



# Cloning and characterization of *ChiMYB* in *Chrysanthemum indicum* with an emphasis on salinity stress tolerance

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**ABSTRACT.** v-myb avianmyeloblastosis viral oncogene homolog (MYB) transcription factors are key regulators of stress responsive gene expression in plants. In this study, the MYB gene, *ChiMYB* (GenBank accession No. KT948997), was isolated from *Chrysanthemum indicum*, and was functionally characterized with an emphasis on salinity stress tolerance. The full *ChiMYB* cDNA sequence (948 bp) encoded a typical R2R3 MYB transcription factor that contained 315 amino acid residues and two MYB domains. The temporal expression pattern of *ChiMYB* was noted in *C. indicum*, and the highest level was detected in the roots, followed by leaves and stems. *ChiMYB* expression was induced by NaCl treatments, and transient expression of the fusion of *ChiMYB* and green fluorescent protein (*GFP*) indicated that the protein was targeted to the nuclei of onion epidermal cells. *Arabidopsis* plants

overexpressing *ChiMYB* displayed improved tolerance to drought and salt stress. When under salt stress conditions, transgenic *Arabidopsis* plants had higher survival rates than non-transgenic wild-type plants. Chlorophyll content, intercellular CO<sub>2</sub> concentration, photosynthetic rate, and stomatal conductance were higher in the transgenic *Arabidopsis* plants than in non-transgenic control plants. Further investigation revealed that *ChiMYB* was able to regulate the expression of *RD29A*, *RAB18*, *COR15*, *ABI1*, and *ABA* genes, which are involved in salt stress signaling pathways. Our findings demonstrated that *ChiMYB* is essential for plant responses to salt stress, and it may have great potential for the improvement of salt tolerance in crops.

**Key words:** *Chrysanthemum indicum*; *Arabidopsis thaliana*; *ChiMYB*; Salt stress; Photosynthesis; qRT-PCR

## INTRODUCTION

Salinity can significantly and negatively affect plant growth, development, and productivity. Serious salt stress can cause the substantial loss of crop production, and it may even lead plant death. Several characteristics of salt-stressed plants lead to the alteration of the photosynthetic parameters of plant leaves (Sudhir and Sistla, 2004), including damaged cytochrome systems, reduced chlorophyll content, closed stomata, decreased CO<sub>2</sub> concentrations, and reduced photosynthetic pigment content (Hoshida et al., 2000; Jin, 2002; Jiang et al., 2008; Shu et al., 2012a). These alterations are often associated with the degree and duration of salt stress (Lakshmi et al., 1996; Misra et al., 1997). Therefore, it has become an urgent need to genetically develop plants with significantly improved tolerance to salinity.

Transcription factors are proteins with special structures and functions that regulate the expression of other genes, and they are essential to plant defensive responses to biotic and abiotic stress (Liu et al., 2008). To date, numerous transcription factors from different families, including v-myb avianmyeloblastosis viral oncogene homolog (MYB), ethylene responsive factor (ERF), basic region/leucine zipper motif (bZIP), and WRKY, were found to be involved in the regulation of stress responses (Schwechheimer et al., 1998; Singh et al., 2002). MYB proteins are one of the largest families of plant transcription factors (Tang and Chen, 2014). Moreover, most MYB members belong to the R2R3-MYB type (Shu et al., 2012b), and they are involved in the regulation of plant secondary metabolism and responses to different stresses (Uimari and Strommer, 1997; Hoeren et al., 1998; Lea et al., 2007; Du et al., 2008). Different members of the MYB family may have varying tempo-spatial expression patterns, and they may function differently in plants (reference needed). For instance, *AtMYB2* regulates responses to dehydration and salt stresses in *Arabidopsis* (Martin and Paz-Ares, 1997). Enhanced expression levels of *OsMYB4* in rice significantly improve the tolerance of transgenic plants to drought, salinity, and UV radiation (Vannini et al., 2004). In addition to drought-induced expression, *BcMYB1* from *Boea crassifolia* also responds to different stresses such as polyethylene glycol (PEG), salinity, and low temperatures (Chen et al., 2005). Furthermore, transcription factors generally contain a short-span nuclear localization signal (NLS) that is often rich in arginine and lysine residues, and it also regulates the entry process of the transcription factor into cell nuclei.

Since the promoters of different stress-induced genes often contain the same *cis* acting elements, these genes can be regulated by the same transcription factors (Liu et al., 2008). A transcription regulator might possibly modulate a set of functional genes responsible for plant tolerance to the stressor, which consequently improves plant stress tolerance. Thus, manipulation of transcription factor expression has great potential for the improvement of plant resistance to stress.

*Chrysanthemum indicum* is a perennial herbaceous species that is spreading widely in China, and it exhibits variably colored flowers and tolerance to different stresses. In this study, the R2R3-MYB gene, *ChiMYB*, was cloned and functionally characterized for a better understanding of its role in plant responses to salt stress and its potential applications to the improvement of stress resistance in crops.

## MATERIAL AND METHODS

### Plants and salt stress

*C. indicum* seeds were introduced to the nursery of the Northeast Forestry University from Benxi City (121°21'E, 41°22'N), Liaoning Province, China. The cutting plants were obtained in November 2014, and were potted (diameter 12 cm) in mixed media (garden soil:vermiculite, 1:1) without additional fertilizer in March 2015. The plants were maintained in a greenhouse under standard growing conditions. Young plants were watered daily and fertilized weekly using half strength Hoagland's nutrient solution. Clipped leaves were frozen in liquid nitrogen and cryopreserved at -80°C for RNA extraction.

*Arabidopsis thaliana* ecotype Columbia (Col-0) plants were cultivated in pots filled with a mixture of perlite:vermiculite:soilrite (v:v:v, 1:1:1) in a growth chamber with 16 h of light (60-100 lx) at 23° and 18°C for day and night, respectively. Relative humidity in the chamber was maintained at 60%, and the plants were watered every four days until floral dip transformation occurred.

