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## ORIGINAL RESEARCH

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# Identification of functional single nucleotide polymorphism of *Populus trichocarpa* *PtrEPSP-TF* and determination of its transcriptional effect

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## Abstract

In plants, the phenylpropanoid pathway is responsible for the synthesis of a diverse array of secondary metabolites that include lignin monomers, flavonoids, and coumarins, many of which are essential for plant structure, biomass recalcitrance, stress defense, and nutritional quality. Our previous studies have demonstrated that *Populus trichocarpa* *PtrEPSP-TF*, an isoform of 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase, has transcriptional activity and regulates phenylpropanoid biosynthesis in *Populus*. In this study, we report the identification of single nucleotide polymorphism (SNP) of *PtrEPSP-TF* that defines its functionality. *Populus* natural variants carrying this SNP were shown to have reduced lignin content. Here, we demonstrated that the SNP-induced substitution of 142nd amino acid (*PtrEPSP-TF*<sup>D142E</sup>) dramatically impairs the DNA-binding and transcriptional activity of *PtrEPSP-TF*. When introduced to a monocot species rice (*Oryza sativa*) in which an EPSP synthase isoform with the DNA-binding helix-turn-helix (HTH) motif is absent, the *PtrEPSP-TF*, but not *PtrEPSP-TF*<sup>D142E</sup>, activated genes in the phenylpropanoid pathway. More importantly, heterologous expression of *PtrEPSP-TF* uncovered five new transcriptional regulators of phenylpropanoid biosynthesis in rice. Collectively, this study identifies the key amino acid required for *PtrEPSP-TF* functionality and provides a strategy to uncover new transcriptional regulators in phenylpropanoid biosynthesis.

## KEYWORDS

DNA binding, EPSP synthase, phenylpropanoid, rice, transcriptional regulation

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## 1 | INTRODUCTION

Rice (*Oryza sativa*) is currently one of the most important cultivated crops in the world, which feeds about half of the human population. Meanwhile, with the production of approximately 1,140 million tons per year worldwide, the lignocellulosic biomass derived from rice straws has emerged as an attractive renewable feedstock for bio-fuels (Sattlewal, Agrawal, Bhagia, Das, & Ragauskas, 2018). In plants, the phenylpropanoid pathway is responsible for the synthesis of secondary metabolites, including lignin monomers, flavonoids, and coumarins, which play essential roles in determining plant structure, biomass recalcitrance, and stress tolerance (Vogt, 2010). Thus, a comprehensive understanding of the phenylpropanoid pathway and its regulation in rice can lead to more sustainable agriculture and energy.

In plants, the phenylpropanoid pathway consists of a set of enzymes (Xie, Zhang, et al., 2018). Phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate: CoA ligase (4CL) catalyzes the first three steps of the general phenylpropanoid pathway to convert phenylalanine into *p*-coumaroyl-CoA, which serves as the precursor for phenylpropanoids. Downstream of 4CL, quinate/shikimate *p*-hydroxycinnamoyltransferase (HCT), *p*-coumaroylshikimate 3'-hydroxylase (C3'H), caffeoyl shikimate esterase (CSE), caffeic acid O-methyltransferase (COMT), and caffeoyl-CoA O-methyltransferase (CCoAOMT) are involved in the remaining steps of the general phenylpropanoid pathway and the biosynthesis of lignin monomers. In addition, enzymes, including cinnamoyl-CoA reductase (CCR), ferulate 5-hydroxylase (F5H), and cinnamyl alcohol dehydrogenase (CAD), specifically catalyze the biosynthesis of lignin monomers.

The phenylpropanoid pathway is tightly regulated by a transcriptional regulatory hierarchy to ensure precise spatial and temporal deposition of phenolics in plant cells. In *Arabidopsis* and grasses (e.g., rice), several MYB transcription factors directly target phenylpropanoid biosynthetic genes via binding to AC elements, which widely exist in the promoters of major phenylpropanoid pathway genes (e.g., *PAL*, *C4H*, *4CL*, *C3H*, *HCT*, *CCoAOMT*, *CCR*, *F5H*, *CAD*) (Rogers & Campbell, 2004). MYB46 and its paralog MYB83 activate the expression of phenylpropanoid biosynthetic genes, as well as other MYBs (Kim, Kim, Ko, Kang, & Han, 2014; Zhong et al., 2011; Zhong & Ye, 2012). In rice, the overexpression of *OsMYB46* was shown to upregulate the expression of *4CL*, *MYB58*, *MYB63*, and *MYB85* (Zhong et al., 2011). Downstream of MYB46/83, clades of MYBs, such as MYB58/63, MYB42/85, and MYB4/32, are also involved in the regulation of the phenylpropanoid pathway (Rao & Dixon, 2018). In rice, overexpression of *OsMYB58/63* or *OsMYB42/85* resulted in the upregulation of the expression of one *CAD* gene and lignin biosynthesis (Hirano, Kondo, et al., 2013). In *Arabidopsis*, genes in the MYB4/32 clade (e.g., *AtMYB4*, *AtMYB32*, and *AtMYB7*) are proposed to negatively regulate the phenylpropanoid pathway via suppressing the expression of *C4H* and *4CL* genes (Fornalé et al., 2014; Jin et al., 2000; Preston, Wheeler, Heazlewood, Li, & Parish, 2004). However, rice *Osmyb4*, one member of MYB4 family, was shown to activate the expression of *PAL* gene and the phenylpropanoid pathway in *Arabidopsis*, *Nicotiana tabacum*, and *Salvia sclarea* (Docimo,

Coraggio, Tommasi, & Leone, 2008; Vannini et al., 2004), suggesting that MYB4/32 genes in *Arabidopsis* and grasses may be involved in different regulatory mechanisms to fine-tune the phenylpropanoid flux. Upstream of MYB46/83, a group of NAC transcription factors, called secondary wall-associated NACs (SWNs), function as master switches for the phenylpropanoid pathway and secondary cell wall biosynthesis (Rao & Dixon, 2018). The promoter of *OsMYB46* contains secondary wall NAC-binding elements (SNBEs) and is directly activated by *OsSWNs* (Zhong et al., 2011). Besides *OsMYB46*, overexpression of *OsSWNs* was able to upregulate the expression of *SND3*, *MYB83*, *MYB85*, *MYB58*, *MYB63*, etc. (Zhong et al., 2011). Furthermore, the existence of feedforward and feedback regulations, as well as the continual discovery of new transcription factors (e.g., *WRKY12*, *E2Fc*, etc.) in the transcriptional regulatory hierarchy (Xie, Zhang, et al., 2018), demonstrates the complexity of the transcriptional regulation of the phenylpropanoid pathway.

5-enolpyruvylshikimate 3-phosphate (EPSP) synthase widely exists in plants, bacteria, and fungi. EPSP synthase has carboxyvinyl transferase activity and catalyzes the conversion of phosphoenolpyruvate and shikimate-3-phosphate to EPSP in the shikimate pathway, which is responsible for the biosynthesis of aromatic amino acids (Maeda & Dudareva, 2012). Recently, we discovered one isoform of EPSP synthase in *Populus* (*PtrEPSP-TF*) that is involved in the regulation of the phenylpropanoid pathway. Besides the EPSP synthase activity, *PtrEPSP-TF* has transcriptional activity and regulates the expression of *PtrMYB021* (Xie, Muchero, et al., 2018), one functional homolog of *MYB46* in *Populus* (Wilkins, Nahal, Foong, Provart, & Campbell, 2009; Zhong, McCarthy, Haghigat, & Ye, 2013).

In this study, we identified a SNP critical for *PtrEPSP-TF* functionality. The SNP-induced substitution of the 142nd amino acid dramatically impairs the DNA-binding and transcriptional activity of *PtrEPSP-TF*, which results in the reduced lignin content in *Populus* natural variants. We also demonstrated the *PtrEPSP-TF*-mediated transcriptional reprogramming of the phenylpropanoid pathway in rice. As a monocot, rice only has an EPSP synthase lacking the DNA-binding helix-turn-helix (HTH) motif, providing a low-background platform to study the transcriptional effect of *PtrEPSP-TF*. We found that the heterologous expression of *PtrEPSP-TF* in rice alters the transcription of a bunch of genes involved in the phenylpropanoid pathway. Furthermore, we uncovered five negative regulators of the phenylpropanoid pathway in rice using this heterologous expression system. Our results provide a strategy to better understand the regulation of the phenylpropanoid pathway in rice and *Populus*, which will complement the strength of the comparative study of *Arabidopsis*, rice, and *Populus* transcriptional regulatory elements.

## 2 | METHODS

### 2.1 | Plant growth conditions

To generate transgenic rice, cDNAs of the archetypic *PtrEPSP-TF*, the BESC-35 *PtrEPSP-TF*, and BESC-876 *PtrEPSP-TF* were cloned into binary vector pANIC10A (Mann et al., 2012) and then transformed into

Japonica rice Taipei 309, using biolistics and the procedures as described in Mann et al., 2011 (Mann et al., 2011). The empty pANIC10A vector was transformed in parallel as a negative control. Positive transgenic events were selected by measuring *PtrEPSP-TF* expression using RT-PCR in hygromycin-resistant plants. RNA was isolated as described (Jacobs, Lawler, LaFayette, Vodkin, & Parrott, 2016). Total RNA was treated with RNase-free DNase I (Thermo Fisher Scientific). Endpoint RT-PCR was performed with the GoScript Reverse Transcription System (Promega) according to the manufacturer's instructions using primers EPSP-1219F (5'-GCTATGACTCTGGCTGTTGTTGC-3') and OCS-R (5'-CAACGTGCACAACAGAATTGA-3'). Rice plants were grown in soil under natural sunlight in a closed greenhouse at 22–32°C with controlled water and nutrient supply and a 16-hr light period.

## 2.2 | RNA-seq experiments

RNAs were extracted from stem tissues of three-month-old rice plants prior to flower emergence (i.e., the vegetative stage). Four biological replicates were collected for each transgenic line. The cDNA libraries were constructed following Illumina standard protocols and sequenced with Illumina HiSeq 2500. After filtering out low-quality reads, RNA-Seq reads were aligned to the *Oryza sativa* genome ([https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org\\_Osativa](https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Osativa)) using TopHat (Kim et al., 2013). Differentially expressed genes (DEGs) were identified using the R package DESeq2 (Love, Huber, & Anders, 2014). Raw *p*-values were adjusted for multiple comparison effects using the false discovery rate (FDR). The cutoff for significant DEGs was set as >2 absolute fold change (FC) and FDR ≤ 0.05.

