Genome-wide identification of *BXL* genes in *Populus trichocarpa* and their expression under different nitrogen treatments

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Abstract

β -D-xylosidase (BXL) hydrolyzes xylobiose and xylo-oligosaccharides into xylose monomers, and is a rate-limiting enzyme in the degradation of hemicellulose in the cell wall. In this study, ten genes encoding putative BXL proteins were identified in the *Populus trichocarpa* genome by bioinformatics methods. In the phylogenetic analysis, the PtBXLs formed two subfamilies. *PtBXL8* and *PtBXL9* were closely related to *AtBXL1*, an important enzyme in the normal development of the *Arabidopsis* cell wall structure. Chromosomal distribution and genome synteny analyses revealed two tandem-duplicated gene pairs *PtBXL3/4* and *PtBXL6/7* on chromosomes II and V, respectively, and six segmental-duplicated gene pairs on chromosomes II, V, VIII, X, and XIV among the *PtBXL* gene family. Tissue-specific expression data from PlantGenIE indicated that *PtBXL2*, 4, 5, and 10 were highly expressed in stems. Quantitative real-time RT-PCR analyses revealed that *PtBXL4*, 5, and 9 were up-regulated in the upper stem in response to the low and high ammonium and nitrate treatments. The influence of nitrogen on the expression of *PtBXL4*, 5, and 9 genes may affect the formation of the plant secondary cell wall. This comprehensive analysis of the *BXL* family in poplar provides new insights into their regulation by nitrogen and increases our understanding of the roles of BXLs in hemicellulose metabolism in the secondary cell wall and during plant development.

Keywords *Populus trichocarpa* · β -D-xylosidase · Nitrogen treatment · Expression pattern · Evolution

Jinyuan Chen and Chunpu Qu contributed to the work equally and should be regarded as co-first authors.

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Introduction

The cell wall is an important structure that surrounds the plant cytoplasm. It not only plays roles in the maintenance of cell morphology and the regulation of cell growth and differentiation, but also participates in intercellular signal

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transduction processes (Miller et al. 1997; Obel et al. 2002; Showalter 1993). The cell wall is composed mainly of cellulose, hemicellulose, and pectin in higher plants (Heredia et al. 1995). After cellulose, hemicellulose is the next most abundant carbohydrate polymer in the plant cell wall. It consists mainly of xylan and xyloglucan and a few mannose-containing polysaccharides, and accounts for 30–35% of the dry weight of the plant cell wall (Bewley et al. 1997; Handford et al. 2003).

Xylan is the main component of hemicellulose, and its hydrolysis requires a number of enzymes, including β -1,4-endoxylanase (EC 3.2.1.8) and β -D-xylosidase (BXL; EC 3.2.1.37). These enzymes synergistically convert xylan into its constituent sugars (Rahman et al. 2003; Tuncer and Ball 2003). Endoxylanase and BXL catalyze the cleavage of the xylan backbone. The insoluble xylan skeleton is hydrolyzed to soluble xylan xylo-oligosaccharides by endoxylanase, and xylo-oligosaccharides and xylobioses are hydrolyzed by BXL from the non-reducing ends to liberate D-xylose (Sunna and Antranikian 1997). The hydrolysis of xylan by endoxylanase is improved by adding BXL. BXL can alleviate the inhibitory effect of the hydrolysates of endoxylanase, and is the key enzyme for the complete degradation of xylan (Sunna and Antranikian 1997).

BXLs have been classified into several glucoside hydrolase (GH) families, GH1, GH3, GH5, GH30, GH39, GH43, GH51, GH52, GH54, GH116, and GH120 (Henrissat 1991) (<http://www.cazy.org/glycoside-hydrolases.html>), based on their substrates, amino acid sequences, three-dimensional structures, and enzymatic reaction mechanisms. Most plant BXLs belong to the GH3 family, whose members retain the anomeric substrate carbon to cleave glucosidic bonds (Knob et al. 2010; Sinnott 1990). The hydrolysis process of BXL is an acid–base catalytic reaction with two key catalytic amino acids at the active site: glutamic acid (Glu), which is the catalytic acid/base, and aspartic acid (Asp), which is the catalytic nucleophile (Collins et al. 2005; Lee et al. 2003; Minic et al. 2004). Amino acid sequence analyses have shown that BXLs have three conserved domains: the N-terminal and C-terminal catalytic domains that are conserved in GH family 3 (Pfam 00933 and Pfam 01915), and a fibronectin type III domain with unknown function (Pfam 14310). Pfam 00933 has conserved WGR and KH motifs, which are considered to be involved in substrate binding (Di Santo et al. 2015; Hrmova et al. 2001, 2002; Minic et al. 2004).

