Dimerization of PtrMYB074 and PtrWRKY19 mediates transcriptional activation of *PtrbHLH186* for secondary xylem development in *Populus trichocarpa*

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**Summary**

- Wood formation is controlled by transcriptional regulatory networks (TRNs) involving regulatory homeostasis determined by combinations of transcription factor (TF)–DNA and TF–TF interactions. Functions of TF–TF interactions in wood formation are still in the early stages of identification.
- *PtrMYB074* is a woody dicot-specific TF in a TRN for wood formation in *Populus trichocarpa*. Here, using yeast two-hybrid and bimolecular fluorescence complementation, we conducted a genome-wide screening for *PtrMYB074* interactors and identified 54 *PtrMYB074*-TF pairs. Of these pairs, 53 are novel.
- We focused on the *PtrMYB074*-PtrWRKY19 pair, the most highly expressed and xylem-specific interactor, and its direct transregulatory target, *PtrbHLH186*, the xylem-specific one of the pair’s only two direct TF target genes. Using transient and CRISPR-mediated transgenesis in *P. trichocarpa* coupled with chromatin immunoprecipitation and electrophoretic mobility shift assays, we demonstrated that *PtrMYB074* is recruited by *PtrWRKY19* and that the *PtrMYB074*-PtrWRKY19 dimers are required to transactive *PtrbHLH186*. Overexpressing *PtrbHLH186* in *P. trichocarpa* resulted in retarded plant growth, increased guaiacyl lignin, a higher proportion of smaller stem vessels and strong drought-tolerant phenotypes.
- Knowledge of the *PtrMYB074*-PtrWRKY19-PtrbHLH186 regulation may help design genetic controls of optimal growth and wood formation to maximize beneficial wood properties while minimizing negative effects on growth.

**Introduction**

Wood is one of the world’s most abundant and renewable resources for energy and value-added products (Sarkanen, 1976; Chiang, 2002; Ragauskas *et al.*, 2006; Chen & Dixon, 2007). Wood formation in angiosperms is a complex process of tissue-specific development and differentiation derived from the vascular cambium, which gives rise to three types of cells, fibre, vessel and ray, accompanied by secondary wall thickening and programmed cell death (Esau, 1965; Evert, 2006). Fibres and vessel elements are the two major cell types and have unique functions (Chen & Evans, 2005; Ohshima *et al.*, 2011). Fibre cells provide support, and vessels transport water and nutrients (Wardrop, 1981; Tyree & Sperry, 1989). The proportion of vessels and fibres in wood is a major factor affecting wood quality and property (Bowyer *et al.*, 2003).

Wood formation is controlled by transcriptional regulatory networks (TRNs) consisting of transcription factors (TFs) and secondary cell wall (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). Secondary wall-associated NAC domain (SND) and vascular-related NAC domain (VND) TFs were suggested as master regulators of the TRN for wood formation (Kubo *et al.*, 2005; Zhong *et al.*, 2006, 2011; Ohtani *et al.*, 2011; Li *et al.*, 2012; Lin *et al.*, 2013, 2017; Chen *et al.*, 2019). MYBs have been identified as SND’s downstream TFs in the TRN for wood formation (McCarthy *et al.*, 2010; Lin *et al.*, 2013; Nakano *et al.*, 2015; Tang *et al.*, 2015; Jiao *et al.*, 2019; Ohtani & Demura, 2019). The *Populus trichocarpa* genome encodes 266 MYB genes (*PtrMYBs*) (Wilkins *et al.*, 2009; Tian *et al.*, 2020). Many of the *PtrMYBs* or their homologs in other *Populus* species directly or indirectly regulate SCW component genes for wood formation, such as *PtrMYB002*, *PtrMYB003*, *PdMYB10*, *PtrMYB020*, *PtrMYB021*, *PtrMYB074*, *PtrMYB90*, *PtrMYB092*, *PtrMYB125*, *PtrMYB152*, *PtrMYB156*, *PtrMYB161*, *PtoMYB170*, *PtrMYB189*, *PtrMYB194*, *PtoMYB216* and *PdMYB221* (also named LTF1) (McCarthy *et al.*, 2010; Tian *et al.*, 2013; Zhong...
et al., 2013; Chai et al., 2014; C. Li et al., 2014; Li et al., 2015; S. Wang et al., 2014; Tang et al., 2015; Wang et al., 2016; Xu et al., 2017; Yang et al., 2017; Chen et al., 2019; Gui et al., 2019; Jiao et al., 2019; Balmant et al., 2020; Wang et al., 2020; B. Liu et al., 2021). Of the studied *Populus* MYBs, PtrMYB074 has been identified as one of the key wood formation regulators in our previously established PtrSND1-directed TRN (Lin et al., 2013; Chen et al., 2019; Wang et al., 2020). In this TRN, all PtrSND1 family members directly regulate PtrMYB074, which directly transactivates the expression of 17 target genes for wood formation (Chen et al., 2019; Wang et al., 2020).

Gene regulation and cell-type determination typically involve multiple TFs that function in a combinatorial manner, enabling a relatively small number of TFs to generate a large diversity of cell types (Small et al., 1992; Halflon et al., 2000; Takahashi & Yamanaka, 2006; Swanson et al., 2010; Vierbuchen et al., 2010; Farley et al., 2015). Heterodimerization between TFs is widely associated with biological diversity and functional specificity for different metabolic processes in plants. For example, a bHLH protein AN1 and a MYB protein AN2 form a heterodimer to activate anthocyanin biosynthetic genes in *Petunia* (Spelt et al., 2000, 2002), while a AN2-PH4 heterodimer activates vacuolar acidification (Quattrocchio et al., 2006). In *Populus*, PaC3H17 interacts with PaMYB199 to attenuate PaMYB199-driven xylem target gene suppression. Auxin promotes the dual regulation of a PaC3H17-PaMYB199 module for xylem formation, resulting in enhanced SCW deposition (Tang et al., 2020). In thePtrSND1-directed TRN, seven TF–TF protein interactions were found to be involved in gene transregulation for wood formation (Chen et al., 2019). Such TF–TF regulations may cooperatively or combinatorially mediate the biosynthesis of specific types of lignin (Chen et al., 2019). However, the underlying regulatory mechanisms of TF–TF protein interactions associated with wood formation remain to be explored.

Yeast two-hybrid (Y2H) is a powerful tool for studying protein–protein interactions due to its ability for large-scale screening of protein pairs (Fields & Song, 1989; Ou et al., 2011). For example, Y2H screening of c. 8000 open reading frames (ORFs) generated an interactome map that includes 6200 protein–protein interactions in Arabidopsis (Arabidopsis Interactome Mapping Consortium, 2011) and a screening of c. 13000 ORFs constructed a human interactome network consisting of 14 000 protein–protein interactions (Rolland et al., 2014). In *P. trichocarpa*, Y2H screening of a secondary xylem cDNA library resulted in 165 protein–protein interactions, including 162 distinct ORFs (Petzold et al., 2017). The Y2H system is useful for the identification of protein–protein interactions *in vivo*. The resulting interactions need to be validated *in vitro*, such as using bimolecular fluorescence complementation (BiFC) assays (Walter et al., 2004).

