



Article

# R2R3-MYB Transcription Factor *PIMYB108* Confers Drought Tolerance in Herbaceous Peony (*Paeonia lactiflora* Pall.)

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**Abstract:** The MYB transcription factor (TF) is crucial for plant growth, development, and response to abiotic stress, but it is rarely reported in the herbaceous peony (*Paeonia lactiflora* Pall.). Here, an MYB TF gene was isolated, and based on our prior mRNA data from *P. lactiflora* samples, it was treated with drought stress (DS). Its complete cDNA structure was 1314 bp, which encoded 291 amino acids (aa). Furthermore, using sequence alignment analysis, we demonstrated that *PIMYB108* was an R2R3-MYB TF. We also revealed that *PIMYB108* was primarily localized in the nucleus. Its levels rose during DS, and it was positively correlated with drought tolerance (DT) in *P. lactiflora*. In addition, when *PIMYB108* was overexpressed in tobacco plants, the flavonoid content, antioxidant enzyme activities, and photosynthesis were markedly elevated. Hence, the transgenic plants had stronger DT with a higher leaf water content and lower H<sub>2</sub>O<sub>2</sub> accumulation compared to the wild-type (WT) plants. Based on these results, *PIMYB108* is a vital gene that serves to increase flavonoid accumulation, reactive oxygen species (ROS), scavenging capacity, and photosynthesis to confer DT. The results would provide a genetic resource for molecular breeding to enhance plant DT.

**Keywords:** MYB; drought stress; flavonoid; ROS; *Paeonia lactiflora*



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## 1. Introduction

As the global climate continues to warm, a large quantity of water evaporates, resulting in a massive reduction in soil water content and subsequent drought conditions [1]. Being a major abiotic stressor, drought severely affects growth, development [2], yield [3], and quality [4] of plants, which in turn negatively affects agricultural supply. In China, the northern regions are mostly dry or semidry. As a result, in these areas, drought remains a major factor restricting landscaping construction. Therefore, the cultivation of drought-tolerant plant varieties and the generation of a low water consumption model and an esthetic plant configuration model are the main directions of green construction in the northern region.

Plants have evolved a variety of survival mechanisms in order to adapt to arid environments. One such mechanism is the regulation of drought-resistant genes [5]. Drought-resistant genes are mainly divided into two types: functional and regulatory. Transcription factors (TFs) belong to regulatory genes, which modulate the levels of multiple functional genes related to drought resistance. Multiple studies revealed that this is the most effective approach to enhancing plant drought resistance [6]. At present, the TF families associated with plant drought resistance mainly include bZIP (Basic leucine zipper), AP2/ERF (APETALA2/Ethylene Response Element Binding Factors), MYB (myeloblastoma), WRKY, NAC, etc. [7–9]. Of this list, MYB is the most prevalent in plants [10]. Its N-terminus has a highly conserved MYB DNA-binding domain, and it encodes proteins with 51–53 amino acid (aa) [11]. The MYB TFs are further categorized into four groups,

namely, 1R-MYB/MYB-related, R2R3-MYB, 3R-MYB (R1R2R3-MYB), and 4R-MYB based on the number of unit repetitions within the MYB domain [12,13]. Among them, the R2R3-MYB TFs contribute greatly to plant growth and development [14] and abiotic stress response [15,16]. In a study by Zhang et al. [17], it was reported that the wheat gene R2R3-MYB TF *TaMYB30-B* improved drought stress (DS) tolerance in transgenic *Arabidopsis*. Similarly, the poplar R2R3-MYB TF *PtrMYB94* modulates abscisic acid signaling to enhance drought tolerance (DT) in plants [18]. These studies indicate that the R2R3-MYB TF can be used as an important target for improving drought resistance in plants.

The herbaceous peony (*Paeonia lactiflora* Pall.) is a perennial herbaceous flower that can survive for long periods of time and can massively reduce landscaping costs. *P. lactiflora* has a long history of cultivation in China, and it is mainly cultivated in the northern regions. In these regions, however, drought is a major stress factor affecting the cultivation of *P. lactiflora*. In *P. lactiflora*, the physiological and biochemical responses under drought stress were investigated [19], and drought stress changed the expression of differentially expressed genes (DEGs) involved in the reactive oxygen species (ROS) system, chlorophyll degradation and photosynthetic capacity, secondary pathways of biosynthesis, and sugar metabolism [20]. However, studies involving the TFs that regulate *P. lactiflora* DT are rarely reported. Herein, an R2R3-MYB TF *PIMYB108* was extracted from *P. lactiflora*, and its function in conferring DT was examined in transgenic tobacco plants.

## 2. Results

### 2.1. Extraction and Sequence Analysis of *PIMYB108*

Based on our previous transcriptome data of *P. lactiflora* treated with drought stress, (SAR: SRP131648) [20], the TF *MYB108* (Unigene0031519), produced the largest difference from the controls; we examined its full-length cDNA via RACE. In addition, the result demonstrates that *MYB108* has an open reading frame (ORF) of 876 bp, a 5' UTR (untranslated region) of 156 bp and a 3' UTR of 282 bp. Moreover, it encodes a 291 aa protein (Figure 1).

