



# Article Transcriptome Identification of R2R3-MYB Gene Family Members in *Pinus massoniana* and *PmMYB4* Response to Drought Stress

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Abstract: One of the largest families of transcription factors in plants, the MYB transcription factors family (Myeloblastosis, MYB TF), plays a vital role in regulating plant biochemical and physiological processes. The role of MYB TF in coping with stresses, such as drought, salt and cold, has been reported. Unfortunately, a comprehensive identification of R2R3-MYB TF in Masson pine (Pinus massoniana) has not been achieved. In this study, a total of 49 sequences were identified as R2R3-MYB TF. The structure, function and phylogenetic relationships of the conserved structural domains of Masson pine R2R3-MYB TF and Populus trichocarpa Torr. & A.Gray ex Hook. TFs were compared using bioinformatics tools. The results showed that Masson pine R2R3-MYB TF was divided into 24 groups, mainly located in the nucleus, and mostly lacking signal peptides and transmembrane structural domains with multiple phosphorylation sites. The drought stress-responsive R2R3-MYB gene, *PmMYB4*, was selected from the drought stress transcriptome based on analysis of the expression pattern and tissue specificity of *PmMYB4* gene under abiotic stress using qPCR. The results showed that *PmMYB4* can be involved in drought stress treatment through ABA signaling, as well as in multiple stress responses such as salt stress, and there were significant differences in the expression of *PmMYB4* in the eight tissues. These results provide a reference scheme for the functional identification of R2R3-MYB transcription factors, which may be involved in plant responses to multiple stresses such as drought, and enrich our understanding of the functions of R2R3-MYB transcription factors in plants.

Keywords: Pinus massoniana; R2R3-MYB; PmMYB4; drought stress

### 1. Introduction

The MYB protein is widely expressed in plants. It usually has two distinct regions, a regulatory region at the C-terminus responsible for regulating protein activity, and a highly conserved N-terminal DNA-binding domain [1]. Based on the number of R structures contained in the structural domain, the MYB TF can be classified into four categories, namely 1R structure (R1-MYB/MYB-related), 2R structure (R2R3-MYB), 3R structure (R1R2R3-MYB), and 4R structure (4R-MYB) [1]. In plants, most MYB belong to the 2R structure, R2R3-MYB, class of transcription factors [2]. Overall, most MYB TF act as early responders to environmental signals and can further induce or repress the expression of relevant genes essential for plant responses to stress. Researchers have identified massive R2R3-MYB TF in *Glycine max* [3], *Arabidopsis* [4] and *Oryza sativa* [5]. They play important roles in response to abiotic and biotic stresses and in regulating secondary metabolite biosynthesis [6].

Water deficit is one of the abiotic stresses that severely inhibit plant development [7,8]. When plants are subjected to drought stress, MYB TF respond to drought signals and



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**Copyright:** © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). regulate the expression of some downstream genes to bring about changes in plant response to biochemical physiological processes, thus exerting a drought-resistant function [9–11]. It was found that *FtMYB9* and *FtMYB13* could activate different stress response signals of *Fagopyrum tataricum* (L.) Gaertn. and increase the response to ABA, thus improving drought tolerance of plants [12,13]. The *HvMYB1* in barley positively regulates plant drought tolerance [14], *ZmMYB3R* in maize improves drought tolerance [15], and overexpression of *NtMYB4a* reduces malondialdehyde content in tobacco, accumulates more proline, and improves plant drought and cold resistance [16]. The *SbMYB8* in *Scutellaria baicalensis* Georgi regulates flavonoid biosynthesis to enhance drought resistance [17], and *PtrMYB94* in poplar acts synergistically with ABA signaling to improve plant drought resistance [18].

*Pinus massoniana* Lamb. (Masson pine) is the most abundant conifer species distributed in the humid eastern subtropical region, and is a major fast-growing and productive timber, lipid-producing and ecologically important protected species, with high economic value in China [19,20]. The frequent occurrence of abnormal climate, such as drought and high temperatures, not only aggravates the deterioration of the habitat of Masson pine, but also seriously threatens its growth and restricts the development of the Masson pine industry [21]. The drought resistance ability of adult Masson pine is strong, but at the seedling stage, at the early stage of large-scale plantation and after woodland tending, the woodland is without shade and relatively dry. Under such conditions, drought is the main limiting factor for forestation survival of Masson pine, resulting in the low survival rate at the seedling stage. Some progress has been made in the physiological mechanism of resistance to drought stress, phosphorus stress and other abiotic stresses of Masson pine, as well as the cloning of resistance genes [22,23]. However, studies on the response of the MYB gene to abiotic stresses in Masson pine are relatively blank.

In this study, we investigated the R2R3-MYB transcription factors of Masson pine, identified the R2R3-MYB gene family, and isolated a drought-inducible gene, *PmMYB4*, by screening. The expression of the *PmMYB4* gene in different environmental stresses, such as drought, was analyzed by q-PCR. The gene functions of the R2R3-MYB genes of Masson pine under stress and their response mechanisms were explored. These results provide a reference for the functional identification of R2R3-MYB TF and enrich our understanding of the functions of R2R3-MYB TF in plants.

## 2. Materials and Methods

#### 2.1. Identification and Classification of R2R3-MYB Genes from Masson Pine

The transcriptome data for each of the four Masson pine species were the drought stress transcriptome (PRJNA595650), the transcriptome under CO<sub>2</sub> stress [24], the transcriptome of pine wood nematode inoculation (PRJNA660087), and the transcriptome of shoots (PRJNA655997) [25]. Protein translation was performed online by ExPASy (https://web.expasy.org/translate/, accessed on 15 April 2022). The Hidden Markov Model (HMM) of the MYB binding domain (PF00249) was obtained from the Pfam database (http://pfam.xfam.org/, accessed on 15 April 2022) and used to identify all MYBs in Masson pine [26]. The transcription factors of the Masson pine MYB family were screened by default parameters of HMMER3 V3.0 software. Using Pfam and the Conserved Domain Search, all domains of the MYB protein sequence and deleted domains that did not have MYB protection sequences that defend the domain, or did not have the complete conserved domain, of MYB were held by MYB TF R2R3-MYB TF, obtained and further analyzed [27,28].

#### 2.2. Characterization of MYB Gene Family Members of Masson Pine

Physicochemical properties of the gene, such as isoelectric point, protein instability coefficient, and molecular mass, were determined using ExPASy (https://web.expasy.org/, accessed on 22 April 2022). Subcellular localization was analyzed by WoLF PSORT (https://psort.hgc.jp/, accessed on 22 April 2022). Protein transmembrane structure was analyzed using TMHMM v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/, accessed on 23 April

2022). NetPhos 3.1 and SignalP 5.0 (https://www.nature.com/articles/s41587-019-0036-z, accessed on 23 April 2022) phosphorylation sites and signal peptides were used [29].