For the functional characterization of *ChiMYB*, non-transformed wild-type plants and transgenic *Arabidopsis* plants that overexpressed *ChiMYB* were treated with 250 mM NaCl. The rosette leaves were excised at 0, 1, 3, 6, 12, and 24 h after the salt treatment, and were then stored at -80°C until use. Twenty-day-old non-transgenic and transgenic plants were used for RNA extraction and gene expression analyses.

### cDNA and gDNA cloning

Total RNA was isolated from *C. indicum* leaves using the RNAiso reagent (TIANGEN, Beijing, China), and cDNA was then synthesized using a Revert Aid™ First Strand reverse transcription kit (TOYOBO Japan). Based on conserved sequence information from homologous *MYB* genes in *Arabidopsis*, alfalfa, and other species in the GenBank database at the National Center for Biotechnology Information (NCBI), degenerate primers (MYB-F and MYB-R; Table 1) were designed using Primer5.0 software. The polymerase chain reaction (PCR) products were purified and cloned into the pMD18-T vector (TaKaRa Japan), which was introduced into *E. coli* DH5 $\alpha$ . The inserts in plasmids isolated from positive clones after PCR screening were sequenced. BLAST analyses, using the isolated *MYB* gene as query against the GenBank database, were performed to obtain further annotation. The FullMYB-F

and FullMYB-R primers (Table 1) were designed using Primer5.0, and were then used to clone the full-length transcript. The cloned gene was then sequenced for confirmation.

**Table 1.** Primers used in qRT-PCR analyses.

Primer	Sequences (5'-3')
<i>MYB</i>	F: GTCCAGACGAGGACGAAATGT R: GTCTTATCGGCATCACATTCG
<i>FullMYB</i>	F: CATCCTCTCATTTCCTTCCAA R: CTACCCTCATCATATCCCATCTC
<i>MYB-1301</i>	F: GGATCCATGGCTAGTGTTTCAA R: CGAGCTCCTAATCTATTTTACTAATCCC
<i>AtUBQ</i>	F: GGTTCGTACCTTTGTCCAAGCA R: CCTTCGTAAACCAAGCTCAGTATC
<i>RD22</i>	F: ACTTGGTAAATATCACGTCAGGGCT R: CTGAGGTGTTCTTGTGGCATACC
<i>RD29A</i>	F: GATAACGTTGGAGGAAGAGTCGG R: TCCTGATTCACCTGGAAATTCG
<i>RAB18</i>	F: GCAGTATGACGAGTACGGAAATCC R: CCTGTCCATCATCCGAGCTAGA
<i>COR47</i>	F: TGACGGATAAAACAAAAGAGG R: GACCCTACTTTGTGGCATCCTT
<i>ABA1</i>	F: GCTATGAAGGTGATCTGCTGTGG R: TTCATACCATTGGAGCATCAGC
<i>ABI1</i>	F: AGCTGCTGATATAGTCGTCGTTGATA R: GAGGATCAAACCGACCATTAACA
<i>HAB1</i>	F: TTCTCGCCATGCTAGGTCCAT R: TTCGACAGACTTCTGGTTGTTC

### Phylogenetic analysis of *ChiMYB*

The open reading frame (ORF) of *ChiMYB* was identified using the ORFfinder program, and the structures of the conserved domains were examined using NCBI BLAST analyses and DNAMAN software. Phylogenetic analyses were performed with MYB protein sequences from other plant species.

### Transgenic *A. thaliana*

The *ChiMYB* coding sequence, with additional *Bam*HI and *Sac*I restriction sites at the 5' and 3' ends, respectively, was amplified using the MYB-1301-F/MYB-1301-R primer pair (Table 1). The amplified product was then purified and cloned into MYB-PMD18-T, which is a pMD18-T vector (TaKaRa). The insert was retrieved using *Bam*HI and *Sac*I, and was inserted into a pCAMBIA1301 vector that was digested with the same enzyme pair. The construct was then introduced into *Agrobacterium tumefaciens* EHA105 for *Arabidopsis* floral dip transformation (Clough and Bent, 1998). The seeds from *Agrobacterium*-infected *Arabidopsis* plants were sown into half-strength Murashige and Skoog (MS) medium that contained 50 mg/L of hygromycin for transgenic plant screening. Surviving plants were re-screened and self-pollinated, and the process was repeated until homozygous T4 transgenic *Arabidopsis* lines were obtained. Total RNA was extracted from the young leaves of transgenic and non-transgenic plants using the TRIZOL extraction method, and transgene expression was quantified using *MYB*-specific primers.

### Subcellular localization of *ChiMYB*

The complete *ChiMYB* ORF, with the addition of *Xba*I and *Spe*I restriction sites at the ends, was amplified via PCR using primers MYB-121-F/R (Table 1). The PCR product (confirmed via sequencing) was digested with *Xba*I and *Spe*I, and was then inserted into pBI121 to create a fusion construct (pBI121-*ChiMYB*-GFP). The same construct, without *ChiMYB* (pBI121-GFP), was used as a control. Onion epidermal cells were bombarded with both the fusion and control vectors using a GJ-1000 Gene Gun System (Scientz, China). After 24 h at 25°C in darkness, the fluorescent signals from GFP and *ChiMYB*-GFP fusion samples were observed using a confocal laser scanning microscope (Zeiss LSM510, Germany).

### Salt stress tolerance assay of *Arabidopsis* plants expressing *ChiMYB*

Germination assays, comprised of 100 transgenic or non-transgenic seeds, were conducted on 1/2 MS media containing 0, 50, 100, 150, or 200 mM NaCl, and each assay was run in triplicate. Water-imbibed seeds were kept in the dark for 48 h at 4°C, and seeds were then germinated in a growth chamber under normal growth conditions for 10 days. The germination rates of seeds (with 2 mm radicles) were presented as the percentage of the germinated seeds over the total number of seeds planted.