Previous studies have shown that *AtBXL1* is expressed mainly in the stem of *Arabidopsis thaliana*. When *AtBXL1* expression was down-regulated in *A. thaliana*, the siliques became shorter and contained fewer seeds, and the cell wall composition changed (Goujon et al. 2003). Further, *AtBXL1* expression was induced by the absence of sucrose and by dark treatment (Lee et al. 2004), and subsequent

experiments confirmed either sugar starvation or darkness induced *AtBXL1* expression (Lee et al. 2007). Fruit ripening is usually accompanied by content changes of naphthaleneacetic acid (NAA), gibberellin (GA_3), abscisic acid (ABA), and other hormones. Compared with the control, the expression of the strawberry *FaXyl1* gene decreased after NAA and GA_3 treatment, whereas ABA treatment stimulated its expression (Bustamante et al. 2009). Previous studies have shown that the production rate of BXL differed when different forms of nitrogen were used as the substrate for yeast fermentation (Rajoka 2007), and that high nitrogen treatment affected the expression of *PtBXL1* in poplar (Euring et al. 2014). To date, most studies on BXL genes have focused on sugar starvation or cell wall softening during fruit ripening, rather than on the effects of nitrogen on BXL expression (Bustamante et al. 2006; Di Santo et al. 2009; Itai et al. 1999).

Populus trichocarpa is a model tree system that has been widely used for genetic research after its genome sequence was published in 2006 (Tuskan et al. 2006). In this study, ten genes in the *P. trichocarpa* genome encoding putative BXL proteins were characterized by bioinformatic methods. Amino acid sequence and multiple sequence alignment analyses confirmed that all of them have the typical N-terminal and C-terminal catalytic domains conserved in GH family 3 and the fibronectin type III domain with unknown function. We also studied the expression characteristics of the BXL genes in response to nitrogen in different forms and at different concentrations (i.e., low and high ammonium and low and high nitrate). This study provides new insights into the regulation of genes encoding BXLs by nitrogen, and into hemicellulose metabolism in the secondary cell wall and during plant development.

Materials and methods

Identification of BXL family genes in *P. trichocarpa* and parameter analysis

To identify BXL proteins from *P. trichocarpa*, the protein database of *P. trichocarpa* was downloaded from Phytozome v12.0 (<https://phytozome.jgi.doe.gov/pz/portal.html>) (Goodstein et al. 2011), and the Hidden Markov Model (HMM) files Glyco_hydro_3 (PF00933), Glyco_hydro_3_C (PF01915), and Fn3-like (PF14310) were downloaded from the Pfam database (<https://pfam.xfam.org/>) (El-Gebali et al. 2018). Using the *hmmsearch* command in HMMER (v3.1) software (Potter et al. 2018), the poplar protein database was searched with Glyco_hydro_3.hmm, Glyco_hydro_3_C.hmm, and Fn3-like.hmm files, and the identified sequences were integrated. In addition, the *Arabidopsis AtBXL1* sequence (Goujon et al. 2003)

was used as a probe to search the *P. trichocarpa* protein database using BlastP online. The parameters were set as: Target type = Proteome; Program = BLASTP-protein query to protein db; Expect (E) threshold = -5 ; other parameters, default. The protein sequences obtained using these two methods were further screened. After screening and identification, 10 PtBXL amino acid sequences of *P. trichocarpa* were obtained. The characteristics of these amino acid sequences, including predicted molecular weight, isoelectric point, number of amino acids, aliphatic index, and grand average of hydropathicity (GRAVY) score were analyzed using the online ExPASy program (<https://www.expasy.org/>) (Gasteiger et al. 2005). The subcellular localization of the PtBXL proteins was predicted by Wolfpsort (https://www.genscript.com/psort/wolf_psort.html) (Horton et al. 2007).