#### 2.3. Phylogenetic Analysis of R2R3-MYB Proteins in Masson Pine

The phylogenetic tree was constructed using MEGA 10.0, and the partial deletion (Pairwise deletion) option was selected according to the R2R3-MYB classification of *Populus trichocarpa* [30] using the neighborhood join method (NJ) p-distance model. The bootstrap method was used with a value of 1000 [28,31], and then embellished using Evolview (https://academic.oup.com/nar/article/47/W1/W270/5494715?login=false, accessed on 24 April 2022). The MEME tool (version 5.3.3) (https://academic.oup.com/nar/article/43/W1/W39/2467905, accessed on 24 April 2022) analyzed the amino acid motifs of the PmMYB protein with 5 motifs sets. Fragment sizes ranged from 6 to 70 amino acids and all other default parameter values. The conserved motifs obtained from the search were annotated using SMART (http://smart.embl-heidelberg.de/, accessed on 25 April 2022) online analysis software [32].

## 2.4. Expression Pattern Analysis of Masson Pine R2R3-MYB Genes

Expression of the R2R3-MYB gene was studied by drought transcriptome data (PR-JNA595650). To estimate the abundance of PmMYB gene transcripts, the number of fragments per kb per exon model per million reads mapping (FPKM) value was used and heat maps of selected genes were plotted and analyzed on a line scale based on the value of log2 (FPKM + 0.01); the visualization process was performed using TBtools (Toolbox for biology) [33] software. The *PmMYB4* (GenBank:MW579325) was selected for real-time quantitative RT-PCR (qRT-PCR) validation.

Drought stress was simulated by soaking 2-year-old Masson pine seedlings with 15% PEG6000, and Masson pine needles were collected at 0 h, 3 h, 6 h, 12 h, and 24 h and immediately stored in liquid nitrogen at -80 °C. The control group was untreated seedlings (0 h). Three biological replicates and three process replicates were performed for all treatments, respectively. After obtaining all samples, sample RNA was extracted and reverse transcribed to obtain cDNA (methods from Vazyme FastPure Plant Total RNA Isolation Kit (Polysaccharides and Polyphenolics-rich) instructions). The α-tubulin (TUA) (GenBank: KM496535.1) [34] gene was used as the internal reference gene and the *PmMYB4* gene was selected for qRT-PCR. Primers for the *PmMYB4* gene (*PmMYB4*-Q-F, *PmMYB4*-Q-R) were designed by Primer Premier 5.0 software. The kit used for qRT-PCR is from YEASEN. The  $2^{-\Delta\Delta CT}$  [35] was used to calculate the relative changes in gene expression levels.

#### 2.5. Subcellular Localization Detection

The PJIT166 vector contained a  $2 \times 35S$  double 35S strong promoter and a GFP green fluorescent reporter gene. Using CE Design software, primers carrying restriction sites were designed using the pJIT166 vector and the ORF sequence of *PmMYB4* (with the termination codon removed) as templates. Next, a  $2 \times 35S$ ::PmMYB4-GFP fusion vector was constructed and transformation performed, followed by positive detection of bacterial solution, sequencing, and screening of the correct positive clones for amplification and culture. Target gene plasmids were extracted with the QIAGEN large plasmid extraction kit and transformed into *Agrobacterium tumefaciens* (EAH105).

Young tobacco leaves (grown for 20 d) were infested with *Agrobacterium*-mediated transient transformation and incubated in an artificial climate incubator for 2 d. Tobacco leaf samples ( $0.5 \times 0.5$  cm) were taken and laid flat on slides. The expression of the fusion vector was observed under a laser confocal microscope (UV excitation wavelength 488 nm), and the results were photographed and recorded.

#### 2.6. Response of PmMYB4 to Stress

Based on the *Escherichia coli* genome codon usage patterns, the codon optimization website ExpOptimizer (https://www.novopro.cn/tools/codon-optimization. html) was used to optimize the codon of the *PmMYB4* gene to a pattern more suitable for expression in *Escherichia coli*, to optimize the structure of its mRNA and regulate the matching degree with the level of host cell tRNA [36–40]. The optimized *PmMYB4*, which we named *PmMYB4*-optimization nucleotide sequence, is shown in Supplementary Materials.

The *PmMYB4*-optimization and pET28a were fused and transformed with *E. coli* TransB-DE3 strain using the same method as in Section 2.5. After incubation in petri dishes overnight, positive clonal colonies were selected and tested on LB plates containing 800 mM d-mannitol, 400 mM NaCl and 20% PEG<sub>6000</sub> to observe their growth.

#### 2.7. Gene Expression Analysis and Promoter Cis-Acting Element Analysis of PmMYB4

Flowers (F), roots (R), xylem (X), phloem (P), old leaves (OL), young leaves (YL), old stems (OS), and young stems (YS) were collected from 15-year-old Masson pine. Phloem and xylem were obtained from developing trunks (phloem was retrieved from inside the phloem and xylem tissue from peeled logs). For abiotic stress treatments of 2-year-old Masson pine, abiotic stress treatments included mechanical damage treatment, 50  $\mu$ M ethylene (ETH) treatment, 400  $\mu$ M abscisic acid (ABA) treatment, 10 mM methyl jasmonate (MeJA) treatment, 10 mM H<sub>2</sub>O<sub>2</sub> treatment, and 1 mM salicylic acid (SA) treatment. The mechanical damage was treated by cutting the upper part of the needles of Marengo pine, and the other treatments were performed by spraying hormone solutions on the plant surface. The specific methods were the same as in Section 2.4.

The local database of *PmMYB4* was established and aligned with the *Pinus taeda* genome. The nucleotide sequence approximately 2000 bp upstream of this fragment was selected as the promoter sequence and design specific primers (Pro-*PmMYB4*-F, Pro-*PmMYB4*-F, Pro-*PmMYB4*-F, Pro-*PmMYB4*-F, Pro-*PmMYB4*-F, Pro-*PmMYB4*-F, Pro-PmMYB4-F, 2.5  $\mu$ L, 2× ApexHF FS PCR Master Mix 25  $\mu$ L and ddH2O 18  $\mu$ L. The reaction was performed at 98 °C for 3 min, kept at 98 °C for 30 s, 60 °C for 5 s, 72 °C for 1 min, 72 °C for 5 min, 35 cycles and stored at 4 °C.

#### 3. Results

#### 3.1. Identification of R2R3-MYB TF in Masson Pine

We mined 57 MYB TF in the four transcriptomes of Masson pine and named them Pm-MYB1 to PmMYB57. The amino acid sequences of the 57 R2R3-MYB sequences are shown in Figure 1. There were three extremely conserved tryptophan (W) in the R2 structure and two extremely conserved tryptophan (W) in the R3 structure of Masson pine, in which the first tryptophan was replaced by leucine (L), isoleucine (I) and phenylalanine (F), as shown in Figure 1. The eight amino acid sequences, PmMYB13, PmMYB15, PmMYB17, PmMYB19, PmMYB31 PmMYB47, PmMYB48, and PmMYB56, did not have the complete MYB-R2R3 structural domain, while the remaining 49 Masson pine sequences MYB structural domains had high similarity and conservation, including the complete R2 and R3 regions.