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1      CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGTACATGGGGAAGAATTAATCCAATTCTCTCTCTC
79     TCTCTCTCTTTAGAAAACCTCTGTATATCGATCATCAAAACCCCTTTTCATTTCAATCAATCAGTCTGTCCAATTACGGGA
157    ATGGATGTTAATGGGAGAGGATCGTCATCAAGCAACTCCACCCCCACTCAAAGTGAAGAAGATCAGATGGATTGAGA
      M D V N G R G S S S S N S T P T Q S E E D Q M D L R
235    AGGGGTCCATGGACTGTTGAAGAAGACCTCACCTCATGAATTACATTGCCACAACCGGTGAAGTCCGCTGGAAATCT
      R G P W T V E E D L T L M N Y I A N N G E G R W N S
313    CTTGCCCGTTGTGCCGGTCTAAACGCACTGGAAGAGCTGCAGATTAAGATGGCTGAATATTACGCTCCAGATGTT
      L A R C A G L K R T G K S C R L R W L N Y L R P D V
391    CGTCGGAACATTACCCCTGAAGAGCAGCTTCTGATTCCTGAGCTTCATTCTCGCTGGGAAATCGGTGGTCGAAA
      R R G N I T L E E Q L L I L E L H S R W G N R W S K
469    ATAGCCAGCACTTGCCTGGAAGAACAGATAACGAGATCAAAAACCTATTGGAGAACCGAGTTCAAAAGCATGCCAAA
      I A Q H L P G R T D N E I K N Y W R T R V Q K H A K
547    CAGCTCAAAATGTGACGTGAACAGCAAGCAATTCAAGGACACCATGCGTTATTATGGATGCCAAGGTTGGTAGAGAGA
      Q L K C D V N S K Q F K D T M R Y L W M P R L V E R
625    ATTCAAGCCGCTCTAATGTTGTTGGAGCTTCTTCATCCTCCACCGTCTTACCACCAACGGCAATCTCCGGCGACTT
      I Q A A S N V V G A S S S S T V L P P T A I S G D F
703    GCCGGGIGCATGTGAATCAACTAGTTGCATTCCCGAGAATTCTAGCACGGGAGTTCTTCTGACTCATTTGGGACC
      A G V H V N S T S C I P E N S S T G V S S D S F G T
781    AGTCAAGTTTCTCCGCTATCTGACCTCGCCGAAAATAATTACAATTTCCCACTTAATGTCTCCGGATAAATTATTTTC
      S Q V S P L S D L A E N N Y N F P L N C H P D N Y F
859    CAAGGCGGTCAAGCTGGTTACTCAGATACGCTAATCAGCCCATCTGGTTATTACAATCCTGCCATGGATTTCAAGCT
      Q G G Q A G Y S D T L I S P S G Y Y N P A M D F Q A
937    ATGGAATATCAAAACAATAACCAAGTATATATCCGACAAITTTGGAATGTTGAAGACAATGTTCTTACAACAACAG
      M E Y Q N N N Q Y I S D N L W N V E D N W F L Q Q C
1015   TTCTCCAGCAATATTTAAGACGGTCCAGGTAGCGGTGACGATAATTAGGATGACGGGTTATGTAGGGTTACTCCATC
      F S S N I *
1093   GTTATCTGAAGATTATGATTTTGAACAAGACTGCGTACAGCGATCCTGAGGTAGCCATTTTATGTATGATAATTTAGG
1171   TTATACCGGACAGAAITTTGTAAGAAAAGACAGTTTTTACAGTTGTATGGATTGTATTTCTGTTTGTACTTTTTTAT
1249   TTGTTGGGTGGGAGAAAATTTGTAATTTGAGATTTGTTAATCTCATTCTTGGAAAAAATAA