Wood formation is controlled by TRNs formed by TF–DNA interactions (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). TF–DNA interactions can be identified *in vivo* by chromatin immunoprecipitation (ChIP; Solomon et al., 1988). There are typically two types of TF–DNA interactions identified by ChIP, the direct binding of a TF to its target and the indirect binding of a TF to its target (i.e. the TF requires other protein partners necessary for DNA binding). ChIP alone is limited in its ability to distinguish between the two types of TF–DNA interactions. Electrophoretic mobility shift assay (EMSA) can be used for the detection of the direct binding of TFs to specific DNA fragments *in vitro* (Fried, 1989). The combined analysis of ChIP assay and EMSA has been used to discover specific regulatory mechanisms of TF–TF protein interactions for target gene transregulation. For example, FLOWERING LOCUS T has no DNA binding activity but associates with API promoters through a direct interaction with FLOWERING LOCUS D, a bZIP TF that directly binds to the *API* promoter (Wigge et al., 2005).

In this study, we performed a Y2H screening and identified 54 PtrMYB074-interacting TFs. Among them, PtrWRKY19 is the most highly expressed xylem-specific interactor. ChIP-seq analyses of PtrMYB074 and PtrWRKY19 reveal that the two proteins co-target a set of genes, including a basic helix–loop–helix (bHLH) TF family gene, *PtrbHLH186*. Our results support a regulatory module in which PtrMYB074 is recruited by PtrWRKY19 to their common target, *PtrbHLH186*, and mediates the activation of this gene. Genetic analysis reveals that the overexpression of *PtrbHLH186* caused abnormal lignification, enhanced vessel cell development and altered wood composition. These data reveal a molecular mechanism for the combined TF–DNA and TF–TF regulations associated with wood formation in *P. trichocarpa*.

**Materials and Methods**

**Plant materials and growth conditions**

All experiments were performed with *Populus trichocarpa* Torr. & Gray (genotype Nisqually-1). Approximately 600 wild-type and transgenic plants generated in this study were grown and maintained in a glasshouse as described (Li et al., 2019). The tissues of healthy 4-month-old plants were used for growth index measurement, RNA isolation, histological analysis and scanning electron microscopy. The stems of 6-month-old wild-type plants were used for stem-differentiating xylem (SDX) protoplast isolation.

**Protein interaction**

Y2H assays were performed according to the Matchmaker Gold Yeast Two-Hybrid System (Clontech, San Francisco, CA, USA). A total of 221 xylem-abundant *P. trichocarpa* TFs were generated for Y2H screening (Supporting Information Table S1; Lin et al., 2017; Yeh et al., 2019). All TFs were fused to the GAL4-activating domain (AD) in the pGADT7 vector, and the full-length PtrMYB074 cDNA and PtrMYB074 N-terminus were amplified and subcloned into the GAL4 binding domain vector (pGBK7). Yeasts (strain Y2HGold) transformed with these two constructs were grown on selective medium SD-LWH/X/A (SD/-Leu/-Trp/-His/X-α-Gal/AbA) and SD-LWH/X/A (SD/-Leu/-Trp/-His/-
Ade/X-α-Gal/AbA) with 40 mg ml⁻¹ X-α-Gal and 350 mg ml⁻¹ aureobasidin A (AbA) to detect their growth status.

For BiFC assays, PtrMYB074, PtrMYB021 and PtrWRKY19 coding regions were cloned into pUGW0 and pUGW2 vectors for gene transient expression, respectively. *P. trichocarpa* SDX protoplast isolation and transfection were carried out following an established protocol (Lin et al., 2014). Each pair of constructs was co-transfected with H2A-1:mCherry into SDX protoplasts. After 12 h of incubation in the dark, transfected SDX protoplasts were collected by centrifugation at 400 g for 5 min and the YFP signal was captured using a Leica DM6 B microscope. Three biological replicates were carried out for each combination of TFs and the negative controls.

**Plant protein extraction and Western blotting**

Nuclear protein isolation from *P. trichocarpa* SDX tissues was carried out following an established protocol (Loziuk et al., 2015). The nuclear protein extracts were separated on 10% (w/v) sodium dodecyl sulphate–polyacrylamide gel electrophoresis and subsequently transferred to a polyvinylidene fluoride membrane (Thermo Scientific, Waltham, MA, USA). The membrane was blocked using nonfat dry milk. The proteins were tested using anti-FLAG (peptide sequence DYKDDDDK) antibodies (F1804; Sigma). The signals were detected using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and an X-ray film (Sigma).

**Chromatin immunoprecipitation assays**

Chromatin immunoprecipitation was carried out on the SDX of 6-month-old *P. trichocarpa* plants following established protocols (Lin et al., 2013; C. Li et al., 2014; W. Li et al., 2014). Briefly, c. 5 g of SDX tissues was cross-linked in 1% (w/v) formaldehyde and used to isolate nuclei and chromatin. The chromatin was sonicated by using a Bioruptor (Diagenode, Liege, Belgium). The purified ChIP-DNAs from immunoprecipitation with anti-FLAG antibodies (Sigma, F1804) were used for ChIP-qPCR and ChIP-seq. The purified ChIP-DNAs immunoprecipitated by anti-IgG (ab205719; Abcam, Cambridge, UK) or anti-PtrMYB074 (custom-made; Abmart, Shanghai, China) antibodies were used for ChIP-qPCR. The anti-PtrMYB074 monoclonal antibody was produced in full-length protein against the chromatin and purified using an IgG affinity chromatography column (Abmart). ChIP-qPCR primers are listed in Table S2. ChIP-seq libraries were constructed using NEBNext® Multiplex Oligos from Illumina® (E7355S; NEB, San Diego, USA) and NEBNext® Ultra™ II DNA Library Prep Kit from Illumina® (E7645S; NEB) according to the manufacturer’s instructions, and were sequenced using an Illumina NextSeq 550 in Beijing.

**ChIP-seq analysis**

The NextSeq platform was used to obtain single-end reads with an average length of 50 bp. Clean data were derived by removing low-quality reads, N-containing reads and joint contamination (FASTX-Toolkit, http://hannonlab.cshl.edu/fastx_toolkit/). Sequencing reads were mapped to the reference genome (*P. trichocarpa* genome v.3.0) using BOWTIE 2 (v2.3.5.1) (Wick et al., 2017). The cross-correlation and irreproducible discovery rate of ChIP-DNA samples were analysed by ENCODE (Landt et al., 2012). Peak calling was used by MACS2 (Zhang et al., 2008) with default parameters (P-value < 1e⁻⁰⁵). Final peaks were annotated using CHIPSEEKER (Yu et al., 2015), which was obtained from HOMER (Heinz et al., 2010).

**Subcellular localization**

The coding region of *PtrbHLH186* was cloned into the pUC19-sGFP vector. The plasmids were extracted by the CsCl density-gradient ultracentrifugation method (Lin et al., 2014), and were transfected into SDX protoplasts with a nuclear marker (pUC19-35S-H2A-1-mCherry) as described previously (Lin et al., 2013, 2014). The signal was captured using a Leica DM6 B microscope.