Exon/intron structure and conserved motifs analysis

The Gene Structure Display Server (GSDS2.0, <http://gsds.cbi.pku.edu.cn>) (Hu et al. 2014) was used to predict the distribution patterns of exons and introns in the *PtBXL* genes. Multiple Em for Motif Elicitation (MEME v5.0.5; <http://meme-suite.org/tools/meme>) (Bailey et al. 2009) was used to identify conserved motifs in the PtBXL proteins.

Multiple sequence alignment and phylogenetic analysis

The PtBXL amino acid sequences were aligned using Clustal X (Thompson et al. 1997) and checked for the presence of the conserved WGR and KH sites. The neighbor-joining method was used to construct phylogenetic trees (1000 bootstrap replications) using MEGA v7.0.14 (Kumar et al. 2016).

Chromosomal location, duplication analysis, and Ka/Ks calculation

Location information for the *PtBXL* genes was retrieved from Phytozome and PopGenIE (<http://popgenie.org/chromosome-diagram>) (Sjödin et al. 2009). The MG2C tool (MapGene-2Chrom web, v2; http://mg2c.iask.in/mg2c_v2.0) was used to construct a chromosome distribution map of the *PtBXL* genes. Chromosome gff3 files of *P. trichocarpa* and other species were obtained from EnsemblPlants (<https://plants.ensembl.org>) (Bolser et al. 2016). Then, gene replication events were analyzed using the Multiple Collinear Scanning toolkit (MCScanX; <http://chibba.pgml.uga.edu/mcscan2/>) (Tang et al. 2008) under the Linux system. The Ka and the Ks values were calculated using DnaSP (v5.0) (Librado and Rozas 2009).

Tissue-specific expression patterns of the *PtBXL* genes

Tissue-specific expression data of the *PtBXL* genes in mature leaves, young leaves, roots, nodes, and internodes were derived from PopGenIE (<http://popgenie.org>), and used to generate visual images.

Plant materials, growth conditions, and nitrogen treatment

Populus trichocarpa plants were grown at the Northeast Forestry University Genetics and Breeding Laboratory, Harbin, China. Seedlings (approximately 15-cm tall) were rooted in hydroponic culture and then grown in vermiculite in a greenhouse under a 16-h light/8-h dark photoperiod at 25 °C. Seedlings were irrigated with improved LA nutrient solution (Cavagnaro et al. 2001) containing 1 mM NH_4NO_3 every 2 days. Each poplar shoot was marked to easily recognize the new parts of the shoot formed during the different nitrogen treatments. The poplar seedlings were then treated with 0.1 mM NH_4Cl or 10 mM NH_4Cl (low or high ammonium treatment),

Table 1 Parameters for the ten identified *BXL* genes and deduced polypeptide sequences present in the *P. trichocarpa* genome