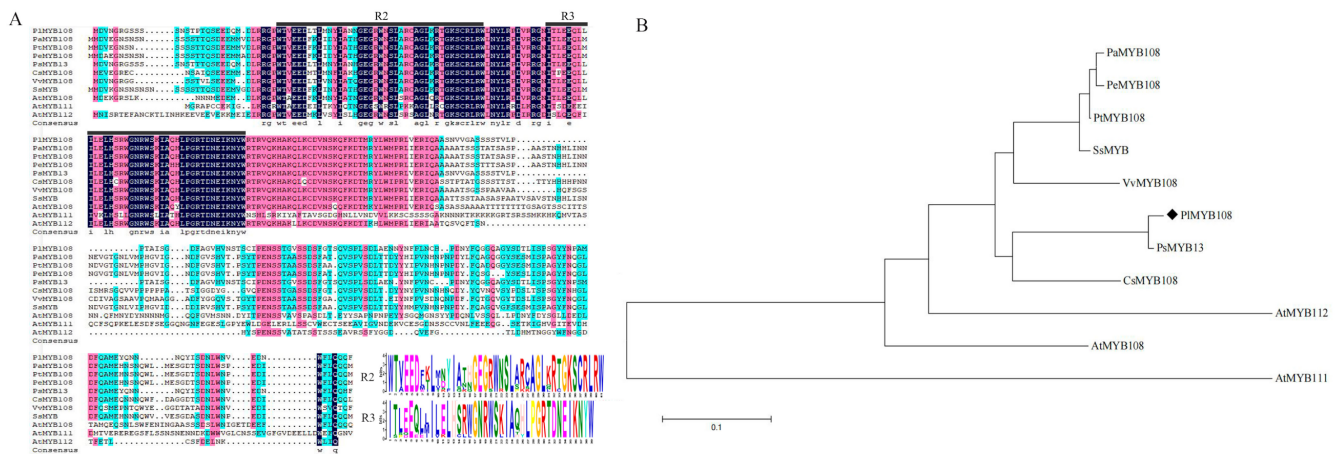
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**Figure 1.** cDNA sequence of *PIMYB108* and its deduced amino acid sequence. The deduced amino acid sequence is shown underneath the corresponding nucleotide sequence, the others are noncoding regions, and the stop code was indicated with \*.

Using the web software ProtParam, we further revealed that *PIMYB108* has a molecular weight of 33,009.52 Da and a theoretical isoelectric point (pI) of 6.22. Based on this evidence, we identified this protein to be both unstable and hydrophilic. Moreover, using the web softwares TMHMM and SignalP 5.0, we estimated that *PIMYB108* had no signal

peptide and transmembrane sites. This suggests that PIMYB108 is likely a nonsecretory and/or nontransmembrane protein.

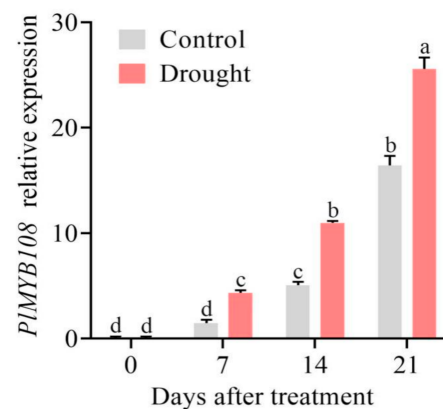
The homologous sequence alignments of PIMYB108 with *Populus alba* MYB108 (XP\_034907007), *Populus trichocarpa* MYB108 (XP\_002316060), *Populus euphratica* MYB108 (XP\_011010946), *Paenonia suffruticosa* MYB13 (QIG55696), *Camellia sinensis* MYB108 (XP\_028115735), *Vitis vinifera* MYB108 (RVW26470), *Salix suchowensis* MYB (KAG5237142), *Arabidopsis thaliana* MYB108 (NP\_187301), *Arabidopsis thaliana* MYB111 (NP\_199744), and *Arabidopsis thaliana* MYB112 (NP\_564519) were performed by DNAMAN5.2.2, and they shared a homology rate of 62.39%. Based on a sequence analysis, PIMYB108 was shown to contain both N-terminal R2 and R3 MYB DNA-binding domains, thereby suggesting that it is an R2R3-MYB TF (Figure 2A). Meanwhile, we also generated a phylogenetic tree via the Neighbor-Joining method (NJ) and MEGA5.05. Additionally, we followed numerous protein sequence alignments using ClustalW. Together, the data demonstrate that PIMYB108 is strongly correlated with PsMYB13 (QIG55696) (Figure 2B).



**Figure 2.** Sequences alignment and phylogenetic tree of PIMYB108 and seven other MYB proteins from plants. (A) Sequences alignment. The R2 and R3 MYB domains shown refer to two repeats of the MYB DNA-binding domain of MYB proteins. (B) Phylogenetic tree. Protein accession numbers are as follows: *Populus alba* MYB108 (XP\_034907007), *Populus trichocarpa* MYB108 (XP\_002316060), *Populus euphratica* MYB108 (XP\_011010946), *Paenonia suffruticosa* MYB13 (QIG55696), *Camellia sinensis* MYB108 (XP\_028115735), *Vitis vinifera* MYB108 (RVW26470), *Salix suchowensis* MYB (KAG5237142), *Arabidopsis thaliana* MYB108 (NP\_187301), *Arabidopsis thaliana* MYB111 (NP\_199744), and *Arabidopsis thaliana* MYB112 (NP\_564519).

