forests

MDPI

Article

Cloning and Functional Analysis of *PmMYB45*, a Transcription Factor in *Pinus massoniana*

Manqing Peng, Yiyun Qin, Yuan He, Xin He, Jinfeng Zhang and Kongshu Ji *

State Key Laboratory of Tree Genetics and Breeding, Key Laboratory of Forestry Genetics & Biotechnology of Ministry of Education, Co-Innovation Center for Sustainable Forestry in Southern China, Nanjing Forestry University, Nanjing 210037, China; pmq@njfu.edu.cn (M.P.); qinyiyun@njfu.edu.cn (Y.Q.); heyuan@njfu.edu.cn (Y.H.); hexin1234567@njfu.edu.cn (X.H.); zhangjinfeng@njfu.edu.cn (J.Z.)
* Correspondence: ksji@njfu.edu.cn

Abstract: MYB transcription factors (TFs) have been shown to be important regulators of plant growth and development, and the R2R3−MYB family plays an important role in the regulation of plant primary and secondary metabolism, growth, and development. In this study, the *PmMYB45* gene sequence was successfully cloned from *Pinus massoniana*, with an open reading frame of 1476 bp, encoding 491 amino acids. The results of subcellular localization and transcriptional self-activation showed that the gene was localized in the nucleus and was self-activating. qPCR showed that the highest expression of *PmMYB45* was found in stems. *PmMYB45* can promote lignin synthesis when overexpressed in tobacco. These results suggest that *PmMYB45* promotes lignin synthesis by regulating the lignin biosynthesis pathway. These findings improve our understanding of the mechanism of lignin biosynthesis in *P. massoniana* and provide a basis for molecular breeding functions. It also provides insights into the role of the MYB transcription factor family in lignin accumulation.

Keywords: *Pinus massoniana*; lignin; MYB transcription factor; biological function

check for updates

Citation: Peng, M.; Qin, Y.; He, Y.; He, X.; Zhang, J.; Ji, K. Cloning and Functional Analysis of *PmMYB45*, a Transcription Factor in *Pinus massoniana*. *Forests* **2024**, *15*, 814. https://doi.org/10.3390/f15050814

Academic Editor: Gaetano Distefano

Received: 27 March 2024
Revised: 26 April 2024
Accepted: 30 April 2024
Published: 6 May 2024

1. Introduction

Secondary metabolites (SMs) produced by plants are significant in various aspects, such as plant growth, metabolic control, signaling pathways, and protection against both biotic and abiotic stressors [1–3]. Lignin, as a widespread class of secondary metabolites in plants, is mainly involved in plant material transportation, defense against pests and pathogens, plant resistance to stunting, and response to various environmental stresses [4–7]. MYB transcription factors are one of the largest families of plant transcription factors, and R2R3-MYB transcription factors (TFs) are the most numerous among them, and they have a significant impact on the growth, development, and regulation of metabolism in plants [8]. MYB transcription factors play a significant role in the modulation of lignin secondary metabolism on a broad scale, the most representative being *AtMYB46* as well as *AtMYB83*, which are the target genes of *SND1*, *VND6*, and *VND7* [9]. *AtMYB20*, *AtMYB43*, and *AtMYB42* activate lignin synthesis-related genes, and the silencing of these genes leads to a significant reduction in lignin synthesis and defective plant growth in *Arabidopsis* [10]. The loss of *AtMYB75* function resulted in increased thickness of wood fibers in *Arabidopsis* inflorescence stems, some genes related to lignin biosynthesis were upregulated, and the S/G ratio of lignin monomer and total lignin content were also affected [11]. Involvement of MYB transcription factors in lignin biosynthesis has also been demonstrated in other species, such as the excessive expression of *CmMYB8* results in the suppression of multiple genes responsible for lignin synthesis and subsequently reduces the lignin content in chrysanthemum plants [12]. *VcMYB4a* resulted in a reduction in lignin biosynthesis via the downregulation of blueberry calli *4CL*, *COMT*, and *CAD* gene expression [13].

Pinus. massoniana, which has been considered the main wood resource for paper and artificial fiberboard, occupies a key position in the development of the forest tree [14].

To date, several genes related to secondary wall synthesis have been cloned and characterized from *P. massoniana*. *PmMYB7* is highly expressed in the xylem and promotes lignin synthesis via interaction with *PmCCoAOMT2* [15]. *PmMYB4* binds to the AC-box motif to directly activate *PAL* and *CCoAOMT* promoters involved in the secondary cell wall (SCW) biosynthesis genes, and overexpression increases tobacco lignin deposition and increases SCW thickness [14]. Transgenic *PmMYB6* upregulates several genes in the lignin biosynthesis pathway, resulting in increased lignin content in transgenic plants [16]. While specific transcription factors have been identified as playing a role in the control of secondary cell wall (SCW) biosynthesis, a complete understanding of this process remains elusive. Investigating additional transcription factors involved in the regulation of SCW biosynthesis in *P. massoniana* is of significant scientific importance for improving wood productivity and quality. It also plays a crucial role in developing new cultivars customized to meet human needs.

In this study, we identified the MYB TF *PmMYB45* and mainly used bioinformatics-related methods to comprehensively analyze the coding protein properties, study the coding sequence features, and analyze the expression pattern of *P. massoniana* tissues, as well as the localization of the protein expression and the detection of transcriptional self-activation activity. We further overexpressed *PmMYB45* in tobacco and analyzed its biological functions by means of cell biology and molecular biology.

2. Materials and Methods

2.1. Plant Material

In this study, we used *P. massoniana* seedlings, which were grown from seeds in the Seed Orchard of Baisha State Forest Farm in Shanghang County, Fujian Province, and cultivated in pots in our laboratory; the seeds of Arabidopsis and tobacco (*Nicotiana* x *sanderae was used for the transgene*, *Nicotiana benthamiana was used for subcellular localization)* used in this experiment were stored at 4 °C in our laboratory for a long time and were grown in a greenhouse at 24 °C under a 16/8 h light/dark cycle and supplemental light (4500lx). The genetic transformation system of *P. massoniana* was not successfully established. We selected Arabidopsis and tobacco for the functional validation of Masson pine genes, which serve as plant biological indicators.

2.2. Cloning of Genes and Promoters

RNA was isolated from the tissues of a 2-year-old *P. massoniana* seedling using the FastPure Universal Plant TotalRNA Isolation Kit (Vazmy Biotechnology, Nanjing, China). cDNA was extracted by reverse transcription of the first strand cDNA from the total RNA using a One-step gDNA Removal and cDNA Synthesis SuperMix kit (Yeasen Biotechnology, Shanghai, China). Specific primers were designed using Primer Premier 5.0 software (Table S1); the ORF sequence of *PmMYB45* was cloned from *P. massoniana* by PCR using a 50 μL PCR reaction system. The polymerase chain reaction (PCR) was conducted with the following specified conditions: pre−denaturation at 98 °C for 3 min, followed by 35 cycles consisting of denaturation at 94 °C for 1 min, annealing at 58 °C for 30 s, and extension at 72 °C for 40 s, holding at 72 °C for 5 min. The ORF fragment was attached to the Blunt Vector using the pClone007 Blunt Simple Vector Kit (Tsingke Biotechnology, Nanjing, China). Using the *Pinus taeda* genome database, we established a local database of *PmMYB45* and compared it with it, the nucleic acid sequence of about 2000 bp upstream of the gene fragment with the highest similarity was selected as the reference sequence, specific primers were designed, the gDNA of *P. massoniana* leaves was used as the PCR template, the PCR reaction procedure was consistent with ORF cloning after it was sent to Tsingke Biotech (Nanjing, China) for sequencing, and finally, it was pieced together using the online software SeqMan1.0.

2.3. Bioinformatics Analysis of the Coding Region of the PmMYB45 Gene

The online software was utilized to predict the hydrophobicity, signal peptide, transmembrane region, secondary structure, and tertiary structure of the PmMYB45 protein such as Expasy−ProtScale3.0 (https://web.expasy.org/protscale, accessed on 28 February 2024), SignalP4.1 server (https://services.healthtech.dtu.dk/service.php?SignalP, accessed on 28 February 2024), TMHMM2.0 Server (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0, accessed on 28 February 2024), SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=%20npsa_sopma.html, accessed on 28 February 2024), SWISS−MODEL (https://swissmodel.expasy.org/, accessed on 28 February 2024). The amino acid sequence of *PmMYB45* and other homologous genes were sequenced using the online software Clustalw2.1 (https://cdnai.gameax.cn/#/home, accessed on 1 March 2024). The MYB protein sequences of other species were downloaded from the NCBI database, and the MEGA5.1 software was used to perform multiple sequence comparisons of protein sequences and construct phylogenetic evolutionary trees. The cis-acting elements of the promoter were analyzed and predicted using the online analysis website PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 28 February 2024).

