A PtrLBD39-mediated transcriptional network regulates tension wood formation in *Populus trichocarpa*

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ABSTRACT

Tension wood (TW) is a specialized xylem tissue formed in angiosperm trees under gravitational stimulus or mechanical stresses (e.g., bending). The genetic regulation that underlies this important mechanism remains poorly understood. Here, we used laser capture microdissection of stem xylem cells coupled with full transcriptome RNA-sequencing to analyze TW formation in *Populus trichocarpa*. After tree bending, *PtrLBD39* was the most significantly induced transcription factor gene; it has a phylogenetically paired homolog, *PtrLBD22*. CRISPR-based knockout of *PtrLBD39/22* severely inhibited TW formation, reducing cellulose and increasing lignin content. Transcriptomic analyses of CRISPR-based *PtrLBD39/22* double mutants showed that these two genes regulate a set of TW-related genes. Chromatin immunoprecipitation sequencing (ChIP-seq) was used to identify direct targets of *PtrLBD39*. We integrated transcriptomic analyses and ChIP-seq assays to construct a transcriptional regulatory network (TRN) mediated by *PtrLBD39*. In this TRN, *PtrLBD39* directly regulates 26 novel TW-responsive transcription factor genes. Our work suggests that *PtrLBD39* and *PtrLBD22* specifically control TW formation by mediating a TW-specific TRN in *Populus*.

Key words: tension wood, cellulose, lignin, transcriptional regulatory network, transcriptomics, chromatin immunoprecipitation sequencing

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INTRODUCTION

Wood is one of the most abundant components of fixed carbon in the terrestrial biosphere and an important sustainable feedstock for biofuels and value-added chemicals (Sarkanen, 1976; Richter et al., 1999; Ragauskas et al., 2006). Wood formation is a complex process of tissue-specific development and differentiation initiated from the vascular cambium, which divides and differentiates into secondary xylem (Esau, 1965; Evert and Eichhorn, 2006). Tension wood (TW) is a specialized xylem tissue in angiosperm trees formed on one side of leaning or bending stems in response to the perception of gravity or mechanical stresses, such as wind, soil downhill creep, bending, and light (Scurfield, 1973; Timell, 1986; Okuyama et al., 1994; Pilate et al., 2004; Déjardin et al., 2010; Mellerowicz and Gorshkova, 2012; Groover, 2016; Wang et al., 2017). The morphology, anatomy, ultrastructure and chemical composition of TW are strikingly different from those of normal wood (NW) (Norberg and Meier, 1966; Côté et al., 1969; Timell, 1969, 1986; Sarkanen and Ludwig, 1971; Wu et al., 2000; Pilate et al., 2004; Liu et al., 2021). TW has smaller and fewer vessel elements and contains fibers with a thick inner gelatinous layer called the G-layer that is highly enriched in cellulose and contains little or no lignin (Onaka, 1949; Norberg and Meier, 1966; Côté et al., 1969; Okuyama et al., 1994; Wu et al., 2000; Pilate et al., 2004; Clair et al., 2010; Mellerowicz and Gorshkova, 2012; Groover, 2016). Some of these chemical features can improve the efficiency of wood conversion for biofuel and material production (Brereton et al., 2011, 2012;
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Sawada et al., (2018). Understanding the molecular and genetic regulation of TW formation can advance the engineering of wood for more efficient material and energy production.

TW formation is a complex developmental process of angiosperm trees. Previous studies have profiled gene expression in TW formation using varied sequencing strategies and have identified a large number of differentially expressed genes (DEGs) in poplar (Déjardin et al., 2004; Andersson-Gunnerås et al., 2006; Chen et al., 2015; Felten et al., 2018; Du et al., 2020), Eucalyptus (Paux et al., 2005; Mizrachi et al., 2015), yellow poplar (Liriodendron tulipifera) (Jin et al., 2011), and Catalpa bungee (Xiao et al., 2020). Many transcriptomic analyses in these studies have revealed downregulated expression of lignin and xylan biosynthetic pathway genes and upregulated expression of cellulose biosynthetic genes and fasciclin-like arabinoxylan proteins (FLAs) in TW relative to NW or the opposite wood (OW). Many transcription factors (TFs) differentially expressed during TW formation were also identified in these studies; however, only a handful of TFs have been confirmed to regulate TW formation by genetic analyses. In Populus alba × P. tremula, Class I KNOX homeodomain TF ARBORKNOX2 (ARK2) affects TW fiber development and gravitropism (Gertulta et al., 2015). In Populus tremula × P. tremuloides, overexpression of ERF139 led to a decrease in vessel diameter, and overexpression of ERF18 and ERF21 increased the carbohydrate content of wood (Vahala et al., 2013). In Populus davidiana × P. b. boreana, PtMYB128 regulates G-layer formation in fiber cells during brassinosteroid-mediated TW formation by activating the expression of PtCesAs (Jin et al., 2020). Recently, we identified two TFs, PtrHSFB3-1 and PtrMYB092, which specifically regulate the expression of secondary cell wall (SCW) component (lignin, cellulose, and hemicellulose) genes during TW formation in Populus trichocarpa (Liu et al., 2021). However, the known TFs that specifically regulate TW development are limited, and the transcriptional regulatory network (TRN) that underlies TW development remains to be explored.

In this study, we used the model woody plant P. trichocarpa for TW induction by artificial stem bending and employed RNA-sequencing (RNA-seq) to assay gene expression in TW-forming cells. Laser capture microdissection (LCM) was used to collect samples of TW-forming cells across multiple time points. These LCM-based RNA-seq analyses identified PtrLBD39 (LATERAL ORGAN BOUNDARIES DOMAIN TF) as the most TW-responsive TF gene. Loss of function of PtrLBD39 and its close homolog PtLBD22 reduced the severity of TW development under bending stress. Transgenesis, together with RNA-seq and chromatin immunoprecipitation sequencing (ChIP-seq) analyses, allowed us to construct a PtrLBD39-mediated TRN that is specific for TW formation.

RESULTS

Identification of TW-related TF genes in P. trichocarpa

TW formation can be induced under gravitational and/or mechanical stress by bending the stem of an angiosperm tree species (Côté et al., 1969; Scurfield, 1973). We induced TW in the stems of 4-month-old greenhouse-grown P. trichocarpa plants by bending the plants at a 90-degree angle for 0, 3, 5, 7, 14, and 21 days (Figure 1A and Supplemental Figure 1). These time-course experiments showed that TW with a G-layer in fiber cells was produced on the upper side of the stems after a 3-day bending. As the duration of bending increased from 3 to 21 days, the TW area increased progressively from 6 to 40 cell layers (Figure 1A). We then used this system to study the transcriptional regulation of TW formation. LCM is a powerful tool for specific sorting and collection of different cell types for molecular analysis (Emmert-Buck et al., 1996; Asano et al., 2002; Tang et al., 2006; Chen et al., 2014). Therefore, to more accurately identify genes that control TW formation, we used LCM to collect the TW-forming cells at the six bent time points (see “methods”; Figure 1B), followed by full transcriptome RNA-seq analysis of the collected cells.