Gene Ontology (GO) enrichment analysis was performed for DEGs using agriGO (Tian et al., 2017). A multiple testing correction was performed using the FDR under dependency (Yekutieli & Benjamini, 1999). GO terms with a corrected *p* ≤ .05 were considered to be significantly enriched. For transcription factor binding sites (TFBS) prediction, 1-kb sequences upstream of the translation start site of DEGs in each cluster were analyzed using PlantPAN (Chow et al., 2016).

## 2.3 | Metabolite analysis

Freeze-dried stems were ground with a micro-Wiley mill, and ~50 mg DW was subsequently twice extracted with 2.5 ml 80% ethanol overnight and then combined prior to drying a 0.5 ml aliquot in a nitrogen stream. Sorbitol was added before extraction as an internal standard to correct for differences in extraction efficiency, subsequent differences in derivatization efficiency and changes in sample volume during heating. Dried extracts were silylated for 1 hr at 70°C to generate trimethylsilyl (TMS) derivatives, which were analyzed after 2 days with an Agilent Technologies Inc 5975C inert XL gas chromatograph-mass spectrometer as described elsewhere (Li et al., 2012; Tschaplinski et al., 2012). Metabolite peak extraction, identification, and quantification were described previously (Tschaplinski et al., 2012), and unidentified metabolites were denoted by their retention time as well as key mass-to-charge (*m/z*) ratios. Data were pooled across four independent

replicates, and treatment means were tested for statistical significance (*p* ≤ .05) using Student's *t* tests.

## 2.4 | Electrophoretic mobility shift assay

The archetypic *PtrEPSP-TF*, BESC-35 *PtrEPSP-TF*, and BESC-876 *PtrEPSP-TF* were cloned into the pGEX-6P-1 vector (GE Healthcare) by BamHI and EcoRI for GST fusion constructs. The constructs were transformed into *E. coli* strain BL21(DE3)pLysS (Invitrogen) for protein expression. GST fusion proteins were extracted and purified as previously described using Glutathione Sepharose 4B beads (GE Healthcare) (Xie, Ren, Costa-Nunes, Pontes, & Yu, 2012). To perform EMSA, proteins were then eluted from beads by incubating with Elution Buffer (50 mM Tris-HCl pH 8.0, 10 mM reduced glutathione) at 4°C for 30 min. For DNA probes, *PtrhAT* promoter DNA was PCR amplified, gel purified, and end labeled with biotin as described previously (Xie, Muchero, et al., 2018). The DNA-binding reaction included 0.25 nM Biotin-labeled probe, 0.4 μg of purified protein, 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 2.5% Glycerol, 5 mM MgCl<sub>2</sub>, 1 μg Poly (dl-dC), 0.05% NP-40. Reactions were incubated at room temperature for 20 min. The reaction mixtures were then resolved in 6% DNA retardation gel (Novex) by electrophoresis at 100 V for 1–2 hr and electrophoretically transferred to Nylon membrane. Signals of biotin were detected using Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific) as suggested by the manufacturer.

## 2.5 | Transcriptional activity assay

The protoplast transfection-based transcriptional activity assay was performed according to the previously described method (Tiwari, Hagen, & Guilfoyle, 2003). Ten μg of effector and reporter plasmids was co-transfected into 100 μl of *Populus* protoplasts using PEG-calcium transfection method and incubated under the darkness for 18–20 hr at room temperature. GUS activity assay was performed as described (Yoo, Cho, & Sheen, 2007). GUS activity was measured using a Fluoroskan microplate reader. To normalize GUS activity, 100 ng of 35S: Luciferase plasmid was co-transfected for each transfection. Luciferase activity was measured using Promega Luciferase Assay System according to the manufacturer's manual.

## 2.6 | Phloroglucinol-HCL staining

The S8 segment of rice internode II was sectioned into 60 μm thickness and then stained with 1% phloroglucinol in HCl for 5 min and immediately observed under a light microscope (Zeiss). The internal diameters of sclerenchyma cells were measured under the microscope using the manufacturer's software (Zeiss).

## 2.7 | Co-expression analysis

The microarray data of rice were downloaded from the NCBI GEO database under the series accession numbers GSE19024 (<https://>

www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE19024; 190 samples corresponding to 39 tissues from two indica varieties; Minghui 63 and Zhenshan 97) (Wang et al., 2010) and GSE51289 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE51289; 59 samples corresponding to 20 data points of internodes during secondary cell wall formation and 143 samples corresponding to 48 tissue and organ types) (Hirano, Aya, et al., 2013). For co-expression analysis, a matrix of DEGs in our RNA-Seq was constructed by calculating pairwise Pearson correlation coefficients based on the normalized expression data across all samples from GSE19024 and GSE51289. Cytoscape (Shannon et al., 2003) was used to visualize the resulting network. Only co-expression relationships with  $p \leq .05$  were indicated in the network.

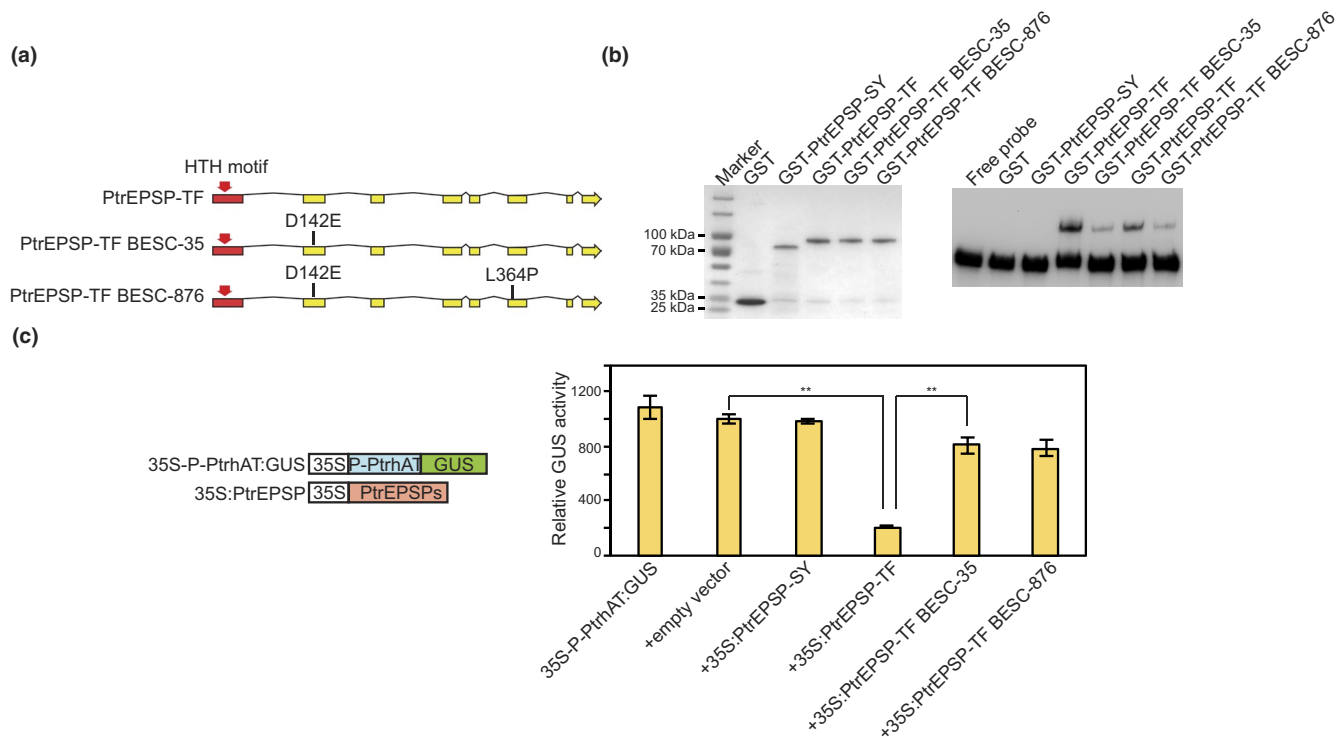
### 3 | RESULTS

#### 3.1 | DNA binding and transcriptional activities of allelic variants from BESC-35 and BESC-876 are impaired

In previous genome-wide association studies, we identified two *Populus* natural variants containing *PtrEPSP-TF* rare alleles: BESC-35

and BESC-876 that showed reduced lignin content compared with the average of the *Populus trichocarpa* population (Xie, Muchero, et al., 2018). To determine how mutations in these genotypes affect *PtrEPSP-TF* protein function, we cloned *PtrEPSP-TF* allelic variants from both genotypes. Sequencing and amino acid alignments revealed that both BESC-35 and BESC-876 variants carry a point mutation that results in the substitution of the 142nd amino acid from aspartic acid (D) to glutamic acid (E) (D142E, Figure 1a). BESC-876 has an additional point mutation at position 364 that leads to the substitution of leucine (L) with proline (P) (L364P, Figure 1a).