2.4. RT-qPCR

RNA was extracted from various tissues and subsequently converted into complementary cDNA. Different tissues, including Roots (R), Young stems (YS), Old stems (OS), Xylem (X), young leaves (YN), old leaves (ON), and Phloem (P). Horsetail pine *PmTUA* (KM496535.1) was used as an internal reference gene in *P. massoniana* [17]. The specific primers for each gene used in the RT−qPCR test can be found in Table S1. The setup was referred to the Hieff UNICON® Universal Blue qPCR SYBR Green Master Mix instructions, and RT−qPCR was performed using the TP700 real-time PCR machine (Takara, Dalian, China); at the end of the reaction, relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method [18].

2.5. Subcellular Localization of PmMYB45 Protein

The ORF fragment of *PmMYB45* was inserted into the PJIT166−eGFP vector containing the 35S promoter. The PJIT166−eGFP vector carrying GFP alone was used as a control. 35S::PmMYB45−eGFP and control vector were transformed into *Agrobacterium tumefaciens* (GV3103), and tobacco leaves (grown for 3–4 weeks) were infested with Agrobacterium-mediated transient transformation. After dark incubation for 12 h, they were grown in an incubator with a photoperiod of 16/8 for 24−36 h. The expression of fusion vectors was detected at the end of the photoperiod by applying the nucleus-specific dye DAPI staining, followed by examination using laser confocal microscopy (UV excitation wavelength of 488 nm).

2.6. Transcriptional Activation Activity Assay

pGBKT7−PmMYB45 fusion vector was constructed by homologous recombination. The pGBKT7−PmMYB45 plasmid and pGADT7 plasmid were co-transformed into AH109 yeast-competent cells as an experimental group. pGBKT7−p53 and pGADT7−largeT co-transfer AH109 were positive control, and pGBKT7−laminC with pGADT7−largeT were negative control and coated to the corresponding defective type screening plates. After PCR examination, positive bacteria were inoculated in 5 mL of SD/-Trp liquid medium, shaken overnight, and spot-coated onto SD/-Trp/-Leu, SD/-Trp/-Leu/-His/-Ade and SD/-Trp/-Leu/-His/-Ade/X-α-Gal plates.

2.7. GUS Staining

This paper describes a slightly improved method of GUS staining with reference to that reported by Chu [19]. First, *A. thaliana* was immersed in a 1/2MS hypertonic solution containing a 25% sucrose mass fraction for 15 min at 25 °C. Subsequently, *A. thaliana* was infected with Agrobacterium-mediated transient transformation and incubated at

120 r/min for 2.5 h at 25 °C. This was followed by placing it in co-culture medium (1/2MS + 120 μmol/L AS + 1.5 mg/L KT + 0.5 mg/L NAA + 3% sucrose, pH = 5.4) for 72 h in the dark at (23 ± 2) °C. Finally, *A. thaliana* was immersed in GUS staining solution (Leagene Biotechnology, Beijing, China), protected from light overnight at 37 °C, and then destained with a solution of V (ethanol):V (acetic acid) = 3:1. After completion of decolorization, a microscope was used to take pictures.

2.8. Genetically Modified Tobacco

According to the ORF sequence of *PmMYB45* and the PBI121 vector sequence, a pair of specific primers was designed using Primer Premier 5 software for PCR amplification, and then the PCR products were connected to the plant binary vector PBI121. We transformed the resulting vector 35S:PmMYB45 into *A. tumefaciens* using the freezing-thawing transformation method. As described previously, tobacco transformation was mediated by *A. tumefaciens* [20]. First, leaf discs were infected with A. tumefaciens cultures containing 35S::PmMYB45 for 8−10 min, placed in medium (MS + 0.2 mg/L NAA + 2 mg/L 6-BA), and incubated in dark conditions for 3 d. Second, leaves were transferred to medium (MS + 0.2 mg/L NAA + 2 mg/L 6−BA + 250 mg/L Cef and 50 mg/L Kan) for screening. Until after small green shoots had grown. They were grown for another 15 days, and then the green shoots were cut and transferred to containing 100 mg/L Cef and 25 mg/L Kan for rooting screening of the resistant plants. Positive transgenic tobacco was identified for further study using PCR and qRT−PCR (S1).

2.9. Chemical Staining of Xylem

Phloroglucinol is a commonly used chemical reagent that can be utilized to detect lignin. When phloroglucinol reacts with lignin, it will appear red or purplish-red, enabling the determination of lignin content based on the depth of the stain. Sections were prepared from stem segments of the stem base and sixth leaf petioles of transgenic and wild-type tobacco. The samples were stained with phloroglucinol and examined under a microscope.

2.10. Determination of Lignin Monomer Content

Tobacco stem segments were taken and put into a mortar grinding. About 0.1 g of the sample was weighed, 2 mL of pre-cooled ultra-pure water was added, and the sample was soaked in a constant temperature water bath at 90 °C for 30 min. Then, the filtrate was taken, the pH was adjusted to 2.0, and it was extract with ethyl acetate three times. A nitrogen-blowing instrument was taken and used for blow drying; the sample was dissolved with a mobile phase and was filtered with a pinhead filter to be measured.

2.11. Scanning Electron Microscope

Stems of 2-month-old wild-type and transgenic tobacco were promptly immersed in FAA tissue fixative for 24 h. Subsequently, the stems were dried and sectioned. Transverse sections of stems were photographed for observation using a scanning electron microscope (FEI Quanta 200, Hillsboro, WA, USA).

2.12. Statistical Analysis

The experimental data were collected from three separate biological replicates, and all graphs were created using Microsoft Excel. Statistical analysis was then conducted using GraphPad Prism 8 software. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3. Results

3.1. Cloning and Structural Analysis of PmMYB45

According to the 57 MYB proteins of *P. massoniana* identified in the previous phase by our research group, *PmMYB45*, located in the S37 subgroup, may participate in lignin biosynthesis [21]. Using PCR technology, the *PmMYB45* gene was cloned from *P. massoniana*. The gene's open reading frame (ORF) was 1317ps long and encoded 438 amino acids

(Supplementary Table S1 and Figure S1). The SMART online program predicted that the protein contains two common SANT structural domains and belongs to the MYB family (Figure 1A). Additionally, the NCBI website's prediction of conserved structural domains revealed that PmMYB45 included the MYB DNA structural domain, along with the PLAN03091, REB1, and SANT structural domains (Figure 1B).

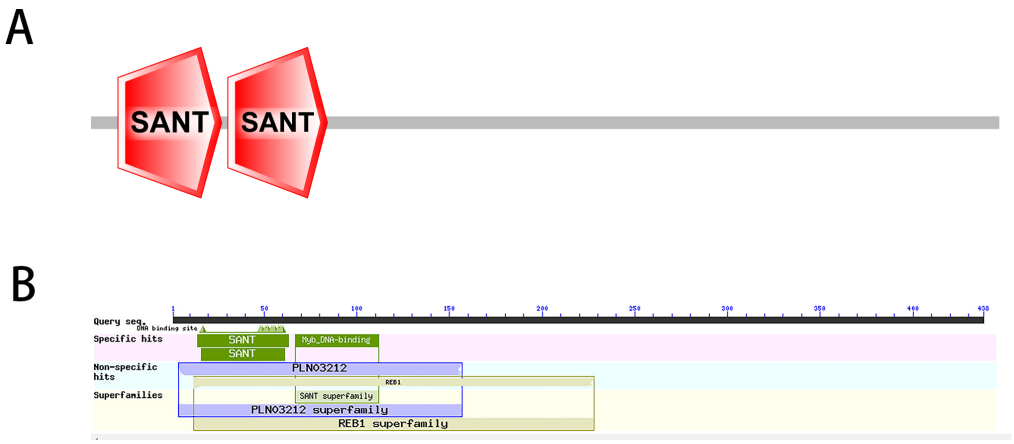

Figure 1. Predicted structure of *PmMYB45*. (**A**) Predicted structure of *PmMYB45* protein. (**B**) Conserved structural domains of *PmMYB45*.

