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Cloning and Expression Analysis of MaMYB308 Gene in Mulberry

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Abstract In order to analyze the function of MYB transcription factors in mulberry abiotic stress and development, the full-length cDNA sequence of MYB transcription factor *MaMYB308* was cloned from the mulberry cultivar 'Guiyou 62'. The amino acid sequence of MYB transcription factor MaMYB308 was analyzed by bioinformatics, and its self activation was verified by yeast single hybridization. The expression pattern of the gene under different tissue and abiotic stresses was detected by real-time fluorescence quantitative PCR. The results showed that the full length of *MaMYB308* gene is 1 266 bp, including an open reading frame of 1 026 bp, which can encode 341 amino acids, and its molecular weight is 39 kD; it has two SANT domains, belonging to typical R2R3 type MYB transcription factor, and has self activating activity. Blast results showed that MaMYB308 had high homology with a variety of R2R3-MYB transcription factors from higher plants, and had the highest homology with MYB308 from *Morus notabilis*, and the consistency of amino acid level was 96.48%. The results of real-time fluorescence quantitative PCR showed that the expression of *MaMYB308* in roots, stems and leaves of mulberry was significantly higher than that in stems and leaves, and it was responsive to abiotic stresses such as high temperature, low temperature, high salt and drought. Among them, the expression of *MaMYB308* was the most significant under low temperature stress, about 40 times of the control. The results of this study could be helpful for further study on the biological function of mulberry *MaMYB308* gene and the stress resistance mechanism of mulberry.
Keywords Mulberry; R2R3 MYB transcription factor; Abiotic stress; Gene expression

MYB (V-myb avian myeloblastosis viral oncogene homolog) transcription factor is one of the largest transcription factor families in plants, widely present in eukaryotes. The N-terminal contains a highly conserved DNA-binding domain, and the C-terminal usually contains a foldable amphiphilic helix for transcriptional activation, responsible for protein regulatory activity. MYB transcription factors can be divided into 4 categories, namely 1R-MYB, R2R3-MYB, 3R-MYB and 4R-MYB, due to the number and location of repeated sequences in their DNA-binding domain. R2R3-MYB is the largest transcription factor. It is extensively involved in primary and secondary metabolism, growth and development regulation, organ formation, hormone signal transduction and stress response of plants (Niu et al., 2016). At present, multiple R2R3-MYB family members have been identified in Arabidopsis and rice (Chen et al., 2006; Dubos et al., 2010), abundant evidence suggests that they can respond to biotic or abiotic stresses, including pests and diseases, and drought. Arabidopsis *AtMYB44* regulates its resistance to green peach aphid and diamondback moth by activating an Ethylene-insensitive protein 2 (EIN2) -mediated defense system in *Arabidopsis thaliana* (Lü et al., 2013). *AtMYB96*, *AtMYB15*, and *AtMYB2* can act as positive regulators to activate transcription of responsive to dehydration 22 (RD22) to enhance drought resistance in *Arabidopsis thaliana* (Abe et al., 2003; Seo et al., 2009). In addition, *AtMYB15* is a positive regulator of drought resistance. It can also improve freeze tolerance by inhibiting the expression of *CBF1*/*DREB1* (C-repeat binding factors/dehydration response element binding factors1) genes. It is known as a negative regulator of freezing tolerance (Agarwal et al., 2006). *OsMYB4* is involved in gene expression of different cold induction pathways in rice. Transient expression of *OsMYB4* can activate cold-induced promoters such as PAL2 and SAD (stearoyl-Δ9 desaturase) in phenylalanine ammonia-lyase gene family (Vannini et al., 2004). The MYB binding element, found in promoters of many functional genes, binds to MYB transcription factors to activate the

expression of stress-responsive genes in response to stress. The functional study of MYB transcription factor has important guiding significance for the mechanism of plant resistance to stress and the cultivation of stress-resistant crops (Albert et al., 2014).

Mulberry (*Morus* spp.) is one of the important cash crops and plays an important role in the silkworm industry chain. Its developed root system and strong adaptability to soil and environment also play an important role in ecological restoration. The *M. notabili* genome was sequenced and about 128 MYB transcription factors were predicted (He et al., 2013; Li et al., 2016), but most members have not been reported in functional studies, and a few studies on the function and regulatory mechanism of MYB transcription factor genes in Mulberry mainly focus on the synthesis of flavonoids and anthocyanins (Li et al., 2016). Currently, there is still a lack of studies on the mechanism of MYB transcription factor in mulberry stress response. In the previous study, we analyzed the transcript*ome of Morus atropurpurea* infected with mulberry vein banding-associated virus (MVBaV) using mulberry leaves without virus infection as control. The differentially expressed MYB transcription factor *MaMYB308* gene of mulberry was screened. In order to further study the function of *MaMYB308* gene, the full-length cDNA sequence of *MaMYB308* was cloned by RACE (Rapid-amplification of cDNA ends) technology in this study. On this basis, bioinformatics analysis of the sequence was conducted, and the expression pattern of this gene was analyzed by fluorescence quantitative PCR. In order to clarify the biological function of MaMYB308 and provide theoretical basis for the genetic improvement of mulberry resistance.