		R2 Stru	icture Domai	n	R3 Structure Domain
PmMYB1			MDEUDY INCLI	DOCUSODI DUTNUL D	DEL VECT I SECRET I TEL HAAT CHEN SET A OT DOPTEMET UN VENTE L'UNE
PmMYB2	OVUVDCINSDEEPE V	I INVITEVOVO	WEEVER VSCIO	PCCKSCPI PWINILR	PDERROLESESEETETETETETETETEN UNDER VIA SETERETEN ETEN SETEVEN SETEVET
PmMYB3	VMCDDCWWDDAEDE V	IDELUEOVEDO N	WNSTAR VINC	DSCREEDED DE D	PDIRKONFSPREELIINLAINVONKWAILASHEPGRIDNEIKAIWASWIKKKLAF
PmMYB4	DVI DVCI WSDEEDD	T TNYMMYNCOC	WSDUAK CACLO	DCCMSCRIPWINGID	ENTINENET IEEEEERLEAANKEIGNEWAANTAKEFFGETENAVENNET VINANET.
PmMYB5	UTN VCANTUEEDD D	TTANTATUCEC C	WDSIDY ANCIN	DCCMSCRIPWINIER	PDL NDCCETEEPDELTTYL HAUT CNUMSTIACDI DCDTDNETWNYMNTHTYDY
PmMYB6	CIN DOWTYDERM T	ISEVUDINCEGC	WENTER VACLY	DCCRECED DWINYID	PDERROGFIEEEDELTIKERAVEGRAVISTIAGREFORIDRETRAINAIRIKKK
PmMYB7	DDTVGDWSDEEDA S	LOPIVORVERP N	WTITSK GIDG	DSCREEDE DWCNOTS	POURHODETDSEDAATIOAHAOHGNEWATTADAIDGDTDNATENHWNSTIDDCDL
PmMYB8	VID VCINSPEEDE V	TUDHTTEVONCHC C	WSAUEK CACLO	PCCKSCPI PWINVI P	DEL VOCTES DOFFNI TUET HSUT ONDESCIATUT DOPTEMETENIENSCIVEV
PmMYB9	CORTROPWSPEEDA A	LOWINEWIGER N	WEITER CIDC	DSCREEDE DWCNOTS	POLYCHDERSDEEDDMINEAUSWHGNUWATIADII DGDTDNAIWNWNSTI DDWCIA
PmMYB10	GLK KOPWIPEEDO I	I TSYTNKHCHC N	WPALPK CAGTM	PCCKSCPIPWTNVIP	PDTKPGNESLKEFOTTTHLHOTIGNPWSATASHT PGPTDNETKNUWNTHLKKP
PmMYB11	MKERCRWOPFEDA	LRAYVKOYGPR. F	WNLVSORMGKALD	RDAKSCLERWKNYLK	PGIKKGSLTEFEGSLVISLOAKYGNKWKKTAAFVEGETAKELGKWWEVEKEKOLKE
PmMYB12	GDRHEVTWSECEDN. M	LRECVRINGTER	WANTASCLKD	KTSRCCRRRWNTYLS	TAYKKGSWSOFEDILLFEAHKKFGNRWTELAKVVOGRTENAVKNRFNALCKKOAKE
PmMYB13	NKLEENALAKEDKOTPO	RWENVATCVPGK.T	PAEVRRHYELLVEDVTV	IESGRVAL PAYSENSYTP.	PELMSDOLGDLT., KOCAVSV, KAPSAKASECERKKGVPWTEFEHRLFLMGLNKYG
PmMYB14	HTN. KGAWTOFFDA R	TVAHTCAHGEGG	WRSLPK AAGL	RCGKSCRLRWINYLR	POLKRONESEEEDELTIKEHSRLONKWSLTARILPORTONETKNHWNTHIKKK
PmMYB15	RERGSMMWGTWEEL	LAGAVERHOTE N	WELTSEELRARTVSPYV	ESPEACHAKYDALR. BREN	GHVSENPENSNDERNSVLLEVLRARRIACIKTOEIP, LKNDSIGSLESELRRLKAG
PmMYB16	KAVKRGEWSPEEDMK	LITYITTHGHGC	WSSLSE. RAGLC	RCGKSCRLRWMNYLR	PGIKEGNESTEEENVIISLOARLNNEWACIAKHLPGETETEIKNYWHSCLKKKAVA
PmMYB17	RKSIMRASMPKTHAG	RFPSGESHVLSV.G	YKAETNNGIPLEVSTGK	RALPSANLNIGSIPTKR	IRSSTIAARCRAAGSLSGGTTGASGLANKTDVSSGDTSSLCEDFNTLSGGSCHE
PmMYB18	GIN.RGAWTASEDKI	LSEYIKTHGVGR	WRCLPIKTGLK	RCAKSCRLRWVNYLR	PDIKRGNISPEEELLIRLYRLLGNRWALIAGRLPGRTDNEIKNYW
PmMYB19	NKIFENALADEDKDTPD	RWEKVAARLPGK.T	ATDVRKHYEDLVEDVTC	IEAGRVALPTYS.NSSCS	LEWLAEKSGAMHGLKCOFGSVGRGOSTKASECERKKGVPWTEEEHROFLMGLRKYG
PmMYB20	PELVKGPWTKEEDEL	IVELVGKYGSKK	WSVISCSLPG	RIGKCCRERWHNHLN	PEIKKDAWSCEEELALIRAHCLYGNKWAEIAKFLPGRTDNSIKNHWNSSIKKKLDS
PmMYB21	GIK.KGPWTPEEDIA	LVSYIQE <mark>H</mark> GQ <mark>G</mark> N	WRLIPARTGLL	RS <mark>S</mark> KSCRLRWTN <mark>YLR</mark>	PGIKHGNFTPYEDRIIIHLCALLGNRWAAIASYLFCRTDNDIKNHWNTHLKKRLTF
PmMYB22	GLN.KGAWSAEEDSL	LGKYIQT <mark>HGEG</mark> N	WRSLPKKAGLR	RCGKSCRLRWLNYLR	PCIKRGNITTDEEELIIRMHALLGNRWSIIAGRVPGRTDNEIKNYWNTNLSKK
PmMYB23	GLH.RGPWTAREDLL	LTKYIEA <mark>HG</mark> EGQ	WRALPKKAGL	RC <mark>G</mark> KSCRLRWMN <mark>YL</mark> R	PDIKRGNITLDEEELIVRLHKLLGNRWSLIAGRLPGRTDNEIKNFWNTHLSKK
PmMYB24	RLCARGHWRPAEDAK	LKELVSQYGPQN	WNLIAEKLEG	RS <mark>G</mark> KSCRLRWFNQLD	PRINRRPFSEEDEEKLLAAHRLYGNKWAMIARLFPGRTDNAVKNHWHVIMARRYR.
PmMYB25	AERIKGPWSPEEDAA	LHKLVEKYGPRN	WSQISRGIPG	RS <mark>G</mark> KSCRLRWCNQLS	PQVEHRPFSPHEDATIIQAHARHGNKWATIARLLPGRTDNAIKNHWNSTLR <mark>RR</mark> YHG
PmMYB26	RLN.RGPWSADEDMI	LLEYIGI <mark>H</mark> GQ <mark>G</mark> R	WSSVPQKAGLK	RCAKGCRLRWVNYVR	FNIKRGNICPNEEELICRLHRLLGNRWSLIAGRLPGRTNQEIKSYWNTHLKRK
PmMYB27	AVH.RGSWTPEEDRI	LKTYIEANGVGR	WSTLPQNAGLM	RR <mark>G</mark> KSCRLRWMNHL <mark>R</mark>	PNVKRGHISADEEELIIRLHNLLGNRWSLIAGRVPGRTDNEVKNYWNTRLSKK
PmMYB28	PMSKKGRWSPDEDNK	LTNYIIK <mark>NG</mark> LLG.G	WNYIAEK <mark>AGI</mark> Q	RS <mark>G</mark> KSSRLRWVN <mark>YLR</mark>	FGLKRGAFTYCEERLIIHLCSILGNRWSFIAKCLFGRTDNELKNYWNSCIKKKLKI