**Recombinant protein production**

The coding sequences of PtrMYB074 and PtrWRKY19 were cloned into the pET101/D-TOPO vector (Invitrogen) with a 6×His tag at the N-terminus according to the manufacturer’s instructions, respectively. To produce recombinant proteins, the constructs were transferred into *E. coli* (E. coli) Rosetta2 (Invitrogen). The recombinant protein was purified using HisPur Ni-NTA Resin (Thermo Scientific) and collected in a concentration buffer using Centrifugal Filter Devices (Millipore) as described previously (Li et al., 2019). Primers for construct generation are listed in Table S2.

**Electrophoretic mobility shift assay**

Electrophoretic mobility shift assay was used for the detection of the direct binding of PtrMYB074 and PtrWRKY19 to DNA fragments of the *PtrbHLH186* promoter. ThePtrMYB074 and PtrWRKY19 proteins were prepared from *E. coli*, respectively, as described above. An empty pET101/D-TOPO vector was used as a negative control. DNA fragments of the *PtrbHLH186* promoter, harbouring a W-box, were biotin-labelled using a Biotin 3’ End DNA Labeling Kit (Thermo Scientific). The W-box in the promoter fragments was also mutated by changing the second T to A for the synthesis of mutated probes. Primers for probe preparation are listed in Table S2. Electrophoretic mobility shift assay was carried out by using a Lightshift Chemiluminescent EMSA Kit (Thermo Scientific) according to the manufacturer’s instructions. The DNA–protein binding reaction was incubated for 20 min in a binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM MgCl₂, 0.01 mM ZnCl₂, 0.05% Nonidet P-40 and 100 ng μl⁻¹ poly (dl-dC)). Protein–DNA mixtures were separated on a 6% (w/v) native PAGE and transferred to a nylon membrane (Thermo Scientific). Signals were detected by chemiluminescence.
Gene expression analysis in SDX protoplasts

The PtrMYB074 and PtrWRKY19 coding regions were cloned into the pUC19-35Spro-RfA-35Spro-sGFP (Li et al., 2012) destination vector. The plasmids were prepared using the CsCl gradient, which provides high-quality plasmid DNA, and transfected into SDX protoplasts as described previously (Lin et al., 2013, 2014). After culturing for 12 h, the protoplasts were collected for RNA extraction and RT-qPCR analysis as described above.

Generation of gene overexpression and CRISPR-edited transgenic P. trichocarpa

Genetic transformations are described in detail in Methods S1 online. The coding regions of PtrMYB074, PtrWRKY19 and PtrbHLH186 were amplified from P. trichocarpa plants and cloned into pBI121 for constructing the plant transformation vector. The three genes were individually overexpressed in P. trichocarpa under the control of a cauliflower mosaic virus 35S promoter with kanamycin selection. The knockout mutants of PtrMYB074 and PtrWRKY19 were generated by the CRISPR-Cas9 system (Ueta et al., 2017). The sgRNA sequences were selected by using CRISPR-P2.0 (http://crispr.hzau.edu.cn/cgi-bin/CRI spontaneously/S2/CRISPR). The sgRNA sequences were synthesized and constructed into pEGP237-2A-GFP and PMgP237-2A-GFP vectors (Ueta et al., 2017). The plasmids were introduced into Agrobacterium tumefaciens strain GV3101 for P. trichocarpa transformation as described previously (Song et al., 2006). The over-expressing transgenic plants were identified by RT-qPCR to determine the expression of the transgenes in SDX tissues. For detection of PtrMYB074 and PtrWRKY19 mutations after transformation, primers flanking the sgRNA target sequences were used for PCR amplification. The PCR products were inserted into the pMD18-T vector (Takara, 6011, Otsu, Japan), and at least 20 colonies were selected for sequencing. All primers and sgRNA sequences are listed in Table S2.

Quantitative RT-PCR

Detailed procedures for RT-qPCR are described in Methods S2 online. Total RNA from SDX or SDX protoplasts of P. trichocarpa was extracted using the Qiagen RNeasy Mini Kit. One microgram of total RNA from each sample was used to synthesize cDNA using the PrimeScript RT Reagent Kit with gDNA Eraser according to the manufacturer’s instructions (Takara). All RT-qPCRs were performed on the Agilent Mx3000P Real-Time PCR System (Agilent, Santa Clara, CA, USA) with FASTSTART Universal SYBR Green Master (Roche) using gene-specific primers (Table S2).

Wood chemistry

All wood assay-related details are described in Methods S3 online. Stem segments of transgenic and wild-type P. trichocarpa were extracted with 90% acetonitrile for 48 h, followed by three additional extractions (each 48 h) using 100% acetonitrile, and air-dried. The air-dried stem segments were used to quantify the wood composition (acid-insoluble lignin, acid-soluble lignin and sugars) and lignin composition (S-lignin, G-lignin and H-lignin) following established procedures (Abraham et al., 2013; Wang et al., 2018) with three replicates for each assay.

Multi-omics model prediction of S and G subunits of lignin biosynthesis in SDX of OE-PtrbHLH186-L4 and wild-type P. trichocarpa

An established multi-omics integrative model for monolignol biosynthesis (Wang et al., 2018) was used to evaluate how changes in the transcript expression of pathway genes mediated by the overexpression of PtrbHLH186 affect the biosynthesis of G and S monolignol subunits. Details of multi-omics integration are described in Methods S4 online. The expression profile of 22 monolignol pathway genes for three wild-type and three OE-PtrbHLH186-L4 samples was used as the input of the model. The transcript abundance, determined using RT-qPCR, was normalized to µM as described in Wang et al. (2018) to predict changes in the corresponding protein abundance, steady-state metabolic fluxes and lignin composition. The output of the model for G and S subunits was normalized as a percentage of wild-type and compared with the measured wood chemistry data from OE-PtrbHLH186-L4 and wild-type trees.

Histological analyses

Histological analyses were carried out as described previously (Wang et al., 2020). Briefly, P. trichocarpa stem internodes were cut into 2-mm fragments and fixed with formalin–acetic acid–alcohol liquid (FAA) solution. The fixed tissues were transferred into a graded ethanol series at 4°C for dehydration and incubated sequentially in 100% ethanol, ethanol/xylene (50:50; v/v) and 100% xylene. The stem sections were then incubated in xylene/paraffin (75:25; v/v) overnight at 42°C and embedded in 100% paraffin (Sigma). The embedded fragments were sectioned to a thickness of 12 µm using a rotary microtome (RM2245; Leica Wetzlar, Germany), and stained with Safranin O and Fast Green to observe the status of lignin and cellulose. Stem cross-sectional micrographs were processed using a scanner M8 (FM34F056; PreciPoint Lang Drake, TX, USA) and the VIEWPOINT (v1.0.0.0) set-up software.

Scanning electron micrograph analysis

Scanning electron micrograph analysis was conducted to analyse cell morphology and cell wall thickness with three plants for each genotype. The 10th internodes of 4-month-old P. trichocarpa were harvested and coated with gold at 10 mA for 60 s. The samples were imaged using a Nanotech JCM-5000 scanning electron microscope.