Regarding the salinity tolerance test, three-week-old non-transgenic Col-0 and overexpressed *ChiMYB Arabidopsis* plants were cultivated in pots filled with a mixture of perlite:vermiculite:soilrite (v:v:v, 1:1:1) in a growth chamber. Plants were exposed to different levels of NaCl (0, 50, 100, 150, and 200 mM), and were watered for 12 days at four-day intervals. Survival rates of *ChiMYB* transgenic and non-transgenic plants were obtained and statistically analyzed.

### Determination of chlorophyll content

The chlorophyll *a* (*chl**a*), chlorophyll *b* (*chl**b*), total chlorophyll, and carotenoid contents in salt-stressed *ChiMYB*-expressing transgenic and non-transgenic *Arabidopsis* plants were determined using the Lichtenhaler and Wellburn (Lichtenhaler and Wellburn, 1983) method.

### Determination of the net photosynthetic rate, stomatal conductance, and intercellular CO<sub>2</sub>

The net photosynthetic rate (P<sub>n</sub>), stomatal conductance (G<sub>s</sub>), and intercellular CO<sub>2</sub> concentration (C<sub>i</sub>) of salt-treated Col-0 and *ChiMYB*-expressing transgenic and non-transgenic *Arabidopsis* plants were measured using a Licor-6400 (Ecotek Technology, United States) at 23°C with 50% relative humidity and a light intensity of 300 lx. Leaf area was measured and calculated using a scanner (YMJ, China) and Leaf Data software, and each treatment was repeated at least four times.

### Quantitative reverse transcription PCR (qRT-PCR)

To determine the expression patterns of *ChiMYB* in *C. indicum* under salt stress

conditions, total RNA was isolated from fresh leaves (homogenized in liquid nitrogen) using the TRNzol Universal Reagent (TIANGEN), and genomic DNA contamination was eliminated using DNaseI. cDNA synthesis was conducted using random primers and a reverse transcription kit (TOYOBO, Japan). Quantitative PCR (qPCR) was performed using SYBR Green I (TOYOBO, Japan) and a LightCycler® 96 Real-Time PCR System (Roche Diagnostics Ltd., Shanghai, PRC). The qRT-PCR mixture (20  $\mu$ L) contained 10  $\mu$ L SYBR Green PCR master mix, 0.4  $\mu$ L of each gene-specific primer (Table 1), and 0.5 ng cDNA. The following qPCR protocol was used: 95°C for 60 s; 40 cycles of 95°C for 15 s; 60°C at 15 s; and 72°C for 45 s. The data are represented by the means  $\pm$  SD of three replicates. The reference *Arabidopsis* gene *AtUBQ* (NM\_116771.5) was used for the normalization of *ChiMYB* expression via the  $2^{-\Delta\Delta Ct}$  method, where  $\Delta\Delta Ct = (Ct \text{ of target gene} - Ct \text{ of reference gene } AtUBQ) \text{ Time}_x - (Ct \text{ of target gene} - Ct \text{ of reference gene } AtUBQ) \text{ Time}_0$  (Livak and Schmittgen, 2001). For the *Arabidopsis* stress-responsive gene expression analyses, RNA was extracted from non-transgenic and *ChiMYB*-expressing plants, and cDNA was synthesized. Transcripts of the stress-responsive genes *RD22*, *RD29A*, *RAB18*, *COR15*, *ABA1*, *ABI1*, and *HAB1* were quantified using gene-specific primers (Table 1). The qPCR protocol for *Arabidopsis* gene expression was identical to that used for *ChiMYB* expression, with the exception of different gene-specific primers.