Given that the HTH motif (amino acids 30–70) is responsible for DNA binding and transcriptional activity of *PtrEPSP-TF* (Xie, Muchero, et al., 2018), we speculated that the amino acid substitution adjacent to the HTH motif (i.e., D142E) may affect the transcriptional function of *PtrEPSP-TF*. To examine this possibility, we analyzed the binding affinity of BESC-35 and BESC-876 variants to the promoter of *PthAT*, which is the direct regulatory target of *PtrEPSP-TF* in *Populus* (Xie, Muchero, et al., 2018). *PtrEPSP-TF* and the variant proteins were tagged with GST and expressed in *E. coli*. After purification, these proteins were subjected to in vitro electrophoretic mobility shift assay (EMSA) to determine their binding affinities to the *PthAT* promoter, which was labeled with biotin for



**FIGURE 1** The reduced DNA-binding and transcriptional activities of BESC-35 and BESC-876 variants. (a) A scheme of point mutations in BESC-35 and BESC-876 variants. The HTH motif is indicated by a red arrow. Amino acid abbreviation: D, Aspartic acid; E, Glutamic acid; L, Leucine; P, Proline. (b) BESC-35 and BESC-876 variants display reduced DNA-binding affinity to *PthAT* promoter. Left panel: purified GST, GST-PtrEPSP-SY (an *EPSP* homolog of *PtrEPSP-TF* that lacks the HTH domain), GST-PtrEPSP-TF, GST-PtrEPSP-TF from BESC-35, and GST-PtrEPSP-TF from BESC-876 were resolved in SDS-PAGE gel and stained with Coomassie Blue. Right panel: EMSA assay of DNA binding to biotin-labeled *PthAT* promoter. Free probe and GST are negative controls. (c) Transactivation assay of repression activities of archetypic *PtrEPSP-TF*, BESC-35 *PtrEPSP-TF*, and BESC-876 *PtrEPSP-TF* on *PthAT* promoter. The repression activity of the blank vector was analyzed in parallel as a negative control. All transfection assays were performed in triplicate to calculate the mean value and standard deviation (error bar).  $P$ -value comparisons (empty vector and *PtrEPSP-TF*, *PtrEPSP-TF* and BESC-35/BESC-876 *PtrEPSP-TF*) were calculated using two-tailed Student  $t$  tests (\*\* $p \leq .01$ )

visualization. As shown in Figure 1b, signals of shift bands of both variants were dramatically reduced compared with that of the archetypic PtrEPSP-TF. These results demonstrate that BESC-35 and BESC-876 variants have significantly reduced DNA-binding activity. Consistent with reduced DNA-binding activity, both PtrEPSP-TF variants display impaired transcriptional repression on the *PthAT* promoter (Figure 1c). In contrast to the archetypic PtrEPSP-TF, neither BESC-35 nor BESC-876 variants can reduce the expression of the *GUS* gene downstream of the *PthAT* promoter in the transactivation assays using the protoplast transient expression system. Thus, the BESC-35 and BESC-876 allelic variants carry mutations impacting the transcriptional repressor activity of PtrEPSP-TF. Given the promoting effects of the archetypic PtrEPSP-TF on *PtrMYB021* (MYB46 close homolog) (Wilkins et al., 2009) expression and lignin biosynthesis in *Populus*, the reduced lignin content of BESC-35 and BESC-876 *Populus* genotypes is likely due to the impaired function of PtrEPSP-TF. As such, BESC-35 and BESC-876 variants can be viewed as loss-of-function alleles of *PtrEPSP-TF*.

### 3.2 | Heterologous expression of *PtrEPSP-TF* in transgenic rice alters sclerenchyma thickening and secondary metabolism

The absence of the HTH motif in monocot EPSP synthases (Xie, Muchero, et al., 2018) prompted us to compare functions of archetypic PtrEPSP-TF with BESC-35 and BESC-876 variants via heterologous expression. We generated transgenic rice plants heterogeneously expressing the functional *PtrEPSP-TF*, or the BESC-35 (*PtrEPSP-TF<sup>D142E</sup>*) and BESC-876 (*PtrEPSP-TF<sup>D142E/L364P</sup>*) alleles. The expression of transgenes is driven by the *ZmUbi1* promoter, which allows for high expression levels of transgenes in monocots (Mann et al., 2012). The heterogeneous expression of the functional *PtrEPSP-TF* or its loss-of-function alleles in rice did not trigger apparent morphological change in leaf and height during the vegetable growth. To visualize secondary cell walls, transverse cross sections of the S8 segment of rice internode II (Lin et al., 2017) were stained with phloroglucinol-HCl, which turns lignin into a red-purple color. Under a low-magnification dissecting microscope, each sclerenchyma cell of the empty-vector control was clearly observed, and their cell walls were stained red (Figure 2a). Different from the empty-vector control, the whole sclerenchyma cell region of rice plants expressing the archetypic *PtrEPSP-TF* stained red and it was hard to distinguish individual sclerenchyma cells (Figure 2a). On the other side, transgenic rice plants expressing *PtrEPSP-TF<sup>D142E</sup>* or *PtrEPSP-TF<sup>D142E/L364P</sup>* exhibit similar staining results as that of the empty-vector control (Figure 2a), suggesting the difference of sclerenchyma cell staining is likely induced by difference in functions between PtrEPSP-TF and *PtrEPSP-TF<sup>D142E</sup>* or *PtrEPSP-TF<sup>D142E/L364P</sup>*. To further determine the cause of the difference of sclerenchyma cell staining, we observed stained stems using a high-magnification compound microscope. As shown in Figure 2b, expression of the functional *PtrEPSP-TF* resulted in thickened secondary cell walls and reduced internal diameter

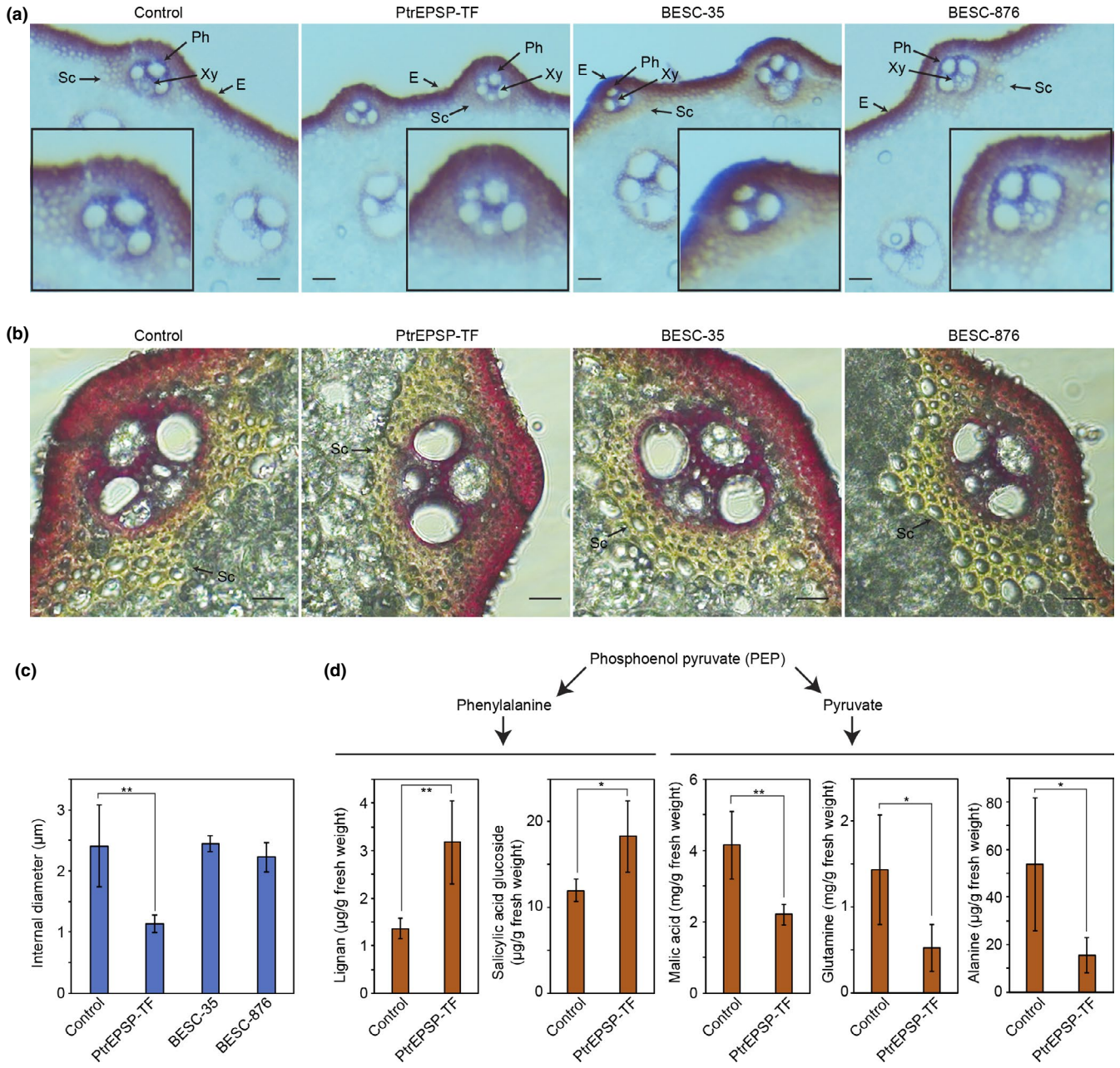
in the sclerenchyma cells that form the cortical layers in bundle sheath fibers surrounding vascular vessels. In contrast, *PtrEPSP-TF<sup>D142E</sup>* and *PtrEPSP-TF<sup>D142E/L364P</sup>* variants resulted in little differences from the empty-vector control in the thickness of secondary cell walls of the sclerenchyma cells (Figure 2b,c). Unexpectedly, the secondary cell wall thickening induced by the archetypic PtrEPSP-TF suggested that PtrEPSP-TF may affect the transcriptional regulation of secondary cell wall biosynthesis in rice as it does in *Populus*, although the EPSP form found in rice lacks the HTH domain and thus lacking transcriptional control.

Besides sclerenchyma thickening, the lignin content of rice expressing archetypic *PtrEPSP-TF* ( $31.98 \pm 0.24$  [% of dry weight]) was significantly higher ( $p \leq .01$ ) than that of the empty-vector control ( $29.11 \pm 0.00$  [% of dry weight]). Gas chromatography-mass spectrometry (GC-MS) analysis revealed that secondary metabolism of transgenic rice was altered by archetypic PtrEPSP-TF, where metabolites in the phenylalanine branch (Figure 2d), including salicylic acid glucoside exhibited 52.3% ( $p = .028$ ) and lignan exhibited 132.5% ( $p = .007$ ) increases, respectively, relative to the empty-vector controls (Figure 2d). This same response occurred in *Populus* overexpressing *PtrEPSP-TF* (Xie, Muchero, et al., 2018). Phenylalanine and pyruvate are synthesized using the same precursor phosphoenol pyruvate (PEP) (Davies, 1979), and phenylalanine is the precursor of the phenylpropanoid pathway (Fraser & Chapple, 2011). Opposite to the pattern of the phenylalanine metabolites, metabolites in the pyruvate branch (Figure 2d), including malic acid, alanine, and glutamine, exhibited significant reductions, 46.8% ( $p = .008$ ), 63.8% ( $p = .039$ ), and 71.3% ( $p = .037$ ) respectively, in rice expressing archetypic *PtrEPSP-TF* (Figure 2d).