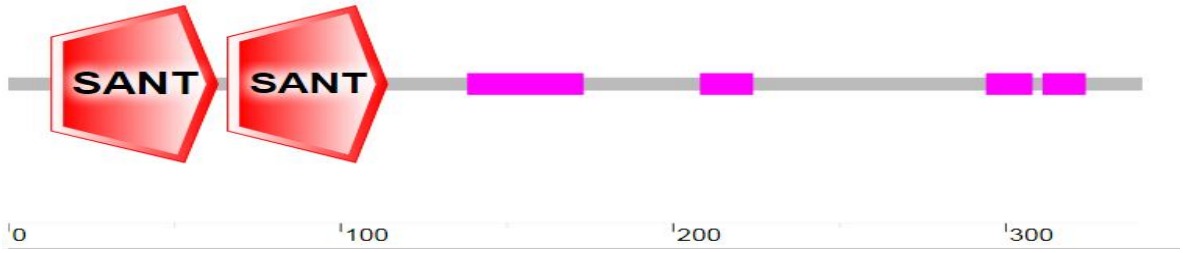
1 Results and Analysis

1.1 Cloning and sequence analysis of full-length cDNA of *MaMYB308* gene from mulberry

Using cDNA obtained by reverse transcription of total RNA from mulberry leaves as template, the 3' and 5' ends of *MaMYB308* were amplified by PCR. After spliced, the full-length cDNA sequence of *MaMYB308* was obtained, with a total of 1 266 bp, including 215 bp of UTR region. The 5' untranslated region was 25 bp, and the coding region was 1 026 bp, encoding 341 amino acids. Online Pfam analysis showed that MaMYB308 had two conserved DNA-binding domains at 14~61 bp and 67~112 bp (Figure 1A), and the gene was predicted to contain two SANT domains by SMATR website (Figure 1B). These results indicate that MaMYB308 is a typical R2R3-MYB transcription factor.

Family	Description	Entry type	Clan	Envelope		Alignment		HMM		HMM length	Bit score	E-value
				Start	End	Start	End	From	To			
Myb_DNA-binding	Myb-like DNA-binding domain	Domain	CL0123	14	61	14	61	1	46	46	48.4	8.0e-13
Myb_DNA-binding	Myb-like DNA-binding domain	Domain	CL0123	67	112	67	112	1	46	46	51.4	8.8e-14

A



B

Figure 1 Prediction of conservative domain of MaMYB308 protein

Note: A: DNA binding site of MaMYB308; B: SANT domain site of MaMYB30

1.2 Bioinformatics analysis of *MaMYB308* gene in mulberry

1.2.1 Physicochemical properties analysis of mulberry MaMYB308

The molecular formula of MaMYB308 is $C_{1689}H_{2667}N_{523}O_{524}S_{10}$ and the theoretical isoelectric point is 9.00, which is an alkaline amino acid, according to ProtParam online software analysis. The protein had 40 negatively charged (Asp+Glu) and 47 positively charged (Arg+Lys) residues. The instability coefficient was 41.16, Classified as unstable proteins and the average fat coefficient was 69.15. ProtScale tool was used to analyze the

hydrophilicity/hydrophobicity of MaMYB308 transcription factor protein, and the results showed that the protein was a hydrophilic protein. Online prediction by TMHMM showed that MaMYB308 protein did not contain transmembrane domain. SignalP online prediction found that there was no signal peptide structure in MaMYB308, indicating that the protein was not a secreted protein. Subcellular localization analysis using Plant-mPLoc software showed that the transcription factor MaMYB308 was located in the nucleus.

1.2.2 Secondary structure and tertiary structure of mulberry MaMYB308

The secondary structure of MaMYB308 protein was predicted by SOPMA online website. The secondary structure of MaMYB308 protein contained 23.75% α-helix structure, 15.84% extended chain structure, 7.33% β-corner structure and 53.08% irregular crimp structure (Figure 2). Homology modeling of MaMYB308 protein was conducted using Swiss-model website, and it was found that the tertiary structure model of MaMYB308 protein was consistent with the predicted results of secondary structure, with more α-helix and irregular crimp structure (Figure 3).