Because we wanted to reveal the genetic regulation of TW formation, we focused on the identification of TF genes that were differentially expressed in response to bending stress at different time points. LCM-based transcriptomes of TW cells from the five bending periods identified a total of 1,506 unique TF genes that were differentially expressed (false discovery rate [FDR] < 0.05) compared with the 0-day (vertical or no bending) control (Supplemental Data 1). These 1,506 TFs are from 56 diverse TF families (Figure 1C), indicating the sensitivity and complexity of the plant’s regulatory responses to gravity and mechanical stress. To begin a more systematic approach to unraveling this complex regulation, we first identified the most responsive and upregulated TF genes. We ranked the 1,506 genes based on their fold changes at each bending time point, and only a handful of TFs were drastically upregulated (Figure 1D). Among these TFs, PtLB39 (Potri.005G097800) was consistently and most significantly upregulated at four time points (four out of five at 5, 7, 14, and 21 days; Figure 1D), with 20.9- to 42.5-fold upregulation (Figure 1E).

In the P. trichocarpa genome, PtLB39 has three homologs in the P. trichocarpa genome (Kong et al., 2017), PtLB49 (Potri.001G081400), PtLB18 (Potri.003G149800), and PtLB22 (Potri.007G067700), with high protein sequence identity (ranging from 70% to 91%) (Supplemental Figure 2). PtLB22 was also upregulated in response to bending stress, but to a much lesser extent than PtLB39 (Figure 1E). However, the expression of the other two PtLB39 homologs, PtLB49 and PtLB18, was not affected by bending stress (Figure 1E; Supplemental Data 2), indicating that PtLB49 and PtLB18 did not contribute to TW formation. The transcripts of PtLB39 and PtLB22 were almost undetectable in P. trichocarpa stem-differentiating xylem (SDX) in the absence of bending treatment, but both were highly induced after bending treatment (Figure 1E; Supplemental Data 2), suggesting that the two genes are intimately associated with TW development and that they may have redundant functions in the control of TW formation. To determine their functions, we generated and characterized loss-of-function mutants in P. trichocarpa.

CRISPR-based editing of PtLB39 and its homolog

PtLB22 reduced TW formation in P. trichocarpa

We generated single-knockout mutants of PtLB39 and double-knockout mutants of PtLB39 and PtLB22 in P. trichocarpa
using a CRISPR/Cas9 genome-editing system (see “methods”; Supplemental Figure 3). We obtained one homozygous single mutant (ptrlb39-2 and ptrlb39-3), and one biallelic double mutant (ptrlb39/22) in which insertions and/or deletions led to frame shifts and premature stop codons (Supplemental Figure 3A and 3B). We selected the homozygous single mutant (ptrlb39) and the double mutant (ptrlb39/22) and clonally propagated them for further analysis (Supplemental Figure 3C).

The single and double mutants had similar height and internode numbers compared with the wild type (Supplemental Figure 3D), and these mutations did not significantly affect the vascular cambium and xylem development (Supplemental Figure 4). Consistently, no significant differences in stem diameter were observed between the mutant and wild-type plants (Supplemental Figure 5). We next used the specific mutants to analyze the roles of PtrLBD39 and PtrLBD22 during TW formation. We bent the stems of 4-month-old ptrlb39, ptrlb39/22, and wild-type plants for 7 and 21 days. We used chlorazol black and safranin to stain G-fibers for observation of the TW area (Gritsch et al., 2015). The intensity of the dark-blue stain normally increases with increasing severity of TW development owing to chlorazol black staining of the G-fibers. In addition, a decrease in vessel density also indicates increased severity of TW formation. Accordingly, we found that after a 7-day bending, the ptrlb39 mutant showed a slight decrease in TW width (by ~12%, Figure 2A–2C) and number of TW G-fiber layers (by ~19%, Figures 2B and 2D), and an increase in vessel diameter (by ~17%, Figure 2B and 2E), compared with the wild type. Double mutations in PtrLBD39 and PtrLBD22 resulted in approximately 36% and 48% decreases in TW width (Figure 2A–2C) and number of TW G-fiber layers (Figure 2B and 2D), and an approximately 57% increase in...
vessel diameter (Figure 2B and 2E). Another indicator of TW development is stem asymmetric growth (Andersson-Gunnerås et al., 2003; Love et al., 2009; Felten et al., 2018), which can be represented by the TW/OW ratio (Seyfferth et al., 2019). The higher the TW/OW ratio, the greater the severity of TW development. In ptrbd39 lines, the TW/OW ratio was reduced by approximately 8% compared with that in wild-type plants, and in ptrbd39/22 lines, the ratio was reduced by approximately 15% (Figure 2A and 2F). We observed no significant differences in stem diameter between the mutant and wild-type plants (Figure 2G). Moreover, the wood width on the OW side was smaller than that on the TW side in the wild type and ptrbd39, and there was no significant change in the ptrbd39/22 mutants (Figure 2H), showing a reduction of TW differentiation in the double mutants. Safranin and fast green staining of stem cross-sections further confirmed the observed reduction in TW formation in ptrbd39 and ptrbd39/22 mutants (Figure 2). Similarly, decreased TW width, TW G-fiber layers, asymmetric growth, and increased stem lift in comparison with the wild type (Supplemental Figure 6). These results indicated that both PtrlBD39 and PtrlBD22 are positive TW regulators and are redundant in function.

We also assayed the effect of PtrlBD39 and PtrlBD22 knockout on gravi-stimulated stem growth and development. The ptrbd39/22 double mutants and wild-type P. trichocarpa were subjected to 90° gravistimulation for a period of 17 days (Supplemental Figure 7A). The ptrbd39/22 double mutants had reduced gravibending stem lift in comparison with the wild type (Supplemental Figure 7B). Histological comparison of stem sections showed that after 17 days of gravibending, the ptrbd39/22 double mutants had decreased TW width and asymmetric growth compared with the wild type (Supplemental Figure 7C and 7D). These results suggest that inhibited TW formation and reduced asymmetric growth in the ptrbd39/22 double mutant decreased its ability to pull the stem into an upright position, providing evidence that TW formation and an asymmetric growth pattern are very important for tree growth in response to gravitational stimulus or mechanical stress.