The 1321 bp promoter sequence upstream of *PmMYB45* was cloned and analyzed using PlantCARE. In addition to core promoter elements and light-responsive elements such as the TATA box and CAAT box, the *PmMYB45* promoter also contains elements for phytohormone-related responses, such as abscisic cis-acting element and salicylic cis-acting element (Figure 2). According to the findings presented, it is postulated that the expression of the PmMYB45 promoter could potentially be influenced by both photoperiodic and phytohormone signaling pathways.

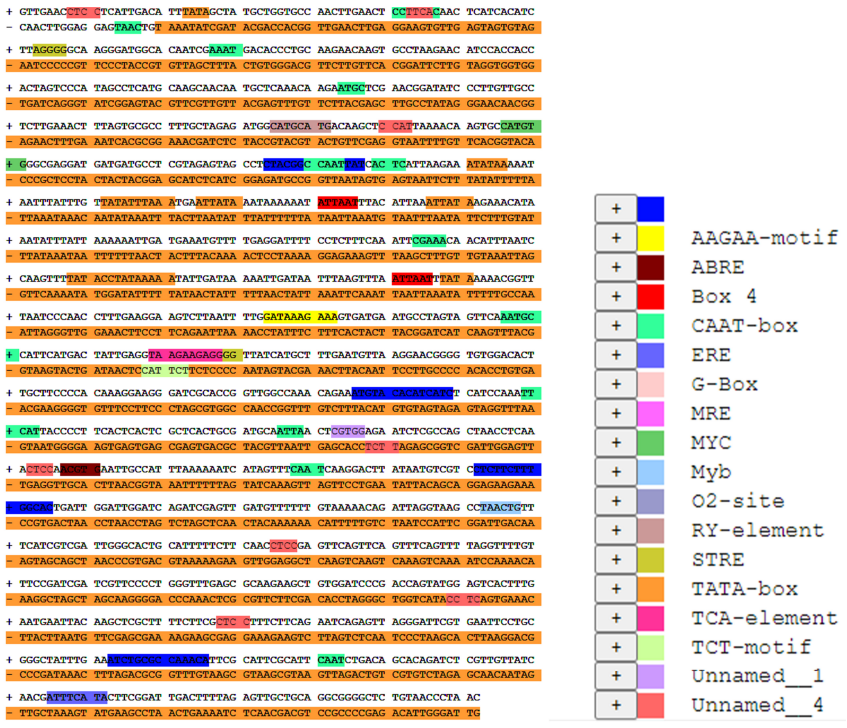

Figure 2. Analysis of cis-acting elements of the *PmMYB45* promoter.

3.2. Bioinformatics Analysis of the PmMYB45

The protein's primary structure was predicted using the online software Expasy-ProtScale, as shown, and it can be observed that the score of *PmMYB45* is mainly in the negative region, indicating that it is a protein with hydrophilicity (Figure 3A). The secondary structure of the protein was analyzed using SOPMA online prediction. The results showed that the secondary structure of the *PmMYB45* protein consisted of random coil, extended strand, and Beta turn. Among them, the random coil accounted for 60.05%; the Alpha helix accounted for 24.66% of the sequence; the extended strand accounted for 9.59% of the sequence; and the Beta turn was 5.71% (Figure 3B). The tertiary structure of the protein was predicted online using the homology modeling method via the SWISS-MODEL website. The main structures constituting it were identified as random coil and random coil, which is consistent with the secondary structure prediction (Figure 3C). The signal peptide and transmembrane structures of *PmMYB45* were analyzed using SignalP1.6 and TMHMM Sever v.2.0 online programs. *PmMYB45* did not contain signaling peptides and transmembrane structures, indicating that it is a nonsecretory protein (Figure 3D,E).

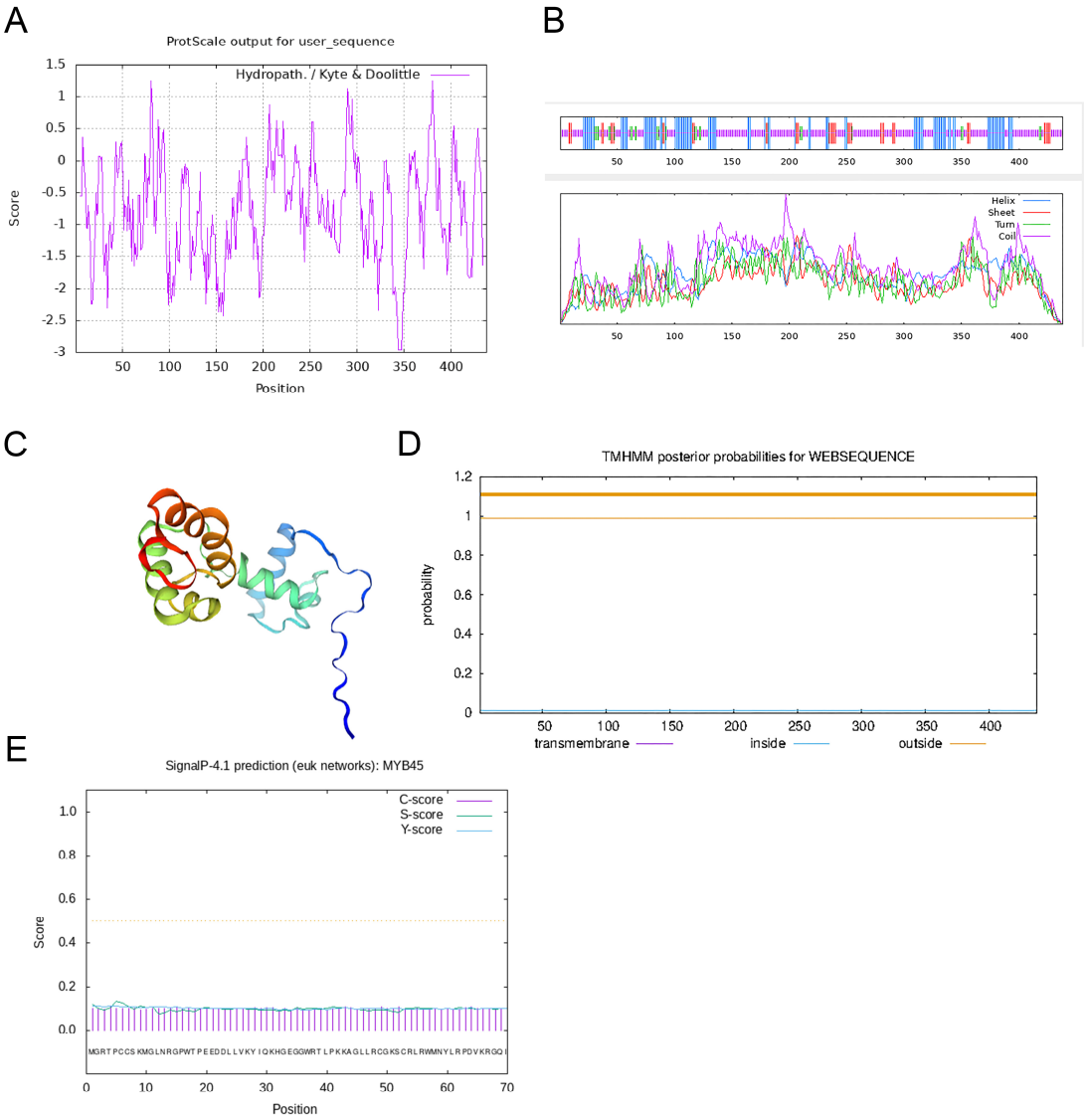

Figure 3. Bioinformatics analysis of *PmMYB45*. (**A**) Hydrophilicity of *PmMYB45* protein. (**B**) Predicted secondary structure of *PmMYB45*. (**C**) Predicted tertiary structure of *PmMYB45*. (**D**) Predicted transmembrane structure of *PmMYB45*. (**E**) Prediction of the signal peptide of *PmMYB45*.