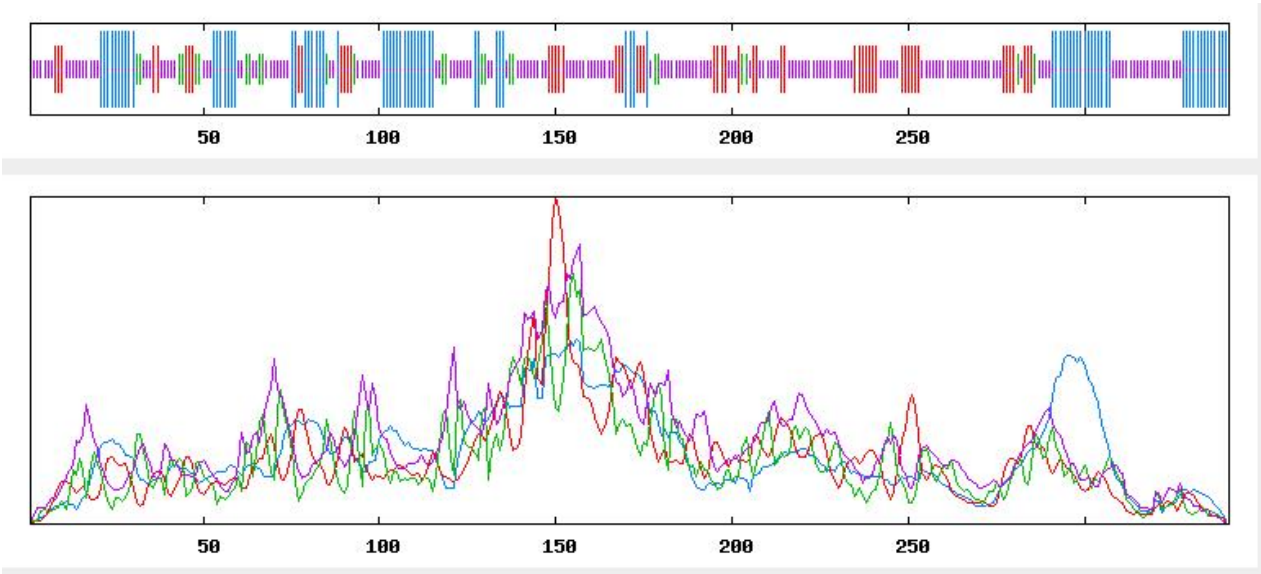


Figure 2 Prediction of secondary structure of MaMYB308

Note: Red: Extended chain structure; Purple: Irregular crimp structure; Blue: α - helix structure; Green: β-corner structure

Figure 3 Predicted 3D structure model of MaMYB308 protein

1.2.3 Homology analysis of mulberry MaMYB308

The amino acid sequence encoded by *MaMYB308* was BLAST in NCBI database. The results showed that MaMYB308 had high homology with R2R3-MYB transcription factors of various higher plants, and the homology with *M. notabilis* was the highest, the consistency of amino acid level was 96.48%. *Ziziphus jujuba*, *Parasponia andersonii*, *Trema orientale*, *Morella rubra*, *Herrania umbratica*, *Theobroma cacao*, *Pistacia vera*, *Durio zibethinus*, *Hibiscus syriacus*, *Cephalotus follicularis*, *Prunus yedoensis* var. Nudiflora and *Prunus avium* are between 53.35%~57.43%. Multiple sequences were compared between MaMYB308 and MYB amino acids of different plants using DNAMAN software, and it was found that the N-terminal amino acids of these proteins were highly conserved, with similar R2 and R3 domains, while the C-terminal sequences were more diversified (Figure 4), in line with the structural characteristics of R2R3 MYB transcription factors. It is speculated that MaMYB308 has the same or similar biological functions as R2R3-MYB transcription factors such as

ZjMYB308L.