Mutations in PtrlBD39 and PtrlBD22 of P. trichocarpa resulted in increased lignin content and decreased cellulose content in TW

TW has many unique features, such as low lignin and high cellulose content (Wardrop, 1964; Côté et al., 1969; Fujita et al., 1974; Timell,
genes
transcriptional regulatory roles of or wood formation-associated TF. We next studied the suggesting that it has a transcriptional regulatory role as a xylem-
(Table 1). The double mutants had an approximately 6% content compared with the TW lignin content of the wild type
22 double mutants after a 21-day bending. We found that analyzed the wood composition of TW in the stem of their absence would increase the lignin content and reduce the upper stem side, as demonstrated above (Figure 2). Therefore,
PtrLBD39 glucose) ( Table 1 ). These results support the notion that reduction in cellulose content (represented by the amount of
nucleus of P. trichocarpa characteristic features of TW (low lignin and high cellulose
these two LBDs and probably encode TFs required for the formation. We also found that a recently discovered negative regulator of TW, the PtrHSFB3-1 TF gene (Liu et al., 2021), was upregulated in the TW of ptrlbd39/22 mutants (Figure 3A) and downregulated in the TW of wild-type plants (Figure 3B). In the TW of ptrlbd39/22 and wild-type plants, ARK2, a Class I KNOX TF gene that affects TW fiber development and gravibending (Gerttula et al., 2015), had an expression pattern similar to that of PtrHSFB3-1 (Figure 3A and 3B). We also performed heatmap analysis of the transcript abundances of TW-related genes based on expression profiles from the LCM-based RNA-seq data of TW in wild-type plants and the RNA-seq data of TW in ptrlbd39/22 and wild-type plants (Supplemental Figure 9).

Plant hormones such as auxin, gibberellin, ethylene, and brassinosteroid are known to play key roles in the regulation of TW formation (Andersson-Gunneräas et al., 2003, 2006; Love et al., 2009; Vandenbussche et al., 2011; Vahala et al., 2013; Gerttula et al., 2015; Groover, 2016; Felten et al., 2018; Du et al., 2020; Jin et al., 2020). Consistently, we found a large number of genes encoding several AUX/IAA auxin-responsive proteins, PIN proteins, LAX auxin influx protein, Ethylene Response Factors (ERFs), ETHYLENE INSENSITIVE 3/ETHYLENE INSENSITIVE3-LIKE1 (EIN3/EIL1), BRASSINOSTEROID ENHANCED EXPRESSION 3 (BEE3), and BR biosynthesis-related CYP85A3 in our DEG population (Supplemental Data 5).

FLAs play important roles in the cell wall architecture of Populus and are necessary for poplar TW formation (Lafarguette et al., 2004; Andersson-Gunneräas et al., 2006; Azri et al., 2014; Wang et al., 2017). We found that the expression levels of FLA genes were downregulated in ptrlbd39/22 lines compared with wild-type plants (Supplemental Data 6). Together, these results suggested that PtrLBD39 and PtrLBD22 may directly or indirectly control the expression of these DEGs to regulate TW formation.

1986; Nugroho et al., 2013; Gritsch et al., 2015; Liu et al., 2021). We speculated that PtrLBD39 and PtrLBD22 were required for TW formation because their absence reduced TW formation at the upper stem side, as demonstrated above (Figure 2). Therefore, their absence would increase the lignin content and reduce the cellulose content at the TW site. To verify this prediction, we analyzed the wood composition of TW in the stem of ptrlbd39/22 double mutants after a 21-day bending. We found that ptrlbd39/22 lines had an average of approximately 18% greater TW lignin content compared with the TW lignin content of the wild type (Table 1). The double mutants had an approximately 6% reduction in cellulose content (represented by the amount of glucose) (Table 1). These results support the notion that PtrLBD39 and PtrLBD22 are required for TW formation and that these two LBDs probably encode TFs required for the characteristic features of TW (low lignin and high cellulose content). Moreover, PtrLBD39 was found to be localized in the nucleus of P. trichocarpa SDX cells (Supplemental Figure 8), suggesting that it has a transcriptional regulatory role as a xylem-wood formation-associated TF. We next studied the transcriptional regulatory roles of PtrLBD39 in TW formation.

**Table 1. Wood composition of tension wood area from the upper side of bent stems in ptrlbd39/22 mutants and wild-type Populus trichocarpa.**

Six-month-old plants were tested. Three biological replicates from independent pools of TW on the upper sides of bent stems in ptrlbd39/22 mutants and wild-type P. trichocarpa were used. Data are means of three independent assays. Asterisks indicate significant differences between ptrlbd39/22 double mutants and wild-type plants based on Student’s t-test (*p < 0.05 and **p < 0.01). Units are g/100 g of dry extractive-free wood.

| Parameter                  | Wild type | ptrlbd39/22 |
|---------------------------|-----------|-------------|
| Glucose                   | 59.62 ± 0.72 | 56.14 ± 0.79* |
| Xylose                    | 7.81 ± 0.51  | 10.28 ± 0.16* |
| Galactose                 | 5.76 ± 0.52  | 6.12 ± 0.35  |
| Arabinose                 | 1.39 ± 0.25  | 0.95 ± 0.05  |
| Total carbohydrate        | 74.60 ± 0.75 | 73.30 ± 1.21 |
| Acid insoluble lignin     | 13.03 ± 0.06 | 14.60 ± 0.52  |
| Acid soluble lignin       | 1.69 ± 0.08  | 2.78 ± 0.44  |
| Total lignin              | 14.72 ± 0.14 | 17.38 ± 0.53** |

** PTRLBD39 and PTRLBD22 regulate a set of TW-related genes**

We carried out RNA-seq on TW tissue scraped from the surface of debarked stem segments from the ptrlbd39/22 double mutant lines and wild-type plants after a 7-day bending. We identified 10,695 DEGs (FDR <0.05; Supplemental Data 3). Of these DEGs, 5,480 were upregulated and 5,215 were downregulated in the ptrlbd39/22 double mutant lines. To identify PtrLBD39-regulated genes, we analyzed LCM-based RNA-seq DEGs (14,850 DEGs representing TW induction effects, 7-day bending, Supplemental Data 4) and RNA-seq DEGs of ptrlbd39/22 mutants (10,695 DEGs representing TW reduction effects, 7-day bending, Supplemental Data 3) and identified 6,200 common genes in these two DEG populations (Supplemental Data 5).

Among these genes, several MYB TF genes were downregulated in bent ptrlbd39/22 lines compared with wild-type plants (Figure 3A) and upregulated in wild-type plants under gravitational stimulus or mechanical stress (Figure 3B); they included PtrMYB28, PtrMYB128, PtrMYB167, and PtrMYB221, which have been reported to participate in TW formation or lignin biosynthesis in poplar (Zhong and Ye, 2009; Zhong et al., 2011; Tang et al., 2015; Jin et al., 2020). We also found that a recently discovered negative regulator of TW, the PtrHSFB3-1 TF gene (Liu et al., 2021), was upregulated in the TW of ptrlbd39/22 mutants (Figure 3A) and downregulated in the TW of wild-type plants (Figure 3B). In the TW of ptrlbd39/22 and wild-type plants, ARK2, a Class I KNOX TF gene that affects TW fiber development and gravibending (Gerttula et al., 2015), had an expression pattern similar to that of PtrHSFB3-1 (Figure 3A and 3B). We also performed heatmap analysis of the transcript abundances of TW-related genes based on expression profiles from the LCM-based RNA-seq data of TW in wild-type plants and the RNA-seq data of TW in ptrlbd39/22 and wild-type plants (Supplemental Figure 9).
**PtrLBD39 and PtrLBD22 change the expression pattern of key cell wall component genes**