Drought treatments

The 4-month-old wild-type and OE-PtrbHLH186-L4 plants were used for short-term drought experiments. All plants...
underwent drought stress, in which the soil relative water content (RWC) was reduced from 60% to 13% by withholding water. The RWC was detected with a Soil Moisture Meter (TDR 250; Spectrum Technologies, Aurora, CO, USA).

**Statistical analysis**

Student’s *t*-test was carried out using the SPSS software (v.19.0) for all the statistical analyses to determine significance (*, *P* < 0.05; **, *P* < 0.01).

**Results**

**PtrMYB074 interacts with a set of xylem-abundant transcription factors**

We first performed Y2H screening using the woody dicot-specific *PtrMYB074* as the bait and a total of 221 xylem-abundant *P. trichocarpa* TFs as preys (Table S1; Lin et al., 2017; Yeh et al., 2019) to screen at the genome level for its direct interactive partners.

In the Y2H system, we found that the full-length *PtrMYB074* would activate the yeast reporter gene (Fig. S1). However, an N-terminal version of *PtrMYB074* (*PtrMYB074N*) does not activate the yeast reporter gene (Fig. S1), and therefore, we used *PtrMYB074N* to screen for its interactive proteins and identified 54 interactors from 221 candidate preys (Figs 1a, S2; Table S3). These interactors belong to 21 diverse TF families (Table S3). Based on the RNA-seq of xylem, phloem, leaf and shoot tissues (Yeh et al., 2019), we ranked these 54 TFs based on their xylem specificity (xylem/phloem count per million (CPM) ratios) and abundance (CPM values) (Table S3). From the top 10 ranked TFs, we selected the most highly expressed and xylem-specific interactor, *PtrWRKY19* (Table S3), to explore how *PtrMYB074*-mediated TF–TF interactions would regulate wood formation.

We then tested whether the *PtrMYB074*–*PtrWRKY19* interaction takes place *in planta* using BiFC in protoplasts of wood-forming tissue from *P. trichocarpa*. *PtrMYB074*:YFP<sup>N</sup> (*PtrMYB074* fused to the N-terminus of yellow fluorescent protein (YFP)) and *PtrWRKY19*:YFP<sup>C</sup> (*PtrWRKY19* fused to the C-terminus of YFP) were co-expressed with the H2A-1:mCherry nuclear marker in SDX protoplasts. The assays confirmed the interaction as indicated by the presence of YFP signals from the interactive pairs, which were co-localized with mCherry in the nucleus (Fig. 1b). We next identified direct regulatory targets of *PtrMYB074* and *PtrWRKY19* to investigate the regulatory specificity of these two TFs and their interactions in wood formation.

**PtrMYB074 and PtrWRKY19 co-target 15 genes in the *P. trichocarpa* genome**

To identify direct transregulatory targets of *PtrMYB074* and *PtrWRKY19*, we generated transgenic *P. trichocarpa* overexpressing either *PtrMYB074* or *PtrWRKY19* as a FLAG epitope-tagged fusion (*PtrMYB074*-FLAG and *PtrWRKY19*-FLAG, respectively). ChIP of the transgenics using anti-FLAG antibodies would then allow for the identification of target chromatin bound by *PtrMYB074* or *PtrWRKY19*. We generated four independent transgenic lines each for *PtrMYB074*-FLAG and *PtrWRKY19*-FLAG. The SDX of the overexpressors, OE-*PtrMYB074*-FLAG and OE-*PtrWRKY19*-FLAG, all showed increased levels of the transgene transcripts (Fig. S3). The exogenous proteins of *PtrMYB074* or *PtrWRKY19* were also detected in SDX of the transgenics using anti-FLAG antibodies (Fig. S4).

We then carried out ChIP sequencing (ChIP-seq) in SDX tissue of OE-*PtrMYB074*-FLAG and OE-*PtrWRKY19*-FLAG transgenics using anti-FLAG antibodies. Four ChIP-seq libraries (ChIP-DNA and input DNA, two biological replicates per library) for each TF were sequenced. We obtained 13,95–71.57 million uniquely aligned reads per library (Table S4). The sequencing depth after removing duplication reads was 1.34×–6.91×, showing 52.59%–66.9% genome coverage (Table S4). Data quality assessment using the irreproducible discovery rate framework with a 1% threshold indicated that the two replicates for each TF showed high reproducibility (Fig. S5). Input DNA libraries were used as a control for ChIP-seq peak calling. We used model-based analysis of ChIP-seq (Zhang et al., 2008) to identify peaks (Table S5), and obtained 1112 peaks for *PtrMYB074* and 4500 for *PtrWRKY19* by overlapping peaks from the two replicates for each TF, respectively. Genes that contain one or more binding sites within the 3-kb upstream putative promoter region were defined as targeted genes.

Based on these criteria, *PtrMYB074* bound to at least one location within the 3-kb promoter region of 222 genes (*P* < 1e<sup>−05</sup>; Table S6), whereas *PtrWRKY19* did the same in 1522 genes (*P* < 1e<sup>−05</sup>; Table S6). Of these 1744 target genes, 15 were the common targets shared by *PtrMYB074* and *PtrWRKY19* (Table S7), including genes encoding TFs. To further investigate the effects of *PtrMYB074* and *PtrWRKY19* regulations, we focused on their common TF targets. This is because we seek to understand how the *PtrMYB074*–*PtrWRKY19* interaction modulates the woody TRN, leading to direct transregulation of cell wall genes in wood formation. Of these 15 target genes, there were only two, *PtrbHLH186* and *PtrvCM2* (Table S7), that encode putative TFs. We selected *PtrbHLH186* (Potri.018G083700) because it is the more xylem-abundant and specific one of the two TFs (Table S7). Furthermore, the function of this TF has not previously been investigated.

We next analysed the subcellular localization of *PtrbHLH186* protein in wood-forming cells. *P. trichocarpa* SDX protoplasts were transfected with 35S-*Ptr bHLH186*-sGFP (green fluorescent protein) and a nuclear marker, 35S-H2A-1:mCherry. The green fluorescence signals of GFP-fused *PtrbHLH186* were co-localized in the nucleus along with the red fluorescence signals of H2A-1:mCherry (Fig. S6), suggesting that *PtrbHLH186* is a nuclear protein associated with wood formation. We then focused on the investigation, *in vivo*, of how *PtrbHLH186* was transregulated by its dual upstream regulators, *PtrMYB074* and *PtrWRKY19*, based on ChIP-seq of OE-*PtrMYB074* and OE-*PtrWRKY19* (Table S6).
PtrWRKY19 is required for the association of PtrMYB074 with the PtrbHLH186 promoter