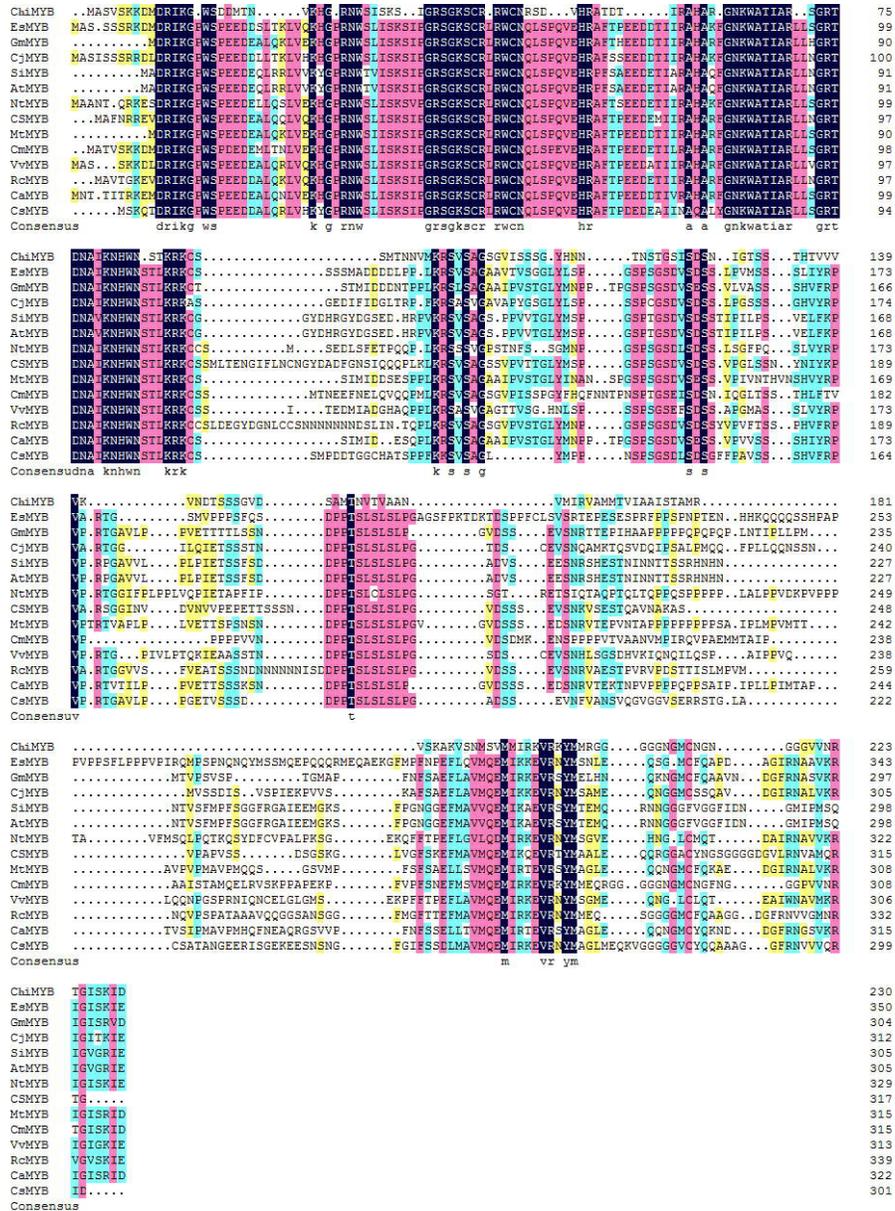
## RESULTS

### Analysis of R2R3-MYB sequences from *C. indicum*

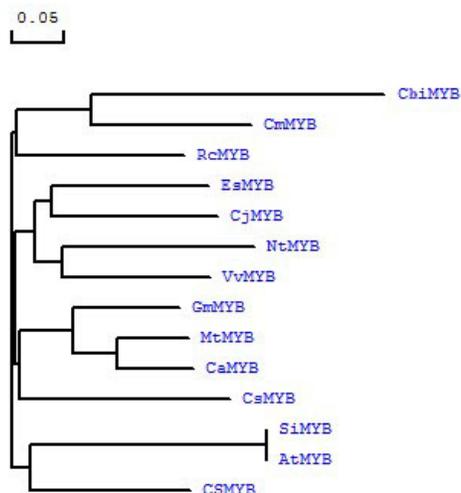
In this study, *ChiMYB* (an MYB gene) was isolated from *C. indicum*, and it contained a full-length ORF (948 bp) that encoded a protein with 315 amino acid residues (GenBank accession No. KT948997). The encoded protein contained two MYB domains, and it functioned as a typical R2R3MYB transcription factor. The sequence alignment analysis indicated that *ChiMYB* shared 95% similarity with chrysanthemum *MYB2* (AE027498.1), 79% with tomato *MYB44* (XP003611666.1), and 76% with an alfalfa homologue (XP003611666.1). Multiple sequence alignment of the *ChiMYB* protein was performed against 13 MYB homologues from different plant species using DNAMAN software (Figure 1), and the results revealed conserved domains across all of the tested MYB proteins.

The reconstructed phylogenetic tree suggested that the tested MYB proteins could be divided into four groups (Figure 2). Group 1 included *Chrysanthemum CmMYB* (*Chrysanthemum x morifolium*, AE027498.1), castor oil *RcMYB* (*Ricinus communis*, XP002524926.1), and a *Chrysanthemum* MYB transcription factor, which are all associated with hormonal responses (Shan et al., 2012). Group 2 contained samples from *Epimedium EsMYB* (*Epimedium sagittatum*, AFH03065.1), *CjMYB* (*Coptis japonica*, BAJ40867.1), grape *VvMYB* (*Vitis vinifera*, XP002285015.1), and tobacco *NtMYB* (*Nicotiana tabacum*, BAC53938.1), which were all involved in the metabolism of flavonoids, abiotic stress, and embryonic development (Masaharu et al., 2003; Huang et al., 2013); Group 3 included soybean *MYB* (*Glycine max*, NP001235142.1), chickpea *CaMYB* (*Cicer arietinum*, XP004511885.1), alfalfa *MtMYB* (*Medicago truncatula*, XP003611666.1), and cucumber *CsMYB* (*Cucumis sativus*, XP004151866.1), which are associated with abiotic stress. Group 4 included *Arabidopsis AtMYB30* (*Arabidopsis thaliana*, CAA90809.1), tomato *SiMYB* (*Solanum lycopersicum*, XP004231882), and orange *CSMYB* (*Citrus sinensis*, ABQ10816.1), which are

involved in the regulation of pigment metabolism (Kirik et al., 1998; Luo et al., 2008; Yang et al., 2008; Antonietta and Paola, 2010). Moreover, the phylogenetic analysis showed that the ChiMYB protein was closely related to the *Chrysanthemum* samples, which suggests that it might function similarly to other *Chrysanthemum* MYB transcription factors (Figure 2).

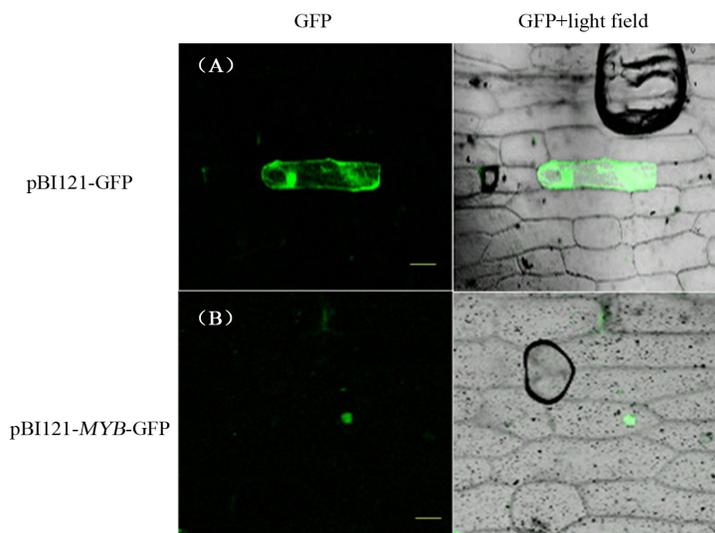


**Figure 1.** Alignment of MYB proteins from different plant species. The conserved SANT DNA-binding domains are highlighted.