RNA-seq analysis of TW tissues (7-day bending) from ptrlbd39/22 and wild-type plants revealed that the expression of five monolignol biosynthetic pathway genes (PtrCCoAOMT2, PtrC4H2, PtrCCR2, PtrPAL1, and PtrPAL3) was upregulated in ptrlbd39/22 mutants (Figure 3C). Among these genes, PtrC4H2, PtrCCR2, PtrPAL1, and PtrPAL3 have been demonstrated to positively regulate lignin content (Wang et al., 2019). Therefore, the upregulation of these five genes is consistent with the increased lignin content in TW of the double mutant compared with wild-type TW (Table 1). All SCW cellulose synthase genes, PtrCesA4, PtrCesA7 (PtrCesA7-A), PtrCesA17 (PtrCesA7-B), PtrCesA8 (PtrCesA8-A), and PtrCesA18 (PtrCesA8-B) (Suzuki et al., 2006; Kumar et al., 2009) were repressed (Figure 3D), consistent with the reduced cellulose content (represented by glucose content) in TW of ptrlbd39/22 compared with wild-type TW (Table 1). The cell-wall composition of TW in ptrlbd39/22 was similar to that of the NW type, suggesting that the absence of PtrLBD39 and PtrLBD22 reduces the severity of TW development under bending stress.

**PtrLBD39 and PtrLBD22 are strong repressors of PtrNAC006, a negative regulator of TW formation**

Among the putative TF DEGs in the ptrlbd39/22 line (Supplemental Data 7) during TW formation, we were particularly interested in a stress-related TF gene we have recently discovered, PtrNAC006, a key regulator that confers strong drought tolerance in *P. trichocarpa* (Li et al., 2019). PtrNAC006 was upregulated in response to gravitational stimulus or mechanical stress in ptrlbd39/22 (Figure 4A). The results suggested that responses to gravitational stimulus, mechanical stress, and drought stress may be coregulated. We then analyzed TW formation in drought-tolerant transgenic *P. trichocarpa* that overexpressed PtrNAC006 (Li et al., 2019), using the same histochemical analysis described above (Figure 2) to evaluate the functional involvement of PtrNAC006 in TW formation. In addition to promoting drought tolerance, overexpression of PtrNAC006 significantly diminished TW formation, as indicated by decreased TW width, fewer TW G-fiber layers, and an increased frequency of vessels in the overexpressor (OE-PtrNAC006) (Figure 4B and 4C). Moreover, the wood width was smaller on the OW side than on the TW side in OE-PtrNAC006 (Figure 4D), indicating a reduction in TW differentiation in the transgenic plant. Consistent with reduced TW formation, wood composition analysis revealed that the stem TW of OE-PtrNAC006 had an approximately 27% reduction in cellulose content, an approximately 28% increase in xylose content, and an approximately 30% increase in lignin content after a 21-day bending compared with the stem TW of the wild type (Figure 4E). Our work revealed that the strong drought tolerance inducer PtrNAC006 is also an important negative regulator of TW formation.

**Genome-wide identification of the direct target genes of PtrLBD39**

Our transgenic and stress experiments suggested a regulatory hierarchy in TW formation. We then tried to reveal the role of PtrLBD39 in this hierarchy more systematically by defining its direct regulatory targets. To investigate which genes are directly regulated by PtrLBD39, we examined genome-wide binding sites of PtrLBD39 by ChIP-seq analysis. *PtrLBD39* was fused with a FLAG tag and overexpressed in *P. trichocarpa* under the control of a CaMV 3SS promoter, enabling binding sites to be identified with FLAG monoclonal antibodies after ChIP. We selected three...
independent transgenic lines (OE-PtrLBD39-FLAG-L1, -L2, and -L3) with the highest transgene transcript levels for further analysis (Supplemental Figure 10). We then carried out ChiP-seq analysis of SDX tissues fromPtrLBD39-FLAG transgenics using FLAG monoclonal antibodies. A total of 1,203PtrLBD39 binding sites were identified on 1,122 putative target genes (p < 1e−0.5; Supplemental Data 8). There were 60, 32, 46, 71, 474, and 520 binding sites in the 5’UTR, 3’UTR, intron, exon, intergenic, and promoter regions of these target genes, respectively (Figure 5A; Supplemental Data 8).

Figure 4. PtnNAC006 has negative effects on TW formation. (A) Transcript abundances of PtnNAC006 determined by RNA-seq. Normalized FPKM indicates normalized transcript abundances expressed as Fragments Per Kilobase of exon model per Million mapped fragments. Error bars indicate the SE of three biological replicates from independent pools ofP. trichocarpa TW tissues. (B) Histological analyses of TW stained with chlorazol black and safranin (upper panel) or safranin O and fast green (lower panel). The cross-sections were taken from the 20th internodes of 4-month-old wild-type (WT) and PtnNAC006-overexpressing (OE-PtnNAC006) plants after bending for 7 days. Black staining and blue staining indicate the presence of gelatinous fibers on the TW side. Scale bars, 200 μm. (C) Statistical analyses of TW area width and number of G-fiber layers of the wild type and OE-PtnNAC006 after bending for 7 days. Asterisks indicate significant differences between transgenic and WT plants based on Student’s t-test (**p < 0.01). Error bars indicate the SE of three independent experiments with threeP. trichocarpa plants of each genotype. (D) Wood width on the TW side and OW side of the wild type and OE-PtnNAC006 after bending for 7 days. Asterisks indicate significant differences between transgenic and WT plants based on Student’s t-test (**p < 0.05). Error bars indicate the SE of three independent experiments with three P. trichocarpa plants of each genotype. (E) TW wood composition after stem bending for 21 days in OE-PtnNAC006 transgenics and WT P. trichocarpa. Error bars indicate the SE of three independent experiments with threeP. trichocarpa plants of each genotype. Asterisks indicate significant differences between transgenic and WT plants based on Student’s t-test (**p < 0.01). Units are g/100 g of dry extractive-free wood. AIR, alcohol-insoluble residue. The wood width on the TW side and OW side was measured based on the xylem width of the TW and OW sides. B7-WT, B7-ptrlbd39/22, and B7-OE-PtnNAC006 represent TW of wild-type, ptrlbd39/22, and OE-PtnNAC006 plants after bending for 7 days; B21-WT and B21-OE-PtnNAC006 represent the TW of wild-type and OE-PtnNAC006 plants after bending for 21 days.
Genome distribution analysis showed that PtrLBD39 binding sites were mainly enriched in promoter regions, accounting for 43.22% of the total number of binding sites identified (Figure 5A). There are 512 PtrLBD39 direct target genes containing one or more PtrLBD39 binding sites within the 3-kb promoter region (p < 1e−0.5; Supplemental Data 9). TFs are known to regulate their target genes by binding to specific regulatory DNA sequences (cis-elements or TF-binding motifs). We analyzed the binding motifs within the promoters of direct PtrLBD39 target genes using DREME (Discriminative Regular Expression Motif Elicitation; Bailey, 2011). This analysis revealed that the “CGGC” core sequence was enriched in PtrLBD39 binding sites (Figure 5B), consistent with a previous finding that LBD proteins preferentially bind to this core sequence (Husbands et al., 2007) and supporting the reliability of our ChIP-seq analysis.