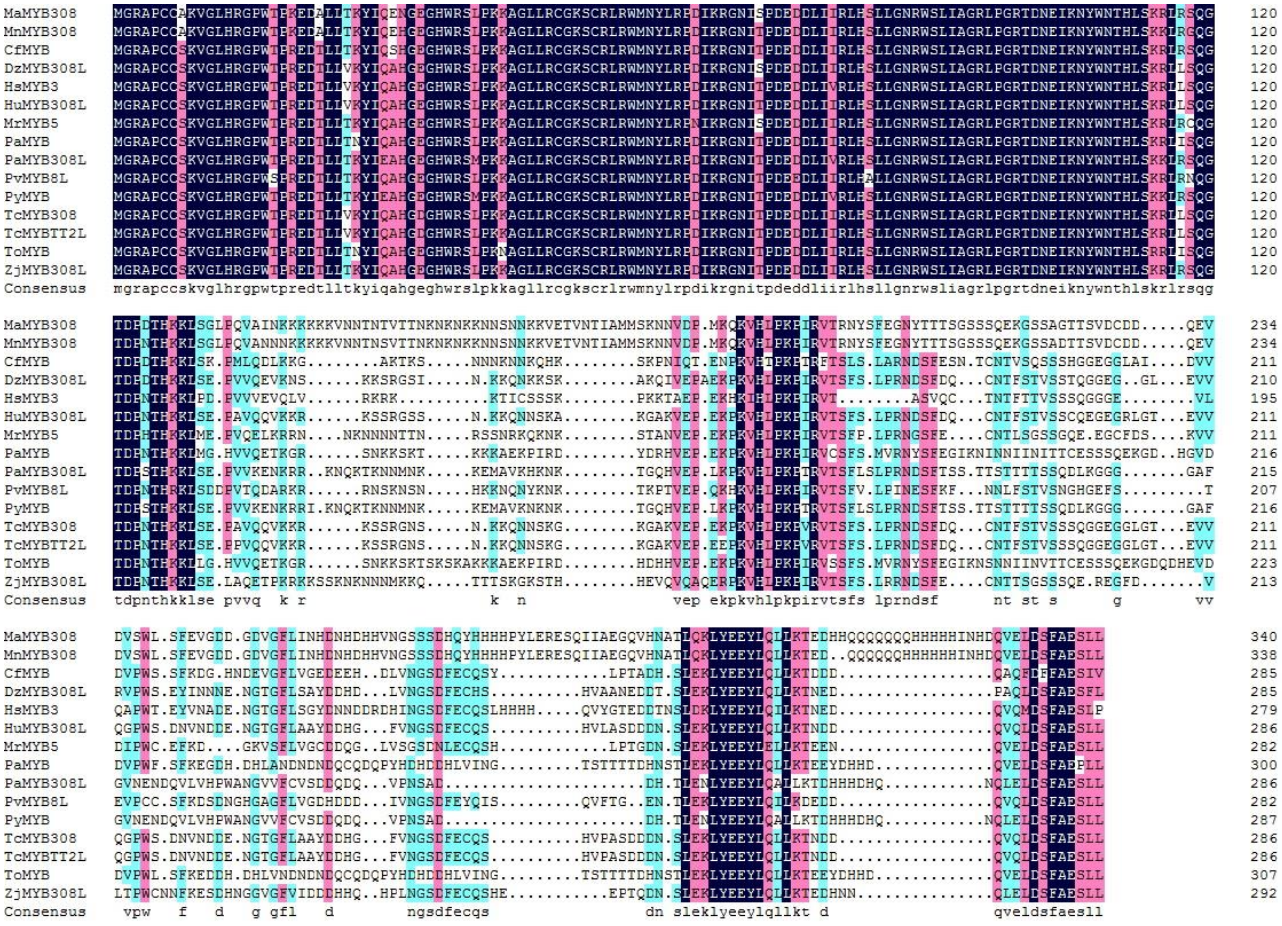


Figure 4 Multiple alignment of amino acid sequence between mulberry MaMYB 308 and other species

Note: MnMYB308: *Morus notabilis* (XP_010100709.1); AtMYB5: *Arabidopsis thaliana* (NP_187963.1); CfMYB: *Cephalotus follicularis* (GAV76866.1); DzMYB308L: *Durio zibethinus* (XP_022769742.1); HsMYB3: *Hibiscus syriacus* (KAE8671353.1); HuMYB308L: *Herrania umbratica* (XP_021280377.1); MrMYB5: *Morella rubra* (KAB1219664.1); PaMYB: *Parasponia andersonii* (PON53991.1); PaMYB308L: *Prunus avium* (XP_021815015.1); PvMYB8L: *Pistacia vera* (XP_031285530.1); PyMYB: *Prunus yedoensis* var. Nudiflora (PQQ21692.1); TcMYB308: *Theobroma cacao* (XP_007051504.1); TcMYBTT2L: *Theobroma cacaor* (ADD51352.1); ToMYB: *Trema orientale* (PON97266.1); ZjMYB308L: *Ziziphus jujuba* (XP_015888701.1)

1.3 Mulberry MaMYB308 has transcriptional self-activation activity

To verify the transcriptional activation activity of MaMYB308, MaMYB308 was linked to the pGBKT7-BD vector with GAL4 DNA binding domain, and the recombinant plasmid PGBKT7-MaMYB308 was obtained. The recombinant plasmid pGBKT7-MaMYB308 was transformed into yeast Y2H gold strain with pGBKT7 (control). The results showed that yeast carrying recombinant plasmid pGBKT7-MaMYB308 and pGBKT7 could grow on single absence medium SD/-TRP. The yeast transferred to pGBKT7 could not grow normally on the triple-deficiency medium SD/-Trp-Ade-His, and the colony was white on the SD/-Trp+X-α-Gal medium, while the yeast transferred to pGBKT7-MaMYB308 could grow normally on the SD/-Trp-Ade-His medium. And the colonies in SD/-Trp+X-α-Gal medium were blue (Figure 5), indicating that MaMYB308 had transcriptional self-activation activity.