**Figure 2.** Phylogeny of *ChiMYB* and samples from other species.

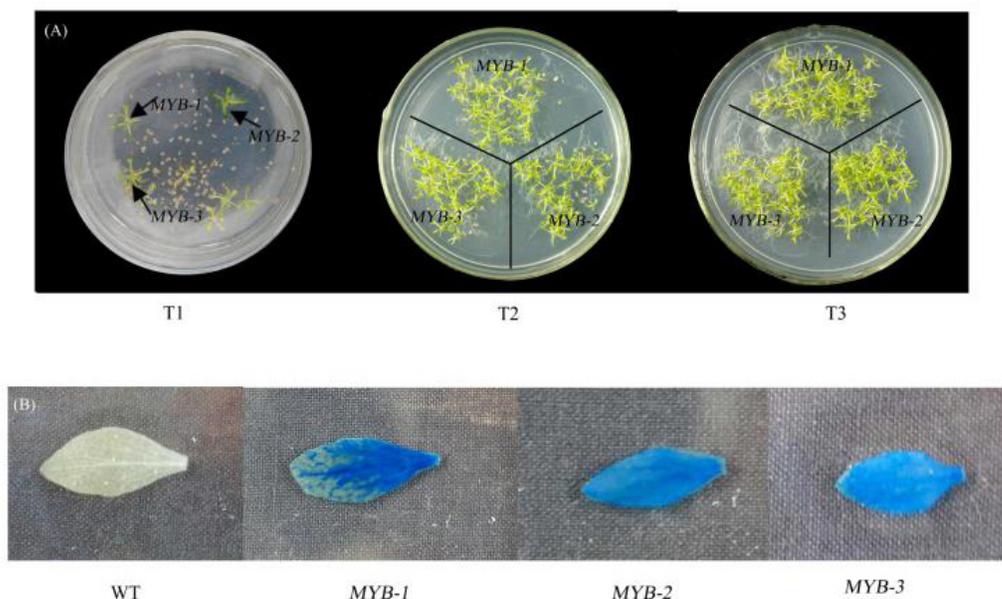
To confirm the subcellular location of *ChiMYB*, 35S:ChiMYB-GFP and 35S:GFP were constructed and introduced into onion epidermal cells. Confocal microscopy observations revealed that the ChiMYB-GFP fusion was localized to the nucleus. However, GFP (alone) was distributed throughout the cell. Therefore, these results indicated that *ChiMYB* was localized to the nucleus, which is consistent with its predicted function as a transcription factor (Figure 3).



**Figure 3.** ChiMYB subcellular localization revealed by GFP fusion. The constructs carrying GFP alone (A) or ChiMYB-GFP were both driven by the CaMV 35S promoter, which was introduced into onion epidermal cells using a gene gun (B). The transformed samples were maintained at 22°C for 24 h, and were observed under a confocal microscope. Scale bars = 50  $\mu$ m.

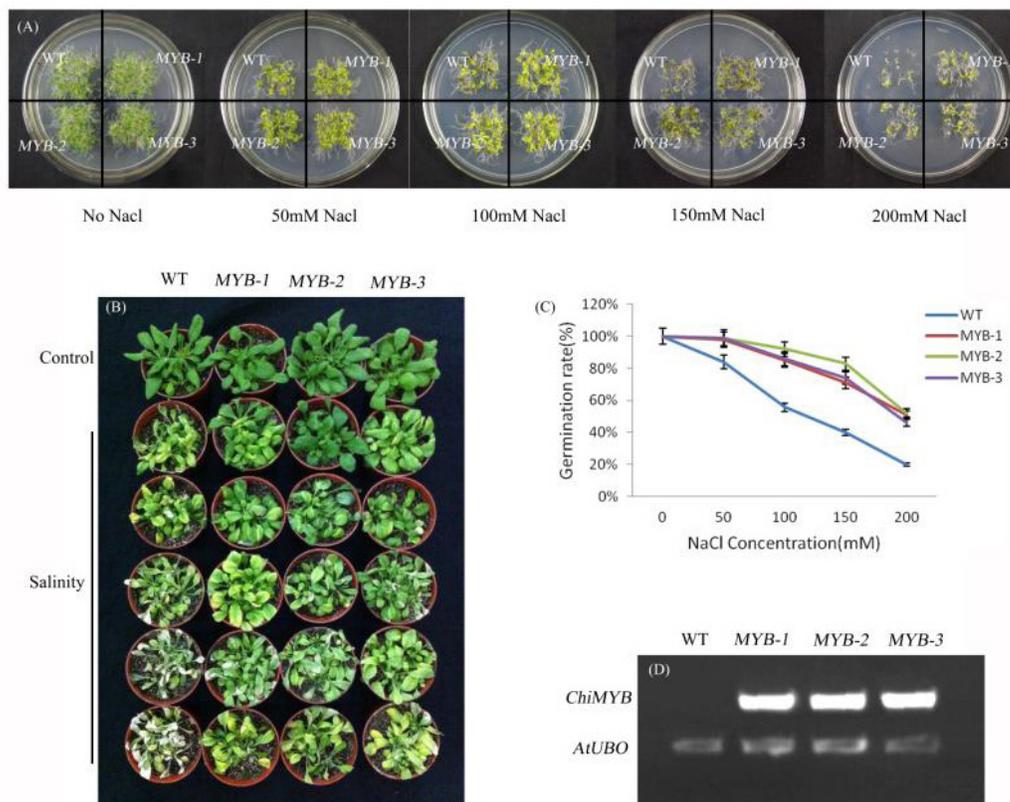
### Alteration of seed germination and plant tolerance in transgenic plants under salt stress

After hygromycin screening of  $T_0$  generation seeds,  $T_1$  seedlings of the three independent lines (*MYB-1*, *MYB-2*, and *MYB-3*) were obtained. Continuous selfing was conducted until  $T_3$  homozygotes of the three lines were acquired (Figure 4A). The results of GUS staining, which was used to screen for transgenic *A. thaliana*, are shown in Figure 4B.



**Figure 4.** Positive transgenic *Arabidopsis* plants in selection media containing hygromycin (A). GUS stain screening for transgenic *A. thaliana* (B).