### 3.3 | Heterologous expression of *PtrEPSP-TF* in rice alters transcriptional regulation of the phenylpropanoid pathway

To explore the molecular effects of the archetypic PtrEPSP-TF in transgenic rice, we extracted total RNAs from stems of three-month-old transgenic rice plants and performed RNA-seq analysis. For each sample, four biological replicates were analyzed, and highly consistent results were obtained (Pearson correlation  $r \geq .93$ ). Differential expression analysis identified a large collection of mRNAs whose abundance was significantly affected (Fold change  $\geq 2$ ,  $p \leq .01$ , false discovery rate [FDR]  $\leq 0.05$ ) by the archetypic PtrEPSP-TF. Compared with transcriptomes of empty-vector control, a total of 3,953 upregulated genes and 1,471 downregulated genes were identified (Figure 3a and Dataset S1). Consistent with results that the *PtrEPSP-TF<sup>D142E</sup>* and *PtrEPSP-TF<sup>D142E/L364P</sup>* variants have impaired DNA-binding and transcriptional repressor activity, differentially expressed genes (DEGs) in BESC-35 and BESC-876 alleles expression lines were far fewer compared to the functional archetypic *PtrEPSP-TF* expression line (Figure 3a). The BESC-35 variant led to 729 upregulated genes and 1,091 downregulated genes, whereas the BESC-876 variant resulted in 353 upregulated and 172 downregulated genes (Figure 3a, Dataset

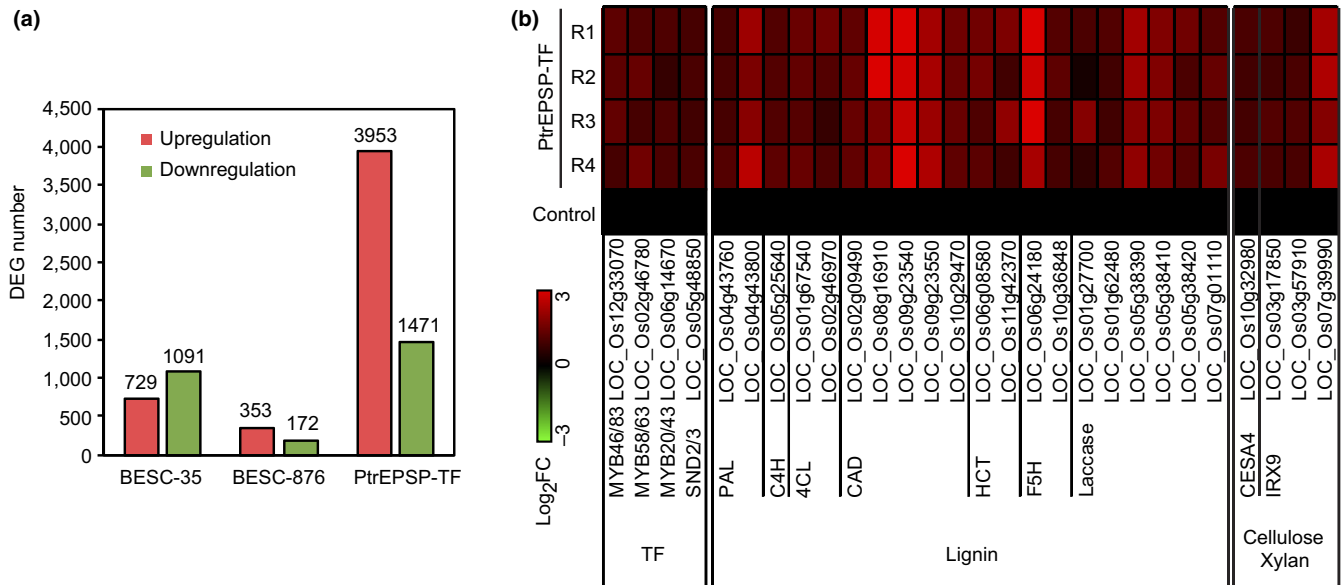




**FIGURE 2** Transgenic rice plants expressing *PtrEPSP-TF* display thickened sclerenchyma and altered secondary metabolism. (a) Dissecting microscopy images of phloroglucinol-HCl stained rice stems from empty-vector control, archetypic *PtrEPSP-TF*, BESC-35 *PtrEPSP-TF*, and BESC-876 *PtrEPSP-TF*. E, Epidermis; Ph, phloem; Sc, sclerenchyma cells; Xy, xylem. Scale bar: 200  $\mu\text{m}$ . (b) Compound microscopy images of phloroglucinol-HCl stained rice stems empty-vector control, archetypic *PtrEPSP-TF*, BESC-35 *PtrEPSP-TF*, and BESC-876 *PtrEPSP-TF*. Scale bar: 10  $\mu\text{m}$ . Sc, sclerenchyma cells. (c) The Sclerenchyma cells of *PtrEPSP-TF* have smaller internal diameter. The internal diameters of ten sclerenchyma cells were measured under a microscope to calculate mean values and standard errors (error bars). *P*-value comparison was calculated using two-tailed Student *t* tests (\*\* $p \leq .01$ ). (d) Rice plants expressing functional archetypic *PtrEPSP-TF* have increased accumulation of phenylpropanoid metabolites, but reduced accumulation of pyruvate metabolites. Four independent replicates were measured to calculate mean values and standard errors (error bars). *P*-value comparison was calculated using two-tailed Student *t* tests (\*\* $p \leq .01$ , \* $p \leq .05$ )

S1). In addition, among the 3,953 upregulated genes by the archetypic *PtrEPSP-TF*, only 134 of them (3.39%) were also upregulated by BESC-35 and BESC-876 variants (Figure S1). Similarly, only 1.63% (24 out of 1,471) downregulated genes by the archetypic *PtrEPSP-TF* were downregulated by BESC-35 and BESC-876 variants (Figure S1).

We then performed Gene Ontology (GO) enrichment analysis on DEGs of rice expressing the functional archetypic *PtrEPSP-TF* to determine the global effect of *PtrEPSP-TF*. Among upregulated ones, genes associated with cell walls, lipids, secondary metabolism, and light reactions were significantly enriched (Figure S2a). Further GO enrichment analysis showed that genes involved in phenylpropanoid,



**FIGURE 3** The heterologous expression of the archetypic *PtrEPSP-TF* in rice induces the upregulation of genes involved in lignin and secondary cell wall biosynthesis. (a) Rice plants expressing archetypic *PtrEPSP-TF* have more DEGs than rice plants expressing BESC35 *PtrEPSP-TF* or BESC876 *PtrEPSP-TF*. Red bar: upregulated genes; Green bar: downregulated genes. (b) Heat map showing expression patterns of PAL, C4H, 4CL, CAD, F5H, LACCASE, CESA4, IRX9, MYB46/83, MYB58/63, MYB20/43, and SND2/3 in four independent replicates (R1 to R4) of the archetypic *PtrEPSP-TF* expression rice.  $\text{Log}_2\text{FC}$  of mean expression levels of the four replicates of empty-vector control was set as 0. The color scale represents the value of  $\text{Log}_2\text{FC}$

flavonoid, and lignin metabolism were overrepresented among up-regulated genes associated with secondary metabolism (Figure S2b). In contrast, downregulated genes did not show any enrichment in metabolic pathways (Figure S2c and d).

To determine whether the differential sclerenchyma cell staining between rice plants expressing the archetypic *PtrEPSP-TF* and BESC-35 or BESC-876 alleles was related to transcriptional reprogramming of secondary cell wall biosynthesis, we looked for genes related to secondary cell wall biosynthesis from DEGs of transgenic rice. A total of twenty lignin, one cellulose, and three xylan biosynthetic genes were found. Interestingly, four master regulators of secondary cell wall biosynthesis were also found (Figure 3b). All of these were upregulated by the functional *PtrEPSP-TF* (Figure 3b). In contrast, expression of these genes only showed modest upregulation or even downregulation in the BESC-35 and BESC-876 lines (Figure S3), suggesting that the archetypic *PtrEPSP-TF*, but not BESC-35 or BESC-876 variants, can trigger the transcriptional reprogramming of secondary cell wall biosynthesis in rice.

Among these 28 upregulated genes involved in secondary cell wall biosynthesis, four genes encode transcription factors (Figure 3b), and their *Arabidopsis* homologs were found to be master regulators of secondary cell wall/lignin biosynthesis, including *LOC\_Os12g33070* (MYB46/83)(Zhong et al., 2011), *LOC\_Os02g46780* (MYB58/63)(Zhou, Lee, Zhong, & Ye, 2009), *LOC\_Os06g14670* (MYB20/43)(Zhong, Lee, Zhou, McCarthy, & Ye, 2008), and *LOC\_Os05g48850* (SND2/3)(Hussey et al., 2011). Downstream of these transcription factors, PAL, C4H, and 4CL catalyze the first three steps of the phenylpropanoid pathway and are essential for the biosynthesis of monolignols (Fraser & Chapple, 2011). We observed that two PAL genes (*LOC\_Os04g43760*

and *LOC\_Os04g43800*), one C4H gene (*LOC\_Os05g25640*), and two 4CL genes (*LOC\_Os01g67540* and *LOC\_Os02g46970*) were upregulated by the archetypic *PtrEPSP-TF* (Figure 3b). In addition, genes critical for monolignol biosynthesis were also upregulated by the archetypic *PtrEPSP-TF*, such as five CADs (*LOC\_Os02g09490*, *LOC\_Os08g16910*, *LOC\_Os09g23540*, *LOC\_Os09g23550*, and *LOC\_Os10g29470*), two HCTs (*LOC\_Os06g08580* and *LOC\_Os11g42370*), and two F5Hs (*LOC\_Os06g24180* and *LOC\_Os10g36848*) (Figure 3b). Additionally, six Laccase genes were upregulated by *PtrEPSP-TF*: *LOC\_Os01g27700*, *LOC\_Os01g62480*, *LOC\_Os05g38390*, *LOC\_Os05g38410*, *LOC\_Os05g38420*, and *LOC\_Os07g01110* (Figure 3b). The four cellulose and xylan biosynthetic genes upregulated by *PtrEPSP-TF* were CESA4 (*LOC\_Os10g32980*) and IRX9 (*LOC\_Os03g17850*, *LOC\_Os03g57910*, and *LOC\_Os07g39990*) (Figure 3b).

Collectively, we demonstrated that the heterologous expression of the archetypic *PtrEPSP-TF* in rice resulted in the thickening of secondary cell wall and increased lignin content, which may be through transcriptional reprogramming of genes critical for lignin/secondary cell wall biosynthesis in rice. This observation is consistent with the analysis of transgenic *Populus* overexpressing *PtrEPSP-TF* (Xie, Muchero, et al., 2018), suggesting that besides *Populus*, *PtrEPSP-TF* is capable of affecting the lignin/secondary cell wall biosynthesis when introduced to rice in which the native rice EPSP synthase lacks the HTH domain.