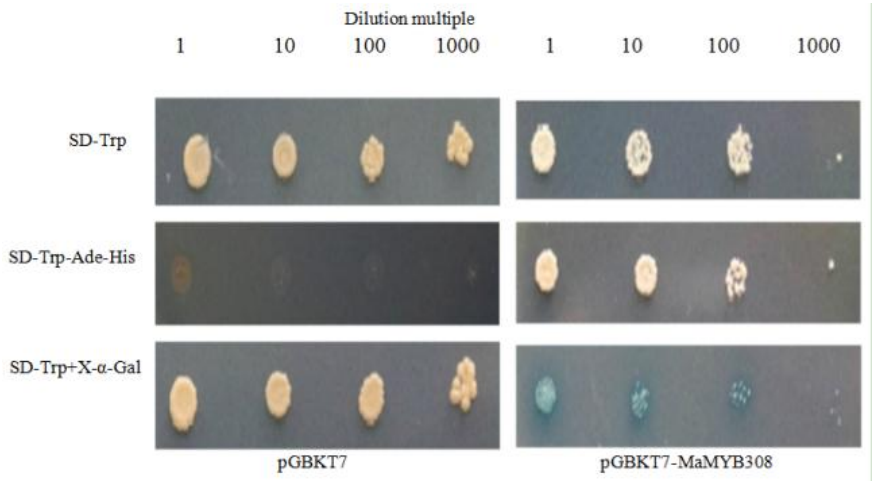


Figure 5 Analysis of transcription activation function of MaMYB308

1.4 Expression characteristics of mulberry *MaMYB308*

1.4.1 Tissue expression characteristics of mulberry *MaMYB308*

In order to explore the expression of *MaMYB308* in different organs, roots, stems and leaves of mulberry seedlings aged 6 to 7 were collected for fluorescence quantitative PCR, and the results showed that *MaMYB308* was expressed in roots, stems and leaves, and the expression level in roots was significantly higher than that in stems and leaves, which was 20 times of that in stems and about 30 times of that in leaves (Figure 6). It is speculated that this gene may play an important role in the formation of mulberry roots.

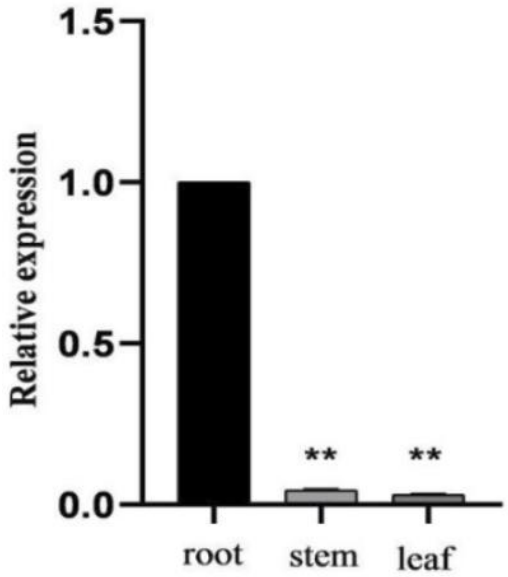


Figure 6 Expression pattern of *MaMYB308* among different tissues of mulberry

Note: ** means very significant different with root at $p<0.01$ level

1.4.2 Expression pattern of *MaMYB308* in mulberry under abiotic stress

The expression of *MaMYB308* in mulberry leaves under abiotic stress was detected by fluorescence quantitative PCR method after 6 to 7 leaf age mulberry seedlings were treated with high temperature, low temperature, high salt and drought. The results showed that under drought stress (Figure 7), the expression level of *MaMYB308* gene in leaves was up-regulated from 1 to 12 h and significantly increased at 12 h, and reached the highest level, which was about 30 times that of the control group. After high salt treatment, the expression level of *MaMYB308* gene in leaves decreased slightly at 1 h, presented an up-regulation trend at 6 h, increased significantly at 12 h and reached the highest point at 12 h, which was about 6 times that of the control group, then decreased sharply and was lower than that of the control group at 24 h. After high temperature treatment, the expression level of *MaMYB308* gene

did not change significantly at 1 h, but increased to 4 times of the control group at 6 h, and decreased to about 2 times of the control group at 12 h, and then increased sharply and reached the highest point at 24 h, which was about 26 times of the control group. After low temperature treatment, the expression level of *MaMYB308* in leaves was significantly upregulated to about 30 times that of the control group at 1 h, and continued to significantly increase to the peak at 6 h, which was 45 times that of the control group. At 12 h, it decreased sharply, which was 20 times that of the control group. At 24 h, the expression level of *MaMYB308* was significantly upregulated to about 40 times that of the control group. After abiotic stress, the expression level of MaMYB308 in leaves showed varying degrees of up-regulation, suggesting that MaMYB308 was involved in the response of mulberry to drought, high salt and temperature stress signals, and was most sensitive to low temperature stress signals.