When salt was not added to the medium, the transgenic *Arabidopsis* plants germinated normally. Furthermore, the germination rate of transgenic *Arabidopsis* seeds in the presence of 50 mM NaCl was over 90%, and similar results were also found for Col-0 seeds. However, when the salt concentration was increased to 100 mM, the transgenic lines still maintained high germination rates (91.8, 91.3, and 93.3% for *MYB-1*, *MYB-2*, and *MYB-3*, respectively). In contrast, the germination rate of Col-0 seeds decreased to 55.6%. When the NaCl concentration was further increased to 150 or 200 mM, the average germination rates of the three transgenic seed lines were 48 and 36%, respectively. However, that of the Col-0 seeds at 150 or 200 mM decreased to 18 and 9%, respectively (Figure 5A). Moreover, as the NaCl concentration increased, the survival rates of transgenic and non-transgenic Col-0 plants gradually decreased. The leaves of transgenic *Arabidopsis* plants wilted slowly, whereas Col-0 leaves wilted rapidly. At a concentration of 250 mM NaCl, the survival rates of the three transgenic lines (*MYB-1*, *MYB-2*, and *MYB-3*) were 72, 83, and 79%, respectively. In contrast, that of Col-0 plants was only 30.1% (Figure 5B).

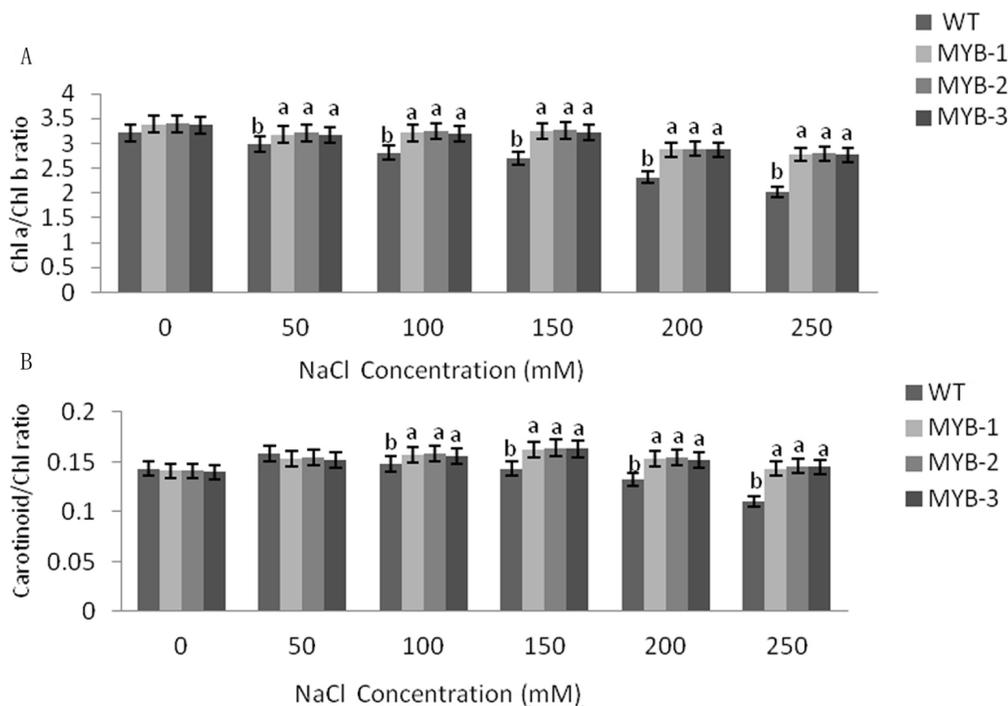


**Figure 5.** Seed germination and growth of overexpressed *ChiMYB* in *Arabidopsis* under different levels of salt stress. Seed germination under different salt stress conditions (A and C), plant tolerance at different salt concentrations (B), PCR-detected presence of transgenes and internal reference genes (*AtUBQ*) in transgenic plants (D).

### Chlorophyll content in transgenic *Arabidopsis* plants

The ratio of *chl a* to *chl b* in both transgenic and non-transgenic *Arabidopsis* plants decreased as the NaCl concentration increased. Compared with that in transgenic *Arabidopsis* plants, the ratio in non-transgenic controls was greatly reduced. When the NaCl concentration was 200 or 250 mM, the *chl a/chl b* values of wild-type *Arabidopsis* plants decreased to the lowest values, while those of transgenic *Arabidopsis* plants slightly declined. These results suggested improved salt tolerance in transgenic plants (Figure 6A).

As the salt stress conditions intensified, the carotenoid/chlorophyll ratios (Car/Chl) of the transgenic *Arabidopsis* plants gradually increased until the salt concentration reached 150 mM and then declined. However, the Car/Chl values of non-transgenic Col-0 plants peaked at 50 mM NaCl before rapidly declining. These results demonstrated that transgenic *Arabidopsis* plants were more tolerant to salt stress conditions than non-transgenic plants ( $P < 0.05$ ) (Figure 6B).



**Figure 6.** Changes in the ratios of chlorophyll a/b (A) and carotenoid/chlorophyll (B) in transgenic and non-transgenic *Arabidopsis* plants under different salt concentrations.

