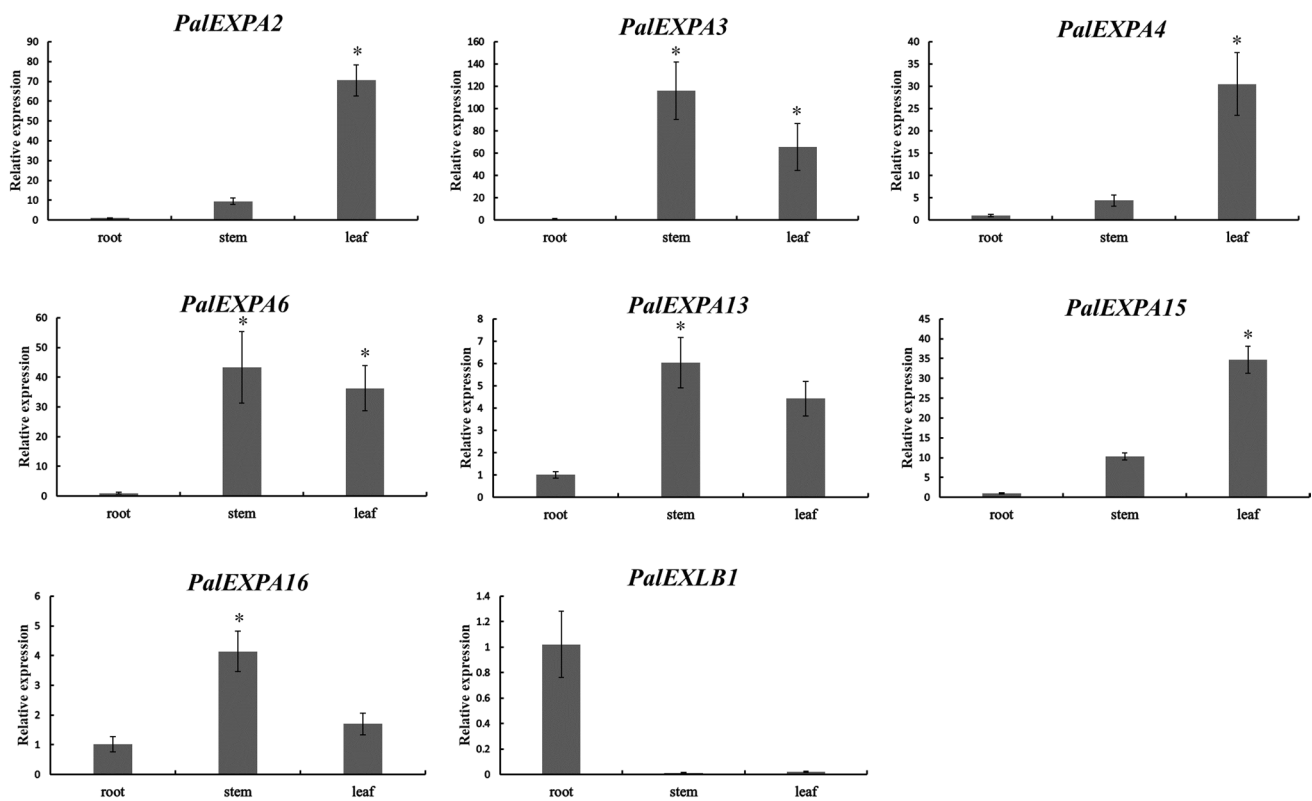


Fig. 8 RT-qPCR analysis of *PalEXP* genes in roots, stems and leaves of *P. alba* 'Berolinensis'. Data are mean values \pm standard error. Asterisks indicate statistically significant differences compared to the ratio obtained by RT-qPCR alone (t-test; $p < 0.05$)

the other two comparison groups (Fig. S5A), implying that bending could induce the expression of related response genes. Meanwhile, the DEGs were significantly highly enriched in cell wall organization or biogenesis, hemicellulose metabolic processes and secondary cell wall biogenesis (Fig. 2), which suggests that gene regulation patterns were modulated in these cell wall development processes during tension wood formation.

Genes which functioned in xylem cell wall development identified by DEG analysis

In this study, unigenes annotated as sucrose synthase (TRINITY_DN10097_c0_g1), cellulose synthase (TRINITY_DN19943_c0_g3, TRINITY_DN23646_c3_g1), and fasciclin-like arabinogalactan protein (TRINITY_DN20133_c1_g2) were all upregulated in tension wood relative to normal wood (Fig. 4). Sucrose synthase (SuSy) is an essential enzyme in plant sucrose metabolism. It plays a crucial role in various metabolic processes, including the synthesis of starch and cellulose, as well as the distribution of carbon sources (Chalivendra et al. 2007; Abid et al. 2012). Research has shown that SuSy is important for vascular development (Lugassi et al. 2022). The *CesA* genes encode the key enzyme of cellulose biosynthesis, and the