PmMYB29	GTLKKGPWTSAEDAI	LVEYVRK <mark>HG</mark> EGN	WNAVQKHSGLS	RC <mark>G</mark> KSCRLRWANHL <mark>R</mark>	PNLKKGAFTAEEERIILELHAKLGNKWARMAAHLPGRTDNEIKNYWNTRIKRRQRA
PmMYB30	HTNNKGAWTKEEDER	LIAHIEA <mark>HG</mark> EGS	WRSLPKA <mark>AGL</mark> L	RC <mark>G</mark> KSCRLRWIN <mark>YLR</mark>	FDLKRGSFSEEEDDLIIKLHSLLGNKWSLIAGRLFGRTDNEIKNYWNTHMKRK
PmMYB31	MFLIGLOKLGKGD	WRGISRNFVPTR.T	PTQVASHAQKYFIRQSN	LTRRKRRSSLFD	ITAEPISCPLP SPALPVLSSQSASDQEEAESGDNSANSADVETLLPQVDETA
PmMYB32	GLN.RGPWTRDEDLR	LKNYIEV <mark>HG</mark> EGG	WRTLPIKAGLL	RCGKSCRVRWMNYLR	PDVKCGHILPEEEDLILRLHRLLGNRWALIAGRIPGRTDNEIKNYWNTH
PmMYB33	.MKERQRWRSEEDSL	LLSYVKRY <mark>G</mark> PRE	WNLISERMNRALD	RDPKSCAERWKNYLK	FGIKKGSLSEDEORLVISLOAKYGNRWKKIAACVFGTTAKRLSKWWEVYREKODKI
PmMYB34	SKLRKGLWSPEEDSK	LINYIMK <mark>N</mark> GLDD.S	WTYVSKQAGLQ	RCGKSCRLRWVNYLR	PDLKRGAFSCOEERLIIHLOSILGNRWSQIAAQLPGRTDNEIKNYWNSAIKKKFKL
PmMYB35	ANVKRGPWSPEEDTI	LKNFVEK <mark>HG</mark> T <mark>G</mark> G.N	WIALPRK <mark>AGL</mark> K	RCGKSCRLRWLNYLR	PDIKHGDFSEEEDDIICTLYTSIGSRWSIIAAQLPGRTDNDIKNYWNTRLKKKLLG.
PmMYB36	PKLRKGLWSPEEDEK	LITYIMNK <mark>G</mark> MVGCS	WTYIAKQAGLQ	RCGKSCRLRWINYLR	PDLKRGSFSPQEEYLIRNLHSILGNRWSQIAASLPGRTDNEIKNYWNTCIKKKKLK
PmMYB37	KLR.KGLWSPEEDEK	LMRYVTT <mark>HG</mark> HGR	WSAVPKHAGLE	RC <mark>G</mark> KSCRLRWIN <mark>YLR</mark>	PDLKRGTFSAHEEKLITDLHAALGNRWSQIATHLPGRTDNEIKNFWNSSIKKK
PMM1B38	DERIKGPWSPEEDAV	LSRLVDKFGARN	WSLIARGIPG	RSGKSCRLRWCNQLN	FGVKRKPFTDEEDRAIVAAHAIHGNKWASIARMLQGRTDNAIKNHWNSTLRRKYLG
PRIMI B39	GVR.RGPWSPEEDRR	LIDYIQKHGFGN	WRAIPRQAGLL	RCGKSCRLRWINYLR	PDLKRGSLSPEEECTIIRLCGVLGNRWSTIASYLPGRTDNEIKNIWNTHIKKR
DmMVR41	PMRRKRLWSPEEDNK	LNSYIMKNGLTR.C	WNYVADQAGIQ	RSGKSCRLRWMNYLR	PGLKRGAFSDQEERLIVHLCSVLGNRWSLIAAEFPGRTDNALKNYWNSRMKKKLEL
DmMVR42	GQRIKGPWSPEEDAA	LQKLVEKLGPRN	WSLISKGIPG	RSGKSCRLRWCNQLS	PCVCHRPFSPEEDRMIMEAHSMHGNKWATIARILPGRTDNAIKNHWNSTLRRKCLA
DmMVD42	HIN. KGAWIKEELEK	LIAYIQAHGEGC	WRSLPKAAGLL	RCGKSCRLRWINYLR	PDLKRGNFSEEEDELIIKLHALLGNKWSLIAGRLPGRTDNEIKNYWNTHIKRK
DmMVB44	GLN.RRAWTAKELMI	LSDYTRINGDCG	WISLPGKAGLK	RSAKSCRLRWLNYLR	PDIDHGNISLDEEELIIRMHRLLGNRWSLIAKRHPGRTDNKIKNHWNTHLSKK
DmMVR45	GVK.KGPWILDELKI	LVDYITKHGHGN	WRALPKQAGLL	RCGRSCRLRWINYLR	PDIKKGNFSPEEEDQVIKLHERIGNRWSTIASYLPGRIDNEIKNVWNTHLKKR
PmMYB46	GLN.RGPWTPEEDDL	LVKYICKHGEGG	WRTLPKKAGLL	RCGRSCRLRWMNYLR	PDVKRGQILPDEEDLILKLHRLLGNKWSLIAGKMPGRIDNEIKNYWNIHLSKK
PmMVB47	GLN.RGPWIPEELLC	LANIILANGLGG	WRILPRRAGIL	RCGRSCRLRWMNILR	POVKHGHILPEEDLILKLEKLLGNKWSLIAGKMPGRIDNEVKNIWNIHLSKK
PmMYB49	CIN NORWIPEPE I	UTVIOFNCHC C	WRAIDD WACLI	COVECDI DWANYI D	PERFIEWING PERFECTION PALICADE STATUT PUTTING THE SUCCESSION OF THE STATUT PUTTING THE ST
PmMYB48	PLENDEDVSUPSAV T	ATTTODNNDDP F	MAATSUDNI PREASUNS	CONCOUNCEST SKNUDMOTU	SUVSSNEDNDI GNSGSVTGDNVTTDHSNKVNAVOSDDI ATTTTEEDSONTDHEDO
PmMYB50	DERVGONTUREDY D	TRIAUMUVGPP 9	WEETAS EVEG	TEVOCDEDWONVTD	DSI KI DEWTEEEDTKI KEAVSI HEVOWAKUAI SUDDETDMOODDWTUI HOOFI AA
PmMYB51	CLK PGANTPSERK T	TTEVIKTHOTO	WDDIDD WAGTD	CERSCRI DWI NVI D	DIVERGNIS DEFERTIVOTHOLIGNOWTITACOT DODTINETKNYWNTOI SKD
PmMYB52	PELVKGPWTKEEPE	TVELVKKHGAC K	WSLISO, SVPG.	RICKCCRERWYNHIN	PETKKEAWTOFFELAT TRAHOTYONKWAFTAKYT PORTONA TKNEWNSSAKDKUAS
PmMYB53	GGLERGEWITTEEDO	LSYHVORHGLC	WRTLPK. LAGLS	RCGKSCRLRWANYLR	PDIKRGTFSVHEDINILRLHRILGNKWSAIASHLPGRTDSEIKNHWNTKLSKR
PmMYB54	PELVKGPWTKVEPE R	IVELVSKYGCK K	WSAIAK. HLPG	RIGKCCRE	
PmMYB55	VRRGPWTVDEDMS	LIRCVTTRGEGR	WNTVAKCAGLK	RTGKSCRLRWLNYLR	PDVKRGNITPEEQLLILELHRLWGNRWSKIARQLPGRTDNEIK
PmMYB56	KRTTDGIYSCOSKK K	RLDESASNAIGG	YSNPPDHSLNOFESNPS	ITNSGRAGDILKLISS	KPSTSSSMESNPOSTSSTOTNACDLLASLLLPNKEKESNKKRKOPOTATSSSK
PmMYB57	DRIKGPWSPEEDA A	LOHEVOKYGPR N	WSLISK, AIPG.	RSGKSCRLRWCNCLS	POVEHEPETPEEDATTVEAHAOHGNKWATTAEMISGETDNATKNHWNSTLERRCOG