In this paper, a phylogenetic tree was constructed using MEGA6.0 software. The *PmMYB45* protein sequences were used to construct the phylogenetic tree along with MYB protein sequences from other species. The analysis revealed that *PmMYB45* shares a high degree of homology with other plant MYBs known to be involved in secondary wall formation (Figure 4A, Supplementary Table S2). The protein sequence closely resembling *PmMYB45* was identified by querying the National Center for Biotechnology Information (NCBI) database using the BLASTN. The results showed that all five proteins have R2 and R3 structural domains, indicating that they were all R2R3−MYB TFs (Figure 4B).

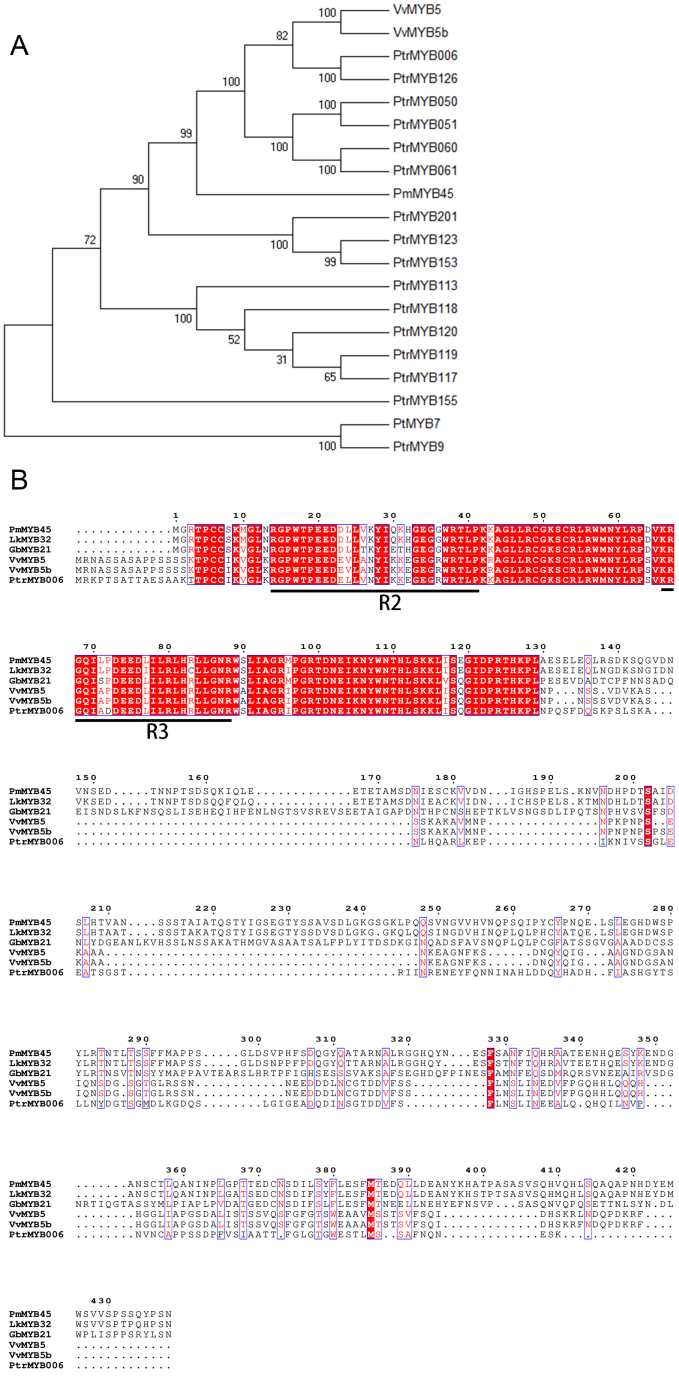

Figure 4. Sequence analysis of *PmMYB45*. (**A**) Phylogenetic relationships of *PmMYB45* with other secondary wall or lignin-associated MYB transcription factors. (**B**) Predicted sequence similarity of the *PmMYB45* with other MYBs.

3.3. Analysis of the Tissue-Specific Expression Pattern of PmMYB45

The qRT−PCR technique was used to evaluate the expression levels of *PmMYB45* in different tissues of *P. massoniana*, with the *PmTUA* gene used as a reference. High levels of *PmMYB45* expression were detected in old and young stems, and the lowest levels were detected in roots. The expression of *PmMYB45* in *P. massoniana* was tissue-specific (Figure 5A). To further investigate the spatial and temporal expression patterns, the 35S::PmMYB45−GUS vector containing the 1.3 kb promoter region upstream of *PmMYB45* was introduced into *Arabidopsis*. Histochemical analysis of GUS expression after the transformation of the plants showed that *PmMYB45* promoter-driven staining was expressed predominantly in stems as well as the veins of mature leaves. This restricted expression pattern suggests that the *PmMYB45* gene demonstrates expression in tissues that are undergoing the process of secondary cell wall thickening (Figure 5B).

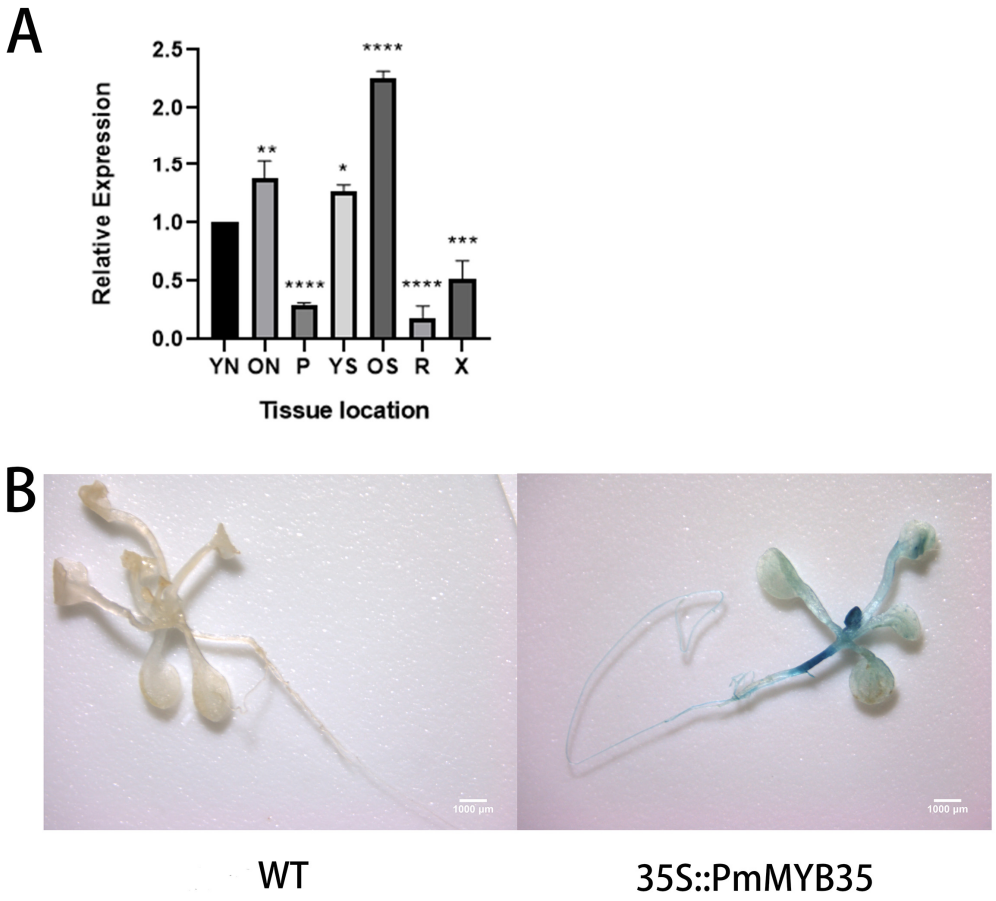

Figure 5. PmMYB45 tissue expression pattern. (**A**) Expression pattern of PmMYB45 in *P. massoniana* YN, young leaves; ON, old leave; P, Phloem; YS, Young stems; OS, Old stems; R, Roots; X, Xylem. (**B**) Expression analysis of PmMYB45 gene promoter in A. thaliana. Different numbers of "*" indicate significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Data are shown as mean ± SE, with three biological replicates.