### 3.4 | *PtrEPSP-TF* mediates a transcriptional regulatory network in rice

In *Populus*, the archetypic *PtrEPSP-TF* positively regulates *PtrMYB021* expression by directly repressing the expression of





*PtrHAT*, a *SLEEPER*-like gene that directly represses *PtrMYB021* expression (Xie, Muchero, et al., 2018). As a close homolog of *Arabidopsis* MYB46, a master regulator of secondary cell wall biosynthesis, *PtrMYB021* functions as a transcriptional activator and activates the biosynthesis pathways for lignin, cellulose, and xylan (Zhong et al., 2013), the major components of secondary cell walls. Similarly, in the present study, we demonstrate that the archetypic *PtrEPSP-TF* is able to upregulate rice *MYB46* (*LOC\_Os12g33070*) and phenylpropanoid/lignin biosynthetic genes (*PAL*, *4CL*, *C4H*, *F5H*, *CAD*, *CCR*, and *LACCASE*) when introduced to rice. Therefore, we hypothesize that an analogous *PtrEPSP-TF*-mediated transcriptional mechanism, in which *PtrEPSP-TF* represses negative regulators to activate lignin/secondary cell wall biosynthesis, may exist in rice.

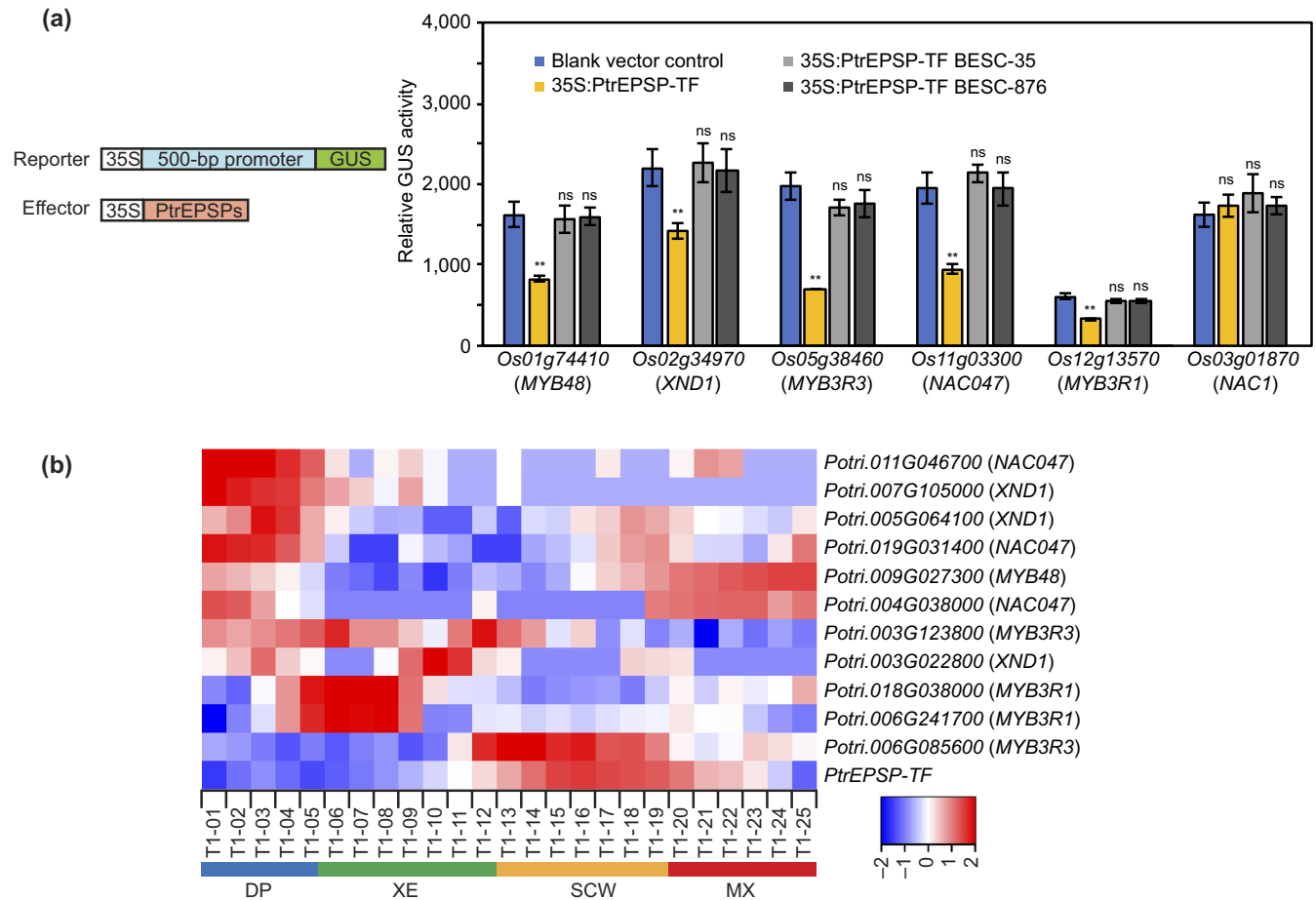
In support of this hypothesis, we found three tandemly duplicated hAT *SLEEPER*-like genes, *LOC\_Os09g21410* ( $-7.3$ ,  $p = .0049$ ), *LOC\_Os09g21420* ( $-3.1$ ,  $p = 2.7E-11$ ), and *LOC\_Os09g21430* ( $-1.7$ ,  $p = 1.1E-06$ ), that were significantly downregulated in the archetypic *PtrEPSP-TF* expression lines (Dataset S1). In contrast, the expression of *LOC\_Os09g21410* and *LOC\_Os09g21420* only had moderate reduction or even no significant change in the BESC-35 and BESC-876 lines (Dataset S1). The expression of *LOC\_Os09g21430* exhibited a higher reduction in the BESC-35 line than that in the line expressing the archetypic *PtrEPSP-TF*, but had no significant change in the BESC-876 line (Dataset S1). Besides hAT *SLEEPER*-like genes, we found an additional 82 transcription factors that were significantly downregulated in the same line (Dataset S2). Among them, we found twelve NAC and MYB transcription factors (Dataset S2). Among the twelve NAC and MYB transcription factors, *XND1* (*LOC\_Os02g34970*) is a homolog of *Arabidopsis* AT5G64530, which has been reported to negatively regulate lignin biosynthesis (Zhao, Avci, Grant, Haigler, & Beers, 2008). *MYB48* (*LOC\_Os01g74410*) has a close homolog *MYB12* in *Arabidopsis*, which was found to activate the biosynthesis of flavonoids, which, interestingly, competes for precursors with the biosynthesis of monolignols (Mehrtens, Kranz, Bednarek, & Weisshaar, 2005). Also, *MYB3R1* (*LOC\_Os12g13570* and *AT4G32730* in *Arabidopsis*) and *MYB3R3* (*LOC\_Os05g38460* and *AT3G09370* in *Arabidopsis*) are putative transcriptional repressors (Kobayashi et al., 2015) and *NAC047* (*LOC\_Os11g03300* and *AT3G04070* in *Arabidopsis*) is associated with cell wall re-organization (Rauf et al., 2013). To determine whether these five transcription factors (*XND1*, *MYB48*, *MYB3R1*, *MYB3R3*, and *NAC047*) are indeed targets of *PtrEPSP-TF* in rice, we tested the repression of their promoter activity by *PtrEPSP-TF* using the *Populus* mesophyll protoplast transient expression system. A 500-bp fragment of the promoter region of each putative target gene ( $-500$  to  $-1$  from start codon) was inserted between the 35S promoter and the GUS gene in reporter constructs. Then, each reporter construct was co-transfected with the effector construct expressing *PtrEPSP-TF* into protoplasts. GUS activity was measured to represent the activity of the tested promoters. As illustrated by the reduced GUS activity, *PtrEPSP-TF* repressed the activity of all five tested promoters (Figure 4a). In contrast, *PtrEPSP-TF* did not repress the promoter of *LOC\_Os03g01870* (*NAC1*) (Figure 4a), a NAC transcription

factor downregulated in all the three transgenic lines (*PtrEPSP-TF*, BESC-35, and BESC-876) (Dataset S1), suggesting that the altered expression of *LOC\_Os03g01870* (*NAC1*) may be from background signals (e.g. sequencing noises). The combined RNA-seq and trans-activation assay results demonstrate that expression of the five rice NAC and MYB transcription factors was negatively regulated by *PtrEPSP-TF*.

To further investigate the analogous *PtrEPSP-TF*-mediated transcriptional mechanism in *Populus* and rice, we analyzed the expression patterns of *Populus* homologs of *XND1*, *MYB48*, *MYB3R1*, *MYB3R3*, and *NAC047* using the AspWood database (<http://aspwood.popgenie.org/>). During *Populus* wood formation, the phenylpropanoid pathway is activated to generate lignified secondary cell wall. AspWood provides high-spatial-resolution gene expression profiles spanning the wood-forming zone of *Populus* (Sundell et al., 2017). As shown in Figure 4b, *PtrEPSP-TF* gene has a peak expression in the secondary cell wall deposition zone, which is similar to expression patterns of positive regulators of the phenylpropanoid and lignin biosynthesis (e.g., *SND1*, *SND2*, *VND7*; Sundell et al., 2017). In contrast, *Populus* homologs of *XND1*, *MYB48*, *MYB3R1*, *MYB3R3*, and *NAC047* (except *Potri.006G085600*, one homolog of *MYB3R3*) have low expression levels in the secondary cell wall deposition zone, but have peak expression levels in the differentiating phloem zone, xylem expansion zone, or the mature xylem zone (Figure 4b). These opposite expression patterns suggest that *PtrEPSP-TF* may also have repression effect on *Populus* *XND1*, *MYB48*, *MYB3R1*, *MYB3R3*, and *NAC047* genes.