Gene name	Locus name NCBI	Locus name Phytozome v12.1	Amino acid no.	Molecular weight (Da)	Isoelectric points	GRAVY	Aliphatic index	Chromosome location	Cellular localization
PtBXL1	PNT53504.1	Potri.001G089100.1	800	87,792.39	8.39	-0.181	84.12	Chr01:7035666..7039543(-)	Vacuole
PtBXL2	PNT57353.1	Potri.001G294700.1	777	84,936.47	5.89	-0.164	84.18	Chr01:29946390..29949590(+)	Cytoplasm
PtBXL3	PNT48761.1	Potri.002G093900.1	779	86,158.50	8.77	-0.294	83.27	Chr02:6717053..6721139(-)	Cytoplasm
PtBXL4	PNT48763.1	Potri.002G094000.1	773	84,897.59	6.96	-0.234	80.00	Chr02:6722676..6727414(-)	Vacuole
PtBXL5	PNT50651.1	Potri.002G197200.1	770	82,832.53	8.23	-0.094	83.70	Chr02:15787688..15793426(+)	Extracellular
PtBXL6	PNT37090.1	Potri.005G168400.1	818	90,161.21	6.98	-0.341	78.45	Chr05:17882350..17888812(-)	Mitochondria
PtBXL7	PNT37091.1	Potri.005G168500.1	773	84,741.46	7.82	-0.211	82.02	Chr05:17892563..17899118(-)	Chloroplast
PtBXL8	PNT23950.1	Potri.008G108100.1	771	83,584.70	8.58	-0.103	86.54	Chr08:6879163..6884696(+)	Extracellular
PtBXL9	PNT16448.1	Potri.010G141400.1	768	83,467.43	8.71	-0.114	87.36	Chr10:15298233..15304245(-)	Cytoplasm
PtBXL10	PNT04419.1	Potri.014G122200.1	770	82,948.48	8.24	-0.122	84.08	Chr14:9455916..9460246(+)	Vacuole

Fig. 1 Multiple alignment of deduced amino acid sequences of 10 PtBXLs. Boxes indicate conserved WGR and KH motifs; blue arrow represents the presumed catalytic nucleophile (Asp₂₆₇) and the pre-

sumed catalytic acid/base (Glu₄₇₁). Green arrow represents alternative catalytic acid/base residue (Glu₄₆₉)

and 0.1 mM or 10 mM NaNO₃ (low or high nitrate treatment) for 28 days after an initial 3 days of nitrogen-free treatment (Xu et al. 2017b). Seedlings treated with 1 mM NH₄NO₃ served as the control. Roots, stems, leaves, and apical buds of the plants with uniform growth were selected, and the stems and leaves were divided into upper parts (formed during the different nitrogen treatments) and lower parts (formed before the nitrogen treatments) (Jiao et al. 2017). The collected samples were immediately frozen in liquid nitrogen kept at -80 °C until analysis. To obtain reliable results, each sample was a mixture of three plants, and three groups were biological repeats.

RNA extraction and qRT-PCR analysis

Total RNA was extracted from the different tissues of the plants using the CTAB method (Gambino et al. 2008). The

extracted RNA (1 µg) was treated with RNase-free DNase I and then used for single-strand cDNA synthesis with a reverse transcription kit (SYBR Premix Ex Taq; Takara, Dalian, China). Real-time PCR was performed using Power Green qRCR Mix reagent (Dongsheng Biotech Co., Ltd., Beijing, China) based on the SYBR Green fluorescence program. The PCR cycling protocol consisted of initial denaturation at 94 °C for 3 min, followed by 45 cycles of 94 °C for 5 s, 55 °C for 5 s, and 72 °C for 10 s. After the last cycle, a melting curve analysis was performed over a temperature range of 55–94 °C in increments of 1 °C to verify the reaction specificity. The *UBQ7* gene (Pettengill et al. 2012; Xu et al. 2017a) was used as an internal reference gene, and relative expression was measured using the 2^{-ΔΔCT} method (Livak and Schmittgen 2001). TBtools (Chen et al. 2018) was used to generate a heatmap of gene expression. The

Fig. 2 Phylogenetic analysis of BXL proteins of *Populus trichocarpa* and *Arabidopsis thaliana*. The phylogenetic tree was constructed using the neighbor-joining method. Blue diamond represents PtBXLs; green dot represents AtBXLs

Fig. 3 Phylogenetic tree and structure analysis of proteins encoded by 10 *PtBXL* genes. **a** Phylogenetic tree based on deduced full-length amino acid sequences of PtBXLs was constructed using the neighbor-joining method. **b** Structure of corresponding *PtBXL* genes. Yellow

indicates protein-coding sequences (CDSs); blue indicates upstream/downstream sequences; black line indicates introns. **c** Motifs in the *PtBXL* amino acid sequences predicted using the MEME tool

significance of differences in gene expression levels was analyzed using the Student's *t* test ($P < 0.05$). The primers used in this study are listed in Table S1.