**Figure 1.** Protein domain analysis of R2R3-MYB in Masson pine. The red lines are the R2 domain, and the green lines are the R3 domain.

## 3.2. Physical Properties of R2R3-MYB TF in Masson Pine

The number of amino acids in MYB proteins ranged from 114 (PmMYB55) to 1721 (PmMYB48), and protein molecular weight ranged from 13.26 kDa (PmMYB55) ~ 189.75 kDa (PmMYB48), with isoelectric points ranging from 4.72 (PmMYB44) to 9.97 (PmMYB18). The protein average hydrophilicity (GRAVY) prediction scores ranged from -1.18 (PmMYB15) ~ -0.227 (PmMYB30), indicating that all R2R3-MYB are hydrophilic proteins. The 29 R2R3-MYB proteins were acidic amino acids with isoelectric points less than 7.0. The remaining 28 proteins with isoelectric points greater than 7.0 were basic amino acids. The instability index was 42.93 (PmMYB56) ~ 71.40 (PmMYB30). Details of physicochemical properties and the length distribution of the R2R3-MYB proteins of Masson pine are shown in Table S2 of the Supplementary Material.

In addition, we found that the R2R3-MYB transcription factors of Masson pine were mainly located in the nucleus and mostly without signal peptides. The PmMYB protein secondary structure was mainly random coils (Table S3), indicating that random coils are the main component of PmMYB protein structure, followed by  $\alpha$ -helices and relatively few extension chains and  $\beta$ -angles. NetPhos3.1 was used to predict the PmMYB proteins' phosphorylation sites. The results showed (Table S4) that serine, tyrosine and threonine phosphorylation sites were present in each gene, and the number of phosphorylation sites was serine > threonine > tyrosine. Based on the identification results of Section 3.1, we selected 49 PmMYBs containing the complete R2R3 structural domain for conserved motif distribution analysis (Figure 2). The results showed five different conserved motifs (Table S1 in supplementary material). All conserved motifs were distributed at the c-terminus of MYB proteins and belonged to the c-terminal conserved structural domain. SMART analysis showed that Motif 1, Motif 2 and Motif 4 were SANT (SWI3, ADA2, N-COR and TFIIIB B) structures, while no functional structural information was obtained for Motif 5 and Motif 3. Motif 1 and Motif 2 were R2 and R3 structural domains, respectively, and there were 47 tf containing Motif 1, accounting for 95.92%. The 45 MYB tf of Motif 2 accounted for 91.84%. In addition, only 15 MYB TF had Motif 3, 5 MYB TF had Motif 4, and 15 MYB TF had Motif 5. In general, although R2R3-MYB genes in the same subclade share common motif characteristics, this does not prove that members of the same subclade have the same or opposite functions.



Figure 2. Analysis of conserved motif elements of Masson pine R2R3-MYB TFs.

To predict the biological functions of the R2R3-MYB transcription factors of Masson pine, we constructed a phylogenetic tree of *Populus trichocarpa* and Masson pine R2R3-MYB transcription factors together. As seen in Figure 3, 49 Masson pine R2R3-MYB TF were classified into 24 subgroups according to the classification of *Populus trichocarpa* R2R3-MYBs [29], and the remaining eight were not classified into taxonomic groups, accounting for 14.29%. This is consistent with previous results of structural analysis of R2R3-MYB proteins in Masson pine.



**Figure 3.** Phylogenetic analysis of the R2R3-MYB TF family in Masson pine and *Populus trichocarpa*. Different background colors and strips are used to distinguish groups. The red asterisks represent Masson pine R2R3-MYB TFs, and the white background refers to hairy poplar R2R3-MYB only, without Masson pine R2R3-MYB is included; blue dots represent unclassified Masson pine R2R3-MYB TFs.

## 3.5. Expression Profile Analysis of Masson Pine R2R3-MYBs

We produced a heat map of the R2R3-MYB genes, derived from RNA-Seq of drought, and divided its expression levels into two groups: rising and falling. As shown in Figure 4, 11 of these genes (*PmMYB4*, *PmMYB12*, *PmMYB8*, *PmMYB33*, *PmMYB40*, *PmMYB29*, *PmMYB27*, *PmMYB34*, *PmMYB43*, *PmMYB23*, and *PmMYB37*) showed an increasing trend.

These results suggest that these genes are responsive to drought in Masson pine. We chose *PmMYB4* as a representative for our study.



**Figure 4.** Heat map of differential expressions of Masson pine *MYBs* in drought stress. The horizontal coordinates T1, T2, T3, T4 indicated the water supply strength, which were 80% ( $\pm$ 5%), 65% ( $\pm$ 5%), 50% ( $\pm$ 5%), and 35% ( $\pm$ 5%), and the ordinate was gene ID. Yellow indicates positive expressions; darker yellow circles indicate higher expression levels. Blue is negative; darker blue circles indicate lower expression.

## 3.6. Expression Profiles of PmMYB4 under Drought Stress

Drought stress was simulated using the 15%  $PEG_{6000}$  treatment. The results showed (Figure 5) that the expression level of *PmMYB4* had a trend of up-regulation followed by down-regulation, peaking at 6 h, which was 3.15-fold up-regulation compared to 0 h. These results indicate that our RNA sequence data are reliable.



**Figure 5.** Expression profiles of *PmMYB4* under 15%  $PEG_{6000}$  mimicing drought stress. Gene expression at 0 h was set to the control value 1. X-axis: 0 h, 3 h, 6 h, 12 h and 24 h are stress durations; *Y*-axis: relative expression. Data in the figure are presented as mean  $\pm$  standard deviation (*n* = 3). The different letters above the bars represent significant differences (*p* < 0.05).