The observed repression of *SLEEPER*-like genes in addition to known negative regulators of cell wall biosynthesis, such as *XND1*, is consistent with the proposed transcriptional regulatory model in *Populus* (Xie, Muchero, et al., 2018), that established that the phenylpropanoid pathway is induced upon overexpression of *PtrEPSP-TF*, which suppresses the expression of a *SLEEPER*-like repressor of the pathway. To confirm this hypothesis, we looked for transcriptional regulators related to cell wall biosynthesis among a total of 221 predicted transcriptional regulators. A total of 43 NAC and MYB transcription factors were upregulated by *PtrEPSP-TF* (Dataset S3). Among these transcription factors, four master regulators of phenylpropanoid biosynthesis were found, including NAC and MYB transcription factors *LOC\_Os12g33070* (*MYB46/83*), *LOC\_Os02g46780* (*MYB58/63*), *LOC\_Os06g14670* (*MYB20*) (Zhong et al., 2008), and *LOC\_Os05g48850* (*SND2/3*). These four transcription factors and/or their *Arabidopsis* orthologs activate lignin and secondary cell wall biosynthesis (Nakano, Yamaguchi, Endo, Rejab, & Ohtani, 2015). For the remaining 39 NAC and MYB transcription factors, we searched for their predicted cis-element-binding sites in promoter regions of 3,423 genes specifically upregulated by the archetypic *PtrEPSP-TF* (Figure S1). Five transcription factors are presented to highlight using this approach (Figure S4 and Dataset S4): *LOC\_Os11g08210* (*NAC081*), *LOC\_Os03g42630* (*NAC058*), *LOC\_Os05g35500* (*MYB4*), *LOC\_Os08g43550* (*MYB4*), *LOC\_Os12g07640* (*MYB4*).

Cis-elements recognized by the five transcription factors were enriched in promoters of these genes. Specifically, the cis-element

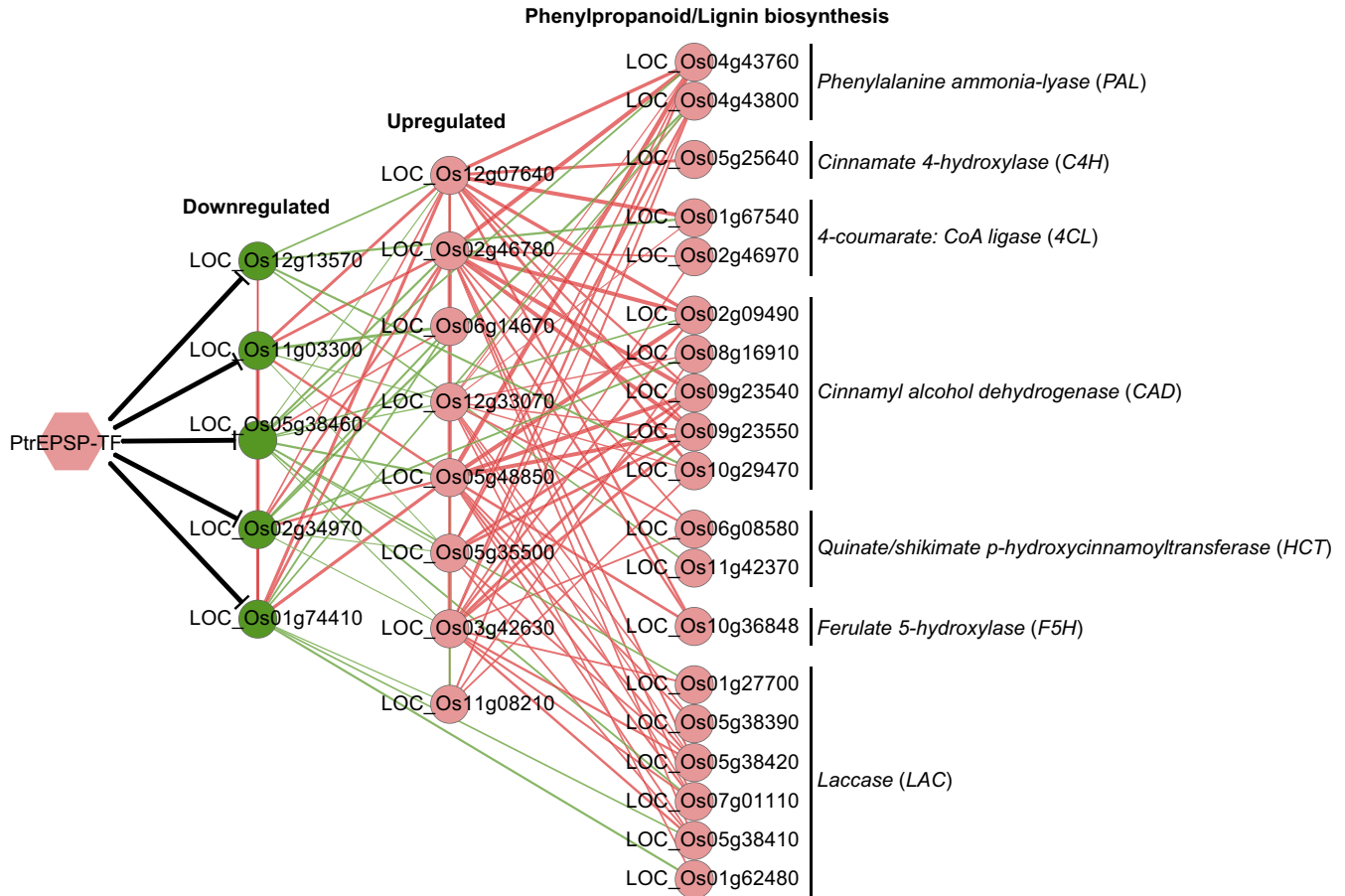


**FIGURE 4** PtrEPSP-TF represses the expression of five genes. (a) Left panel: a scheme of reporter and effector constructs used in transactivation assays. Right panel: transactivation assays determining the transcriptional repression of PtrEPSP-TF on *LOC\_Os02g34970* (*XND1*), *LOC\_Os01g74410* (*MYB48*), *LOC\_Os12g13570* (*MYB3R1*), *LOC\_Os05g38460* (*MYB3R3*), and *LOC\_Os11g03300* (*NAC047*). All transfection assays were performed in triplicate to calculate the mean value and standard deviation (error bar). *P*-value comparison with blank vector control was calculated using two-tailed Student *t* tests (\*\**p* ≤ .01, \**p* ≤ .05, ns *p* > .05). (b) Heatmap of expression levels of *PtrEPSP-TF*, *Potri.003G022800* (*XND1*), *Potri.005G064100* (*XND1*), *Potri.007G105000* (*XND1*), *Potri.009G027300* (*MYB48*), *Potri.018G038000* (*MYB3R1*), *Potri.006G241700* (*MYB3R1*), *Potri.003G123800* (*MYB3R3*), *Potri.006G085600* (*MYB3R3*), *Potri.011G046700* (*NAC047*), *Potri.004G038000* (*NAC047*), and *Potri.019G031400* (*NAC047*) in wood-forming zones (<http://aspwood.popgenie.org>). DP, differentiating phloem zone; XE, xylem expansion zone; SCW, secondary cell wall deposition zone; MX, mature xylem zone. Color bar at the bottom depicts the relative expression levels

(TFmatrixID\_0382) targeted by *LOC\_Os11g08210* (*NAC081*) was found in 1,952 of the 3,423 (57.03%; Figure S4). *LOC\_Os03g42630* (*NAC058*) targets the cis-element TFmatrixID\_0391, which was found in promoters of 984 out of the 3,423 (28.75%; Figure S4). The cis-element TFmatrixID\_0336 targeted by the four MYB4 paralogs *LOC\_Os05g35500*, *LOC\_Os08g43550*, and *LOC\_Os12g07640* was found in promoters of 963 out of the 3,423 genes (28.13%; Figure S4). Overall, we propose that these five transcription factors in addition to *LOC\_Os12g33070* (*MYB46/83*), *LOC\_Os02g46780* (*MYB58/63*), *LOC\_Os06g14670* (*MYB20*), and *LOC\_Os05g48850* (*SND2/3*) may function downstream of the *MYB48*, *XND1*, *MYB3R3*, *MYB3R1*, and *NAC047*, which are individually repressed by the archetypic *PtrEPSP-TF* (Figures 4 and 5).

To validate this proposed *PtrEPSP-TF*-mediated transcriptional network, we conducted co-expression analysis using existing

developmental transcriptome datasets of rice (GEO: GSE51289 (Hirano, Aya, et al., 2013) and GEO: GSE19024 (Wang et al., 2010); Dataset S5). In the co-expression analysis, *LOC\_Os08g43550* (*MYB4*) and *LOC\_Os06g24180* (*F5H*) were not present in the two microarray datasets and were not included in the proposed network (Figure 5). Co-expression analyses of the rest of genes except *LOC\_Os12g33070* (*MYB46/83*) were performed using dataset GEO: GSE51289 because *LOC\_Os12g33070* (*MYB46/83*) is not included in the dataset. We used dataset GEO: GSE19024 to analyze the co-expression of *LOC\_Os12g33070* (*MYB46/83*) (Hirano, Aya, et al., 2013) with other members in the *PtrEPSP-TF*-mediated network. Expressions of most Layer one genes (downregulated by *PtrEPSP-TF*) were negatively co-related with expressions of Layer two genes (upregulated by *PtrEPSP-TF*) and even some lignin/phenylpropanoid biosynthetic genes (green lines,



**FIGURE 5** Co-expression analysis of members of PtrEPSP-TF-mediated transcriptional network in rice. For co-expression relationships, only correlation coefficient ( $r$ )  $>0.1$  or  $<-0.1$  and  $p \leq .05$  are shown. Black edges indicate experimentally validated relationships. Red edges indicate positive co-relationship. Green edges indicate negative co-relationship. The strength of co-relationship ( $r$ ) is represented by the thickness of edges

Figure 5). As predicted, expressions of Layer two genes displayed significant positive co-expression with expression of PALs (LOC\_Os04g43760, LOC\_Os04g43800), C4H (LOC\_Os05g25640), 4CLs (LOC\_Os01g67540, LOC\_Os02g46970), CADs (LOC\_Os02g09490, LOC\_Os08g16910, LOC\_Os09g23540, LOC\_Os09g23550, LOC\_Os10g29470), HCTs (LOC\_Os06g08580, LOC\_Os11g42370), F5H (LOC\_Os10g36848), and Laccases (LOC\_Os01g27700, LOC\_Os01g62480, LOC\_Os05g38390, LOC\_Os05g38410, LOC\_Os05g38420, LOC\_Os07g01110) (Figure 5).

Collectively, our transcriptomics analysis and transactivation assay identified five repression targets of PtrEPSP-TF, which may negatively regulate lignin and phenylpropanoid biosynthesis.