Results

Identification and sequence analysis of BXL genes in *P. trichocarpa*

To identify *BXL* genes in *P. trichocarpa*, *AtBXL1* from *Arabidopsis* was used as the probe sequence. Ten *PtBXL* genes were identified. *PtBXL1*–*PtBXL10* were named

according to their locations on chromosomes I to XIV. Details of these 10 *PtBXL* genes, including locus name, chromosome location, number of amino acids, molecular weight, isoelectric point, aliphatic index, and GRAVY score are listed in Table 1. The putative *PtBXL* protein sequences contained 768–818 amino acids and had isoelectric points ranging of 5.89–8.77. All were determined to be hydrophilic proteins. The proteins were predicted to be located in the cytosol, chloroplast, mitochondria, vacuoles, and extracellular space.

Multiple alignment of the 10 *PtBXL* protein sequences revealed that all of them contained the conserved WGR and KH motifs (Fig. 1) that are thought to be associated with

Fig. 4 Chromosomal distribution of *PtBXL* genes. Yellow strips represent chromosomes. Chromosome numbers are shown above the bar chart; *BXL* genes are on both sides of the chromosome. Scale on the left indicates chromosome length (Mb)

substrate binding (Minic et al. 2004). In addition, all ten proteins contained conserved amino acids at the two key active sites for xylose hydrolysis: Asp₂₆₇ as the catalytic nucleophile and Glu₄₇₁ as the catalytic acid/base, as well as Glu₄₆₉ as an alternative catalytic acid/base (Di Santo et al. 2015).

Gene structure and phylogeny analysis of *PtBXL* genes

The BXL amino acid sequences of *P. trichocarpa* and *A. thaliana* were aligned and a phylogenetic tree was constructed (Fig. 2). Members of the BXL family clustered into two subfamilies, SI (PtBXL2, 5, 8, 9, 10 and AtBXL1, 2, 3, 4, 5) and SII (PtBXL1, 3, 4, 6, 7 and AtBXL6, 7).

To further understand the relationships among the *PtBXL* gene family members, a phylogenetic tree of the *P. trichocarpa* BXL genes was constructed (Fig. 3a), and the intron and exon structures were compared. We found that *PtBXL5* and *PtBXL10* contained seven exons, *PtBXL6* contained eight exons, and the other *PtBXL* genes contained six exons (Fig. 3b). The conserved motifs of the PtBXL family were analyzed using the online tool MEME and a schematic map was constructed to represent the structure of the proteins (Fig. 3c). All the PtBXL proteins contain ten conserved motifs. The conserved WGR and KH motifs were located in motif2 and motif1, respectively, and the catalytic nucleophile and catalytic acid/base are located in motif8 and motif9. The motif sequences are listed in Table S2.

Chromosomal distribution and synteny analysis of *PtBXL* genes

To determine the distribution of the *PtBXL* genes, we mapped their positions on the poplar chromosomes. The 10 *PtBXL* genes were distributed on six chromosomes: three on chr II, two on each of chr I and V, and one on each of chr VIII, X, and XIV (Fig. 4).

The results of a genome-wide analysis of *P. trichocarpa* indicated that *PtBXL3/4* and *PtBXL6/7* are tandem duplications on chr II and chr V, respectively. Segmental duplications of the BXL genes were analyzed using MCScanX. We identified six segmental duplication events in six *PtBXL* genes located on duplicated segments on chr II, V, VIII, X, and XIV (Fig. 5). These results indicate that some *PtBXL* genes may have arisen by gene duplication events, and suggest that these replication events are the main drivers of the amplification of the *PtBXL* gene family.