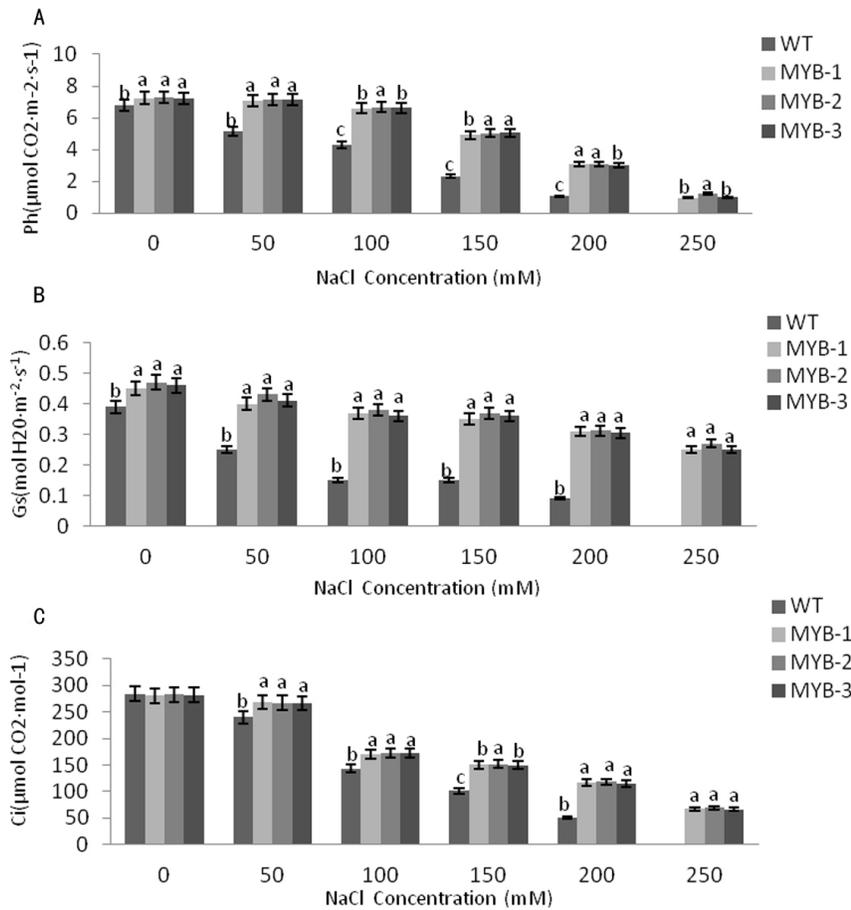
### Photosynthetic rates of transgenic *Arabidopsis* plants

As the NaCl concentration increased, Gs, Ci, and Pn values decreased in Col-0 and the three transgenic *Arabidopsis* lines (Figure 7). Compared to the transgenic *Arabidopsis* plants, a greater reduction in the above parameters occurred in non-transgenic plants (Col-0). When NaCl concentration was 50 mM, transgenic *Arabidopsis* plants maintained Pn values compared to those not under salt stress conditions. However, when the NaCl concentration increased to over 100 mM, the Pn values of the three transgenic lines declined, but a sharper decline in Pn occurred in non-transgenic plants. At a concentration of 250 mM NaCl, the Gs value of Col-0 decreased significantly. However, at concentrations up to 250 mM NaCl, the Gs values of the three transgenic *Arabidopsis* lines did not change significantly. The Ci concentrations of Col-0 and the three transgenic *Arabidopsis* plants decreased with increasing NaCl concentrations. For instance, at a concentration of 100 mM NaCl, the Ci rates of both transgenic and non-transgenic plants were significantly lower, and they continued to decline with increasing NaCl concentrations. However, a faster decline in Ci values was observed in Col-0 than for the transgenic strains.

### Differential *ChiMYB* expression in transgenic *Arabidopsis* plants

Transcript quantification results indicated that the expression levels of *ChiMYB*

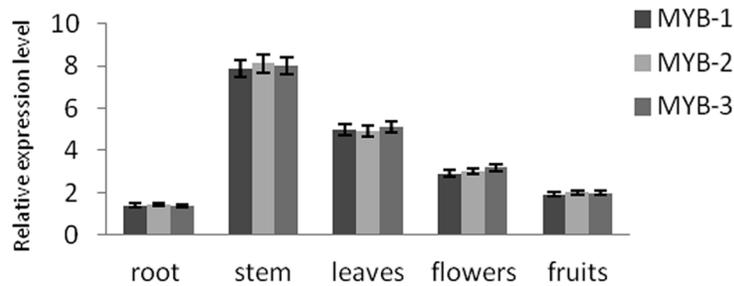
differed in the tested plant organs. The highest transcription was found in the stems, and the lowest was found in the roots (Figure 8).



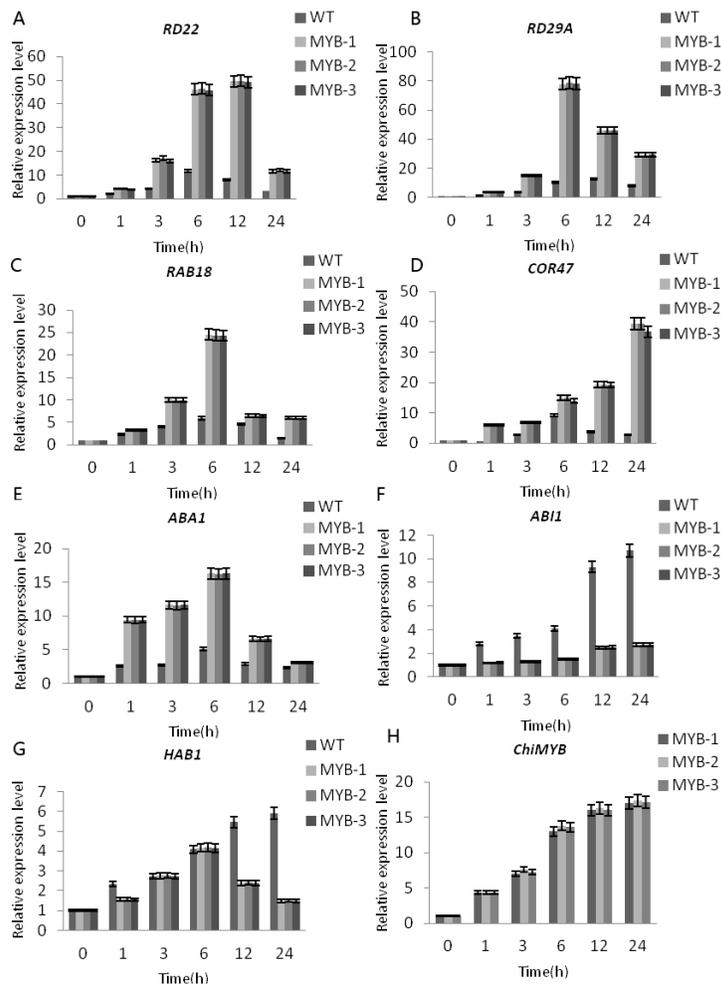
**Figure 7.** Changes in photosynthetic parameters Pn (A), Gs (B), and Ci (C) in transgenic and wild-type *Arabidopsis thaliana* plants five days after salt treatment.