Our ChIP-seq analysis of OE-PtrMYB074-FLAG and OE-PtrWRKY19-FLAG in SDX showed that both PtrMYB074 and PtrWRKY19 TFs were associated with the upstream 1-kb promoter sequence of PtrbHLH186, where a W-box (TTGACT) motif, a binding site for WRKY TFs, exists (Ciolkowski et al., 2008) (Fig. 2a). To verify whether such an association was a consequence of PtrMYB074’s or PtrWRKY19’s binding to PtrbHLH186, we performed ChIP-qPCR and EMSA. Because of the presence of a WRKY’s binding motif (the W-box) in PtrbHLH186’s gene promoter, we first tested OE-PtrWRKY19-FLAG using its SDX to perform FLAG antibody-based ChIP-qPCR assays with a primer set that would amplify a fragment harbouring the W-box (Fig. 2a). We detected an approximately eightfold enrichment of the targeted fragment in the ChIP assay of OE-PtrWRKY19-FLAG (Fig. 2b), confirming that PtrWRKY19 binds to the PtrbHLH186 gene promoter. We then performed EMSA to validate this binding. Retardation of DNA probe mobility and competition analyses demonstrated that PtrWRKY19 could bind directly to the W-box motif in the PtrbHLH186 promoter (Fig. 2c,d). In addition, we also included a W-box motif sequence that harbours a single nucleotide mutation as a competitor to further confirm that W-box is a necessary and specific binding site for PtrWRKY19 to bind to PtrbHLH186 (Fig. 2c,d). Based on the in vivo (Fig. 2a,b) and in vitro (Fig. 2c,d) evidence, we concluded that PtrWRKY19 targets PtrbHLH186 by directly binding to its W-box motif in the promoter.

Next, we used the SDX from OE-PtrMYB074-FLAG for FLAG antibody-based ChIP-qPCR assays with the same primer set as we used for assaying OE-PtrWRKY19-FLAG. We detected a 2-fold enrichment of the W-box-containing promoter fragment of PtrbHLH186 (Fig. 2e), suggesting that PtrMYB074 also binds to PtrbHLH186 at the same location as PtrWRKY19 does. However, EMSA uncovered that PtrMYB074 does not bind to the W-box-containing promoter fragment of PtrbHLH186 (Fig. 2f). The positive ChIP-qPCR and negative EMSA results suggest that the ChIP-seq-inferred association of PtrMYB074 with PtrbHLH186 (Table S6; Fig. 2a) may be due to an indirect binding of PtrMYB074 to PtrbHLH186. The suggestion is

Fig. 1 PtrMYB074 Interacts with a Set of Transcription Factors. (a) Y2H demonstrated that PtrMYB074 interacts with 54 xylem-abundant TFs. Each bait and prey pair was co-expressed in yeast cells and selected on the SD/-Leu/-Trp/-His/-Gal/AbA (-LW/X/A), SD/-Leu/-Trp/-His/-X-Gal/AbA (-LWH/ X/A) and SD/-Leu/-Trp/-His/-Ade/X/-Gal/AbA (-LWHA/X/A) medium. BD-53/AD-Lam were used as negative controls. BD-53/AD-T were used as positive controls. (b) Bimolecular fluorescence complementation (BiFC) demonstrated that PtrMYB074 interacts with PtrWRKY19 in vivo. PtrMYB074-YFP and PtrWRKY19-YFP (I) co-expressed into Populus trichocarpa stem-differentiating xylem protoplasts gave a positive BiFC signal for heterodimerization, which are co-localized with H2A-1:mCherry signal in the nucleus. PtrMYB021, an interacting protein of PtrMYB074 (Chen et al., 2019), was used as a positive control (II). As negative controls, the transformation of single TF-YFP/PtrWRKY19-YFP (I) alone did not yield any YFP signals (III and IV). Green represents the YFP signals from protein interactions, red indicates the nuclear marker H2A-1:mCherry, and yellow shows the merged signals from YFP and mCherry. Bar, 10 µm. YFP, yellow fluorescent protein.
supported by the dimerization of PtrMYB074-PtrWRKY19 (Fig. 1a,b), where PtrMYB074 may be recruited by PtrWRKY19 and become indirectly associated with PtrbHLH186. The EMSA results confirmed that PtrWRKY19 binds to the biotinylated probes, but PtrMYB074 alone does not (Fig. 2f). Furthermore, the presence of both PtrWRKY19 and PtrMYB074 resulted in two shift bands, suggesting that the probes may be bound to not only PtrWRKY19 alone but also the PtrMYB074-PtrWRKY19 dimer. These results support that PtrMYB074 is bound to the biotinylated probe by interaction with PtrWRKY19 (Fig. 2f).

**In vitro evidence and in planta evidence support the binding ofPtrMYB074 toPtrbHLH186 through dimerization withPtrWRKY19**

The EMSA experiments validated *in vitro* that the association of PtrMYB074 with *PtrbHLH186* depends on the presence of PtrWRKY19. We then tested this dependence *in vivo* using transgenic *P. trichocarpa* that lacks a functional PtrWRKY19. We generated two independent biallelic mutants (*ptrwrky19-1* and *ptrwrky19-2*) and one homozygous mutant (*ptrwrky19-3*) using

Fig. 2 PtrWRKY19 Is Required for the Association of PtrMYB074 with the *PtrbHLH186* Promoter. (a) A schematic diagram of the *PtrbHLH186* loci. Triangles show the two primers used for the ChIP-qPCR analysis. The wedge represents the W-box in the *PtrbHLH186* promoter. (b, e) Chromatin immunoprecipitation (ChIP) assays showing that PtrMYB074 and PtrWRKY19 associate with the promoter of the *PtrbHLH186* gene. Wild-type (WT), *PtrWRKY19-FLAG* and *PtrMYB074-FLAG* transgenic *Populus trichocarpa* plants were used for the ChIP assays with anti-FLAG antibodies, and the precipitated DNA was quantified by qPCR. Enrichment of DNA was calculated as the ratio between *PtrWRKY19-FLAG* and wild-type or between *PtrMYB074-FLAG* and wild-type, normalized to that of the *PtrActin* gene. (c) Nucleotide sequences of the wild-type W-box and a mutated W-box (mW-box). Core sequences are shaded in black, and the mutated nucleotide is shaded in grey. (d) Electrophoretic mobility shift assay (EMSA) analysis of PtrWRKY19 binding to the W-box in the *PtrbHLH186* promoter. (f) PtrMYB074 alone failed to bind to the W-box in the *PtrbHLH186* promoter, and PtrWRKY19 is required for the association ofPtrMYB074 with the *PtrbHLH186* promoter. The arrow shows the shifted band representing the protein–DNA complex. PET101-His was used as a negative control. The *PtrbHLH186* promoter fragment was labelled with biotin. Fragments without biotin labelling were used as competitors. Wild-type or mutated W-box competitors were used in a molar excess of 50×, 100× or 200×. (g) ChIP assays at the promoter of *PtrbHLH186* in WT and *ptrwrky19* with anti-IgG and anti-PtrMYB074 antibodies, and the precipitated DNA was quantified by qPCR. Error bars in b, e and g indicate SE from three biological replicates, and the asterisk indicates significant differences between the control fragment (*PtrActin*) and the fragment containing a W-box motif (*, *P < 0.05, Student’s *t*-test).
Co-expression of PtrMYB074 and PtrWRKY19 is required for the transcriptional activation of *PtrbHL186*

The above *in vitro* and *in vivo* experiments revealed unique interactions among three TFs, encompassing the protein–protein dimerization ofPtrMYB074 and PtrWRKY19 (Fig. 1a,b) and protein–gene promoter interaction betweenPtrWRKY19 and *PtrbHL186* (Fig. 2). These interactions would apparently result in transregulating *PtrbHL186*. But whether such transregulation induces an activation or repression of *PtrbHL186* was unknown. It was also unknown whether a direct binding ofPtrWRKY19 to *PtrbHL186* alone is sufficient for gene transregulation. We then carried out additional experiments to elucidate the interactions related to transregulations.