3.4. Subcellular Localization and Transcriptional Activation Analysis

To verify whether the protein encoded by the *PmMYB45* gene can localize in the nucleus of plant cells and thus bind to the promoters of downstream genes to achieve its transcriptional regulatory role, a 35S::PmMYB45−eGFP fusion gene was generated and transiently transformed in tobacco by *A. rhizogenes*. The findings indicated that the expression of PmMYB45 was localized specifically within the nucleus, while the green fluorescent protein (GFP) activity produced by the control 35S::eGFP transgene was observed to be dispersed throughout the entire cell. (Figure 6A). To determine the transcriptional activity of

PmMYB45, its ORF was fused to PGBKT7 and transferred into the yeast strain AH109. Yeast transformants carrying the PGBKT7::PmMYB45 fusion gene were able to grow on selective media and induce the expression of the α-galactosidase reporter gene (Figure 6B). Suggesting that *PmMYB45* has transcriptional activity, consistent with *PmMYB45* functioning as a transcription factor.

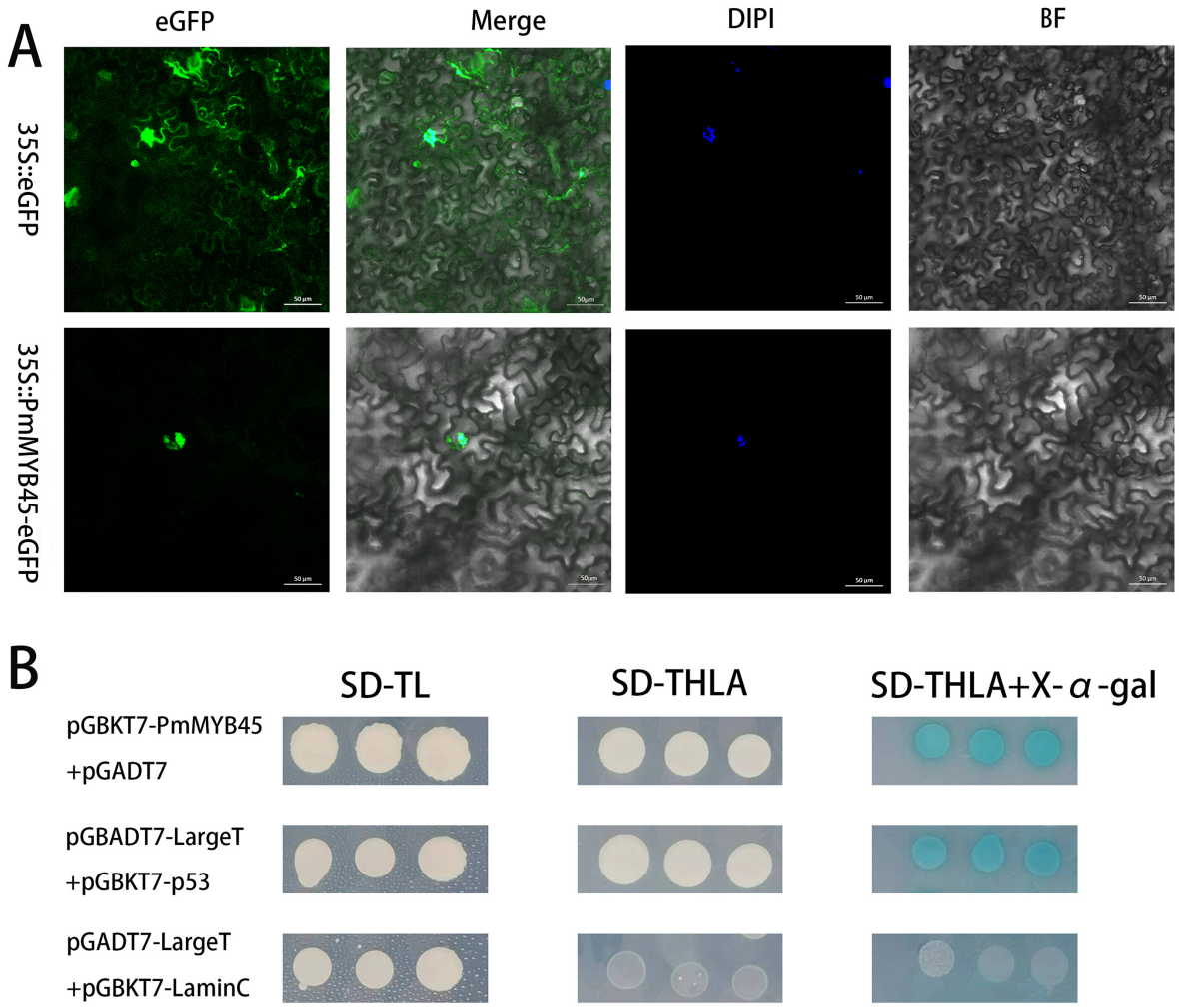

Figure 6. Nuclear localization and transcriptional self-activation of *PmMYB45*. (**A**) 35S::GFP and 35S::PmMYB45−GFP localization in tobacco epidermis. (**B**) Analysis of transcriptional activation of *PmMYB45*.

3.5. Overexpression of PmMYB45 in Tobacco Promotes Lignin Accumulation

To preliminarily investigate the function of *PmMYB45*, it was first overexpressed in tobacco. Several transgenic plants were identified and screened by DNA and RNA levels (Supplemental Figure S1). The expression level of *PmMYB45* was detected by qRT−PCR. Two lines (five replicates per line) with good growth status were randomly selected for further analysis. The results showed that it was successfully expressed in the transgenic lines, with expression levels 179 and 27-fold higher than that of the wild type (Supplemental Table S1, Figure 7B). There were some morphological differences between the transgenic plants and the wild-type control. Phenotypic observations revealed that the transgenic plants exhibited a slightly declined growth, and all of them were smaller in plant height compared to the wild type but had increased stem thickness (Figure 7C,D). These findings suggest that the overexpression of *PmMYB45* may play a role in the secondary growth of tobacco.