FLA genes are G-layer-specific genes (Seifert 2018). *AtCesA8* in particular is a secondary wall-associated *CesA* gene from *Arabidopsis* (Brown et al. 2005). As homologous transcripts of *AtCesA8*, the upregulation of two *CesA* transcripts (TRINITY_DN19943_c0_g3, TRINITY_DN23646_c3_g1) indicated they were involved in the promotion of cellulose biosynthesis in tension wood of *P. alba* 'Berolinensis'. In addition to xylem development, *PtrFLA40/45* modulates SCW synthesis in wood (Zhen et al. 2023). *PopFLA1* to *PopFLA10* identified from *P. tremula* \times *P. alba* have been strongly expressed in tension wood (Gorshkova and Pojiaeva 2021). Among them, *PopFLA10*, which appears slightly higher in mature xylem cells than in differentiating xylem, shares the closest sequence similarity to *FLA* (TRINITY_DN20133_c1_g2) in this study and shares 86% amino acid sequence similarity. These results indicate that the *CesA*, *FLA* and *SuSy* transcripts identified in our RNA-seq data participated in xylem cell wall development in the TW of *P. alba* 'Berolinensis'.

The phenylpropanoid biosynthesis pathway plays a crucial role in the formation of lignin, a major component of xylem cell walls (Liu et al. 2018). The synthesis of cinnamic acid from phenylalanine is the first step of the phenylpropane lignin-synthesis pathway; CAD and CCR are important enzymes response for the third step of the lignin-specific

pathway, monomer synthesis (Xiao et al. 2021b). The decrease in lignin content is possibly caused by the inhibition of the lignin biosynthetic pathway, from *PAL* to *CAD* (Chanoca et al. 2019). Lignin biosynthesis repression is an important pathway for proper tension wood formation (Liu et al. 2021). Therefore, the significant downregulation of the expression of *PAL* (TRINITY_DN21468_c0_g2), *CAD* (TRINITY_DN11567_c0_g1) and *CCR* (TRINITY_DN21635_c0_g4) transcripts in TW vs. OW implies that lignin biosynthesis was repressed during tension wood formation and that these transcripts are vital to this process.

Genes responding to environmental changes like bending

In addition to participating in regulating cell wall formation, there were also some DEGs responsive to common environmental changes. The universal stress protein (USP) family has been found to play roles in resisting diverse stress factors. Additionally, these proteins also regulate growth and development of plants and can function independently or in collaboration with other interacting partners. Studies have shown that *AtUSP* overexpressed *A. thaliana* shows organ enlargement, cell size increase, and positive regulation of *EXP* and other genes encoding cell wall components (Lee et al. 2022). In our study, a transcript coding USP (TRINITY_DN13429_c0_g3) was significantly up-regulated in both TW and OW compared to NW, indicating that *USP* gene performs diverse functions and might play roles in xylem response to bending and gravitational stimulation or tension wood development. There may be a gene regulation mechanism in response to stress in developing xylem under bending.

Genes involved in MAPK signaling pathway and plant hormone signal transduction pathways and the formation of TW.

In the formation of *Hevea brasiliensis* reaction wood, most DEGs were enriched in hormone signal transduction, zeatin biosynthesis and plant–pathogen interaction pathways, which indicates genes involved in these pathways are more likely to influence the formation of xylem cell walls (Meng et al. 2021). In our study, most DEGs were enriched in the MAPK signaling pathway and plant hormone signal transduction (Fig. 3). The MAPK is a cascade phosphorylation process (Dóczy and Bogre 2018). In the present study, most DEGs, like gene encoding pathogenesis-related protein (PR, DN8203_c0_g1) and mitogen-activated protein kinase (MAPK, DN14470_c3_g2), involved in the MAPK signaling pathway were upregulated in OW vs. NW, downregulated in TW vs. OW (Fig. 4C), implying that phosphorylation events occurred when the xylem responded to bending stimuli. Previous studies have shown that cis-zeatin (cZ) and indoleacetic acid (IAA) play key

roles in the formation of tension wood (Xiao et al. 2020). The upregulated RPKM value of auxin-responsive family protein in different periods of xylem development suggests that auxin participates in xylem development (Zhao et al. 2021). In our study, the DEGs were most enriched in plant hormone signal transduction pathways. For example, genes encoding the auxin influx carrier (TRINITY_DN13055_c3_g1), auxin transporter-like protein (TRINITY_DN13055_c3_g3), and auxin-responsive protein (TRINITY_DN16827_c1_g1), were up-regulated in TW vs. NW and TW vs. OW. This suggests the key role of hormone signal transduction pathways, especially the auxin signaling pathway, in tension wood development. These results also provide a valuable reference for exploring the pathways and molecular mechanisms of xylem cell wall formation in the future.

PalEXPs in fiber and vessel cell wall extension of TW.