To further understand the gene replication mechanisms of the BXL gene family in *P. trichocarpa*, we constructed five comparative syntenic maps of poplar associated with five representative species; one monocot (*Zea mays*) (Fig. 6a) and four dicots (*A. thaliana*, *Glycine max*, *Medicago truncatula*, and *Brassica oleracea*) (Fig. 6b). The poplar BXL genes showed a high degree of evolutionary divergence compared with the BXL genes in other dicotyledonous plants. Some BXL genes were associated with at least three syntenic gene pairs, such as *PtBXL9* and *PtBXL10*. These genes may have played an essential role in the evolution of the BXL gene family in poplar. To better understand the evolutionary constraints on the BXL gene family, the Ka/Ks ratios of the BXL gene pairs were calculated (Tables S3–S7). A pair

Fig. 5 Schematic representations of segmental duplications of *PtBXL* genes. Gray lines indicate all syntenic blocks between each chromosome in the poplar genome; thick green lines indicate duplicated *BXL*

gene pairs. Gene names are shown in red font. Chromosome number is indicated at the bottom of each chromosome. Scale bars marked on each chromosome indicates chromosome length (Mb)

of sequences with $Ka/Ks > 1$ implies positive or Darwinian selection; $Ka/Ks = 1$ indicates that both sequences are drifting neutrally; and $Ka/Ks < 1$ implies purifying selection (Navarro and Barton 2003). The selection stress analysis showed that the duplicated gene pairs were mainly under purifying selection ($Ka/Ks < 1.0$).

Tissue-specific expression pattern of *BXL* genes in *P. trichocarpa*

Data for the tissue-specific expression of *PtBXL* genes in mature leaves, young leaves, roots, nodes, and internodes were obtained from the online Plant Genome Integrative

Explorer tool (<https://PlantGenIE.org>) and used to generate the images shown in Fig. 7. *PtBXL3* was highly expressed in young leaves, *PtBXL9* was highly expressed in mature leaves, *PtBXL7* was highly expressed in young leaves and mature leaves, *PtBXL2*, 4, 5, and 10 were highly expressed in the node and internode, and *PtBXL1*, 6, 8 showed low expression levels in all the tissues.

Expression pattern of *PtBXL* genes under different nitrogen treatments

Previous studies have shown that nitrogen can affect plant cell wall components. To investigate the functions of *PtBXLs*, the

Fig. 6 Synteny analysis of *BXL* genes between poplar and other plants. **a** Monocotyledonous plant maize. **b** Dicotyledonous plants *Arabidopsis*, soybean, barrel medic, and kale. Gray lines in background indicate collinear blocks within poplar and other plant genomes; blue lines indicate syntenic *BXL* gene pairs. Species names: *Zea mays*, *Arabidopsis thaliana*, *Glycine max*, *Medicago truncatula*, *Brassica oleracea*, and *Populus trichocarpa*. Red or green bars represent chromosomes with number labeled at top or bottom

Fig. 7 Tissue-specific expression profiles of *PtBXL* genes. Visual images of *PtBXL* genes in *Populus trichocarpa* were generated using tissue-specific expression data of mature leaves, young leaves, roots, nodes, and internodes derived from <https://PlantGenIE.org>

expression patterns of the *PtBXL* genes in response to nitrogen in different forms and at different concentrations (i.e., low and high ammonium and low and high nitrate) were quantitatively analyzed by qRT-PCR (Table S8). As shown in Fig. 8, in the apical buds, the transcript levels of *PtBXL2*, 3 decreased under the high ammonium and nitrate treatments and those of *PtBXL7*, 10 decreased under high nitrate treatment, compared with the controls. The other genes showed up-regulated expression levels under the four treatments; *PtBXL1*, 2, 7 expression increased significantly under the low ammonium treatment; *PtBXL4*, 5, 9, 10 expression increased significantly under the low nitrate treatment; and *PtBXL4* expression was increased significantly under the high ammonium and nitrate treatments. In the upper stems, the transcript levels of *PtBXL1*, 8 were slightly decreased under the low ammonium and high nitrate treatments, and the other genes were up-regulated under all four treatments. The transcript levels of *PtBXL4*, 5, 9 were increased significantly under all four treatments compared with the controls. In the lower stems, the *PtBXL* gene expression patterns were different under the four treatments. The low nitrate treatment resulted in significant down-regulation of *PtBXL4*, 5, 6, 9, 10, and the expression of *PtBXL8* was also decreased but not significantly. The high ammonium treatment up-regulated all *BXLs* except *PtBXL7*; the expression levels of *PtBXL1*, 2, 4, 5 increased slightly, whereas that of *PtBXL6* increased significantly. In the upper leaves, all the *BXLs* except for *PtBXL1* were down-regulated under the high nitrate treatment; *PtBXL5*, 10 showed significantly decreased