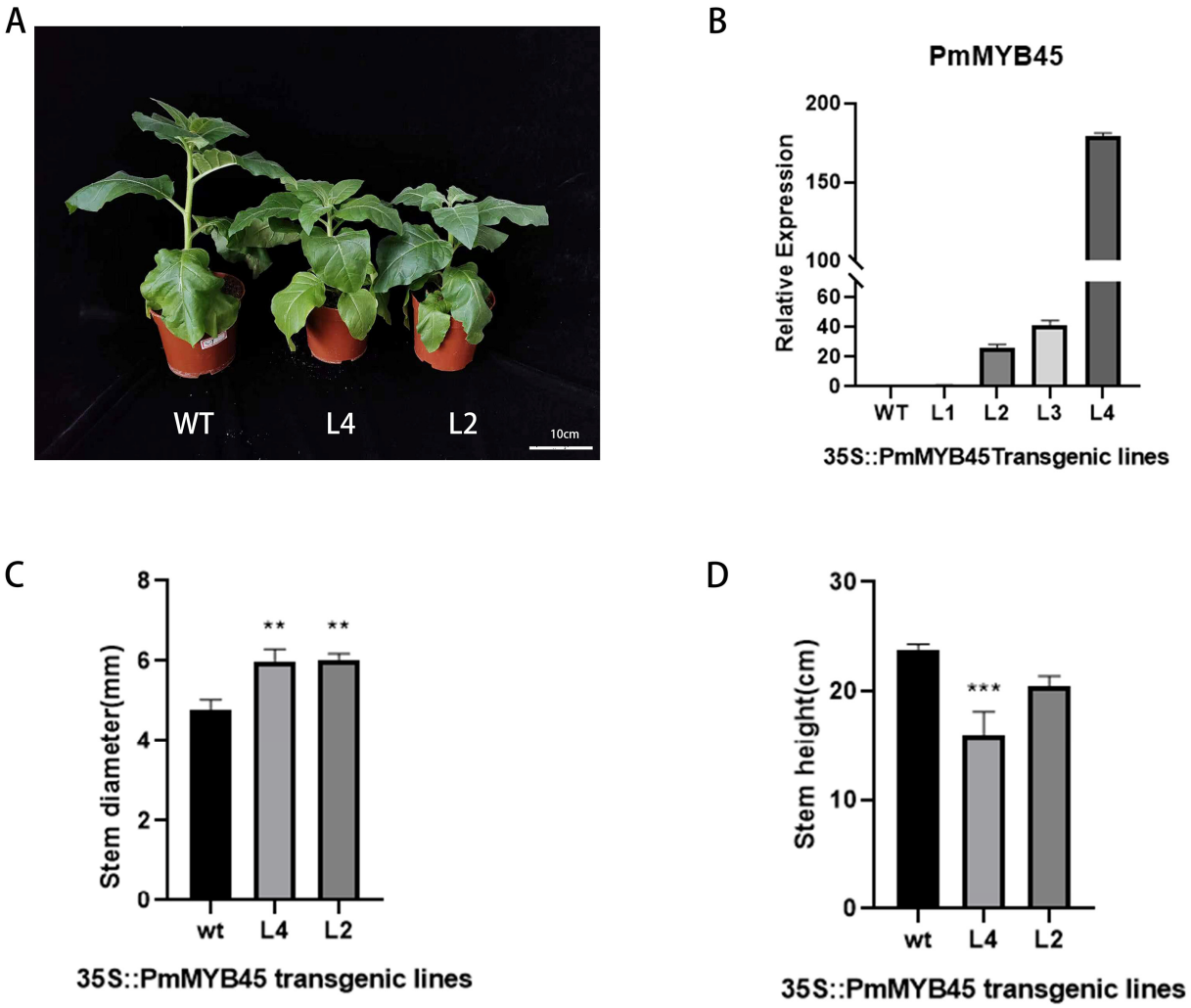

Figure 7. Phenotypes of *PmMYB45* transgene in tobacco. (**A**) Morphological comparison of 2−month−old WT and *PmMYB45*−OE lines. (**B**) Expression level of *PmMYB45* in WT and *PmMYB45*−OE lines. (**C**) Comparison of stem diameter between 2-month-old WT and *PmMYB45*−OE. (**D**) Comparison of stem height between 2−month−old WT and *PmMYB45*-OE. Different numbers of "*" indicate significant differences (** $p < 0.01$, *** $p < 0.001$). Data are shown as mean ± SE, with three biological replicates.

To evaluate the influence of transgenic plants on lignin biosynthesis, the stems and petioles of PmMYB45 transgenic plants were sectioned and subjected to phloroglucinol staining to analyze the distribution of lignin. The results showed that the number of lignin cell layers in the petiole of overexpression plants was significantly increased (Figure 8A), and the thickness of lignin in the stems of *PmMYB45* transgenic plants was increased in transgenic plants compared with that in the wild type (Figure 8B), which indicated that *PmMYB45* could promote the synthesis of lignin in the transgenic plants. The xylem cell wall thickness of the transverse section was observed by scanning electron microscopy (SEM). It was found that the cell wall thickness of transgenic tobacco increased significantly compared with that of WT (Figure 8C,D).

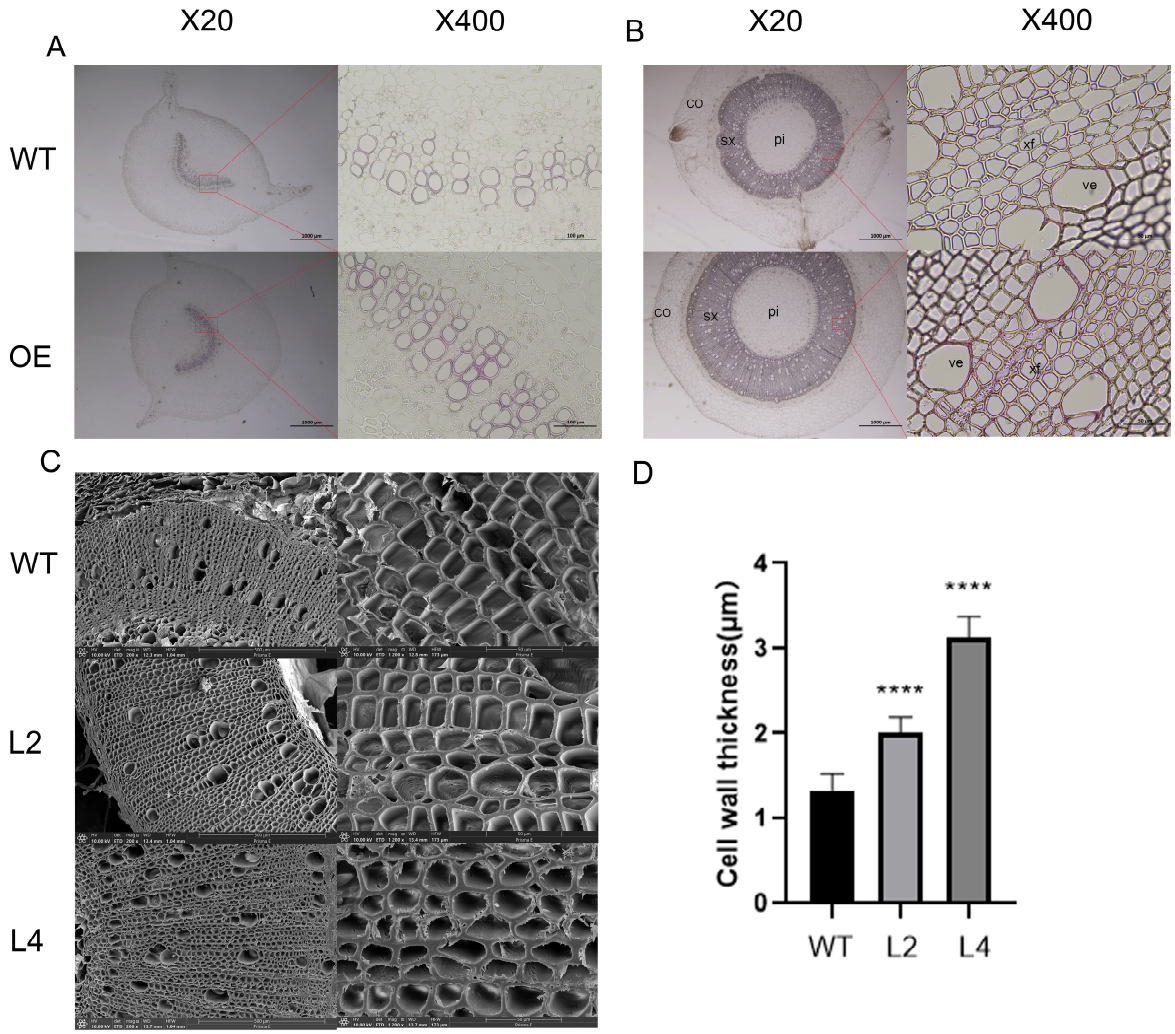

Figure 8. Lignin staining of *PmMYB45* in transgenic tobacco. (**A**) Phloroglucinol staining of wild WT and *PmMYB45*−OE in petioles. (**B**) Phloroglucinol staining of WT and *PmMYB45*−OE in stems. Xf, xylem fiber; ve, vessel; co, cortex; sx, secondary xylem; pi, pith; Xy, xylem. (**C**) Scanning electron microscopy of WT and *PmMYB45*-OE in stems. (**D**) Cell wall thickness measurement. Different numbers of "*" indicate significant differences (**** $p < 0.0001$). Data are shown as mean ± SE, with three biological replicates.

3.6. The Lignin Monomers in Stems of Wild Type and Transgenic Tobacco

Since *PmMYB45* transgenic plants exhibit higher lignin content than wild−type plants, we conducted an assay to determine if the change in lignin content leads to a change in lignin monomer content. We analyzed the lignin monomer content in the stems of both WT and transgenic plants. Our results are shown as follows: G lignin content increased, S lignin also increased, and the G/S ratio changed significantly (Table 1).

Table 1. Contents of lignin monomers in tobacco stems.

	WT	L2	L4
G Lignin (ng/g)	63.28 ± 0.13	113.72 ± 4.18 ****	283.12 ± 7.28 ****
H Lignin (ng/g)	11.42 ± 0.12	20.99 ± 1 *	118.35 ± 4.39 ****
S Lignin (ng/g)	66.92 ± 1.39	64.77 ± 0.73	91.48 ± 1.11 ****
S/G	1.06 ± 0.02	0.58 ± 0.01 ****	0.32 ± 0.01 ****

The values shown in the table are mean ± SE (n = 3). Different numbers of "*" indicate significant differences (* $p < 0.05$, **** $p < 0.0001$). Data are shown as mean ± SE, with three biological replicates.