We overexpressed *PtrMYB074*, *PtrWRKY19* and *PtrMYB074*+*PtrWRKY19* in *P. trichocarpa* SDX protoplasts to determine their regulatory effects on *PtrbHL186* transcript expression. Stem-differentiating xylem protoplast is an efficient system for elucidating complex transregulation responses associated with wood formation (Lin *et al*., 2013, 2014, 2017; Chen *et al*., 2019; Li *et al*., 2019; Yeh *et al*., 2019; Wang *et al*., 2020; C. Liu et al., 2021). Compared with the control (overexpression of GFP), the overexpression of *PtrMYB074* by itself only slightly activated *PtrbHL186* expression (Fig. 3a). The overexpression of *PtrWRKY19* alone had no effect on *PtrbHL186* expression (Fig. 3a). In contrast, co-overexpressing the *PtrMYB074* and *PtrWRKY19* induced an up to a fourfold increase in *PtrbHL186* expression compared with the control and the independent overexpression of *PtrMYB074* or *PtrWRKY19* (Fig. 3a). Therefore, the co-expression of *PtrMYB074* and *PtrWRKY19* is required for transactivating *PtrbHL186* gene expression.

To test this requirement further, we studied the transcript expression of *PtrbHL186* in knockout mutants lacking either *PtrMYB074* or *PtrWRKY19*. Because we have already generated *PtrWRKY19* knockouts (e.g. *Ptrwryk19-3*, Fig. S7), we then generated two independent biallelic mutants (*Ptrmyb074-1* and *Ptrmyb074-2*) and one homozygous mutant (*Ptrmyb074-3*) using CRISPR-Cas9 genome editing (Fig. S8). We measured the transcript abundance of *PtrbHL186* in SDX of *PtrMYB074* and *PtrWRKY19* mutants and wild-type plants. The results showed that compared with wild-type plants, the transcript levels of *PtrbHL186* were reduced when either *PtrMYB074* or *PtrWRKY19* was mutated (Fig. 3b). These results suggest that the presence of both *PtrMYB074* and *PtrWRKY19* is essential for the transactivation of *PtrbHL186* *in vivo*. We next explored the function of this transactivation pathway in wood formation. *PtrbHL186* was overexpressed under the control of a 35S promoter in *P. trichocarpa*, and changes in tree growth, cell morphology and wood formation were evaluated.

Elevated transcript levels of *PtrbHL186* in *P. trichocarpa* reduce plant growth, induce abnormal lignification and enhance vessel cell development

We overexpressed *PtrbHL186* in *P. trichocarpa* under the control of a CaMV 35S promoter and obtained four independent transgenic lines with 8- to 10-fold increases in *PtrbHL186* transcript abundance compared with the wild-type (Fig. 4a,b). All these OE-*PtrbHL186* lines had similarly stunted growth with a reduced height, number of internodes and stem basal diameter (Fig. 4c-e). These stunted phenotypes were sustained throughout the 4 months of glasshouse growth before the trees were harvested for analyses (Fig. S9).

We focused on OE-*PtrbHL186*–L4, the line with the highest level of transgene overexpression for phenotypic characterization. Histochemical analysis of the stem cross sections revealed early
and more enhanced lignification during stem wood development in OE-PtrbHLH186-L4, as compared to the wild-type plants (Fig. S10). The irregularity of lignification observed was further evidenced by wood composition quantification (Table 1). Total lignin content was increased by approximately 20% in OE-PtrbHLH186-L4 transgenic line (Table 1). Of particular interest was the clear increase (by c. 20%) in the quantity of guaiacyl (G) monolignol subunits in the transgenic lignin (Table 2). The increases in the G and total lignin were consistent with the elevated expression of monolignol biosynthetic genes associated with the G-lignin pathway (Fig. 5a), such as PtrCCoAOMT2 and PtrCCoAOMT3, and those control total lignin content, such as PtrPAL2, PtrPAL4, PtrCCR2 and PtrCAD1 (Wang et al., 2014, 2018, 2019).

We next attempted to understand more quantitatively how the perturbation of these many monolignol genes would affect the overall biosynthesis of the G-lignin as measured by wood chemical analysis. To do this, we applied an established multi-omics integrative model (Wang et al., 2018, 2019) of the pathway to estimate lignin composition (Fig. 5b,c) based on the transcript abundance of the monolignol genes in OE-PtrbHLH186-L4 and wild-type. The model quantitatively predicted the lignin S and G subunit abundance with an R² of 0.89 compared with the measured data (Fig. 5d). The model showed that the transregulation of monolignol genes mediated by PtrbHLH186 overexpression led to a consistent increase in G subunits (Fig. 5b), and S

Table 1 Wood composition of OE-PtrbHLH186-L4 transgenic and wild-type Populus trichocarpa

| Plants                  | WT    | OE-PtrbHLH186-L4 |
|-------------------------|-------|------------------|
| Acid-insoluble lignin   | 18.25 ± 0.39 | 23.13 ± 1.14**   |
| Acid-soluble lignin     | 3.56 ± 0.09  | 3.12 ± 0.05**    |
| Total lignin            | 21.81 ± 0.30 | 26.25 ± 1.12**   |
| Glucose                 | 54.36 ± 0.13 | 36.19 ± 1.83**   |
| Xylose                  | 10.33 ± 0.06 | 9.20 ± 1.01      |
| Galactose               | 0.89 ± 0.09  | 0.60 ± 0.02*     |
| Arabinose               | 2.01 ± 0.06  | 0.93 ± 0.09**    |
| Total carbohydrate      | 67.33 ± 0.29 | 46.92 ± 2.85**   |
| C : L ratio             | 3.10 ± 0.04  | 1.78 ± 0.04**    |

Four-month-old plants were tested. Three biological replicates from independent pools of OE-PtrbHLH186-L4 and wild-type (WT) stems were carried out. Data are means of three independent assays with ±SE values. Asterisks indicate significant differences between OE-PtrbHLH186-L4 transgenics and wild-type plants by Student’s t-test (*, P < 0.05; **, P < 0.01). Units are g per 100 g of dry extractive-free wood. The C : L ratio indicates the ratio of carbohydrate to lignin. OE, overexpressing.
Table 2  Lignin composition of OE-PtrbHLH186-L4 transgenic and wild-type *Populus trichocarpa*

| Plants          | WT                  | OE-PtrbHLH186-L4          |
|-----------------|---------------------|---------------------------|
| S-Lignin        | 61.64 ± 0.57%       | 57.85 ± 0.12%**           |
| G-Lignin        | 30.19 ± 0.39%       | 36.16 ± 0.17%**           |
| H-Lignin        | 8.16 ± 0.72%        | 5.99 ± 0.08%*             |
| S/G ratio       | 2.04 ± 0.03         | 1.60 ± 0.01**             |

Four-month-old plants were tested. Three biological replicates from independent pools of OE-PtrbHLH186-L4 and wild-type (WT) stems were carried out. Data are means of three independent assays with ± SE values. Asterisks indicate significant differences between OE-PtrbHLH186-L4 transgenics and wild-type plants by Student’s t-test (*, P < 0.05; **, P < 0.01). G, G subunits; H, H subunits; OE, overexpressing; and S, S subunits. Values indicate the percentage weight of total lignin.