## 4 | DISCUSSION

*Populus* plants containing rare mutations at PtrEPSP-TF locus show reduced lignin content (Xie, Muchero, et al., 2018). However, the underlying molecular mechanism had not been established. Here, by functionally analyzing two natural variants of PtrEPSP-TF, we unveiled that the reduced lignin content and increased sugar

release (Lu, Hao, et al., 2019) are likely caused by the reduced DNA binding and subsequently reduced transcriptional activities of the variant PtrEPSP-TF. An EMSA assay demonstrated that the two PtrEPSP-TF variants occurring in genotypes BESC-35 and BESC-876 have reduced DNA-binding affinity to the *PthrAT* promoter, which is the direct target of PtrEPSP-TF. Moreover, the transcriptional repression effects of the BESC-35 and BESC-876 variants on *PthrAT* promoter displayed significant reduction in transactivation assays. The HTH motif of PtrEPSP-TF was shown to be responsible for its DNA-binding activity (Xie, Muchero, et al., 2018). Given BESC-35 and BESC-876 variants share the same point mutation close to the HTH motif leading to the D142E amino acid substitution, the 142nd amino acid may play an important role in assisting the proper function of the HTH motif (e.g., protein conformation, target recognition). Although PtrEPSP-TF BESC-876 has another point mutation leading to the L364P amino acid substitution, it did not display additional reduction of DNA-binding activity, suggesting that this point mutation may not affect the DNA binding of PtrEPSP-TF. In fact, the L364P substitution is further away from the HTH domain than is the D142E substitution.



Although dicots and monocots have numerous morphological differences in the cell wall, the transcriptional regulatory mechanism appears to be relatively conserved, especially surrounding the critical role of NAC and MYB transcription factors (Nakano et al., 2015; Rao & Dixon, 2018). The ortholog of PtrEPSP-TF in rice lacks the DNA-binding HTH motif, thus lacking the transcriptional activity. By heterogeneously expressing *PtrEPSP-TF* in rice, we observed altered thickness of sclerenchyma cell wall, lignin level, secondary metabolism, and transcript levels of genes associated with lignin/phenylpropanoid biosynthesis, which are similar to *Populus deltoides* plants overexpressing *PtrEPSP-TF* (Xie, Muchero, et al., 2018). As the major mechanical tissues in rice stem, sclerenchyma fibers have heavily thickened secondary cell walls and are the major contributor of straw biomass. As such, increasing sclerenchyma cell wall thickness is a viable option to enhance the production of straw biomass.

We also observed that lignin/phenylpropanoid biosynthetic genes including *PAL*, *4CL*, *C4H*, *F5H*, *CAD*, *CCR*, and *LACCASE* were significantly upregulated by PtrEPSP-TF in rice. Moreover, master regulators of lignin/phenylpropanoid biosynthesis, such as MYB46/83, MYB58/63, MYB20/43, and SND2/3 also displayed increased expression levels in transgenic rice expressing *PtrEPSP-TF*. Collectively, we conclude that PtrEPSP-TF may have a relatively conserved effect on the lignin/phenylpropanoid biosynthesis in *Populus* and rice. The fact that transgenic rice expressing *PtrEPSP-TF* does not exhibit as many upregulated cellulose and xylan biosynthetic genes as *Populus deltoides* overexpressing *PtrEPSP-TF* (Xie, Muchero, et al., 2018) further demonstrates the divergence of secondary cell wall biosynthesis in dicots and monocots. Other regulatory mechanisms of secondary cell wall biosynthesis contain both conserved and divergent parts between dicots and monocots (Rao & Dixon, 2018).

The current understanding of the regulation of rice secondary cell wall biosynthesis mainly derives from comparative studies of the main transcriptional regulatory mechanism defined in *Arabidopsis*. Based on molecular and genetic analyses, we were able to identify five repression targets of PtrEPSP-TF in rice: MYB48 (LOC\_Os01g74410), XND1 (LOC\_Os02g34970), MYB3R3 (LOC\_Os05g38460), NAC047 (LOC\_Os11g03300), and MYB3R1 (LOC\_Os12g13570). Studies on *Arabidopsis* homologs of these repression targets of PtrEPSP-TF suggest that these five transcription factors may negatively affect lignin biosynthesis (Kobayashi et al., 2015; Mehrtens et al., 2005; Rauf et al., 2013; Zhao et al., 2008). However, their detailed regulatory roles in lignin and secondary cell wall biosynthesis remain undefined. Based on RNA-seq and co-expression analyses, we defined their negative regulatory relationships with positive regulators (i.e., MYB46/83, MYB58/63, MYB20/43, SND2/3, NAC081, NAC058, and MYB4) and biosynthetic genes (i.e., *PAL*, *4CL*, *C4H*, *F5H*, *CAD*, *CCR*, and *LACCASE*) of lignin and secondary cell wall, which demonstrates the link of the five repression targets of PtrEPSP-TF and the regulation of lignin and secondary cell wall biosynthesis.

With the production of approximately 1,140 million tons biomass per year worldwide, rice straw has been considered as an attractive biomass feedstock for producing second-generation biofuels and bioproducts (Sattlewal et al., 2018). However, to date, the transcriptional

regulatory network of lignin and secondary cell wall biosynthesis in rice remains poorly studied. The discovery of the five new regulators extends our understanding of the regulatory mechanism of lignin and secondary cell wall biosynthesis in rice and will potentially be bioengineered to improve rice biomass for the production of biofuels and bioproducts. It remains unknown though, which native transcription factors in rice (equivalent to the function of PtrEPSP-TF) regulate the transcription of these five new regulators.

Collectively, our studies of the heterologous expression of *PtrEPSP-TF* and its two alleles defined one critical amino acid for PtrEPSP-TF transcriptional function and uncovered five negative regulators of secondary cell wall biosynthesis in rice, which enrich the toolbox of the bioengineering for an improved biofuel feedstock. Additionally, the integration of heterologous expression and systems biological analyses in this study demonstrates a strategy complementing the approach of referring the established transcriptional network in *Arabidopsis* to understand the regulation of the phenylpropanoid pathway in rice.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest associated with the work described in this manuscript.

## AUTHOR CONTRIBUTIONS

W.M., J.-G.C., G.A.T., and M.X. designed experiments. M.X., W.M., and J.-G.C. wrote the manuscript. G.A.T., T.J.T., and F.C. edited the manuscript. M.X. and S.S.J performed molecular experiments. J.Z. and M.X. performed co-expression analysis. M.J.M., P.R.L., and W.P. generated transgenic plants. N.E. and T.J.T. performed metabolomic analysis. C.D. and M.F.D analyzed lignin levels. J.Z., V.R.S., E.L., and J.S. analyzed the data.

## REFERENCES

Chow, C. N., Zheng, H. Q., Wu, N. Y., Chien, C. H., Huang, H. D., Lee, T. Y., ... Chang, W. C. (2016). PlantPAN 2.0: An update of plant promoter