expression; *PtBXL7* was significantly down-regulated under the low ammonium treatment, and the other genes were differentially induced by the treatments. In the lower leaves, *PtBXL2*, 7 were down-regulated under the low ammonium treatment. There were significant increases in the expression levels of *PtBXL1*, 5, 6, 9, 10 under the high ammonium treatment, *PtBXL5*, 8 under the low nitrate treatment; and *PtBXL3*, 5 under the high nitrate treatment. Other genes responded differently to the different treatments. In the roots, all the *PtBXLs* except *PtBXL5* were up-regulated under the low ammonium treatment, and showed different expression patterns under the nitrate treatments.

Discussion

Nitrogen is an essential nutrient for plant growth and development (Kraiser et al. 2011). The nitrogen absorbed and utilized by plants is derived mainly from soil, so the available nitrogen content in the soil affects plant growth and morphogenesis (Dickson 1989; Geiger et al. 1996). In this study, we identified 10 *BXL* gene family members in *P. trichocarpa*, and their expression patterns in response to nitrogen in different forms and concentrations were analyzed. Our results revealed the regulation of *BXL* expression by nitrogen and provided new insights into how nitrogen affected hemicellulose metabolism in the secondary cell wall and during plant development.

Fig. 8 Expression patterns of *PtBXL* genes in different tissues under different nitrogen treatments. Expression pattern of *PtBXL* genes in **a** apical buds, **b** upper stems, **c** lower stems, **d** upper leaves, **e** lower leaves, **f** roots under different nitrogen treatments (low and high ammonium (0.1 mM and 10 mM NH_4Cl) and low and high nitrate (0.1 mM and 10 mM NaNO_3). Transcript levels of *PtBXLs* were calculated using the $2^{-\Delta\Delta\text{CT}}$ method. For each *PtBXL* gene, $\log_2$ (sample/control) values under the different nitrogen treatment conditions were calculated as relative expression levels. Scale bars are shown at bottom right. Colors of cells in the heatmaps indicate up-regulated or down-regulated expression in treated samples compared with controls

BXL genes have been identified in a number of plants (Decou et al. 2009; Di Santo et al. 2015; Goujon et al. 2003; Lee et al. 2003) and have been studied in detail in the herbaceous plant *Arabidopsis*. However, fewer studies have functionally analyzed *BXL* genes from woody plants, especially the model tree *P. trichocarpa*. In this study, we identified 10 *BXL* genes in *P. trichocarpa*. Evolutionary analysis of *AtBXLs* and *PtBXLs* indicated that the *BXL* gene family comprises two subfamilies (Fig. 2). Sequence analyses showed that the conserved motifs of the *BXL* protein family were identical, indicating that *BXLs* have a highly conserved protein structure (Fig. 3). All the *BXL* sequences contained the conserved WGR and KH motifs in motif2 and motif1 (Table S2). These motifs are thought to be involved in substrate binding in *Arabidopsis* *BXLs* (Minic et al. 2004). Therefore, we speculate that *PtBXL* proteins may have similar functions.

In general, gene families expand by tandem duplication and segmental duplication (Cannon et al. 2004). A genome-wide analysis indicated that *PtBXL* genes were subjected to whole-genome replications and tandem repeat events. *PtBXL3/4* and *PtBXL6/7* are tandem duplications on chr II and chr V, respectively, and six tandem repeat events between six genes may have contributed to the evolution of the *BXL* gene family (Lan et al. 2013; Liu et al. 2012; Xu et al. 2017a). The selection stress analysis indicated that continuous purification selection ($K_a/K_s < 1$) has played a key role in determining the number of *BXL* genes in *P. trichocarpa* (Zhang et al. 2015).