Discussion

In this study, we reported a MYB074-WRKY19-bHLH186 regulatory pathway in *P. trichocarpa* involving a coordinated function of PtrMYB074 and PtrWRKY19 in activating the expression of *PtrbHLH186*. Based on a large-scale Y2H screening, we identified a PtrMYB074 and PtrWRKY19 TF–TF interaction and the interaction was verified by BiFC in a wood-forming cell system (Fig. 1). Using transgenesis (Fig. S3) and ChIP-seq (Table S7), we found that PtrMYB074 and PtrWRKY19 shared 15 common direct targets including *PtrbHLH186* and *PtrVCM2*, the only two TFs in this target population. We focused on *PtrbHLH186* in this study. Transient and stable transgenic *P. trichocarpa* systems coupled with ChIP-qPCR and EMSA analyses demonstrated that PtrMYB074 is recruited by PtrWRKY19 for this TF–TF dimer to bind to the promoter of *PtrbHLH186* through PtrWRKY19 and the dimerization is required to transactivate *PtrbHLH186* (Figs 2, 3). Transgenesis showed that the elevated expression of *PtrbHLH186* through this TF regulatory pathway would result in retarded growth (Fig. 4), increased G-lignin (Table 2), promoted vessel cell development and strong drought-tolerant phenotypes (Fig. 6). *P. trichocarpa* co-overexpressing PtrMYB074 and PtrWRKY19 may exhibit a similar phenotype as OE-PtrbHLH186 transgenics.

PtrMYB074 is a woody dicot-specific MYB TF in a TRN for wood formation in *P. trichocarpa* (Lin et al., 2013; Chen et al., 2019; Wang et al., 2020). Reported homologs of *PtrMYB074* include *PtrMYB74* in *P. tomentosa* (Li et al., 2018) and *EgMYB137* in *Eucalyptus* (Ployet et al., 2019). Overexpressing *EgMYB137* in *Eucalyptus* altered vessel density and SCW deposition, and constitutive expression of *PtrMYB74* in *P. tomentosa* increased SCW thickness of vessels and changed SCW compositions. These findings support that PtrMYB074 and its homologs in woody dicots have important roles in the regulation of wood formation.

While this study focused on functions of one TF–TF (PtrMYB074–PtrWRKY19) interaction, our large-scale Y2H screening discovered many other putative PtrMYB074–TF interactions that may also provide new insights into regulations associated with wood formation. For example, PtrWOX4a and PtrWOX4b (Table S3), each is a direct interactor of MYB074, are the WOX homologs that are the known key players in the TF-mediated regulatory pathway controlling the development of meristematic cells into phloem and vascular xylem in plants (Kucukokgu et al., 2017; Zhang et al., 2019). As a woody dicot-specific TF (Chen et al., 2019), PtrMYB074 may regulate these WOX TFs through dimerization for a more rigorous TF-mediated pathway for stem cell differentiation in wood development.

In addition to PtrWRKY19, three PtrWRKY family members, PtrWRKY12/13/25, also interact with PtrMYB074 (Table S3). The four PtrWRKY members belong to one subgroup, with PtrWRKY19/12 as phylogenetically paired homologs, and PtrWRKY13/25 as the other paired homologs (Jiang et al., 2014), suggesting that the three PtrWRKY members are likely to have similar functions to PtrWRKY19 in wood formation.
Zhong et al. (2011; Yang et al., 2016). Other TFs that interact with PtrMYB074, such as PtrC3H18 (Chai et al., 2014), also have functions implicated in SCW biosynthesis for wood formation. Functions of the homologs of most of PtrMYB074’s partners (Table 2) have been studied in Populus or other species typically as individual TFs. Regulatory mechanisms triggering or affecting these functions were largely unknown but can now be explored starting from aspects associated with interactions with MYB074.

Our study revealed that interactions between PtrMYB074 and PtrWRKY19 were associated with functions of only a handful of target genes (15 of them; Table S7), while as individual TFs, each may potentially regulate hundreds of target genes (222 for PtrMYB074 and 1522 for PtrWRKY19; Table S6). These results suggest that PtrMYB074–PtrWRKY19 interactions would exert specific combinatorial functions. In particular, PtrMYB074-PtrWRKY19 targeted, on a genome-wide basis, only two TF genes (PtrbHLH186 and PtrVCM2). While functions of VCM2...
have recently been tested in a hybrid Populus (P. deltoides × P. euramericanica) (Zheng et al., 2020), the function of PtnVCM2 had not previously been studied. VCM2 and its homolog (VCM1) are negative regulators of vascular cambium activity and wood formation, and function by mediating the expression of PIN5b, an auxin-related gene to influence the cambial IAA flux (Zheng et al., 2020). These studies and our current results suggest a PtnMYB074-PtnWRKY19-mediated TF pathway that leads to a PtnVCM2-guided association with auxin signalling through regulation of PtnPIN5b expression for wood development.

Fig. 6 Overexpressing PtnbHLH186 Affects the Size and Number of Vessels in Xylem Tissue and Improves Drought Tolerance of Populus trichocarpa. (a) Scanning electron micrographs of wild-type (WT) and OE-PtnbHLH186-L4 (L4) with the 10th internode imaged at ×500 (left) and ×1500 (right) magnification. Bar, 20 µm. (b) Stem cross sections and magnified images of 4-month-old OE-PtnbHLH186-L4 and wild-type plants with the 10th internode (IN). The cross sections were stained with Safranin O and Fast Green. Bar, 100 µm. (c) Stem cross sections of OE-PtnbHLH186-L4 and wild-type plants showing the size and number of vessel cells in xylem tissues. Vessel cells are marked with green. Bar, 100 µm. (d) Magnified images of tangential longitudinal sections stained with toluidine blue. Bar, 100 µm. F, xylem fibre; V, vessel element. (e–i) Statistical analyses of the number of fibres and vessels per cross-sectional area (mm²; e), mean lumen area of individual vessel (µm²; f), length of fibres and vessels (µm; g), area of vessels (µm²) per cross-sectional area (mm²; h) and cell wall thickness of fibres and vessels (µm; i) within the 10th internode. Error bars represent SE values of three independent replicates with at least 200 vessel cells and 3000 fibre cells for each genotype in each replicate, and asterisks indicate significant differences between the transgenic and wild-type plants by Student’s t-test (**, P < 0.01). (j) Statistical analysis of survival rates after drought treatment and recovery (rehydrated for 3 d). Error bars represent SE values from three biological replicates. Asterisks indicate significant differences between the transgenics and wild-type plants (**, P < 0.01, Student’s t-test). OE: overexpressing.
We reported here a PtrMYB074–PtrWRKY19–PtrbHLH186 pathway associated with wood formation. PtrMYB074 and PtrWRKY19 work combinatorially as an activator of PtrbHLH186 in trans through the binding of PtrWRKY19 to the PtrbHLH186 gene promoter. The binding of PtrWRKY19 alone to PtrbHLH186 was insufficient to transactivate PtrbHLH186 (Fig. 3a). PtrMYB074 must be recruited by PtrWRKY19 for transactivation (Fig. 3a,b). The ‘function-less’ binding of PtrWRKY19 alone to PtrbHLH186 may imply that such binding took place first, followed by the recruitment of PtrMYB074, which completes the functional PtrMYB074–PtrWRKY19–PtrbHLH186 pathway. It is possible that PtrWRKY19 may also recruit other TFs for transregulating PtrbHLH186, where such recruitments may be part of a more complex regulatory network. Nonetheless, the discovery of the PtrMYB074–PtrWRKY19–PtrbHLH186 pathway represents a novel component of the complex regulatory network in wood formation.