### Enhanced expression of stress responsive genes in transgenic *Arabidopsis* plants

The expression of *RD22*, *RD29A*, *RAB18*, *COR47*, and *ABA1*, which are all stress responsive genes, was strongly enhanced in the overexpressed *ChiMYB* transgenic plants (Figure 9A-H) as compared to the non-transgenic plants treated with 250 mM NaCl. When salt stress was not applied, the expression of these genes was quite low, and no significant differences were detected between transgenic and non-transgenic plants. However, the expression of *ABII* and *HABI* in the overexpressed *ChiMYB* transgenic plants was lower than that observed in non-transgenic Col-0 plants (Figure 9F-G). Nevertheless, the expression of *RD22*, *RD29A*, *RAB18*, *COR47*, and *ABA* in transgenic plants was significantly enhanced compared to non-transgenic plants, and *ChiMYB* expression gradually increased.



**Figure 8.** *ChiMYB* gene expression patterns in different organs under natural conditions.



**Figure 9.** The expression of stress-responsive genes and *ChiMYB* in transgenic and non-transgenic *Arabidopsis* plants.

## DISCUSSION

### ChiMYB transcription factor can improve plant tolerance to salt stress

MYB transcription factors are essential for plant defense responses to biotic and abiotic stress. In this study, *ChiMYB* from *C. indicum* was isolated and expressed in *Arabidopsis* to characterize its function with regard to salt stress tolerance. Our results revealed that under salt stress, the survival rate of non-transgenic plants was approximately 30%, while the transgenic plants exhibited normal growth and higher survival rates. Therefore, our data demonstrated that *ChiMYB* expression increased *Arabidopsis* plant tolerance to high salt concentrations. It is interesting to note that when under salt stress, the germination rate of the transgenic lines was slightly higher than that of non-transgenic control plants. These data suggest that *ChiMYB*-induced tolerance to salt stress might be associated with specific plant developmental stages. However, this hypothesis must be investigated further.

### ChiMYB transcription factor can increase plant chlorophyll content and photosynthetic efficiency

The chl/*chl<sub>b</sub>* ratio is associated with the degree of chloroplast thylakoid stacking. Therefore, when the *chl<sub>a</sub>/chl<sub>b</sub>* value is low, the level of thylakoid stacking might be low, which consequently leads to poor thylakoid membrane stability. Ultimately, the light energy distribution between photosystems PSI and PSII will be negatively affected, and photosynthetic activity will decrease. The Car/Chl value is related to plant stress tolerance and photosynthetic activity (Liu and Liu, 1991). Our data revealed that the *chl<sub>a</sub>/chl<sub>b</sub>* values of both transgenic and non-transgenic plants declined quickly when subjected to high NaCl concentrations. When the NaCl concentration was below 150 mM, the *chl<sub>a</sub>/chl<sub>b</sub>* ratio of transgenic *Arabidopsis* plants did not change (Figure 6A). However, when under high salt concentrations, the ratio of *chl<sub>a</sub>/chl<sub>b</sub>* in non-transgenic plants reduced rapidly while that of transgenic plants did not change significantly, and this suggested that the transgenic plants were more tolerant to salt stress than non-transgenic control plants. As shown in Figure 6B, the Car/Chl ratio of the transgenic *Arabidopsis* plants increased gradually as the salt concentration increased, but the ratio decreased when subjected to a 150 mM salt treatment. In contrast, the ratio of Car/Chl of non-transgenic plants peaked at a concentration of 50 mM NaCl, which was followed by a quick decline as the salt concentration increased. The change in the Car/Chl ratio suggested that transgenic *Arabidopsis* plants were more tolerant to high salt concentrations than non-transgenic control plants. Therefore, our data indicate that *ChiMYB* improved the tolerance of plants to salinity.

The mechanisms underlying salt stress suppression of plant photosynthesis are not fully understood at this point. Under salt stress, the values of Pn, Gs, and Ci in the three transgenic *Arabidopsis* lines decreased, but the decrease was not as pronounced as that observed in the non-transgenic control. These results suggested that photosynthesis in transgenic *Arabidopsis* plants was not as strongly affected as that in the control plants. Furthermore, our results revealed that the *ChiMYB* transcription factor plays a positive regulatory role in improving photosynthesis. For instance, the stomatal factor might be critical to decreased photosynthetic capacity in *A. thaliana* (Xu and Chen, 1999).

### Subcellular localization of *ChiMYB*

In this experiment, *ChiMYB* was fused to the *GFP* reporter gene under the control of a CaMV 35S promoter, and it was transiently expressed in onion epidermal cells. The *ChiMYB*-*GFP* fusion protein was localized to the nucleus, indicating that *ChiMYB* targeted the nucleus as expected.

### *ChiMYB* regulates the expression of downstream genes involved in plant stress responses

Several studies confirmed that MYB transcription factors are essential for signal transduction, regulation of downstream gene expression, and stress responses (Liu et al., 2001). Our data indicated that *ChiMYB* induced the expression of downstream genes in salt stress signaling pathways (*RD22*, *RD29A*, *RAB18*, *COR47*, *AB11*, *ABA1*, and *HAB1*), which suggested that *ChiMYB*-enhanced salt tolerance results from the induction of these resistance genes.

In summary, salinity is a serious problem that affects crop production, and the improvement and enhancement of transcription factor regulatory functions could be an effective approach for the improvement of crop stress tolerance. The application of transcription factors for this purpose has attracted a lot of attention. However, studies of MYB transcription factors mainly focus on single stress conditions, but additional efforts should focus on their regulatory functions and interactions between multiple signaling pathways. Further investigations of MYB transcription factor regulatory mechanisms that control multiple signaling pathway interactions will help elucidate plant responses to stress.

### Conflicts of interest

The authors declare no conflict of interest.

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