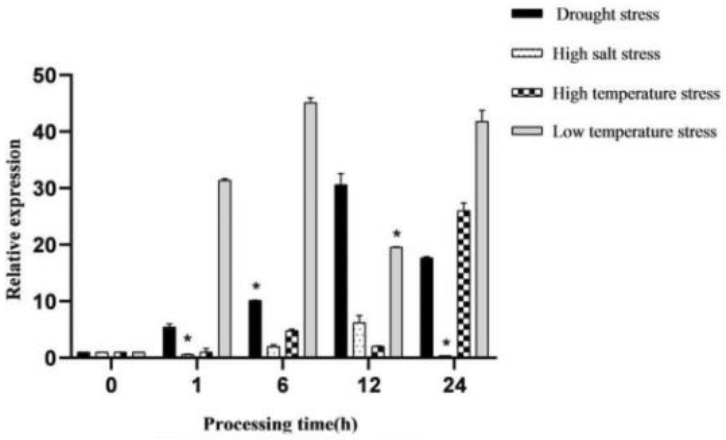


Figure 7 Expression analysis of *MaMYB308* gene under four abiotic stresses
Note: * means very significant different with root at 0.05 level

2 Discussion

Most plant MYB genes encode R2R3-MYB transcription factors, which are widely involved in primary and secondary metabolism, development processes and responses to biological and abiotic stresses in plants (Dubos et al., 2010). In this study, *MaMYB308* gene was cloned from Mulberry variety 'Guiyou 62', which has two DNA binding domains and is a typical R2R3 MYB transcription factor. It was confirmed that *MaMYB308* gene was expressed in mulberry root, stem and leaf tissues, and the highest expression level was found in roots. Abiotic stress such as high temperature, low temperature, high salt and drought can induce its expression.

The spatio-temporal expression patterns of some genes in plants are specific and closely related to their biological functions. In *M. multicaulis*, three R2R3-MYB genes (*MmMYB1*, *MmMYB2*, *MmMYB3*) showed a significant increase in root primordia differentiation in hardwood cuttings, and played a positive role in the differentiation of basal thin-wall tissue of cuttings. Thus, promoting adventitious root formation (Du, 2016). In this study, *MaMYB308* had the highest expression level in roots, suggesting that it might play a regulatory role in morphogenesis of mulberry root. However, in *M. notabilis*, the 6 R2R3-MYB genes related to anthocyanin synthesis were highly expressed only in mulberries, and the expression level was very low in roots or even no corresponding transcripts were detected (Li et al., 2016), indicating that different R2R3-MYB genes may be involved in different life processes of mulberry. Cloning more R2R3-MYB genes from mulberry trees will help to fully understand the function of these genes.

Many plant R2R3-MYB transcription factors respond to abiotic stress. Transformation of *Jatropha curcas JcMYB1* gene can significantly improve the tolerance of transgenic tobacco to drought and salt stress (Li et al., 2014). Previous studies have cloned *AmMYB44* from *Astragalus membranaceus*, which has multiple cis-elements on its promoter related to stress and hormone response, and plays an important role in regulating *Astragalus membranaceus* against abiotic stress (Li et al., 2019). In this study, after abiotic stress (high salt, drought, high temperature and low temperature) treated mulberry seedlings, the expression level of *MaMYB308* was

up-regulated to varying degrees, and the up-regulated level was the most significant after low temperature treatment. It is speculated that *MaMYB308* can combine with corresponding cis-acting elements to regulate the expression of downstream related genes after drought, high salt stress and low temperature induction, and *MaMYB308* is more sensitive to low temperature signal, so it is speculated that *MaMYB308* gene plays an important role in cold resistance of mulberry. Most of the existing studies showed that plant *MYB308* was involved in the regulation of secondary metabolite synthesis. Pomegranate *Pg MYB*308 gene may play an important role as a negative regulator of lignin synthesis in pomegranate grains (Huang et al., 2017). Apple *Md MYB308* affects the accumulation of anthocyanin through its binding with MdbHLH3 (Wang et al., 2017). *TrMYB308* in *Trifolium repens* L. may be involved in the regulation of flavonoid biosynthesis (Tan et al., 2019), and there are few reports on the involvement of *MYB308* gene in abiotic stress response. In this study, the *MaMYB308* gene of mulberry was cloned and its expression pattern in abiotic stress was clarified, which laid a foundation for future research on the function of *MaMYB308* gene and its mechanism in the process of mulberry resistance to stress.