Although what triggers this PtrMYB074–PtrWRKY19–PtrbHLH186 pathway to function remains to be explored, the result is elevated PtrbHLH186 transcript levels and the regulation is likely to be driven by PtrMYB074 specificity of the pathway originator, PtrMYB074 (Chen et al., 2019). As shown by the overexpression of PtrbHLH186 in P. trichocarpa, these results included significantly retarded plant growth (Fig. 4), drastically enhanced development of more and smaller vessel cells (Fig. 6), early and elevated lignification with increased quantity in total lignin (by c. 20%) and its G subunits (by c. 20%) (Figs 5, S10; Tables 1; 2), and marked reduction (by c. 30%) in polysaccharides (Table 1), and strong drought-tolerant phenotypes (Fig. 6).

Drought resilience and growth/development traits are interrelated. Plants develop tolerance to drought by reducing transpiration and water use based on reprogrammed growth, cell division, cell wall thickening, and synthesis of lignin, cellulose and hemicelluloses (Skirycz & Inzé, 2010; Le Gall et al., 2015; Li et al., 2019; Cao et al., 2020; Ha et al., 2021). Under drought conditions, plants alter their physiology to reduce growth and enhance drought tolerance for adaptation (Halevy & Kessler, 1963; Kasuga et al., 1999; Skirycz & Inzé, 2010; Yoshida et al., 2014; Li et al., 2019). Under drought, the stem xylem of trees has smaller vessels (Tyree & Sperry, 1989; Hacke & Sauter, 1996; Fisher et al., 2007; Hacke et al., 2007). Plants with smaller vessel diameters can reduce water potential to prevent xylem cavitation, allowing for more efficient upward water transport (Tyree & Sperry, 1989; Fisher et al., 2007). We previously discovered in P. trichocarpa an epigenetic modification-mediated regulatory pathway for drought tolerance and identified the pathway’s positive regulators (Li et al., 2019). All the drought-resistant transgenic P. trichocarpa genotypes with enhanced expression of these positive regulators have more and smaller vessel cells.

Transgenic plants modified for lower overall lignin content or lower lignin quantity in vessel elements are more drought-sensitive than their wild-type counterparts (Cao et al., 2020; Yan et al., 2021). It is possible that, being hydrophobic, more lignin is needed to help mitigate xylem cavitation and thus facilitate upward water transport (Yan et al., 2021). The key traits in OE-PtrbHLH186 transgenics that are consistent with PtrbHLH186 overexpression are all conducive to drought adaptation. OE-PtrbHLH186 transgenics are a useful biological system to understand the complex co-regulation of growth, cell-type biosynthesis in wood formation and stress tolerance.

Accession numbers

The ChIP-seq data have been deposited in the National Center for Biotechnology Information Sequence Read Archive under accession no. SRR15246566–SRR15246573. Sequence data from this article can be found in P. trichocarpa genome v3.0 (Phytozome, https://phytozome.jgi.doe.gov/pz/portal.html). All gene identifier numbers in this article are listed in Supporting Information Table S6.

Acknowledgements

We thank Drs Ying-Chung Jimmy Lin, Guanzheng Qu and Guifeng Liu for assisting us to clone the 221 TFs. This work was supported by the National Key Research and Development Program of China (no. 2021YFD2200700 and no. 2016YFD0600106). We also acknowledge the financial support from the Fundamental Research Funds for the Central Universities of China (grant nos. 2572018CL01 and 2572018CL02) and Heilongjiang Touyan Innovation Team Program (Tree Genetics and Breeding Innovation Team). No conflict of interest is declared.

Author contributions

WL, VLC and HL designed the research. HL, JG, JS, SL, BZ, ZW and DBS conducted the experiments. HL, CZ, JPW, VLC and WL analysed the data. WL, VLC and HL wrote the manuscript with input from all co-authors.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**Supporting Information**

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** The N-terminal of MYB074 was selected for Y2H screening.

**Fig. S2** Y2H screening of *PtrMYB074* with 221 xylem abundant proteins.

**Fig. S3** Transgene expression levels in *OE-PtrMYB074-FLAG* and *OE-PtrWRKY194-FLAG* transgenics.

**Fig. S4** MYB074 and WRKY19 expressed in the poplar *PtrMYB074-flag* and *PtrWRKY19-flag* were detected by western blot.

**Fig. S5** The irreproducible discovery rate (IDR) framework for assessing the reproducibility of *PtrMYB074* and *PtrWRKY19* ChIP-seq.

**Fig. S6** Subcellular localization of *PtrbHLH186*.

**Fig. S7** Identification of CRISPR-edited mutations in *PtrWRKY19* of *P. trichocarpa*.

**Fig. S8** Identification of CRISPR-edited mutations in *PtrMYB074* of *P. trichocarpa*.

**Fig. S9** Growth phenotypes of wild-type and *OE-PtrbHLH186* transgenic plants.

**Fig. S10** Stem cross sections of 4-month-old wild-type (WT) and *OE-PtrbHLH186-L4* plants with different internodes.

**Fig. S11** Drought response test of wild-type and *OE-PtrbHLH186-L4* transgenic plants.

**Methods S1** Generation of gene overexpression and CRISPR-edited transgenic *P. trichocarpa*.

**Methods S2** Quantitative RT-PCR.

**Methods S3** Wood chemistry.

**Methods S4** Multi-omics model prediction of S and G-subunits of lignin biosynthesis in SDX of *OE-PtrbHLH186-L4* and wild-type *P. trichocarpa*.

**Table S1** List of 221 xylem-abundant transcription factors.

**Table S2** List of primers used in this article.

**Table S3** The expression of 54 interacting proteins in different tissues.

**Table S4** List of reads and sequence coverage for ChIP-seq.

**Table S5** The full list of peak regions identified for *PtrMYB074* and *PtrWRKY19*.

**Table S6** List of *PtrMYB074* and *PtrWRKY19* binding genes.

**Table S7** List of *PtrMYB074* and *PtrWRKY19* common binding genes.

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