### 3.7. Subcellular Localization of PmMYB4

To understand the spatial information of *PmMYB4* protein in cells, the control vector  $2 \times 355$ ::GFP and the experimental vector  $2 \times 355$ ::PmMYB4-GFP were structured and transfected into tobacco leaves by *Agrobacterium* infestation. The results (Figure 6) indicate that the gene signal comes from the nucleus and there is no fluorescent signal in the cytoplasm and cell membrane, indicating that the protein worked in the nucleus, consistent with the previously predicted subcellular localization.



**Figure 6.** The subcellular localization of *PmMYB4*. (**a**) GFP, green represents the nucleus of the cell; (**b**) DAPI, blue represents the nucleus of the cell; (**c**) bright field; (**d**) merged, the overlap point is the nucleus.

#### 3.8. Response of PmMYB4 to Salt and Drought Stress

The recombinant pET28a-PmMYB4 and the control pET28a were inoculated in four different LB solid media. The results (Figure 7) showed that the growth status of the vector in LB solid medium was consistent with that of the recombinant bacteria under the treatment of 800 mM D-mannitol, 20% PEG<sub>6000</sub> and 400 mM NaCl, while the number of recombinant bacteria was significantly higher than that of the control bacteria.



**Figure 7.** Growth of recombinant bacteria (TransB/pET28a-*PmMYB4*) and control bacteria (TransB/pET28a) on solid LB medium.

### 3.9. The Expression Patterns of PmMYB4 in Masson Pine

To investigate the tissue specificity of *PmMYB4*, the expression levels of the *PmMYB4* gene were examined in 15-year-old Masson pine flowers (F), roots (R), xylem (X), phloem (P), old leaves (OL), young leaves (YL), old stems (OS) and young stems (YS) by qRT-PCR (Figure 8). The expression of the *PmMYB4* gene was highest in the phloem, 120-fold higher than in the flowers, followed by the young stems and young leaves. The *PmMYB4* gene expressed was lower in old leaves, old stem and roots, and almost absent in xylem.



**Figure 8.** Gene expression patterns of *PmMYB4*. The expression level in the flower was set to the value 1. *X*-axis: F, flower; R, root; P, phloem; X, xylem; OL, old leaves; YS, young leaves; OS, old stems; YS, young stem. *Y*-axis: relative expression. Data in the figure are presented as mean  $\pm$  standard deviation (n = 3). The different letters above the bars represent significant differences (p < 0.05).

## 3.10. Promoter Cis-Acting Element Analysis of PmMYB4

The obtained promoter was analyzed using PlantCARE and PLACE online tools. As shown in Figure 9, the sequence contains 33 cis-acting elements in addition to the CAAT-box (40 sites) and TATA-box (77 sites) core elements typical of eukaryotic promoters. It also contains the optical response element G-box, the cis-acting element essential for anaerobic induction (ARE), cis-acting element for MeJA response (CGTCA-motif, TGACG-motif), cis-acting element for ABA response (ABRE), element associated with phenylpropanoid synthesis (SNBE), and MYB binding site for flavonoid synthesis gene regulation (MBSI). Thus, this suggests that the regulation of *PmMYB4* promoter expression may be regulated by photoperiodic and phytohormone signaling, and involved in the regulation of plant stress resistance.

