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Overexpression of the *Panax ginseng MYB4* gene enhances stress tolerance in transgenic *Arabidopsis thaliana*

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Abstract

The myeloblastosis (MYB) transcription factors are essential for plant stress responses. They can enhance plant tolerance to abiotic stresses (*e.g.*, drought, salinity, and cold) *via* improved physiological and biochemical responses including the accumulation of metabolites. In this study, we constructed a *Panax ginseng MYB4* (*PgMYB4*) gene expression vector and established the stable transgenic *Arabidopsis thaliana* lines to study the effects of this gene on plant stress tolerance. The germination rate and seedling taproot length were greater for the *PgMYB4*-overexpressing plants than for the wild-type plants. Accordingly, the overexpression of *PgMYB4* in *Arabidopsis* enhanced seedling tolerance to drought, salt, and cold conditions. Under drought stress, the relative chlorophyll content decreased less, the proline content increased more, and the water loss rate decreased more in the transgenic plants than in the wild type. The expressions of stress-related genes *responsive to dehydration 19A*, *responsive to dehydration 22*, *responsive to desiccation 29A*, *cold-regulated 15A*, *cold-regulated 47*, and *pyrroline-5-carboxylate synthase 1* were significantly upregulated in the transgenic *Arabidopsis* plants. Under high salt stress, the *kinesin* 1 (*KIN1*) expression was significantly upregulated in the transgenic plants. In response to the low temperature stress, the *dehydration-responsive element binding protein 2A* and *KIN1* expressions increased dramatically in the transgenic *Arabidopsis* plants. Thus, *PgMYB4* positively regulated the stress tolerance gene networks, which promoted the expression of anti-stress effector genes. This gene may be useful for ginseng breeding programs aiming to develop new cultivars with enhanced stress tolerance.

Keywords: cold stress, drought, gene expression, salinity.

Introduction

The MYB gene family, which is one of the largest transcription factor (TF) families in plants, plays a vital role in plant growth and responses to environmental stresses. The first *MYB* gene (v-Myb proto-oncogene) was discovered in the avian myeloblastic tumor virus genome (Klepnauer *et al*. 1982). Homologs of the *MYB* gene were subsequently identified in vertebrates, insects, and fungi (Lipsick *et al*. 1996, Weston *et al*. 1998). Of the *MYB* homologs in plants, *MYBC1* in maize, was the first identified (Paz-Ares *et al*. 1987), with a function related to pigment synthesis. Many *MYB* genes have been isolated and identified in plants (Marocco *et al*. 1989, Stracke *et al*. 2001, Du *et al*. 2013, Yamagishi *et al*. 2020, Yuan *et al*. 2020), and there is considerable interest in functionally characterizing these genes. A highly conserved DNA binding domain defines the MYB TFs at the N-terminus (Du *et al*. 2012). The binding domain contains 1 - 4 imperfect repeating sequences (Rs), each R segment comprises approximately 52 amino acids residues and includes three regularly spaced tryptophan residues, which together

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Abbreviations: ABA - abscisic acid; COR15A - cold-regulated 15A; COR47 - cold-regulated 47; DREB2A - dehydration-responsive element binding protein 2A; KIN1 - kinesin-1; P5CR - pyrroline-5-carboxylate reductase; P5CS1 - pyrroline-5-carboxylate synthase 1; RD19A - responsive to dehydration 19A; RD22 - responsive to dehydration 22; RD29A - responsive to desiccation 29A; TF - transcription factor.
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form three α-helices. The second and third α-helices of each repeat fold into a helix-turn-helix structure, and the third helix of each repeat directly intercalates in the major groove of DNA (Chen *et al*. 2006). The MYB TF family contains four main subfamilies, which are grouped by the number of tandem repeats they possess: 1RMYB/MYB-related, R2R3-MYB, R1R2R3-MYB, and 4R-MYB (Dubos *et al*. 2010, Zhao *et al*. 2019). Among these groups, the R2R3-MYB subfamilies are the most common in plant MYBs (Stracke *et al*. 2001).

The R2R3-MYB TFs are involved in various physiological and biological processes, such as secondary metabolism,cellularmorphogenesis,cellulardifferentiation, and cell-cycle control (Du *et al*. 2009, Gujjar *et al*. 2014). Some also play essential roles in responses to abiotic stresses (Cao *et al*. 2013). In *A. thaliana*, some *R2R3-MYB* genes, *AtMYB44* TF regulates ABA-mediated stomatal closure, which confers abiotic stress tolerance (Jung *et al*. 2008); while the *AtMYB15* TF is involved in the regulation of *C-repeat binding factor* genes under cold stress, and it plays a role in enhanced freezing tolerance (Agarwal *et al.* 2006). The ectopic expression of rice *Osmyb4* in *Arabidopsis* results in the upregulated expression of the pyrroline-5-carboxylate synthetase gene *P5CS1*, which increases proline content and improves the tolerance of the transgenic *Arabidopsis* plant to drought stress (Mattana *et al.* 2005). The CsMYB0 and CsMYB2 proteins from *Cucumis sativus* are involved in the responsiveness to ABA signalling, high salinity, and low temperature (Li *et al*. 2012). In *Gossypium hirsutum*, *GhMYB108*-like is an important regulatory gene in response to drought and salt stresses (Ullah *et al*. 2020). The BplMYB46 TF from *Betula platyphylla* was reported to improve salt and osmotic stress tolerance. The BplMYB46 TF inhibits the expression of proline degradation genes, which can result in elevated proline content and improved abiotic stress tolerance (Guo *et al*. 2017). In a recent study involving an *in vitro* ginseng root tissue culture model, *PgMYB1* gene transcription increased by up to 4-fold in response to cold or high salinity (Afrin *et al*. 2015). Although MYB TFs are well studied, their functional roles are not entirely understood. Furthermore, many members of the MYB TF family are still not described.