### 2.2. PIMYB108 Expression Pattern Analysis in *P. lactiflora* Exposed to DS

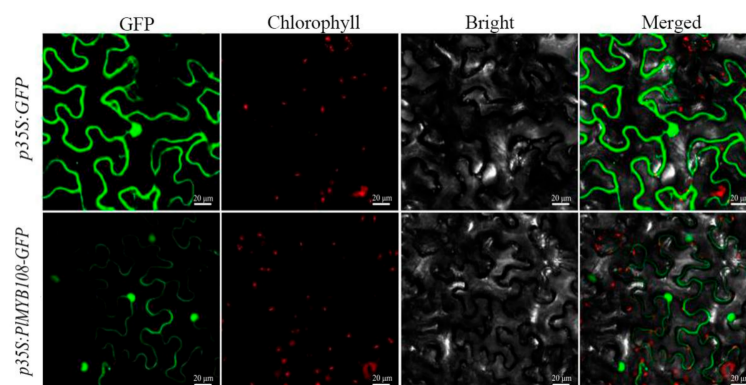
The *PIMYB108* expression pattern was determined in *P. lactiflora* exposed to DS (Figure 3). With increasing duration, the *PIMYB108* levels increased remarkably in controls, whereas under natural DS, the *PIMYB108* levels still increased, reaching their highest expression on the 21st day post exposure. Relative to the controls, the *PIMYB108* levels in *P. lactiflora* exposed to DS were markedly high.



**Figure 3.** Relative expression levels of *PIMYB108* in *P. lactiflora* under drought stress. The values represent the mean  $\pm$  SD, and different letters indicate significant differences according to Duncan's multiple range test ( $p < 0.05$ ).

### 2.3. Examining Localization of the *PIMYB108* Protein

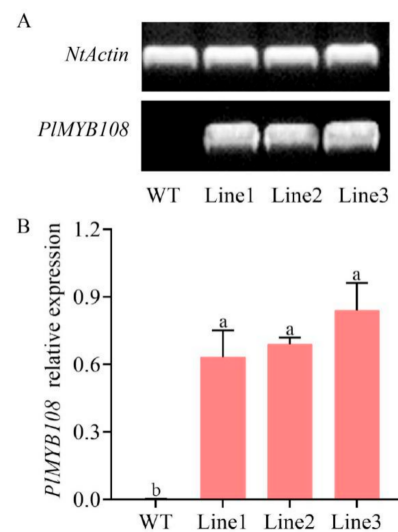
To examine the localization of the *PIMYB108* protein, the constructed plasmid *p35S:PIMYB108-GFP* was transformed in tobacco leaves. Based on our data, the emitted fluorescence is strongly expressed in the nucleus, which indicates that *PIMYB108* is primarily a nuclear protein (Figure 4).



**Figure 4.** Subcellular localization of *PIMYB108* in tobacco leaves. Note: From left to right are superimposed photos of GFP (green fluorescent protein), chloroplast autofluorescence, bright field, and three channels of target gene/empty vector, respectively.

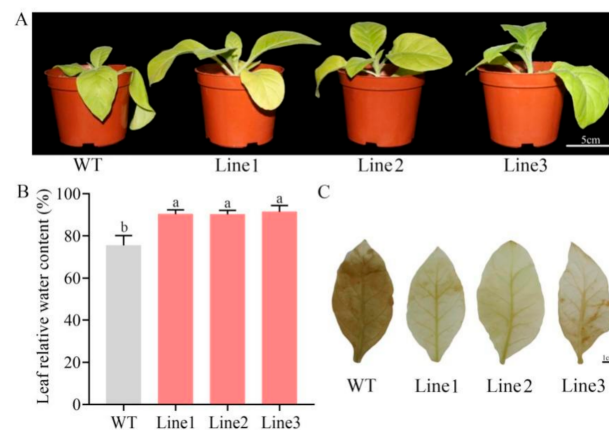
### 2.4. *PIMYB108* Conferred DS Tolerance in Tobacco

PCR and qRT-PCR were used for the identification of transgenic tobacco plants in this study. After 2 months of cultivation, the leaves of all the tobacco plants were employed as materials for identification. The PCR analysis revealed that all the tobacco plants produced a single bright band of *NtActin*, and the products of *PIMYB108* were detected only in the transgenic lines (TL) but not in WT (Figure 5A). In addition, we performed a further analysis using qRT-PCR. We demonstrated that the *PIMYB108* levels in TL were markedly elevated relative to WT (Figure 5B). These results suggest that *PIMYB108* was successfully transformed in tobacco plants.



**Figure 5.** Identification of transgenic tobacco lines. (A) PCR analysis of *PIMYB108* mRNA; (B) Relative expression level of *PIMYB108* in leaves using qRT-PCR; the values represent the mean  $\pm$  SD, and different letters indicate significant differences according to Duncan's multiple range test ( $p < 0.05$ ).