A previous study showed that *AtBXL1* is expressed mainly in the stem of *Arabidopsis* (Goujon et al. 2003). Compared with wild-type plants, *AtBXL1* antisense transgenic lines showed differences in development under the same growth conditions, with significant phenotypic changes in the leaf and siliques and decreased height. This indicated that *AtBXL1* is an important enzyme in the normal development and cell wall structure of *Arabidopsis*, and may participate in the loosening of glucuronoarabinoxylan during cellulose deposition in the secondary cell wall, thus promoting the polymerization of lignin in the polysaccharide matrix (Goujon et al. 2003). *AtBXL1* transcripts were shown to be

strongly induced in *Arabidopsis* leaves after sugar depletion, which indicated that *AtBXL1* may play roles in the release of xylose from cell wall macromolecules. The released xylose can be used as a carbon source and may be re-mobilized to storage organs (Lee et al. 2004, 2007). In another study, *BXL* activity was detected in stems of *Arabidopsis* at different developmental stages by chromatography on CM-trisacryl, and was found to be highest in stems of plants 3–4 cm in height. Those findings suggested that *AtBXL1* and *AtBXL4* might be related to stem development (Minic et al. 2004). In a study of tension wood of poplar, *PtaBXL1* was found to be specifically expressed in the cambium and xylem of the secondary wall, whereas the other three *BXL* genes *PtaBXL2*, *PtaBXL3*, and *PtaBXL4* were not transcribed in the xylem and cambium, but only in leaves, roots, and flowers. The results of the qRT-PCRs showed that *PtaBXL1* was expressed most strongly during the formation of the secondary cell wall. In addition, *PtaBXL1* was down-regulated during the formation of tension wood, which was thought to be essential for maintaining the plasticity of the cell wall during stem bending (Decou et al. 2009).

Previous studies have shown that, compared with low nitrogen conditions, high nitrogen conditions can lead to a decrease in lignin content, but higher cellulose and hemicellulose contents (Novaes et al. 2009). These changes are often accompanied by changes in secondary cell wall morphology, such as a thinner cell wall and wider vessel lumina under high nitrogen conditions (Luo et al. 2005; Novaes et al. 2009). In other studies, the fibers produced under a high nitrogen treatment had a significantly greater diameter and slightly shorter length than those produced under lower nitrogen conditions, and the newly formed fibers developed thicker cell walls with altered structure (Pitre et al. 2007, 2010). Low nitrogen conditions can lead to reduced xylem width, fewer cell layers in the xylem, narrower lumina of vessels and fibers, thicker double fiber walls (walls between two adjacent fiber cells), enhanced hemicellulose and lignin deposition, and reduced cellulose accumulation in poplar wood (Lu et al. 2019).

In this study, the qRT-PCRs showed that the transcript levels of *PtBXL6* under the high ammonium treatment and *PtBXL3* under the high nitrate treatment increased significantly in the lower stem, and that the transcript levels of *PtBXL4*, 5, 9 in the upper stem were up-regulated in the four different nitrogen treatments (Fig. 8). Tissue-specific expression data showed that *PtBXL4* and *PtBXL5* were highly expressed in the internode (Fig. 7). The phylogenetic analysis showed that *AtBXL1* is closely related to *PtBXL8* and *PtBXL9* (Fig. 2). Therefore, we speculate that nitrogen affects the expression of *PtBXL4*, 5, 9, and that this may affect the formation of the plant secondary cell wall. The expression patterns of the *PtBXL* genes in other tissues were

different, which may reflect a complex regulation mechanism of *PtBXLs* in different tissues. Further research is required to study the *PtBXL* expression patterns and regulation in more detail.

Conclusions

We identified 10 *BXL* genes by bioinformatics methods and studied changes in their transcript levels in response to nitrogen in different forms and at different concentrations. Our results provide important information about the *PtBXL* genes and the new insights into their regulation by nitrogen. Their regulation patterns may affect hemicellulose metabolism in the secondary cell wall and during plant development.

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Author contributions JC and CQ conceived and designed the study, JC and CQ performed most of the experiments, RC and JS conducted the sampling, JY and XS performed bioinformatics calculations, GL and ZX processed and analyzed the data, and JC, CQ, and ZX wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests.

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