3.7. Effect of PmMYB45 Overexpression on Key Enzyme Genes for Secondary Cell Wall Formation in Tobacco

To further demonstrate that *PmMYB45* regulates lignin biosynthesis, we utilized RT-qPCR to analyze the relative expression of enzyme genes that are specifically involved in lignin monomer biosynthesis. The results showed that *4CL5*, *PAL1*, *CAD*, and *HCT* were significantly upregulated in all transgenic tobacco compared with wild-type plants (Figure 9). These data suggest that *PmMYB45* is indeed involved in regulating lignin biosynthesis.

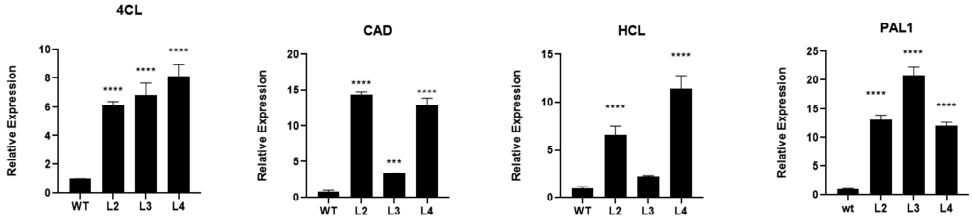

Figure 9. RT−PCR analysis of transcript accumulation of genes related to lignin synthesis in transgenic tobacco. Different numbers of "*" indicate significant differences (*** $p < 0.001$, **** $p < 0.0001$). Data are shown as mean ± SE, with three biological replicates.

4. Discussion

Cell walls are the main component of wood biomass, and studying the process of cell wall formation can help humans obtain new species that fulfill their needs. Numerous MYB TFs involved in the regulation of secondary wall biosynthesis have been extensively and intensively studied in the herb *A. thaliana* and the woody plant *Populus tremula* [22]. In contrast, there is limited research on the identification of transcription factors responsible for controlling the process of secondary wall biosynthesis in *P. massoniana*. In order to comprehend the molecular regulation mechanism of secondary wall biosynthesis in *P. massoniana*, we validated the *PmMYB45* TF associated with SCW synthesis based on the identified R2R2-MYB TFs. We employed transgenesis to carry out a functional study with a novel perspective for investigating related metabolite contents.

In analyzing its tissue expression and promoter analysis, we also noted that the *PmMYB45* gene is also abundantly expressed in tobacco-lignified tissues, such as stems as well as the veins of mature leaves (Figure 5B), where a number of structural genes and transcription factors involved in lignin biosynthesis are also specifically expressed, such as *PtoMYB158*, *PtoMYB189*, and *PtoMYB152* [23,24]. This suggests that *PmMYB45* may be involved in the regulation of lignin biosynthesis. The sites of expression of the TFs overlap with those of lignin synthesis, and the two may be related. Previous studies have shown that *PmMYB45* belongs to subfamily 37, along with *PtrMYB009*, *PtrMYB123*, *PtrMYB153*, *PtrMYB115*, *PtrMYB201*, *PtrMYB006*, *PtrMYB126*, *PtrMYB050*, *PtrMYB051*, *PtrMYB060*, and *PtrMYB061.* Branch and members of subfamily 37 have been reported to play key roles in lignin biosynthesis [25,26]. In this branch, *PtrMYB6* inhibits secondary cell wall development in *poplar* and *A. thaliana* [27]. *PtrMYB061* is homologous to *AtMYB46* and activates cellulose and hemicellulose synthesis [28]. Overexpression of *PmMYB45* in tobacco resulted in changes in plant growth traits, with a significant decrease in plant height, an increase in basal stem diameter, and an increase in the thickness of xylem ductal cell wall, which is consistent with the results of *PtMYB8* overexpression in spruce(*Picea asperata*), which resulted in slow growth of the plant, dwarfing, and an increase in the accumulation of lignin [29]. Overexpression of *PmMYB45* resulted in a significant upregulation of genes related to the lignin synthesis pathway (*4CL*, *CAD*, *HCT*, *PAL1*) in tobacco, suggesting that PmMYB45 can influence multiple target genes to regulate their expression. It was shown that overexpression and upregulation of secondary wall biosynthesis genes resulted in the accumulation of transcripts, stronger xylem lignification, and thicker secondary walls in plants [30]. The *PtoMYB170* promoter was able to activate the expression of the *CCOAOMT1* and *CCR2* genes. Knockdown of *PtoMYB170* resulted in the weakening of lignin deposition, as well as the loosening and collapsing of the xylem structure. This indirectly indicates

that a target gene can be regulated by multiple TFs simultaneously, leading to varying expression levels of the same gene in different plants [31]. In addition, the non-specific binding to the gene promoter caused by MYB overexpression may be indirectly affected by ectopic MYB overexpression [29]. Furthermore, the non-specific attachment to the gene promoter induced by the overexpression of MYB could potentially be indirectly influenced by the ectopic overexpression of MYB. Therefore, there are many reasons for the upregulation of downstream/target genes in *PmMYB45* overexpressing plants.

In summary, *PmMYB45* functions as a transcriptional activator that stimulates the expression of crucial enzyme genes in the lignin biosynthesis pathway, thereby enhancing lignin biosynthesis. However, the mechanism of how to realize the regulatory function is not clear. Delving deeper into its function and exploring its potential interaction with other TFs to create a network map of secondary wall formation in coniferous species would be a promising area for further research.

5. Conclusions

In this study, we isolated the MYB transcription factor *PmMYB45* and functionally characterized it. Our results indicate that *PmMYB45* is a transcriptional activator localized in the nucleus. Overexpression in tobacco promotes the expression of lignin biosynthesis genes, resulting in an increase in xylem thickness in transgenic plants. *PmMYB45* is involved in the regulation of lignin biosynthesis during the formation of the secondary cell wall. This factor may be a specific regulator. To prove this, the application of antisense techniques can be considered in future experiments to more accurately determine the function of the gene.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f15050814/s1, Figure S1: Cloning of the open reading frame of *PmMYB45*.M:NormaLRunTM 250bp -IIDNA ladder; ORF: open reading frame for *PmMYB45*. Figure S2: Cloning of the *PmMYB45* promoter. Figure S3: PCR assay for overexpression of *PmMYB45* tobacco. M: DL 5000 plus DNA Marker; +: positive control. -: wild type (negative control); L1–L4: transgenic strains. Table S1: Primer sequences used in this study. Table S2: Gene name and GenBank ID in this study. Table S3: Summary of acronyms.

Author Contributions: Conceptualization, M.P. and Y.H.; data curation, M.P.; formal analysis, M.P.; funding acquisition, K.J.; investigation, J.Z.; methodology, K.J., M.P. and X.H.; software, Y.Q.; validation, M.P.; visualization, X.H. and J.Z.; writing—original draft, M.P.; writing—review and editing, K.J., Y.H. and Y.Q. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the National Key R&D Program of China (2022YFD2200202), and the project was funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Data Availability Statement: All data are reported in this manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Jan, R.; Asaf, S.; Numan, M.; Lubna, L.; Kim, K.M. Plant secondary metabolite biosynthesis and transcriptional regulation in response to biotic and abiotic stress conditions. *Agronomy* **2021**, *11*, 968. [CrossRef]
2. Ramakrishna, A.; Ravishankar, G.A. Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signal. Behav.* **2011**, *6*, 1720–1731. [PubMed]
3. Yadav, B.; Jogawat, A.; Rahman, M.S.; Narayan, O.P. Secondary metabolites in the drought stress tolerance of crop plants: A review. *Gene Rep.* **2021**, *23*, 101040. [CrossRef]
4. Li, Q.; Fu, C.; Liang, C.; Ni, X.; Zhao, X.; Chen, M.; Ou, L.J.A. Crop lodging and the roles of lignin, cellulose, and hemicellulose in lodging resistance. *Agronomy* **2022**, *12*, 1795. [CrossRef]
5. Dowd, P.F.; Sarath, G.; Mitchell, R.B.; Saathoff, A.J.; Vogel, K.P.; Evolution, C. Insect resistance of a full sib family of tetraploid switchgrass Panicum virgatum L. with varying lignin levels. *Genet. Resour. Crop Evol.* **2013**, *60*, 975–984. [CrossRef]
6. Besseau, S.; Hoffmann, L.; Geoffroy, P.; Lapierre, C.; Pollet, B.; Legrand, M. Flavonoid accumulation in *Arabidopsis* repressed in lignin synthesis affects auxin transport and plant growth. *Plant Cell* **2007**, *19*, 148–162. [CrossRef]