**PtrLBD39 mediates a TW-related TRN**

The ChIP-seq based identification of 512 direct PtrLBD39 targets allowed us to continue to explore the role of PtrLBD39 in a hierarchical regulatory network for TW formation. We next analyzed which of these PtrLBD39 target genes’ expression was affected by gravitational stimulus or mechanical stress to identify PtrLBD39’s key direct and indirect effector genes in the regulatory network for TW formation. To do this, we first analyzed LCM-based RNA-seq DEGs (14,850 DEGs representing TW induction effects, 7-day bending; Supplemental Data 4) and RNA-seq DEGs of ptrlbd39/22 double mutants (10,695 DEGs representing TW reduction effects; Supplemental Data 3). The common genes from these two DEG populations would then be those most responsive to TW stress. We identified 6,200 such genes (Supplemental Data 5). Overlapping the 512 direct targets of PtrLBD39 with the 6200 TW-responsive genes revealed a “core” set of 198 genes (Figure 5C, Supplemental Data 10) that are likely to be regulated directly by PtrLBD39 during TW formation. Of these 198 genes, 26 encoded TFs.

Next, we focused on these 26 TW-responsive TF genes to construct a PtrLBD39-mediated TRN, in which PtrLBD39 directly activates the expression of five TF genes and represses 21 TF genes (Figure 6). In this TRN, we also included PtnAC006 and PtnHSFB3-1, two negative regulators of TW formation, and 10 cell wall component genes (Figure 6). The expression of these two TF genes and the five monolignol biosynthetic pathway genes (PtrCCoAOMT2, PtrC4H2, PtnCCR2, PtrPAL1, and PtrPAL3) is indirectly repressed by PtrLBD39, and the five SCW cellulose synthase genes (PtrCesA4, PtnCesA7, PtnCesA17, PtnCesA8, and PtnCesA18) are indirectly activated. The TRN was extended using a computational approach to identify downstream genes of the 26 PtrLBD39 direct target TFs. Among these 26 TFs, 18 were predicted to regulate 16 secondary cell wall biosynthetic genes, including six monolignol, four cellulose, and six hemicellulose genes (Supplemental Figure 11). Together, we discovered a PtrLBD39-mediated TRN that is important for the...
characteristic features of TW (asymmetric growth, increased fibers, low lignin, and high cellulose).

**DISCUSSION**

In this study, we analyzed the transcriptomes of *P. trichocarpa* TW-forming cells isolated by LCM (Figure 1A and 1B). This transcriptomic analysis of TW responses in cells representing various stages of TW development provided a comprehensive view of TW formation. We identified 1,506 unique differentially expressed TF genes (Figure 1C) that are potentially important regulators of TW formation. Moreover, we examined the transcriptomic response at multiple time points and identified the most significantly induced TF gene, *PtrLBD39*, and its homologous gene, *PtrLBD22* (Figure 1). We generated CRISPR-edited *PtrLBD39/22* mutants (Supplemental Figure 3) for further characterization. Editing of these two TF genes led to reduced TW formation (Figure 2 and Supplemental Figure 6) with low cellulose and high lignin content (Table 1). These results suggested that *PtrLBD39* and *PtrLBD22* are key positive regulators of TW formation. Furthermore, we generated genome-wide transcriptomes for the *PtrLBD39/22* mutants and carried out ChIP-seq using *PtrLBD39-FLAG* transgenics (Figures 3 and 5). We then combined RNA-seq data with ChIP-seq data to reveal a *PtrLBD39*-mediated TRN that is involved in TW formation. The identification of two TFs that specifically regulate TW formation and the characterization of their regulatory roles are important for improving wood properties and developing new feedstocks for materials and bioenergy.

**PtrLBD39 and PtrLBD22 redundantly regulate TW formation in *P. trichocarpa***

The LBD proteins are a plant-specific TF family with a highly conserved LATERAL ORGAN BOUNDARIES domain (Shuai et al., 2002; Majer and Hochholdinger, 2011); they have diverse functions, such as the definition of lateral organ boundaries and the regulation of secondary growth in *Arabidopsis*, poplar, and Eucalyptus (Yordanov et al., 2010; Xu et al., 2016; Lu et al., 2018; Lee et al., 2019; Zhang et al., 2020). In this study, we identified a responsive LBD gene, *PtrLBD39*, after tree bending (Figure 1). *PtrLBD39* transcripts were almost undetectable in SDX tissues under normal growth conditions but were highly induced when the plant was subjected to gravitational stimulus or mechanical stress (Figure 1E).

Phylogenetic analysis showed that *PtrLBD39* and its LBD homolog *PtrLBD22* have an *Arabidopsis* ortholog, *AtLBD4* (*At1g31320*) (Supplemental Figure 2). *AtLBD4* is expressed at the boundary between phloem and procambium and affects the shape of vascular bundles (Smit et al., 2020). The *PtrLBD22* ortholog in *Populus tremula × P. alba*, *PtaLBD4*, is expressed mainly in phloem and participates in the regulation of secondary phloem development in the stem (Yordanov et al., 2010). We found that *PtrLBD39* had no significant effects on primary and secondary growth in *P. trichocarpa* plants based on CRISPR-based knockout experiments (Supplemental Figures 3 and 4). However, under gravitational stimulus or mechanical stress, the deletion of *PtrLBD39* expression resulted in inhibited TW formation (Figure 2 and Supplemental Figure 6). These results are consistent with the expression pattern of *PtrLBD39* during TW formation. Previous studies and our findings suggested that different LBD proteins have distinct functions and that *PtrLBD39* specifically regulates TW development. CRISPR-based knockout of the *PtrLBD39* and *PtrLBD22* gene pair did not significantly affect plant growth (Supplemental Figure 3) but did result in more severe inhibition of TW formation compared with the *PtrLBD39* single knockouts (Figure 2 and Supplemental Figure 6). The double knockouts had low cellulose and high lignin content compared with the bent wild type (Table 1). These results suggest that *PtrLBD39* and *PtrLBD22* are key positive regulators specific for TW development and exert redundant functions in the regulation of this process. However, a small amount of TW can still be formed in the *ptrlbd39/22* double mutants, supporting the possibility that other, similar LBD genes function redundantly in

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**Figure 6.** *PtrLBD39*-mediated TRN for TW formation in *P. trichocarpa.*

The solid lines represent direct regulation, and the dashed lines represent indirect regulation. The arrows indicate activation, and the blunted lines indicate repression.
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TW formation. In the *P. trichocarpa* genome, *PtrLBD39* and *PtrLBD22* have high protein sequence identity with *PtrLBD49* and *PtrLBD18* (ranging from 70% to 91%; Supplemental Figure 2). Although the transcript levels of *PtrLBD49* and *PtrLBD18* genes were not significantly induced by bending stress, these two genes may have compensatory functions in TW formation in the absence of *PtrLBD39* and *PtrLBD22* in *P. trichocarpa*.