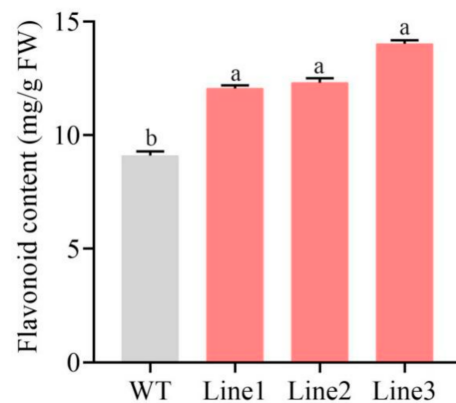
Next, the tobacco plants were exposed to natural DS for 10 days. Based on our results, the leaves of WT and TL showed massive differences. The WT leaves exhibited obvious wilting and drooping, whereas the TL ones exhibited sustained growth (Figure 6A). We also evaluated the relative leaf water content (RLWC) and  $H_2O_2$  accumulation to assess the growth status of the TL post DS. Based on our results, the TL exhibited markedly increased RLWC and lower  $H_2O_2$  accumulation when compared to the WT (Figure 6B,C).



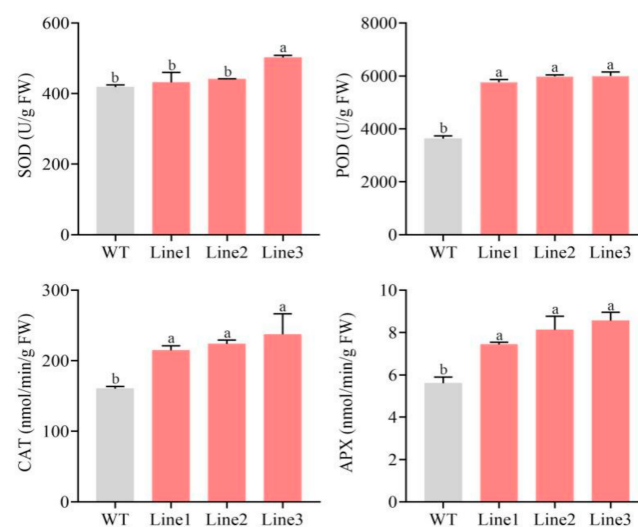
**Figure 6.** Phenotype and stress physiological indices of WT and transgenic lines under drought stress. (A) Phenotype of WT and transgenic lines under drought stress; (B) Relative leaf water content; (C)  $H_2O_2$  accumulation was detected by DAB staining. Tobacco plants were stressed by drought for 10 days. The values represent the mean  $\pm$  SD, and different letters indicate significant differences according to Duncan's multiple range test ( $p < 0.05$ ).

### 2.5. *PIMYB108* Increased Flavonoid Accumulation and Antioxidant Enzyme Activities

To clarify how *PIMYB108* confers DS tolerance within tobacco plants, the flavonoid content and antioxidant enzyme activities were assessed. As shown in Figure 7, the flavonoid content was markedly elevated ( $\sim 40.52\%$ ) in TL compared to WT. Moreover, the antioxidant enzyme activities, including superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POD, EC 1.11.1.7), catalase (CAT, EC 1.11.1.6), and ascorbate peroxidase (APX, EC 1.11.1.11) were markedly increased, with values that were respectively 1.09, 1.63, 1.40, and 1.43 times that of WT (Figure 8).



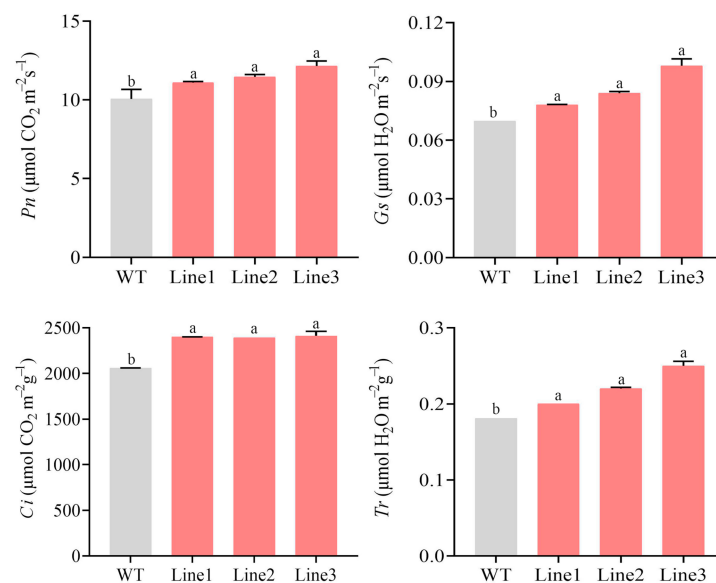
**Figure 7.** Flavonoid content of WT and transgenic lines under drought stress. Tobacco plants were stressed by drought for 10 days. The values represent the mean  $\pm$  SD, and different letters indicate significant differences according to Duncan's multiple range test ( $p < 0.05$ ).