Ginseng is one of the primary medicinal plants in East Asia. Some of the medical benefits of ginseng include helping to balance metabolic activities, protecting the nervous system, and enhancing the endocrine system (Yue *et al*. 2007, Cho *et al*. 2014). However, the cultivation of ginseng usually requires 3 - 20 years. Its growth and development are greatly affected by irradiance, water, soil, temperature, and other environmental conditions. Among environmental factors, drought, high salinity, and low temperatures are the main abiotic stresses that limit ginseng yield and quality (Wu *et al*. 2005, Lee *et al*. 2019). In this study, we isolated a new ginseng *MYB TF*, named *MYB4*, and investigated whether it is important for abiotic stress responses by overexpressing ginseng MYB4 in *Arabidopsis*. Furthermore, we examined the mechanism by which ginseng MYB TFs improve plant tolerance to drought, high salt, and freezing conditions.

Materials and methods

Plants and cultivation: We collected five-year-old *Panax ginseng* C.A Meyer plants from Fusong County in Jilin Province, which is the leading commercial ginseng-producing region in China. We placed *Arabidopsis thaliana* L. (Columbia-0 ecotype) seeds on half-strength Murashige and Skoog (MS) medium in Petri plates. Plants were cultivated in an artificial climate growth chamber at a temperature of 22 °C, a 16-h photoperiod, and an irradiance of 100 μmol m^{-2} s^{-1}.

Extraction of RNA and cDNA synthesis: We used liquid nitrogen to homogenize the ginseng roots to a fine powder, and we isolated the total RNA with the *TRIzol* reagent (*Invitrogen*; *Thermo Fisher Scientific*, Waltham, MA, USA), according to an improved *TRIzol* method (Wolfe-Simon *et al*. 2006). We examined the RNA purity and concentration using a spectrophotometer (*Shimadzu*, Kyoto, Japan). We used electrophoresis on 1 % (m/v) agarose gel to determine the quality of the RNA. We performed reverse transcription using a *TaKaRa* reverse transcription kit (*Takara Biotechnology Co.,* Dalian, China), and the first strand of cDNA was synthesized according to the manufacturer's protocol and stored at -80 °C for further use.

Cloning *PgMYB4* cDNA: We used the reverse transcription-polymerase chain reaction (RT-PCR) to clone the *PgMYB4* cDNA. The *PgMYB4* gene sequence was obtained from the ginseng transcriptome database (http://blast.ncbi.nlm.nih.gov/Blast.cgi, PRJNA659400), after which gene-specific primers were designed with the *PRIMER 5.0* software (MYB4-F: TGCTCTAGAATGGTGAGAGCTCCTTGCTGTG; MYB4-R: CGCGGATCCGAATTCAGGTAAGTCCC CAGCT). The Beijing Genomics Institute (BGI) synthesized the primers. We performed the PCR reaction under the following program: initial denaturation at 94 °C for 2 min, then 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 1 min, followed by a 5-min extension at 72 °C. The total volume of the PCR reaction mixture was 50 mm^3. The mixture contained 10 mm^3 of 5×PCR buffer, 28.75 mm^3 of ddH_2O, 0.25 mm^3 of *TaKaRa Ex Taq*® Hot Start Version, 0.5 mm^3 of each primer, 10 mm^3 of cDNA template. We selected the specific PCR product using electrophoresis on 2 % agarose gel.

Construction of a plant expression vector and transformation of *Arabidopsis* plants: The PCR products were linked to the pMDTM18-T vector (*Takara*, Dalian, China). After digestion with *BamHI* and *XbaI*, the specific DNA fragment was incorporated into the pCAMBIA1303 binary plant expression vector (*Beijing Dingguo Changsheng Biotechnology Co.,* Beijing, China), which contains a CaMV 35S promoter and NOS terminator. We sequenced the recombinant plasmids at the BGI and analyzed with the *DNAMAN* software.

The recombinant pCAMBIA1303 plasmid carrying

PgMYB4 was inserted into *Agrobacterium tumefaciens* strain Agl0, and then used to infect *Arabidopsis* plants according to a floral dip method (Clough *et al*. 1998). We screened the transgenic plants with 60 mg dm^{-3} hygromycin in half-strength MS medium. Then, we tested the seeds with hygromycin from the putative *PgMYB4*-carrying transgenic plants for another two generations to obtain T3 transgenic *Arabidopsis* plants. The RT-qPCR confirmed the expression of *PgMYB4* in the T3 generation of plants.