7. Tu, M.; Wang, X.; Yin, W.; Wang, Y.; Li, Y.; Zhang, G.; Li, Z.; Song, J.; Wang, X. Grapevine VlbZIP30 improves drought resistance by directly activating VvNAC17 and promoting lignin biosynthesis through the regulation of three peroxidase genes. *Hortic. Res.* **2020**, *7*, 150. [CrossRef]
8. Dubos, C.; Stracke, R.; Grotewold, E.; Weisshaar, B.; Martin, C.; Lepiniec, L. MYB transcription factors in *Arabidopsis*. *Trends Plant Sci.* **2010**, *15*, 573–581. [CrossRef] [PubMed]
9. Kubo, M.; Udagawa, M.; Nishikubo, N.; Horiguchi, G.; Yamaguchi, M.; Ito, J.; Mimura, T.; Fukuda, H.; Demura, T. Transcription switches for protoxylem and metaxylem vessel formation. *Genes Dev.* **2005**, *19*, 1855–1860. [CrossRef]
10. Geng, P.; Zhang, S.; Liu, J.; Zhao, C.; Wu, J.; Cao, Y.; Fu, C.; Han, X.; He, H.; Zhao, Q. *MYB20*, *MYB42*, *MYB43*, and *MYB85* regulate phenylalanine and lignin biosynthesis during secondary cell wall formation. *Plant Physiol.* **2020**, *182*, 1272–1283. [CrossRef]
11. Bhargava, A.; Mansfield, S.D.; Hall, H.C.; Douglas, C.J.; Ellis, B.E. *MYB75* functions in regulation of secondary cell wall formation in the *Arabidopsis* inflorescence stem. *Plant Physiol.* **2010**, *154*, 1428–1438. [CrossRef]
12. Zhu, L.; Guan, Y.; Zhang, Z.; Song, A.; Chen, S.; Jiang, J.; Chen, F. *CmMYB8* encodes an R2R3 MYB transcription factor which represses lignin and flavonoid synthesis in chrysanthemum. *Plant Physiol. Biochem.* **2020**, *149*, 217–224. [CrossRef] [PubMed]
13. Yang, B.; Li, Y.; Song, Y.; Wang, X.; Guo, Q.; Zhou, L.; Xue, X.; Zhang, C. Genomes, The R2R3-MYB transcription factor *VcMYB4a* inhibits lignin biosynthesis in blueberry (*Vaccinium corymbosum*). *Tree Genet. Genomes* **2022**, *18*, 27. [CrossRef]
14. Yao, S.; Chen, P.; Yu, Y.; Zhang, M.; Wang, D.; Liu, J.; Hao, Q.; Ji, K. *PmMYB4*, a transcriptional activator from *Pinus massoniana*, regulates secondary cell wall formation and lignin biosynthesis. *Forests* **2021**, *12*, 1618. [CrossRef]
15. Chen, P.; Li, R.; Zhu, L.; Hao, Q.; Yao, S.; Liu, J.; Ji, K. Characterization and interaction analysis of the secondary cell wall synthesis-related transcription factor *PmMYB7* in *Pinus Massoniana Lamb*. *Int. J. Mol. Sci.* **2022**, *23*, 2079. [CrossRef]
16. He, Y.; Hao, Q.; Chen, P.; Qin, Y.; Peng, M.; Yao, S.; He, X.; Yu, Q.; Agassin, R.H.; Ji, K. Cloning of *PmMYB6* in *Pinus massoniana* and an analysis of its function. *Int. J. Mol. Sci.* **2023**, *24*, 13766. [CrossRef] [PubMed]
17. Xia, L.; Wu, F.; Ji, K. Cloning and Expression Analysis on PmPGK1 and PmGPIC Genes in Pinus massoniana. *J. Trop. Subtrop. Bot.* **2021**, *29*, 339–348.
18. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method. *Methods* **2001**, *25*, 402–408. [CrossRef]
19. Chu, J.; Xu, N.; Zhang, Q.; Wang, S.; Zhou, Z.; Jin, G. Establishment of an Agrobacterium tumefaciens-mediated genetic transformation system of *Lilium* speciosum. *Acta Prataculturae Sin.* **2011**, *20*, 164.
20. Heidari Japelaghi, R.; Haddad, R.; Valizadeh, M.; Dorani Uliaie, E.; Jalali Javaran, M. High-efficiency agrobacterium-mediated transformation of tobacco *(Nicotiana tabacum*). *J. Plant Mol. Breed.* **2018**, *6*, 38–50.
21. Lou, X.; Yao, S.; Chen, P.; Wang, D.; Agassin, R.H.; Hou, Y.; Zhang, C.; Ji, K. Transcriptome Identification of R2R3-MYB Gene Family Members in *Pinus massoniana* and *PmMYB4* Response to Drought Stress. *Forests* **2023**, *14*, 410. [CrossRef]
22. Zhao, Q.; Dixon, R.A. Transcriptional networks for lignin biosynthesis: More complex than we thought? *Trends Plant Sci.* **2011**, *16*, 227–233. [CrossRef] [PubMed]
23. Jiang, P.F.; Xu, H.; Guan, C.N.; Wang, X.X.; Wu, A.M.; Liu, Y.J.; Zeng, Q.Y. Evolution, Functional divergence of *Populus MYB158* and *MYB189* gene pair created by whole genome duplication. *J. Syst. Evol.* **2022**, *60*, 169–185. [CrossRef]
24. Li, C.; Wang, X.; Lu, W.; Liu, R.; Tian, Q.; Sun, Y.; Luo, K. A *poplar* R2R3-MYB transcription factor, *PtrMYB152*, is involved in regulation of lignin biosynthesis during secondary cell wall formation. *Plant Cell Tissue Organ Cult.* **2014**, *119*, 553–563. [CrossRef]
25. Tohge, T.; Nishiyama, Y.; Hirai, M.Y.; Yano, M.; Nakajima, J.; Awazuhara, M.; Inoue, E.; Takahashi, H.; Goodenowe, D.B.; Kitayama, M. Functional genomics by integrated analysis of metabolome and transcriptome of *Arabidopsis* plants over-expressing an MYB transcription factor. *Plant J.* **2005**, *42*, 218–235. [CrossRef]
26. Zhou, J.; Lee, C.; Zhong, R.; Ye, Z.H. *MYB58* and *MYB63* are transcriptional activators of the lignin biosynthetic pathway during secondary cell wall formation in *Arabidopsis*. *Plant Cell* **2009**, *21*, 248–266. [CrossRef] [PubMed]
27. Wang, L.; Lu, W.; Ran, L.; Dou, L.; Yao, S.; Hu, J.; Fan, D.; Li, C.; Luo, K. R2R3-MYB transcription factor *MYB6* promotes anthocyanin and proanthocyanidin biosynthesis but inhibits secondary cell wall formation in *Populus tomentosa*. *Plant J.* **2019**, *99*, 733–751. [CrossRef] [PubMed]
28. Zhong, R.; Richardson, E.A.; Ye, Z.H. The *MYB46* transcription factor is a direct target of *SND1* and regulates secondary wall biosynthesis in *Arabidopsis*. *Plant Cell* **2007**, *19*, 2776–2792. [CrossRef] [PubMed]
29. Bomal, C.; Bedon, F.; Caron, S.; Mansfield, S.D.; Levasseur, C.; Cooke, J.E.; Blais, S.; Tremblay, L.; Morency, M.; Pavy, N. Involvement of *Pinus taeda MYB1* and *MYB8* in phenylpropanoid metabolism and secondary cell wall biogenesis: A comparative in planta analysis. *J. Exp. Bot.* **2008**, *59*, 3925–3939. [CrossRef]
30. Tang, F.; Jiao, B.; Zhang, M.; He, M.; Su, R.; Luo, K.; Lan, T. PtoMYB031, the R2R3 MYB transcription factor involved in secondary cell wall biosynthesis in poplar. *Front. Plant Sci.* **2024**, *14*, 1341245. [CrossRef]
31. Xu, C.; Fu, X.; Liu, R.; Guo, L.; Ran, L.; Li, C.; Tian, Q.; Jiao, B.; Wang, B.; Luo, K. *PtoMYB170* positively regulates lignin deposition during wood formation in poplar and confers drought tolerance in transgenic Arabidopsis. *Tree Physiol.* **2017**, *37*, 1713–1726. [CrossRef] [PubMed]