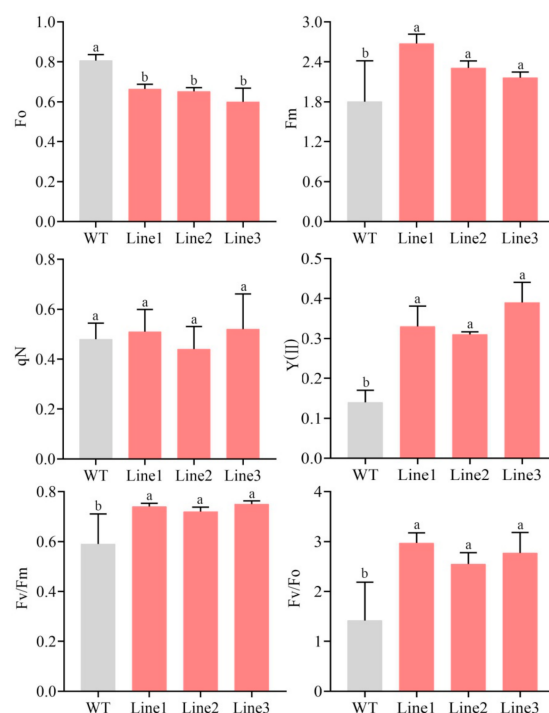
**Figure 8.** Antioxidant enzyme activities of WT and transgenic lines under drought stress. Tobacco plants were stressed by drought for 10 days. The values represent the mean  $\pm$  SD, and different letters indicate significant differences according to Duncan's multiple range test ( $p < 0.05$ ).

### 2.6. P1MYB108 Enhanced Photosynthesis

Subsequently, the photosynthetic characteristics, including net photosynthesis rate ( $P_n$ ), stomatal conduction ( $G_s$ ), intercellular  $CO_2$  concentration ( $C_i$ ), and transpiration rate ( $T_r$ ) were analyzed. Compared to WT,  $P_n$ ,  $G_s$ ,  $C_i$ , and  $T_r$  increased by 14.98%, 12.44%, 16.56%, and 24.06% in TL, respectively (Figure 9). Additionally, the chlorophyll fluorescence parameters, including the minimum fluorescence ( $F_o$ ), the maximum fluorescence ( $F_m$ ), the non-photochemical quenching ( $q_N$ ), the actual photosynthetic efficiency of light system II ( $Y(II)$ ), the variable fluorescence to maximum fluorescence ratio ( $F_v/F_m$ ), and the variable fluorescence to minimum fluorescence ( $F_v/F_o$ ) ratio, were also measured. Compared to WT,  $F_m$ ,  $Y(II)$ ,  $F_v/F_m$ , and  $F_v/F_o$  were all remarkably elevated in TL compared to WT. Moreover,  $q_N$  was slightly higher, while  $F_o$  was significantly lower (Figure 10), in TL than in WT.



**Figure 9.** Photosynthetic characteristics of WT and transgenic lines under drought stress. Tobacco plants were stressed by drought for 10 days. The values represent the mean  $\pm$  SD, and different letters indicate significant differences according to Duncan's multiple range test ( $p < 0.05$ ).



**Figure 10.** Chlorophyll fluorescence parameters of WT and transgenic lines under drought stress. Tobacco plants were stressed by drought for 10 days. The values represent the mean  $\pm$  SD, and different letters indicate significant differences according to Duncan's multiple range test ( $p < 0.05$ ).

### 3. Discussion

Water shortage is a major problem facing the world today, and many garden flowers cannot develop under drought conditions. Therefore, cultivating drought-tolerant varieties is of great significance for landscaping in arid conditions. Multiple factors modulate plant drought resistance. When plants are drought-stressed, they resist and adapt to drought in different ways [21]. However, TFs that modulate the expression of genes related to plant resistance and stress have gained much attention [6]. MYB is among the highly prevalent

TF families in plants [10]. Among them, the R2R3-MYB subtype is widely expressed and contributes greatly to plant growth and development [14] and response to abiotic stress [15,16]. At present, the R2R3-MYB TFs have been cloned in numerous plants. For example, Wang et al. [22] isolated a strawberry R2R3-MYB TF *FvMYB24* that possessed the full-length coding region and encoded an estimated protein of 284 aa. Similarly, Zhang et al. [23] isolated *PmMYBa1* from *Prunus mume*, which carried a conserved R2R3 MYB domain and belonged to the anthocyanin-associated subgroup 6 of the R2R3-MYB family. In this study, the full-length *PIMYB108* cDNA was retrieved via RACE technology, which encoded a protein of 291 aa. Furthermore, using sequence analysis, we demonstrated that *PIMYB108* carried both the N-terminal R2 and R3 MYB DNA-binding domains. This suggests that *PIMYB108* is an R2R3-MYB TF with a high homology with *MYB* from other plants, as reported in a prior study [24].

In recent years, there have been extensive studies on the function of *MYB* in plant abiotic stress response. For example, Qin et al. [25] found that the expression of *TaMYB33* in wheat leaves was induced by NaCl, PEG, and ABA treatments. Likewise, Wu et al. [26] also showed that PEG6000, NaCl, and ABA treatments upregulated *ZmMYB3R* levels in maize. These results suggest that *TaMYB33* and *ZmMYB3R* might modulate plant stress response. Here, we detected *PIMYB108* levels using qRT-PCR. Under natural DS, *PIMYB108* levels were markedly upregulated in *P. lactiflora*, which might positively correlate with DT in *P. lactiflora*. Subsequently, we investigated the subcellular localization of *PIMYB108* and found that it primarily resided in the nucleus, which mirrors the *RhMYB108* study in rose plants [27]. Based on this evidence, *PIMYB108* is a nuclear protein, which might serve as a TF that plays a regulatory role in the nucleus.