- analysis navigator for reconstructing transcriptional regulatory networks in plants. *Nucleic Acids Research*, 44, D1154–D1160.
- Davies, D. (1979). The central role of phosphoenolpyruvate in plant metabolism. *Annual Review of Plant Physiology*, 30, 131–158. <https://doi.org/10.1146/annurev.pp.30.060179.001023>
- Docimo, T., Coraggio, I., De Tommasi, N., & Leone, A. (2008). Enhancing phenylpropanoid secondary metabolites in *Nicotiana tabacum* and *Salvia sclarea* by overexpression of a rice Myb4 transcription factor. *Planta Medica*, 74, PG87. <https://doi.org/10.1055/s-0028-1084839>
- Fornalé, S., Lopez, E., Salazar-Henao, J. E., Fernández-Nohales, P., Rigau, J., & Caparros-Ruiz, D. (2014). ATMYB7, a new player in the regulation of UV-sunscreens in *Arabidopsis thaliana*. *Plant and Cell Physiology*, 55, 507–516. <https://doi.org/10.1093/pcp/pct187>
- Fraser, C. M., & Chapple, C. (2011). The phenylpropanoid pathway in *Arabidopsis*. *The Arabidopsis Book*, 9, e0152. <https://doi.org/10.1199/tab.0152>
- Hirano, K., Aya, K., Morinaka, Y., Nagamatsu, S., Sato, Y., Antonio, B. A., ... Matsuoka, M. (2013). Survey of genes involved in rice secondary cell wall formation through a co-expression network. *Plant and Cell Physiology*, 54, 1803–1821. <https://doi.org/10.1093/pcp/pct121>
- Hirano, K., Kondo, M., Aya, K., Miyao, A., Sato, Y., Antonio, B. A., ... Matsuoka, M. (2013). Identification of transcription factors involved in rice secondary cell wall formation. *Plant and Cell Physiology*, 54, 1791–1802. <https://doi.org/10.1093/pcp/pct122>
- Hussey, S. G., Mizrahi, E., Spokevicius, A. V., Bossinger, G., Berger, D. K., & Myburg, A. A. (2011). SND2, a NAC transcription factor gene, regulates genes involved in secondary cell wall development in *Arabidopsis* fibres and increases fibre cell area in *Eucalyptus*. *BMC Plant Biology*, 11, 173. <https://doi.org/10.1186/1471-2229-11-173>
- Jacobs, T. B., Lawler, N. J., LaFayette, P. R., Vodkin, L. O., & Parrott, W. A. (2016). Simple gene silencing using the trans-acting si RNA pathway. *Plant Biotechnology Journal*, 14, 117–127. <https://doi.org/10.1111/pbi.12362>
- Jin, H., Cominelli, E., Bailey, P., Parr, A., Mehrtens, F., Jones, J., ... Martin, C. (2000). Transcriptional repression by AtMYB4 controls production of UV-protecting sunscreens in *Arabidopsis*. *The EMBO Journal*, 19, 6150–6161. <https://doi.org/10.1093/emboj/19.22.6150>
- Kim, D., Perteza, G., Trapnell, C., Pimentel, H., Kelley, R., & Salzberg, S. L. (2013). TopHat2: Accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biology*, 14, R36. <https://doi.org/10.1186/gb-2013-14-4-r36>
- Kim, W.-C., Kim, J.-Y., Ko, J.-H., Kang, H., & Han, K.-H. (2014). Identification of direct targets of transcription factor MYB46 provides insights into the transcriptional regulation of secondary wall biosynthesis. *Plant Molecular Biology*, 85, 589–599. <https://doi.org/10.1007/s11103-014-0205-x>
- Kobayashi, K., Suzuki, T., Iwata, E., Nakamichi, N., Suzuki, T., Chen, P., ... Ito, M. (2015). Transcriptional repression by MYB3R proteins regulates plant organ growth. *The EMBO Journal*, 34, 1992–2007.
- Li, Y., Tschaplinski, T. J., Engle, N. L., Hamilton, C. Y., Rodriguez, M. Jr, Liao, J. C., ... Graham, D. E. (2012). Combined inactivation of the *Clostridium cellulolyticum* lactate and malate dehydrogenase genes substantially increases ethanol yield from cellulose and switchgrass fermentations. *Biotechnology for Biofuels*, 5, 2. <https://doi.org/10.1186/1754-6834-5-2>
- Lin, F., Williams, B. J., Thangella, P. A. V., Ladak, A., Schepmoes, A. A., Olivos, H. J., ... Bartley, L. E. (2017). Proteomics coupled with metabolite and cell wall profiling reveal metabolic processes of a developing rice stem internode. *Frontiers in Plant Science*, 8, 1134. <https://doi.org/10.3389/fpls.2017.01134>
- Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, 15, 550. <https://doi.org/10.1186/s13059-014-0550-8>
- Lu, K., Hao, N., Meng, X., Luo, Z., Tuskan, G. A., & Ragauskas, A. J. (2019). Investigating the correlation of biomass recalcitrance with pyrolysis oil using poplar as the feedstock. *Bioresource Technology*, 289, 121589. <https://doi.org/10.1016/j.biortech.2019.121589>
- Maeda, H., & Dudareva, N. (2012). The shikimate pathway and aromatic amino acid biosynthesis in plants. *Annual Review of Plant Biology*, 63, 73–105. <https://doi.org/10.1146/annurev-arplant-042811-105439>
- Mann, D. G., King, Z. R., Liu, W., Joyce, B. L., Percifield, R. J., Hawkins, J. S., ... Mazarei, M. (2011). Switchgrass (*Panicum virgatum* L.) polyubiquitin gene (PvUbi1 and PvUbi2) promoters for use in plant transformation. *BMC Biotechnology*, 11, 74.
- Mann, D. G. J., LaFayette, P. R., Abercrombie, L. L., King, Z. R., Mazarei, M., Halter, M. C., ... Neal Stewart Jr, C. (2012). Gateway-compatible vectors for high-throughput gene functional analysis in switchgrass (*Panicum virgatum* L.) and other monocot species. *Plant Biotechnology Journal*, 10, 226–236. <https://doi.org/10.1111/j.1467-7652.2011.00658.x>
- Mehrtens, F., Kranz, H., Bednarek, P., & Weisshaar, B. (2005). The *Arabidopsis* transcription factor MYB12 is a flavonol-specific regulator of phenylpropanoid biosynthesis. *Plant Physiology*, 138, 1083–1096. <https://doi.org/10.1104/pp.104.058032>
- Nakano, Y., Yamaguchi, M., Endo, H., Rejab, N. A., & Ohtani, M. (2015). NAC-MYB-based transcriptional regulation of secondary cell wall biosynthesis in land plants. *Frontiers in Plant Science*, 6, 288. <https://doi.org/10.3389/fpls.2015.00288>
- Preston, J., Wheeler, J., Heazlewood, J., Li, S. F., & Parish, R. W. (2004). AtMYB32 is required for normal pollen development in *Arabidopsis thaliana*. *The Plant Journal*, 40, 979–995. <https://doi.org/10.1111/j.1365-313X.2004.02280.x>
- Rao, X., & Dixon, R. A. (2018). Current models for transcriptional regulation of secondary cell wall biosynthesis in grasses. *Front Plant Sci*, 9, 399. <https://doi.org/10.3389/fpls.2018.00399>
- Rauf, M., Arif, M., Fisahn, J., Xue, G. P., Balazadeh, S., & Mueller-Roeber, B. (2013). NAC transcription factor speedy hyponastic growth regulates flooding-induced leaf movement in *Arabidopsis*. *The Plant Cell*, 25, 4941–4955.
- Rogers, L. A., & Campbell, M. M. (2004). The genetic control of lignin deposition during plant growth and development. *New Phytologist*, 164, 17–30. <https://doi.org/10.1111/j.1469-8137.2004.01143.x>
- Satlewal, A., Agrawal, R., Bhagia, S., Das, P., & Ragauskas, A. J. (2018). Rice straw as a feedstock for biofuels: Availability, recalcitrance, and chemical properties. *Biofuels, Bioproducts and Biorefining*, 12, 83–107. <https://doi.org/10.1002/bbb.1818>
- Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., ... Ideker, T. (2003). Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Research*, 13, 2498–2504. <https://doi.org/10.1101/gr.1239303>
- Sundell, D., Street, N. R., Kumar, M., Mellerowicz, E. J., Kucukoglu, M., Johnsson, C., ... Nilsson, O. (2017). AspWood: High-spatial-resolution transcriptome profiles reveal uncharacterized modularity of wood formation in *Populus tremula*. *The Plant Cell*, 29, 1585–1604.
- Tian, T., Liu, Y., Yan, H., You, Q., Yi, X., Du, Z., ... Su, Z. (2017). agriGO v2.0: A GO analysis toolkit for the agricultural community, 2017 update. *Nucleic Acids Research*, 45, W122–W129.
- Tiwari, S. B., Hagen, G., & Guilfoyle, T. (2003). The roles of auxin response factor domains in auxin-responsive transcription. *The Plant Cell*, 15, 533–543. <https://doi.org/10.1105/tpc.008417>
- Tschaplinski, T. J., Standaert, R. F., Engle, N. L., Martin, M. Z., Sangha, A. K., Parks, J. M., ... Mielenz, J. R. (2012). Down-regulation of the caffeic acid O-methyltransferase gene in switchgrass reveals a novel monolignol analog. *Biotechnology for Biofuels*, 5, 71. <https://doi.org/10.1186/1754-6834-5-71>
- Vannini, C., Locatelli, F., Bracale, M., Magnani, E., Marsoni, M., Osnato, M., ... Coraggio, I. (2004). Overexpression of the rice Osmyb4 gene



- increases chilling and freezing tolerance of *Arabidopsis thaliana* plants. *The Plant Journal*, 37, 115–127.
- Vogt, T. (2010). Phenylpropanoid biosynthesis. *Molecular Plant*, 3, 2–20. <https://doi.org/10.1093/mp/ssp106>
- Wang, L., Xie, W., Chen, Y., Tang, W., Yang, J., Ye, R., ... Zhang, Q. (2010). A dynamic gene expression atlas covering the entire life cycle of rice. *The Plant Journal*, 61, 752–766. <https://doi.org/10.1111/j.1365-313X.2009.04100.x>
- Wilkins, O., Nahal, H., Foong, J., Provart, N. J., & Campbell, M. M. (2009). Expansion and diversification of the *Populus* R2R3-MYB family of transcription factors. *Plant Physiology*, 149, 981–993.
- Xie, M., Muchero, W., Bryan, A. C., Yee, K. L., Guo, H.-B., Zhang, J., ... Payyavula, R. S. (2018). A 5-enolpyruvylshikimate 3-phosphate synthase functions as a transcriptional repressor in *Populus*. *The Plant Cell*, 30, 1645–1660.
- Xie, M., Ren, G., Costa-Nunes, P., Pontes, O., & Yu, B. (2012). A subgroup of SGS3-like proteins act redundantly in RNA-directed DNA methylation. *Nucleic Acids Research*, 40, 4422–4431. <https://doi.org/10.1093/nar/gks034>
- Xie, M., Zhang, J., Tschaplinski, T. J., Tuskan, G. A., Chen, J.-G., & Muchero, W. (2018). Regulation of lignin biosynthesis and its role in growth-defense tradeoffs. *Frontiers in Plant Science*, 9, 1427. <https://doi.org/10.3389/fpls.2018.01427>
- Yekutieli, D., & Benjamini, Y. (1999). Resampling-based false discovery rate controlling multiple test procedures for correlated test statistics. *Journal of Statistical Planning and Inference*, 82, 171–196. [https://doi.org/10.1016/S0378-3758\(99\)00041-5](https://doi.org/10.1016/S0378-3758(99)00041-5)
- Yoo, S. D., Cho, Y. H., & Sheen, J. (2007). *Arabidopsis* mesophyll protoplasts: A versatile cell system for transient gene expression analysis. *Nature Protocols*, 2, 1565–1572. <https://doi.org/10.1038/nprot.2007.199>
- Zhao, C., Avci, U., Grant, E. H., Haigler, C. H., & Beers, E. P. (2008). XND1, a member of the NAC domain family in *Arabidopsis thaliana*, negatively regulates lignocellulose synthesis and programmed cell death in xylem. *The Plant Journal*, 53, 425–436. <https://doi.org/10.1111/j.1365-313X.2007.03350.x>
- Zhong, R., Lee, C., McCarthy, R. L., Reeves, C. K., Jones, E. G., & Ye, Z. H. (2011). Transcriptional activation of secondary wall biosynthesis by rice and maize NAC and MYB transcription factors. *Plant and Cell Physiology*, 52, 1856–1871. <https://doi.org/10.1093/pcp/pcr123>
- Zhong, R., Lee, C., Zhou, J., McCarthy, R. L., & Ye, Z. H. (2008). A battery of transcription factors involved in the regulation of secondary cell wall biosynthesis in *Arabidopsis*. *The Plant Cell*, 20, 2763–2782.
- Zhong, R., McCarthy, R. L., Haghghat, M., & Ye, Z. H. (2013). The poplar MYB master switches bind to the SMRE site and activate the secondary wall biosynthetic program during wood formation. *PLoS ONE*, 8, e69219. <https://doi.org/10.1371/journal.pone.0069219>
- Zhong, R., & Ye, Z. H. (2012). MYB46 and MYB83 bind to the SMRE sites and directly activate a suite of transcription factors and secondary wall biosynthetic genes. *Plant and Cell Physiology*, 53, 368–380. <https://doi.org/10.1093/pcp/pcr185>
- Zhou, J., Lee, C., Zhong, R., & Ye, Z. H. (2009). MYB58 and MYB63 are transcriptional activators of the lignin biosynthetic pathway during secondary cell wall formation in *Arabidopsis*. *The Plant Cell*, 21, 248–266.

## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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