As noted previously, based on the anatomical analysis of *P. alba* 'Berolinensis', there was a noticeable decrease in the number and diameter size of vessels in tension wood compared to opposite and normal wood. However, there was an increase in the length of fibers and xylem vessels in the tension wood of the poplar stems (Jourez et al. 2001). These results suggest complex cell expansion mechanisms during tension wood development. As an important regulator of cell wall extension, expansins can induce cell wall loosening and cellulose destruction and cooperate with cellulase to enhance cellulose hydrolysis (Cosgrove 2005; Wang et al. 2011). Study found that transgenic lines overexpressing *PttEXPA1*, a gene related to cell wall extension, showed increased stem internode elongation, leaf expansion, larger cell sizes in the leaf epidermis, increased fiber diameter growth, and slightly increased vessel length in aspen (Gray-Mitsumune et al. 2008). In our transcriptome analysis, eight *PalEXP* genes were identified, and four showed differential expression between three different cell types (TW, OW, and NW) (Fig. 4). *PalEXPA2* (TRINITY_DN7305_c0_g1), *PalEXPA4* (TRINITY_DN16617_c0_g3) and *PalEXPA15* (TRINITY_DN15609_c1_g2) were significantly up-regulated in TW compared to OW, but *PalEXLB1* (TRINITY_DN8270_c0_g1) was significantly down-regulated (Fig. 4). Combined with the changes in vessel diameter and length, different *PalEXPs* may play different roles in fiber and vessel cell wall extension in tension wood. Interestingly, according to the tissue-specific expression among leaves, stems and roots, *PalEXPA2*, *PalEXPA4* and *PalEXPA15* were highly expressed in leaves but at low levels in stems and roots. It is hypothesized that their lower expression level helps to ensure the normal development of xylem cell walls in stems and roots. When stimulated by external signals, these genes may be induced to express in response to stimuli, resulting in changes in traits. At the same time, we suspect that *PalEXPA2*, *PalEXPA4* and *PalEXPA15* function cooperatively.

Specific expression in roots and *PalEXLB1* involvement in cell wall development.

PalEXLB1 (TRINITY_DN8270_c0_g1) was significantly up-regulated in tension wood compared with opposite wood (Fig. 4) and specifically expressed in roots (Fig. 8). This unique expression pattern sets it apart from the other *PalEXP* genes. Phylogenetic analysis of the *PalEXP* proteins also showed the uniqueness of *PalEXLB1*; only *PalEXLB1* was clustered into the EXLB subfamily, and the remaining *PalEXP* proteins belonged to the EXPA subfamily. EXPA subfamily exhibits unique sequence conservation at both the N- and C-termini of mature proteins. These proteins have the same "GACGYG" structure at the N-terminus, but in *PalEXLB1*, the sequence is instead "GACGFG", which clustered it into the EXLB subfamily (Fig. S6). This difference indicates that the conserved sequence is important for the characteristics of *EXP* proteins and that genes in different subfamilies may have different functions. All the results confirm that EXLB could respond to bending and most likely by being involved in cell wall modification (Cosgrove 2015). The specific expression in roots of *PalEXLB1* suggested it might also function in root development, and it is worthy of an in-depth study due to its specific expression in the roots of *P. alba* 'Berolinensis'.

bHLHs and WRKYs as an upstream regulation factors of PalEXPs and the regulation of tension wood formation

Transcriptional regulation plays a key role in xylem development. At present, the upstream regulation pathway of *EXP* is not clear. A transcriptional regulatory network of *EXP* upstream TFs has been constructed and bHLH, WRKY, ERF and NFY were predicted as upstream TFs of *PalEXPs* (Fig. 7). Some transcription factors appear more frequently in the network, especially the WRKY and bHLH families. Song et al (2020) reported that *MabHLH7* seems to play a role in activating the promoters of cell wall-modifying-related genes like *MaEXPs*. Jang and Li (2018) found that transgenic *Arabidopsis* overexpressing *OsWRKY24* showed a small plant body with decreased cell size, and expression of *EXPs* was also reduced in the transgenic plants. The *bHLHs* and *WRKYs* have been confirmed to participate in wood formation (Yang et al 2016; Song et al 2023), thus the *bHLHs* and *WRKYs* identified in our network are likely to participate in the regulation of wood formation as an upstream regulation factor of *PalEXPs*. However, the expression patterns of TFs and their positive/negative regulation on downstream genes need further study.

Conclusion

Tension wood, opposite wood, and normal wood transcriptomes were constructed using the developing xylem of *P. alba* 'Berolinensis' under artificial bending. A total of 4489 DEGs were identified using RNA-seq and DEG analysis. Most of the DEGs were enriched in cell wall and secondary cell wall biogenesis based on GO enrichment analysis and were enriched in hormone signal transduction and phenylpropanoid biosynthesis pathways according to KEGG pathway analysis. Four *PalEXPs* were identified with different expression patterns and play different roles in xylem cell wall expansion. WRKY, bHLH and ERF were predicted as the upstream TFs of four *PalEXPs* under artificial bending.

Author contributions LF wrote the manuscript and performed some of the assays. YH conducted the plant treatment and sectioning. XL did the data visualization processing. MZ provided the plant materials. CW secured funding and designed the study and revised the manuscript. All authors have read and approved the final version of the manuscript.

Data availability The transcriptome data for TW, OW and NW in *P. alba* 'Berolinensis' reported in this paper are available at the NCBI Short Read Archive (SRA) under accession number PRJNA954564.

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