**PtrLBD39 and PtrLBD22 regulate asymmetric growth in *P. trichocarpa***

The absence of *PtrLBD39* and *PtrLBD22* reduced the asymmetric growth of *P. trichocarpa* in response to bending treatments (Figure 2), suggesting that the cambial activity of the *ptrlbd39/22* double mutants was attenuated in response to gravitational stimulus or mechanical stress. LCM-based RNA-seq analyses of TW formation in the wild-type plant revealed that the transcript levels of *PtrLBD39* and *PtrLBD22* in the TW-forming cells were markedly increased after bending treatments (Figure 1E). This result supported the possibility that loss of *PtrLBD39* and *PtrLBD22* function may inhibit differentiation of cambial cells into xylem cells in response to stem bending, ultimately leading to a decrease in asymmetric growth in the *ptrlbd39/22* double mutants.

The decreased asymmetric growth of the *ptrlbd39/22* mutants in response to bending is similar to previous observations of reduced asymmetric growth in ethylene-insensitive leaning trees (Love et al., 2009). We compared the ethylene-dependent DEGs identified from ethylene-insensitive trees (Seyfferth et al., 2019) with the DEGs in TW of *ptrlbd39/22* (Supplemental Data 3). The analysis revealed many common genes between these two DEG populations that were related to pathways of hormones such as ethylene. Moreover, the *PtrLBD39* ortholog in hybrid aspen was found among the ethylene-dependent genes, and its transcript level was highly induced in TW (Seyfferth et al., 2019), consistent with our LCM-based RNA-seq analyses of TW formation in *P. trichocarpa* (Figure 1E, Supplemental Data 1). Ethylene has been demonstrated to act as an endogenous regulator of meristem growth that stimulates cambial activity and influences many of the features that characterize TW formation (Love et al., 2009; Groover, 2016; Felten et al., 2018). *PtrLBD39* and *PtrLBD22* may regulate TW formation and asymmetric growth through crosstalk with the ethylene pathway under gravitational and mechanical stresses. However, the precise functions of *PtrLBD39* and *PtrLBD22* in mediating the ethylene pathway require further characterization.

Other hormones were also demonstrated to play key roles in the regulation of TW formation and asymmetric growth. For instance, brassinosteroid (BR) can promote TW formation during the stem gravitropic response of poplar (Du et al., 2020; Jin et al., 2020). Overexpression of the BR biosynthetic pathway gene *PagDET2* elevated endogenous BR levels in the TW side of the stem during gravity stimulation (Du et al., 2020), suggesting that a BR concentration gradient can affect asymmetric growth during gravity stimulation. In our study, RNA-Seq of the TW from *ptrlbd39/22* revealed that the *P. trichocarpa* ortholog of PagDET2, *PtrDET2*, was downregulated in these mutants (Supplemental Data 3). These transcriptome analyses of genes associated with hormone pathways suggest that *PtrLBD39* and *PtrLBD22* may be part of complex signaling and regulatory networks that co-mediate multiple growth and adaptation processes for the biosynthesis of hormones and the formation of secondary cell walls.

**PtrLBD39 and PtrLBD22 regulate a set of TW-related genes for TW formation in *P. trichocarpa***

Integrative analysis of the RNA-seq DEGs of *ptrlbd39/22* mutants (Supplemental Data 3) and the LCM-based RNA-seq DEGs (Supplemental Data 4) revealed 6,200 *PtrLBD39*-regulated TW-responsive genes (Supplemental Data 5). Of these genes, *PtrMYB128*, *PtrMYB221*, *PtrMYB167*, and *PtrMYB28* were downregulated in the TW of *ptrlbd39/22* lines (Figure 3A) and upregulated in the TW of wild-type plants (Figure 3B). All these MYB TF genes could activate the expression of SCW biosynthesis genes and affect TW formation (Zhong and Ye, 2009; Zhong et al., 2011; Tang et al., 2015; Jin et al., 2020; Liu et al., 2021). The TW of *ptrlbd39/22* mutants had more lignin and less cellulose (Table 1). Changes in the expression of these MYB genes may contribute to alterations in lignin and cellulose content in the TW of *ptrlbd39/22* lines.

The transcript levels of *ARK2*, *PtrNAC006*, and *PtrHSFB3-1* were upregulated in *ptrlbd39/22* mutants (Figures 3A and 4A). Overexpression of *ARK2* was associated with the formation of fewer TW fibers (Gerttula et al., 2015). The decrease in TW G-fiber layers in *ptrlbd39/22* lines (Figures 2B and 2D) may have resulted in part from upregulation of *ARK2*. *PtrNAC006* was previously reported as a drought-tolerance effector gene involved in the control of vessel development in *P. trichocarpa* (Li et al., 2019). In this study, we found that overexpression of *PtrNAC006* resulted in significantly decreased TW formation (Figure 4B–4D). Together with the observation that CRISPR-based knockout of *PtrLBD39* and *PtrLBD22* led to elevated transcript levels of *PtrNAC006* (Figure 4A), our findings suggest that upregulation of *PtrNAC006* contributed in large part to the reduced TW formation in *ptrlbd39/22* lines (Figure 2). *PtrHSFB3-1*, another upregulated gene in *ptrlbd39/22* mutants (Figure 3A), has been recently reported for its role in affecting cell wall biosynthesis (Liu et al., 2021). Reduced levels of *PtrHSFB3-1* transcripts caused reduced lignin quantity during TW formation, and elevated levels of *PtrHSFB3-1* restored lignin biosynthesis in TW (Liu et al., 2021), suggesting that the increased TW lignin content of the *ptrlbd39/22* lines (Table 1) was partly attributed to upregulation of *PtrHSFB3-1*.

Low lignin content and high cellulose content are unique features of TW (Wardrop, 1964; Coté et al., 1969; Fujita et al., 1974; Timell, 1986; Nugroho et al., 2013; Gritsch et al., 2015; Liu et al., 2021). Our RNA-seq results from *ptrlbd39/22* mutants showed that five monolignol pathway genes were activated and all SCW cellulose synthase genes were repressed in the absence of *PtrLBD39* and *PtrLBD22* (Figure 3C and 3D). This finding suggested that *PtrLBD39* and *PtrLBD22* affect lignin and cellulose content in TW by regulating these key cell wall component genes.