	+ CATTGCCCAT ATTAAAAAAA CTATAAAAAA TAACTAAATT ATAACCTTAT TTTAAATTAT TATTATTATT
	– G <mark>TAAC</mark> GGGTA TAATITITITI GATATITITA AITGATITAA TATIGGAA <mark>TA AAA</mark> TITAATA ATAATAATAA
	+ AATTATAAAA AGAATTAATA TGAGTTAAAC ATATATACAT TTAAAAAAATA TGTAACTAAA TTCTTAACCA
	- TTAATATTTT TC <mark>TTAAT</mark> TAT ACTCAATTTG TATATATGTA AATTTTTTAT ACATTGATTT AAG <mark>AATTGG</mark> T
	+ TGAAGATTCA TCCCATTTTA CCAATAAAAT TCTTAATATG TAGATAGTTA TTGTTTTGAG CTTTTGTATC
	- ACTECTANGE AGGGEAAAAT GGTTATTTTA AGAATTATAC ATCTATCAAT AACAAAACTC GAAAACATAG
	CTTCATABAT BATTATTCTT SCARTCCAT CCCCCATATAC CATCCCTTAT CARTACCACA TCCCCATATAT
	- GAACTATTA TTAATAACAA TGTTAAGGTA CCCGGTTATG GTACCGAATA GTTATGCTGT ACGGGTTATA
	+ GACATGIGTT GATCAATATG AAATTITCTT TAATTICTTA ATTIGAGATA AATTAATTAA AAGAGCATAA
AAGAA-motif	- CIGIACACAA CIAGIIAI <mark>AC IIIAAA</mark> AGAA AIIAAAGAAI <mark>IAAAC</mark> ICIAI IIAAIIAAII IICICGIAII
ABRE	+ TTAATCGGAA GATAAATCTT AACCCTAGAT CTATGAATAC TACTACAAAT ATTTAAACAT TCATGGAAG
ABRE3a	- AATTAGC <mark>UIT CHAITHAG</mark> AA IIIGGGAICIA GAIACIIAIG AIGAIGIIITA IAAAIIIGIA AAGIACCIIC
ABRE4	+ TTAAAATCTCT ATCACCCACC TAATAATTAA AAAAATTGTA AAAATTAATTT TTAAAAATTAA CAATATATTT
AE-box	- AATTTAGAGA TAGTGGGTGG ATTATTAATT TTTT <mark>TAAC</mark> AT TTTAATTAA <mark>A AATTTTA</mark> ATT GTTATATAAA
ARE	+ TAGTTATCTC ACTAAATGTC CTTATAAAAA CAAACATCGT CATTATTTAT ATGAAAAAAA CCCTTATATT
ATCT-motif	- ATCAATAGAG TGATTTACAG GAATATTTTT GTTTGTAGCA GTAATAAATA TACTTTTTTT GGGAATATAA
ATCI-MOUII	+ TITTAAAACT ATATTATAAT TITTATAAA ATGCTTTTGA TAACTACATA TAAACCTACA TATTAATAAT
AT~ABRE	- AAAATTTTGA TATAATATTA AAAAATATTT TACGAAAACT ATTGATGTAT ATTTGGATGT ATAATTATTA
AT~TATA-box	
Box 4	+ AIGIAIAIAI AIAAAAIAAA AIAIAIAIIG IIAIAGUAIC GCIIGIICG <mark>A GAAALAAIIG</mark> GGIAGIIAGC
CAAT-box	
CGTCA-motif	+ TTAGACATTG ACTTT <mark>GAAAG AA</mark> TGAAGTTG TTGAAGATAG ACCTTGACAT GTACATGGCA TAATGTAAAC
ERE	- AATCIGIAAC IGAAACITIC ITACITCAAC AACITCIATC IGGAACIGIA CAIGIACCGI AITACAITIG
G-Box	+ CACTITIGITA TAAAACTCIC ITTACITCGI ICTATATAAG ATTATAAAAG ATTAGIIIGII ATACCITATA
G-box	- GTGAAACAAT ATTTTGAGAG AAATGAAGCA AGATATATTC TAATATTTC TAATCAACAA TATGGAATAT
CTI matif	+ CGAAGGTTGT CCTAATTCAT TTTAAGGAGG GACAATCATA AAAAAATTCA GTGTTATGAT ATACCAGGAT
GII-motii	- GCTTCCAACA GGATTAAGTA AAATTCCTCC CTGTTAGTAT TTTTTTAAGT CACAATACTA TATGGTCCTA
MBS	+ TEGATTEATE ATERATING CAMPETERS CTAATEATTT AAAAAATATA GAGCATTESE ATETATEATA
MBSI	- ACCTAACTAG TACGTTAGAG GTTTCATGTG GATTAGTAAA TTTTTTATAT CTCGTAACCG TACATACTAT
MYB	
MYB-like sequence	- TIGGGANGTA TCANTACGTA ATATATCACT ACTATTATTT CATTATTAC GGTATATATA CONTACGCA
MYC	
Myb	+ ATTATATTTG TACGTGTCGG TGCATGCACC GACATCAAAA ACAAACTTAT TGGGGGGGTAA AATTTCGCTG
Myb-binding site	- TAATATAAAC AIGCACAGCC ACGTACGIGG CIGTAGIIII IGIIIGAATA ACCCCCCATI TIAAAGCGAC
тата	+ GGCATTCCTT TCGTGCGTGA CGACATTCCT TTCACTCTAA TTCAAAGGGA GAACTTGTTT GAGGGCCAAG
TATA boy	- CCGTAAGGAA AGCACGCACT GCTGTAAGGA AAGTGAGATT AAGTTTCCCT CTTGAACAAA CTCCCGGTTC
TATA-DOX	+ TCGTGTCGAG ACTGTTGTTG CGTTGAAAGC ACATCTTAT <mark>T ATAAT</mark> GATGA TGAGTAGAGA AGGGATTGAT
TCA	- AGCACAGCTC TGACAACAAC GCAACTTTCG TGTAGAATAA TAT <mark>TACTACT ACT</mark> CATCTCT TCCCTAACTA
TCCC-motif	+ TAACTTAAGA GTGAAGAAAC ATACGGACAG ATAGATTAGA TTTGGCACGA ATGATTTATG CTTGTACAGG
TGACG-motif	- ATTGAATTCT CACTTCTTTG TATGCCTGTC TATCTAATCT AAACCGTGCT TACTAAATAC GAACATGTCC
Unnamed_1	+ AACACTGAAG TAAAATCCCT GCTCATCCTT CGATTTACCG TTAACGAATG AATAGTTGAA GTTGACGCTG
Unnamed4	- TIGTGACTIC ATTITAGGGA CGAGTAGGAA GCTAAATGGC AATTGCTTAC TIATCAACTI CAACTGCGAC
WRE3	
as-1	+ GCGAATAICIT ATAAICITCI GGITTGCGGC CICCAAIGGC GGAATICCIT IGGAGAAAAG AAIGATTAGC
	- COLITATADA TATTADANDA CLAMACOCCO DADDITACCO CLITANDAR ACCICITITO TIRCIARICO
	+ TGTTGATGAG AAATATAATA ATGTACAACA ATGATCCGTA TTGAAAGCAA AGAGTTGGTT GCCCACGTTG
	- AUAAUIAUIU IIIAIATTAT TACATGTTGT TACTAGGCAT AACITICGIT TCTCAACCAA CG <mark>GGTGC</mark> AAC
	+ TAATGATTGA AGATTGACGA TGGGAGAAAA TG <mark>ATGTTCTC TGAGGG</mark> TACC CACCATACAA CACACGTT
	- ATTACTAACT TCTAACTGCT ACCCICTTTT ACTACAAGAG ACTCCCATGG GTGGTATGTT GTGTGTGCAA
	+ TGATGIGIGI GIGITIGAAT AAAIGAACGG AGCCCIGAGC IGCICAGIIG CIGGIGAGCG CACITAAGAA
	- ACTACACACA CACAAACTTA TTTACTTGCC TCGGGACTCG ACGAGTCAAC GACCACTCGC GTGAATTCTT
	+ CIGACACAAG CACGGCCATT CCGTTCATTC TGCGGGTGAA AATGAGCTGC ALANDAGGAG GALLCHCCCCC
	- GACTGIGTTC GTGCCGGTAA GGCAAGTAAG ACGCCCACTT TTACTCGACG TGTTGTCCTC CTGAGAGAGAG
	+ TOCCAPCTCC PARCORNEC TARCEARCG
	A CONSTRUCT TALOUTROL AND A CONTROL

Figure 9. Cis-acting element analysis of *PmMYB4*.

## 3.11. Expression Profiles of PmMYB4 under Abiotic Stress

The corresponding abiotic stresses were applied to 2-year-old Masson pine under the analysis of cis-acting elements of the promoter. As shown in Figure 10, the *PmMYB4* gene showed a down-regulation trend under ABA treatment within 24 h. After ETH treatment, the *PmMYB4* expression exhibited a trend of first down-regulation and then up-regulation,

peaking at 24 h, which was 1.52 times higher than that at 0 h. The *PmMYB4* gene expression was down-regulated under SA treatment and up-regulated with increasing stress time at 24 h, but not greater than 1. The *PmMYB4* expression was significantly down-regulated under  $H_2O_2$  stress induction. After injury stress treatment, the *PmMYB4* expression was first up-regulated and then down-regulated, with a peak at 6 h, which was 6.48 times higher than that at 0 h.



**Figure 10.** Analysis of *PmMYB4* gene expression under different stress. Gene expression at 0 h was set to the control value 1. *X*-axis: 0 h, 3 h, 6 h, 12 h and 24 h are stress durations; *Y*-axis: relative expression. Data in the figure are presented as mean  $\pm$  standard deviation (n = 3). The different letters above the bars represent significant differences (p < 0.05).

# 4. Discussion

The R2R3-MYB TF is considered to be a pivotal transcription factor in plants [41] for response to abiotic stresses. It has been reported that a large variety of plants contain 70~200 R2R3-MYB TF [29,42]. In this study, a total of 49 R2R3-MYBs were authenticated in four transcriptome data and the number of PmMYBs was lower than in *Glycine max* (244) [43], *Populus trichocarpa* (192) (167) [44], *Linum usitatissimum* (167) [45], *Oryza sativa* (102) [46], *Prunus salicina* (96) [47] and *Ananas comosus* (94) [48]. These results indicate that the number of R2R3-MYB genes varies widely among species. Furthermore, in terms of structural domain structure, not all MYB proteins have a complete R2R3-MYB structural domain, which is consistent with the findings of Song [49].