R2R3-MYB transcription factors can also respond to biotic stress and participate in plant disease resistance. Overexpressed R2R3-MYB transcription factor MYB49 gene in tomato showed significant resistance to *Phytophthora infestans* (Cui et al., 2018). The infection of *Tobacco etch virus* induces the expression of MYB transcription factor genes (Agudelo-Romero et al., 2008). Our previous study found that mulberry vein band-associated virus can induce the expression of *MaMYB308* gene. Whether this gene is involved in the antiviral response of mulberry and its mechanism are worthy of further study. In order to further clarify the function of *MaMYB308* gene, the laboratory has conducted genetic transformation of ordinary tobacco to construct RNAi and overexpressed transgenic plants of *MaMYB308* homologous gene. Later, the stress resistance and disease resistance of transgenic plants will be observed, and the regulatory mechanism will be studied.

3 Materials and Methods

3.1 Test materials

The plant test material is mulberry variety 'Guiyou 62' (Guangdong mulberry, donated by Lin Qiang extension researcher of Guangxi Sericulture Technology Extension Station). Restriction enzyme were purchased from Thermo Fisher Scientifific. PCR amplification enzyme 2×phanta Master Mix, DNA purification and recovery kit and plasmid extraction kit were purchased from Vazyme Biotech Co., Ltd. RNA extraction Kit, PrimeScript ™ RT Master Mix (Perfect real time) Kit, SMARTer RACE 5′/3′ Kit RACE kit, Real-time quantitative PCR Kit were purchased from TaKaRa. The vectors used for cloning were pEASY-T1 Cloning Kit, seamless ligase 2×Assembly Mix and yeast Y2H gold, SD/-Trp culture medium, SD/-Trp-Ade-His culture medium, X-α-Gal, PEG6000, NaCl, YPDA, culture medium were purchased from TransGen Biotech Co., Ltd. pGBKT7-BD vector and *Escherichia coli* strain DH5α were all stored in the laboratory. PCR primer synthesis and DNA sequencing were completed by Beijing AUGCT Co., Ltd.

3.2 Extraction of total RNA and synthesis of cDNA

According to TaKaRa total RNA extraction kit, total RNA was extracted from mulberry roots, stems, leaves and leaves under different treatments. The concentration and purity of RNA were detected by Thermo Fisher NanoDrop 2000 ultra-micro nucleic acid protein analyzer and agarose gel electrophoresis. The first strand of cDNA was synthesized by PrimeScript ™ RT Master Mix (Perfect real time), and 1 000 ng total RNA was added into 20 μL reaction system, and stored at -20℃ for later use.

3.3 Full-length cDNA cloning of *MaMYB308* gene

A pair of specific primers (Table 1) were designed based on the transcriptome data of mulberry in the laboratory. The ORF sequence of *MaMYB308* was amplified by PCR using cDNA obtained by reverse transcription of total RNA from the leaves of 6~7 leaf mulberry seedlings as template. The PCR reaction system was 50 μL, including 2×phanta Master Mix 25 μL, 2 μL of upstream primer and downstream primer (0.01 mol/L), 2 μL of cDNA template, adding ddH_2O to supplement 50 μL. The PCR reaction procedure was as follows: pre-denaturation at 95℃ for 3 min, denaturation at 95℃ for 15 s, annealing at 58℃ for 30 s, extension at 72℃ for 1 min, 30 cycles,

extension at 72℃ for 5 min. After agarose gel electrophoresis, the PCR product was purified and recovered using gel recovery kit. After connecting with pEASY-T1 vector, the PCR product was transformed into *Escherichia coli* DH5α competent cells and cultured on ampicillin resistant medium. Positive clones were selected and sequenced. Using the correct sequence as a template, Primer 5 software was used to design 3' and 5 'specific primers on the intermediate sequence (Table 1), and RACE PCR was performed according to SMARTer RACE 5′/3′ Kit RACE instructions. The PCR products obtained were recovered and sequenced according to the above procedure, and the correct fragments of the three obtained sequences were electronically spliced to obtain the full-length cDNA sequence of the gene.