A previous study reported cloning *TaPIMPI*, with *MYB*-typical characteristics, based on the homologous sequence of *Arabidopsis AtMYB108*. Furthermore, its overexpression in wheat markedly enhanced drought resistance in plants [28]. To further confirm the DT activity of *PIMYB108* in *P. lactiflora*, *PIMYB108* was incorporated into a model tobacco plant, as evidenced by PCR and qRT-PCR. Next, the tobacco plants underwent natural DS for 10 days. Following this, the WT leaves showed significant wilting and drooping, whereas the TL remained normal in its development. This phenotypic difference strongly suggests that *PIMYB108* confers DT in tobacco. Moreover, relative leaf water content and H<sub>2</sub>O<sub>2</sub> accumulation were markedly elevated in the TL compared to WT, which revealed that *PIMYB108* can effectively maintain a normal water requirement and remove ROS.

Flavonoids represent a class of secondary metabolites that exert either positive or negative effects on plant growth. Nakabayashi et al. reported that overexpressing *AtMYB12* and *AtMYB75* in *Arabidopsis* remarkably enhanced DT via the overaccumulation of flavonoids [29]. In *P. lactiflora*, DS significantly increased flavonoid accumulation [20]. Moreover, in our study, the flavonoid content in TL was markedly increased (40.52%) compared to WT. This, in turn, exerted positive effects on the DS of tobacco plants. In addition, antioxidant enzyme activities in TL were significantly elevated in our study relative to WT, which, in turn, increased the ROS scavenging capacity and reduced H<sub>2</sub>O<sub>2</sub> accumulation.

Photosynthesis provides both energy and material resources for plants. In this study, *Pn*, *Gs*, *Ci*, and *Tr* were markedly increased in TL versus WT. This suggests that *PIMYB108* alleviates the DS-mediated suppression of photosynthesis, which is in accordance with results from the *P. ostii* caffeoyl-CoA O-methyltransferase overexpression examinations [30]. In addition, chlorophyll fluorescence parameters are used as internal indices of plants' adaptation to the eco-environment, and their changes during environmental stress can be used to judge the degree of damage to photosynthetic organs. In this study, *Fm*, *Y(II)*, *Fv/Fm*, and *Fv/Fo* in TL were markedly elevated, relative to WT, and the value of *Fv/Fm* was approximately 0.75, suggesting that DS caused little damage to the PSII reaction center in TL compared to WT. Therefore, the TLs were better at maintaining normal photosynthesis during DS. Our conclusions would provide a theoretical basis for future studies attempting to enhance DS tolerance in *P. lactiflora* via genetic engineering.



## 4. Materials and Methods

### 4.1. Extraction and Bioinformatic Analysis of PIMYB108

Total *P. lactiflora* RNA isolation was done with a MiniBEST Plant RNA Extraction Kit (TaKaRa, Kyoto, Japan). The entire cDNA was retrieved via rapid-amplification of cDNA ends (RACE) using a 3' full RACE Core Set v2.0 (TaKaRa, Kyoto, Japan) and a SMARTer™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) with targeted primers (Table S1). The PCR product was then separated on an agarose gel, excised, and sent for sequencing.

The aa composition, protein molecular weight, pI, stability, and hydropathicity were estimated via the ProtParam (<http://web.expasy.org/protparam/>, accessed on 20 August 2021) software. The transmembrane domain was analyzed via the TMHMM server 2.0 software (<http://www.cbs.dtu.dk/services/TMHMM>, accessed on 20 August 2021). The signal peptide was estimated via the SignalP 5.0 Server (<http://www.cbs.dtu.dk/services/SignalP/>, accessed on 20 August 2021). The homologous PIMYB108 sequence alignment with other species was done via DNAMAN5.2.2, and the phylogenetic tree was generated via NJ of MEGA5.05.

### 4.2. Expression Pattern Analysis of PIMYB108 *P. Lactiflora* Exposed to DS

Five-year-old *P. lactiflora* plants in potting soil (loam:peat:perlite, 1:1:1) were employed for the DS-stimulated PIMYB108 expression pattern analysis. The *P. lactiflora* plants were placed in a glass greenhouse on the Wenhui Road campus of Yangzhou University. They were separated into 2 groups, which each contained six plants: one group received water at 17:00 daily and served as the control group, and the other group received natural DS. The top leaves were collected at 0, 7, 14, and 21 days post exposure for an assessment of the PIMYB108 levels, using qRT-PCR via the BIO-RAD CFX Connect™ Optics Module (Bio-Rad, Hercules, CA, USA). All values were computed via the  $2^{-\Delta\Delta C_t}$  relative threshold cycle (Ct) method [31]. Primer sequences are summarized in Table S2.