A group of FLAs, which are involved in the regulation of cellulose microfibril angle and the adhesion of cell wall components, were highly upregulated in the TW-forming tissues of poplar (Lafarguette et al., 2004; Andersson-Gunnerås et al., 2006; Azri
PTR-LBD39 mediates a TW-specific TRN in *P. trichocarpa*

Wood formation is regulated by a TRN that includes TFs and SCW component genes (Zhong et al., 2010, 2011; Ohtani et al., 2011; Li et al., 2012; Lin et al., 2013; Lu et al., 2013; Chen et al., 2019; Yeh et al., 2019; Wang et al., 2020). We previously unraveled a TF PtrSND1-B1-mediated TRN consisting of 57 TF-DNA and nine TF-TF (protein-protein) interactions through 17 TFs in a four-layer regulatory hierarchy that transregulates 27 effector genes for wood formation (Lin et al., 2013; Chen et al., 2019). Recently, we found that the previously constructedPtrSND1-B1-directed TRN is regulated by all PtrSND1 family members as the top-layer regulators that are controlled through feedback regulation mediated by PtrMYB161 (Wang et al., 2020). We also discovered a TW-specific TRN mediated by PtrHSFB3-1 and PtrMYB092 that transactivates the expression of 31 genes involved in the biosynthesis of lignin, cellulose, and hemicelluloses (Liu et al., 2021).

In this study, we constructed a PtrLBD39-mediated TW TRN in which PtrLBD39 directly regulates 26 TF genes and indirectly regulates two TW negative regulators (PtrHSFB3-1 and PtrNAC006), five SCW cellulose synthase genes (PtrCesA4, PtrCesA7, PtrCesA17, PtrCesA8, and PtrCesA18), and five monolignol biosynthesis genes (PtrCCoAOMT2,PtrC4H2,PtrCCR2,PtrPAL1, andPtrPAL3) (Figure 6). Of the 26 direct target TFs,PtrMYB128, which is activated by PtrLBD39, can activate the promoter activities of the PtrCesA genes (Zhong et al., 2011; Jin et al., 2020). The activation of the PtrCesA genes in this TRN may be a result of PtrLBD39 directly activatingPtrMYB128, which in turn activates thesePtrCesA genes. The precise functions of the 26 novel TFs remain to be characterized in order to gain a more comprehensive understanding of the PtrLBD39-mediated TRN for TW formation. In addition,PtrHSFB3-1 is a negative regulator of TW and can mediate transcriptional reprogramming to repress monolignol biosynthesis (Liu et al., 2021). In this TRN,PtrLBD39 may repress the expression of the monolignol genes by repressingPtrHSFB3-1. In the current work, we discovered a PtrLBD39-mediated TRN (Figure 6) that leads to the establishment of characteristic TW features, such as asymmetric growth, increased fiber numbers, low lignin content, and high cellulose content. This TRN is distinct from the NW regulatory network (Lin et al., 2013; Chen et al., 2019; Wang et al., 2020). These findings suggest thatPtrLBD39 mediates a TW-specific TRN in *P. trichocarpa*. The ortholog ofPtrLBD39 in Arabidopsis, LBD4, marks the phloem-procambium boundary and defines the shape of the vascular bundle to control vascular development via regulation by TDF-PXY, WOX14, and TMO6 in a PXY-mediated transcriptional network (Smit et al., 2020). However, when comparing the direct targets ofPtrLBD39 and AtLBD4, we found that they do not share any common direct targets. These data suggest thatPtrLBD39 is functionally divergent from AtLBD4, and the functions ofPtrLBD39 have evolved for TW formation.

**METHODS**

**Plant materials and growth conditions**

All experiments were performed using the *P. trichocarpa* genotype Nisqually-1. Wild-type, knockout mutant, and transgenic plants were grown in a walk-in growth chamber as described previously (Li et al., 2019). Four-month-old *P. trichocarpa* plants were used for artificial stem bending. RNA extraction, and histological analysis. Stems of 6-month-old plants were used for wood composition analysis and ChIP analysis. To induce TW, the *P. trichocarpa* plants were bent at a 90-degree angle for 0, 3, 5, 7, 14, or 21 days.

**Laser capture microdissection**

Four-month-old greenhouse-grown *P. trichocarpa* were bent at the 20th internode (counted from the top) to a 90-degree angle to induce TW formation. After treating for 3, 5, 7, 14, or 21 days, approximately 1.5-cm lengths of the greatest bent degree stems (as shown in the red circle in Supplemental Figure 1) and the upright stems (from untreated trees) were collected and cut into 0.6-cm segments. The ends of the stem segments (about 1/2 length of each 0.6-cm segment) were embedded in OCT embedding matrix (SAKURA, 4583) and fixed onto a freezing platform for cryosectioning (Leica, DM6000B). Cross-sections (16 µm) were placed onto a PET membrane nuclelease-free frame slide (Leica, 11 505 190) with 100% ethanol to dehydrate for 3 min. The solution was removed with RNase-free paper, and the slide was air dried completely at room temperature. The SDX cells of upright stems and TW areas on the upper side of the bent stems were collected using a Leica LMD7000 laser microdissection system (Chen et al., 2014), and the collected tissue was placed directly into RNeasy Lysis Buffer (Qiagen, RNeasy Plant RNA Isolation Kit, 74 904) for RNA extraction.

**Total RNA extraction and RNA amplification**

Total RNA was isolated from SDX and LCM-collected samples using the RNeasy Plant RNA Isolation Kit (Qiagen, 74 904). RNA concentration was determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific), and RNA quality and integrity were assessed with an Agilent 2100 Bioanalyzer (Agilent). For RNA amplification, 750 pg of RNA from each sample was used to generate cDNA using the Ovation RNA-Seq System V2 Kit (NuGEN, 7102) according to the manufacturer’s instructions.

**RNA-seq and data analysis**

For identification of genes associated with TW formation, RNA-seq was performed with total RNA of xylem cells isolated from the upper side of the bent stems by LCM. The RNA-seq libraries were constructed from three biological replicates per sample using the Illumina TruSeq RNA Sample Prep Kit (Illumina, RS-122-9001DOC). A total of 18 libraries were sequenced using the Illumina HiSeq 4000 platform to generate paired-end reads. For examination of gene expression in *ptrlbd39/22* double mutant lines, SDX in the TW area on the upper sides of 7-day bent stems was collected by scraping lightly with a razor blade. RNA-seq libraries of mutants and wild-type plants were constructed from three biological replicates per sample using the MGIEasy RNA Library Preparation Reagent Kit V3.0 (BGI, 1 000 006 383). A total of six libraries were sequenced on the BGISEQ-500 platform to
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generate paired-end reads. For all libraries, average read lengths of 150 base pairs (bp) were generated by sequencing. After removing the library index sequences from each read, the clean reads were mapped to the *P. trichocarpa* genome v.4.0 ([https://phytozome-next.jgi.doe.gov/](https://phytozome-next.jgi.doe.gov/)) using HISAT2 ([Kim et al., 2015](#)). The raw counts were determined and standardized following our established analysis pipeline ([Lin et al., 2013](#)). DEGs were identified using DESeq2 ([Love et al., 2014](#)) with FDR <0.05. A heatmap was drawn using TBtools software based on the FPKM values of 33 genes ([Chen et al., 2020](#)). A co-expression network was constructed with the R (v3.6.1) package WGCNA using the PtrLBD39 direct target genes, the SCW biosynthetic genes, and Pearson correlation analysis with absolute values of correlation greater than 0.99. The network was visualized using Cytoscape (v3.6.0).