Table 1 Full length amplification of MaMYB308, yeast single hybridization and fluorescence quantitative primer sequence

Primer name	Primer sequence (5'-3')
MaMYB308-F	GTAGAGAGAGAACATGGGAAGAGC
MaMYB308-R	CCACTTGAACCCTTAATACACGACG
3RMaMYB308-F	CTGAGCTTCGAAGTCGGTGA
5RMaMYB308-R	GAGACTCGCGCTCCAAATATGGATGGTG
Y1HMYB308-F	ATGGAGGCCGAATTCATGGGAAGAGCTCCATGTTGTGCC
Y1HMYB308-R	GCAGGTCGACGGATCCTCAGATCAATAATGACTCCGCAAA
QMaMYB308-F	GCATTCTCTTCTGGGCAACC
QMaMYB308-R	CTTTTGCTAAGGTGGGTGTTCC
QMaActin-F	ACTGGCTCTGGTTTGGGGTCCTTG
QMaActin-R	TGTCGTAAATAGCTTCATTGTCCAAC

3.4 Bioinformatics analysis of MaMYB308

In the NCBI web site (https://www.ncbi.nlm.nih.gov/) for *MaMYB308* gene nucleotide and amino acids. Protein domain analysis was performed using SMART website (http://smart.embl-heidelberg.de/) and Pfam website (https://pfam.xfam.org/). Using SignalP-5.0 Server online (http://www.cbs.dtu.dk/services/SignalP/) analysis protein hydrophobicity N-terminal signal peptide. Using ProtScale tools (http://web.Expasy.org/protscale/) to carry out hydrophilic/hydrophobic MaMYB308 protein analysis. In the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) website online prediction proteins across the membrane structure. The online software ExPaSy- SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/secpred_sopma.pl) was used to predict the secondary structure of proteins. In the SWISS-MODEL website (https://swissmodel.expasy.org/interactive) protein spatial model is set up. On the ProtParam online software (https://web.expasy.org/protparam/), the protein composition and physicochemical properties were analyzed. Multiple sequence alignment using DNAMAN8 software. Using Plant-mPLoc online website (https://omictools.com/plant-mploc-tool) to predict the subcellular localization.

3.5 Yeast single hybridization

A specific Y1HMYB308F/R primer was designed to amplify the ORF sequence of *MaMYB308* gene using the full-length cDNA of MaMYB308 as the template (Table 1). The PCR reaction system and procedure were the same as that of "full-length cDNA clone of MaMYB308 gene". DNA gel was recovered after agarose gel electrophoresis. The vector pGBKT7-BD was cut into linear vector with *Bam*H I and *Eco*R I double enzyme, and the linear vector was seamlessly connected with the target fragment. The linear vector was transformed into *E. coli* DH5α strain. The positive colonies were selected for culture, and the plasmid DNA was extracted and verified by sequencing. The constructed recombinant plasmid pGBKT7-MaMYB308 and empty vector pGBKT7 were transformed into yeast Y2H gold capable cells, respectively, and coated on SD/-TRP plate. Single colonies were selected and suspended for shaking culture with LIQUID medium YPDA containing Kan. Positive yeast liquid was absorbed after PCR detection. They were placed on SD/-Trp medium, SD/-Trp-Ade-His medium and SD/-Trp+X-α-Gal medium, and the results were observed 48 h later.

3.6 Mulberry seedling planting management and treatment

Mulberry seedlings that grew to 6~7 leaf age and were healthy and disease-free in the constant temperature culture room were selected as materials and treated with drought, high salt and high and low temperature, with 15 seedlings in each treatment group. Under drought and high salt stress, the control group and the treatment group were treated with 10% PEG6000 times solution and 100 mmoL NaCl solution, respectively. Under high and low temperature stress, the control group and the treatment group were cultured in 42℃ and 4℃ constant temperature incubator, respectively. Other treatment conditions and culture were kept the same. The leaves of mulberry seedlings were sampled at 0 h, 1 h, 6 h, 12 h and 24 h after treatment and total RNA was extracted.

3.7 Real-time fluorescence quantitative PCR

Specific primers were designed according to the ORF sequence of MaMYB308 (Table 1). Mulberry *Actin* gene was selected as the internal reference gene, and 3 biological replicates were set for each treatment. SYBR Green method was used for fluorescence quantitative PCR detection, and each sample was repeated three times. The expression level of the target gene was calculated by RQ (relative expression level)=$2^{-\Delta\Delta CT}$. The expression level of *MaMYB308* in mulberry root and the $2^{-\Delta\Delta CT}$ value of the expression level of *MaMYB308* in different treatments at 0 h were assigned to 1. The expression levels of *MaMYB308* were calculated at 1 h, 6 h, 12 h and 24 h in different tissues and under different treatment conditions.

Authors' contributions

WYM was the experimental designer and executor of this study, completed data collation and the writing of the first draft of the paper. ZXR, LYJ, WYN and LJQ participated in some experiments. MJR was the project leader, supervising experimental design, data statistics, paper writing and revision. All authors read and approved the final manuscript.

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