### 4.3. Subcellular PIMYB108 Localization

This analysis was done using tobacco leaves and confocal laser microscopy (Nikon C2-ER, Tokyo, Japan). The *p35S:PIMYB108-GFP* gene fusions were synthesized to retrieve the PIMYB108 product (Figure S1). The ORF of PIMYB108 was amplified using primers carrying *BsmB* I restriction sites (forward 5'-CAGTGGTCTCACAACATGGATGTTAATGGGAG AGG-3', reverse 5'-CAGTGGTCTCATACAAATATTGCTGGAGAAGACTGTT-3'), which were then digested and introduced to the expression vectors with T4 DNA ligase (TaKaRa, Kyoto, Japan) to produce a set of *p35S:PIMYB108-GFP* fusions, which were then sequenced for further validation. Subsequent transformations of the *p35S:PIMYB108-GFP* and the empty *p35S:GFP* vector were conducted as reported previously [32].

### 4.4. PIMYB108 Overexpression in TL Tobacco

The *p35S-PIMYB108* plasmid was incorporated into the *Agrobacterium tumefaciens* strain *EHA105* using the freeze-thaw procedure. In turn, the strains were incorporated into tobacco (*Nicotiana tabacum* 'k326') via the leaf disc transformation procedure. Plasmid transformation was then verified using PCR and qRT-PCR. The primers used are summarized in Table S3.

### 4.5. DS Exposure Affecting PIMYB108 in TL Tobacco

The TL and WT tobacco plants were maintained for 2 months prior to use in natural DS experiments. The TL tobacco plants contained three lines, each line contained six plants. The plants underwent DS (i.e., no water) for 10 days. Next, the photosynthetic characteristic and chlorophyll fluorescence parameters were measured, and essential physiological variables such as RLWC, H<sub>2</sub>O<sub>2</sub>, flavonoid content, and antioxidant enzyme activities were assessed. Following this, the leaves were kept at −80 °C with liquid nitrogen until further gene expression analyses.

#### 4.6. Measurements of Physiological Indexes, Photosynthetic Characteristics, and Chlorophyll Fluorescence Parameters

An oven (Shanghai Jinghong Laboratory Instrument Co., Ltd., Shanghai, China) and balance (Suzhou Science Instrument Co., Ltd., Suzhou, China) were used to evaluate the RLWC. In brief, the leaf weight was measured and documented as fresh weight (FW). Next, the leaves were heated to 105 °C for 5 min in an oven, then cooled to 65 °C for > 2 h. The dried leaf weight was weighed again and recorded as dry weight (DW). Lastly, the RLWC was computed as follows:  $(FW - DW) / FW \times 100\%$ .

Diaminobenzidine (DAB) staining was employed for the detection of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) accumulation. In short, leaves were submerged in 0.1 mg/mL DAB in 50 mM Tris-acetate buffer (pH 5.0) in the dark at 25 °C. Following a 24 h incubation, the samples were removed and boiled in 95% (v/v) ethanol for  $\geq 15$  min, before capturing images with a camera (Canon 50D, Tokyo, Japan).

The flavonoid content and antioxidant enzyme activities, namely SOD, POD, CAT, and APX, were assessed with reagent kits (Suzhou Corning Biotechnology Co., Ltd., Suzhou, China). Finally, a portable photosynthesis system (Li-Cor LI-6400, Nebraska, USA) and a chlorophyll fluorescence spectrometer (Heinz Walz GmbH 91090 Effeltrich, Nuremberg, Germany) were employed for the measurement of photosynthetic and chlorophyll fluorescence, respectively.

#### 4.7. Statistical Analysis

The data presented are the means of at least three randomized replicates with standard deviations. A variance analysis was done with the SAS/STAT statistical analysis package (v6.12, SAS Institute, Cary, NC, USA).

### 5. Conclusions

*PIMYB108*, a R2R3-MYB TF, was extracted from *P. lactiflora*, and its protein was located in the nucleus. The *PIMYB108* expression was positively correlated with DT in *P. lactiflora*. Based on our analysis, *PIMYB108* is an essential gene that markedly enhances flavonoid accumulation, ROS scavenging capacity, and photosynthesis and eventually confers DT in tobacco. Our conclusion would provide a theoretical basis for future studies attempting to enhance DS tolerance in *P. lactiflora* via genetic engineering.

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## Abbreviations

MYB	Myeloblastoma
TF	Transcription factor
DS	Drought stress
aa	Amino acids
DT	Drought tolerance
WT	Wild-type
ROS	Reactive oxygen species
bZIP	Basic leucine zipper
ORF	Open reading frame
DEGs	Differential expression genes
RLWC	Relative leaf water content
RACE	Rapid-amplification of cDNA ends
DAB	Diaminobenzidine
qRT-PCR	Quantitative real-time polymerase chain reaction

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