Phylogenetic analysis

A phylogenetic tree was reconstructed with MEGA 7 using the neighbor-joining (NJ) method and 1,000 bootstrap replicates.

RT-qPCR

One microgram of total RNA was reverse-transcribed using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara, RR047A). RT-qPCR was performed with FastStart Universal SYBR Green Master Mix (Roche, 4 913 914 001) on an Agilent Mx3000P QPCR System (Agilent) according to the manufacturer’s instructions. The primers are listed in Supplemental Table 1.

Generation of *P. trichocarpa* transgenic and knockout mutants

The full length of *PtrLBD39* was cloned from *P. trichocarpa* plants using the primers listed in Supplemental Table 1. 35S::PtrLBD39:FLAG constructs were generated using the pB121 vector. Single-knockout mutants of *PtrLBD39* and double-knockout mutants of *PtrLBD39* and *PtrLBD22* genes were produced using the CRISPR/Cas9 system ([Jeta et al., 2017](#)). The single guide RNA (sgRNA) was designed using CRISPR-P 2.0 ([http://crispr.hzau.edu.cn/cgi-bin/CRISPR2/CRISPR](http://crispr.hzau.edu.cn/cgi-bin/CRISPR2/CRISPR)). The sgRNA sequences were synthesized and ligated into the pEgP237-2A-GFP vector ([Jeta et al., 2017](#)). All constructs were transformed into *P. trichocarpa* plants using Agrobacterium tumefaciens using a rapid genetic transformation method as described previously ([Li et al., 2017, 2018](#)). The expression of *PtrLBD39* in the transgenic plants was detected by RT-qPCR as described above. To detect the editing of knockout mutants, primers flanking the sgRNA target sequence were designed for PCR amplification. The PCR products were ligated into the pMD18-T vector, and at least 20 colonies were selected for sequencing. The primers are listed in Supplemental Table 1.

Histochemical and histological analyses

Four-month-old greenhouse-grown *OE-PtrNAC006* ([Li et al., 2019](#)), *ptrlbd39/22*, and wild-type plants were bent at the 20th internode to a 90-degree angle to induce TW formation. After being treated for 7 and 21 days, approximately 1.5-cm lengths of the greatest bent degree stem segments (as shown in the red circle in Supplemental Figure 1) were cut into 5-cm segments (as indicated by the double-headed arrow in Supplemental Figure 1) and then divided into TW- and OW-side wood using a razor blade. Wood was carefully excised from the TW area using a razor blade to obtain TW-side wood for chemical analysis. The TW area wood was immersed in 90% (v/v) acetone for 2 days and then transferred to 100% (v/v) acetone for 14 days at room temperature. The air-dried TW area wood was used for quantitative analysis of wood components and lignin components following established procedures ([Abraham et al., 2013; Wang et al., 2018](#)).

ChIP assays

The SDX tissues of 6-month-old *OE-PtrLBD39-FLAG* transgenic plants were scraped for ChIP assays following our established protocol ([Lin et al., 2013; Li et al., 2014, 2019](#)). Anti-FLAG antibody (Sigma, F1804) was used to immunoprecipitate the fragmented chromatin. Four ChIP-seq libraries (ChIP-DNA and input DNA, two biological replicates per library) were constructed using the NEBNext Multiplex Oligos for Illumina (NEB, E7335S) and NEBNext Ultra II DNA Library Prep Kit for Illumina (NEB, E7645S) following the manufacturer’s protocol and were sequenced using an Illumina Genome Analyzer.

ChIP-seq data analysis

Single-end reads with an average length of 50 bp were obtained using the Nextseq platform. FASTX-Toolkit (v0.0.14) ([http://hannonlab.cshl.edu/fastx_toolkit/](http://hannonlab.cshl.edu/fastx_toolkit/)) was used to filter the raw data by removing N-containing reads and adapters, thereby generating clean data. The clean data were mapped to the *P. trichocarpa* genome v.4.0 ([https://phytozome.jgi.doe.gov/](https://phytozome.jgi.doe.gov/)) using Bowtie 2 (v2.3.5.1) ([Wick et al., 2017](#)). Alignments with no more than one mismatch were retained for further analysis. Multiple aligned paired reads were filtered with Samtools ([Li et al., 2009](#)), and duplicated reads were removed with Picard ([http://broadinstitute.github.io/picard](http://broadinstitute.github.io/picard)) to obtain unique mapped reads. Peak calling was carried out using MACS2 ([Zhang et al., 2008](#)) with default parameters (p-value < 1e−5). Analysis of cross-correlation and irreproducible discovery rate of measurements made perpendicular to each other for each vessel cell. The average of 200 vessel cell measurements was used as one biological replicate, and the final mean value was considered to be the vessel cell diameter. The distance from the center of the pith to the cambium on both the upper (TW) and lower (OW) sides was measured to calculate the TW/OW ratio ([Seyfferth et al., 2019](#)). The xylem width of the TW and OW sides was measured as the wood width of the TW side and OW side. Data are presented as the means and standard errors of three plants, and the statistical significance of mean differences was assessed using Student’s t-test.

Four-month-old wild-type and *ptrlbd39/22* double mutant plants were subjected to 90° gravi-stimulation for a period of 17 days. The vertical distance from the base of the stem to the apex of curvature was measured to calculate the stem lift ([Ju et al., 2020](#)). The greatest bent degree stem was collected for histological observation and statistical analysis as described above.

Wood composition analysis

Six-month-old greenhouse-grown *ptrlbd39/22*, *OE-PtrNAC006* transgenic, and wild-type plants were bent at the 20th internode to a 90-degree angle to induce TW formation. After 21 days, the bent stems were cut into 5-cm segments (as indicated by the double-headed arrow in Supplemental Figure 1) and then divided into TW- and OW-side wood using a razor blade. Wood was carefully excised from the TW area using a razor blade to obtain TW-side wood for chemical analysis. The TW area wood was immersed in 90% (v/v) acetone for 2 days and then transferred to 100% (v/v) acetone for 14 days at room temperature. The air-dried TW area wood was used for quantitative analysis of wood components and lignin components following established procedures ([Abraham et al., 2013; Wang et al., 2018](#)).
Transcriptional network for tension wood formation

IP samples were performed using phantomepeakqualtools-1.2 and IDR-2.0.4.2 of ENCODE (Landt et al., 2012). Final peaks were obtained using HOMER (two biological repeated peak summit distances <500 bp were defined as the same peak) (Heinz et al., 2010) and annotated by ChiPseeker (Yu et al., 2015).

To examine TF-binding motifs enriched within PtrLBD39-binding peaks, DREME (Bailey, 2011) was used to discover putative motifs within a 500-bp window centered on the peak center. Fisher’s exact test (Fisher, Andersson-Gunnerås, S., Mellerowicz, E.J., Love, J., Segerman, B., Andersson-Gunnerås, S., Hellgren, J.M., and Bj...)

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