The physicochemical properties of 49 R2R3-MYB proteins of Masson pine were analyzed, and they were found to be hydrophobic proteins with large instability coefficients and unstable proteins, of which 29 R2R3-MYB proteins were acidic proteins. The subcellular localization results showed that R2R3-MYB is mainly located in the nucleus, indicating that R2R3-MYB mainly functions in the nucleus, also consistent with the findings of Song [50]. Most of them do not contain transmembrane structures and signal peptides, suggesting that their main working location is in the nucleus. The conserved motifs of Vaccinium R2R3-MYB genes [51] were reported to be essentially the same for the same subfamily of genes, and this was further supported by the conserved motif analysis in this study. Primary structures in subgroups are strongly correlated with the biological functions of proteins, so identifying homologous genes among plants based on phylogenetic relationships can help predict gene function [52]. A phylogenetic tree was constructed together with *Populus* trichocarpa [29] R2R3-MYB with the aim of further studying the biological functions of R2R3-MYB in Masson pine. All PmMYB proteins were clustered into functional groups of Populus trichocarpa, which were divided into 24 subfamilies. As shown in Table S5, members of subpopulation S1 are response to biotic and abiotic stresses [31], members of subpopulation S5 are involved in anthocyanin biosynthesis [53], members of subpopulation S9a are involved in trichome development [54], and members of subpopulations S13 and

S37 play key roles in lignin biosynthesis [29,55], among others. Therefore, it is hypothesized that R2R3-MYBs in these subgroups of Masson pine also have similar functions.

The ABA is an important drought stress messenger molecule. Under drought stress, plant ABA concentration increases and the root system transmits signals to aboveground leaves and stems, regulating stomatal closure and reducing transpiration; ABA can participate in the plant drought response process by inducing differential expression of downstream drought genes. The ABA-dependent signaling pathway initiates cis-acting elements containing similar elements mainly through binding to NAC, MYB, MYC, and ABRE downstream gene expression to counteract drought; for example, OsMYB2 [56], CcMYB-R48 [57] and *PtrMYB94* [18] regulate downstream gene responses that mediate plant responses to ABA and drought. The MYB has been demonstrated to be related to drought stress in plants [58]. Under adversity stress, 42 MYB genes showed transient up-regulated or down-expression in wheat [59], indicating that their gene expression levels are temporally and spatially specific under adversity. In the present study, there were a grand total of 18 R2R3-MYB genes performed as upward or downward adjustment, among which the expression of PmMYB4 was particularly significant, so we selected the PmMYB4 gene for analysis. We found ABA-responsive elements in the promoter of this gene along with MYB and MYC elements, indicating that in gene expression, it received ABA regulation. The above conclusion is further supported by the significant down-regulation of the gene in ABA hormone stress treatment. Thus, PmMYB is engaged in the ABA-dependent regulation of signaling pathways.

However, not all drought genes are regulated by ABA and a considerable number of drought genes are not controlled by ABA, thus drought signaling pathways can be broadly classified into ABA-dependent and ABA-independent [60] based on whether they depend on ABA signaling pathways. The ABA-independent signaling pathways, on the other hand, initiate downstream gene expression through DRE and ABRE elements [61]; for example, *OsM1D1* [61] and *OsMYB6* [62] regulate drought-related genes, but are not responsive to exogenous ABA. Coincidentally, we also found ABRE elements in the *PmMYB4* promoter, and therefore hypothesized that *PmMYB4* genes are jointly involved in non-ABA-dependent and ABA-dependent drought stress response signaling pathways. In addition, extensive research has indicated that lignin deposition also enhances drought resistance in plants. The tube walls of drought-tolerant plants are thicker than those of less drought-tolerant plants [63]. Both *MdMYB88* and *MdMYB124* can enhance drought adaptation in apple plants through improved lignin deposition [64]. Coincidentally, Yao's study showed that *PmMYB4* is homologous to *AtMYB46*, and overexpression of *PmMYB4* in tobacco leads to xylem thickening and ectopic lignin deposition [65]. While MYB46 has also been shown to be associated with salt tolerance in apple [66], birch MYB46 can cope with drought and salt stresses [67], suggesting a potential function of *MYB46* under stress induction. By analyzing the expression of *PmMYB4* in different tissues, it was found that *PmMYB4* expression was highest in the phloem, followed by young stems and young leaves, which may be related to the involvement of *PmMYB4* in lignin deposition. This result can also support the above conclusion, indicating that PmMYBs are involved in drought regulation in multiple ways.

After constructing the prokaryotic expression vector, the gene was initially characterized using *E. coli*, which has the advantages of short time and easy operation [68]. Gong [69] constructed a Lavandula pET28a *DXS* expression vector and was successfully expressed in TransB (DE3). To further investigate the role of the *PmMYB4* gene in drought tolerance, TransB/PET28a *PmMYB4* was successfully induced in this study. The growth condition of both bacteria was similar on normal LB solid plates, while the recombinant strain was stronger on 800 mM D-mannitol, 20% PEG6000 and 400 mM NaCl in LB solid plates. This shows that, under drought stress, the *PmMYB4* gene can promote the growth of *E. coli*. These results provide further evidence that R2R3-MYB transcription factors play a function in drought response. In addition, it showed positive regulation in response to drought stress, which is consistent with the results of abiotic stress response experiments. It is hypothesized that this gene plays a part in the stress response of Masson pine.

Plants are never homogeneous in their resistance to adversity, but rather have a diverse and complex regulatory network [70]. Under drought stress conditions, reactive oxygen content increased more in poorly tolerant plants, and overexpression lines were more drought resistant than wild type [71]. In apple, overexpression of *MdMYB46* could improve salt tolerance. Different MYB46 knockout lines were able to maintain normal secondary growth, but pathogen-induced *Ep5C* gene expression levels were increased in these mutants, and Arabidopsis exhibited greater resistance to Boreal fungus [71]. These results seem to prove the existence of different regulatory modes of MYB46 in response to stress. In addition, MeJA, anaerobic induction and abiotic stress-related elements are present in the *PmMYB4* promoter, and the expression of the *PmMYB4* gene was significantly altered after MeJA, SA, ETH and H<sub>2</sub>O<sub>2</sub> treatment, indicating that the gene was also involved in other stress regulatory networks. In addition, the promoter of this gene contains MBSI, the MYB binding site regulated by the flavonoid biosynthetic gene. These results seem to demonstrate that in response to stress, *PmMYB4* has different regulatory modes, further suggesting that the stress modulation network of the MYB gene family is complex and diverse, with multiple modes interacting to counteract stress.

#### 5. Conclusions

In the transcriptome of Masson pine, 49 R2R3-MYB transcription factors were characterized and divided into 24 sub-groups. They contained complete R2 and R3 structures, and more than 95.92% of R2R3-MYB TF contained conserved motif SANT structure. Transcriptome analysis showed that 18 genes were responsible for drought stress in Masson pine, *PmMYB4* being one of them, which could promote the growth of recombinant bacteria in a drought environment. The expressed of *PmMYB4* was dramatically increased by PEG6000 under drought stress. The expression of *PmMYB4* was found in many parts of Masson pine, and the highest amount of expression was observed in the phloem. The *PmMYB4* was triggered by drought, ABA, MeJA and SA. These results suggest that *PmMYB4* is implicated in the response to drought stress, other abiotic stresses and exogenous hormone responses in Masson pine.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14020410/s1.

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