Analysis of the *PgMYB4* gene: We used the *DNAMAN* software to perform the gene sequence analysis of the PgMYB4 protein. We identified the homology of PgMYB4 using protein *BLAST* in the *NCBI* database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and conducted a phylogenetic analysis using the *MEGA 7.0* program and the neighbor-joining method. The strength of nodes in the tree was set by bootstrap analysis (1 000 replicates). We used the website *ExPASy* (http://www.expasy.org) to analyze the relative molecular mass and isoelectric point.

Analyses of RNA and protein: Total RNA was extracted from the transgenic *Arabidopsis* plants according to an established cetyltrimethyl ammonium bromide (CTAB) method (Hu *et al*. 2002), after which cDNA was synthesized by RT-PCR. The transcriptions of several abiotic stress tolerance genes (*i.e.*, *responsive to dehydration 19A* (*RD19A*), *responsive to dehydration 22* (*RD22), responsive to desiccation 29A* (*RD29A*), *cold-regulated 15A* (*COR15A*), *cold-regulated 47* (*COR47*), and *pyrroline-5-carboxylate synthase 1* (*P5CS1*), *dehydration-responsive element binding protein 2A* (*DREB2A*), and *kinesin-1* (*KIN1*) were analyzed by RT-qPCR, with *Arabidopsis thaliana ubiquitin* as a reference gene. Details regarding the PCR primers are provided in Table 1 Suppl. We examined the protein extracts by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. A Western blot was performed with a polyclonal rabbit antiserum against a PgMYB4 peptide (CDNSGDTTDFNRGA) as the primary antibody and AP-labeled goat anti-rabbit IgG (1:1000; v/v) as the secondary antibody. We combined nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate to produce protein bands, and we took images of the bands.

Drought tolerance: *Arabidopsis* seedlings were grown for 4 weeks and then they were not supplied with water until more than 50 % of the plants showed varying degrees of wilting. After rehydrating the plants, we calculated their survival rates. Samples were collected before and after the drought stress treatment and immediately frozen in liquid nitrogen for an RT-qPCR analysis. The drought-induced gene transcriptions were measured.

The chlorophyll content of seedling leaves at similar positions on plants was determined with a portable chlorophyll meter (*SPAD-502*, *KONICA MINOLT*, Tokyo, Japan) on the 5th day of drought stress. At the same time, the free proline content was determined as previously described (Bates *et al*. 1973).

Plants were grown for 4 weeks, after which the shoots were collected. Under a constant temperature (25 ± 1 °C) and a humidity (60 %), the samples were weighed every 20 min up to 180 min. This analysis was completed with three replicates, each with three plants. The average water loss rate of the leaves was calculated based on the fresh mass basis.

Osmotic stress tolerance: Four-week-old seedlings were irrigated with 100, 200, or 300 mM NaCl every 4 d, and then they were watered normally for 1 week, after which phenotypic changes were observed. Samples were collected before and after the salt stress treatment and immediately frozen in liquid nitrogen for an RT-qPCR analysis.

Arabidopsis seeds were placed on half-strength MS medium containing various concentrations of NaCl (100, 200, or 300 mM) or mannitol (100, 200, or 300 mM). After 3 d of vernalization at 4 °C, the seeds were incubated in an artificial climate growth chamber under an irradiance of 100 µmol m^{-2} s^{-1} for 7 d. Seeds were considered to be germinated if sprouting was detected (at least 1 mm long).

Arabidopsis seeds were placed on normal half-strength MS medium in Petri plates and vernalized at 4 °C for 3 d. The plates were then placed vertically in an artificial climate growth chamber under irradiance of 100 µmol m^{-2} s^{-1} for 5 d. After the roots reached 1 cm in length, the seedlings were transferred to half-strength MS medium containing various concentrations of NaCl or mannitol. The root lengths were measured after 11 d. All the stress treatments were performed with independent three biological replicates.

Freezing tolerance: Healthy transgenic plants were incubated at -6 °C, and then they recovered for one week, after which the phenotypic changes were observed. Samples were collected before and after the low temperature stress treatment and immediately frozen in liquid nitrogen for a RT-qPCR analysis.

Statistical analysis: Statistical analyses were performed using the *SPSS* software (*SPSS Inc.*, Chicago, IL, USA). We analyzed the significance level, and we considered differences between means to be significant and extremely significant when *P* values were less than 0.05 and 0.01, respectively.

Results

The *PgMYB4* gene was cloned from ginseng roots by RT-PCR. The total length of the *PgMYB4* gene was 735 bp, and it encoded 245 amino acid (AA) with an isoelectric point of 5.41 (Fig. 1 Suppl.). The predicted molecular mass of the PgMYB4 protein was 27.9 kDa. The *PgMYB4* gene sequence was accessed in the *GenBank* (https://www.ncbi.nlm.nih.gov/genbank, ID MN998542).

Next, we generated transgenic *Arabidopsis thaliana* plants carrying *PgMYB4* gene under the control of the CaMV 35S promoter. PCR analysis of the putative transgenic plants confirmed the amplification of an

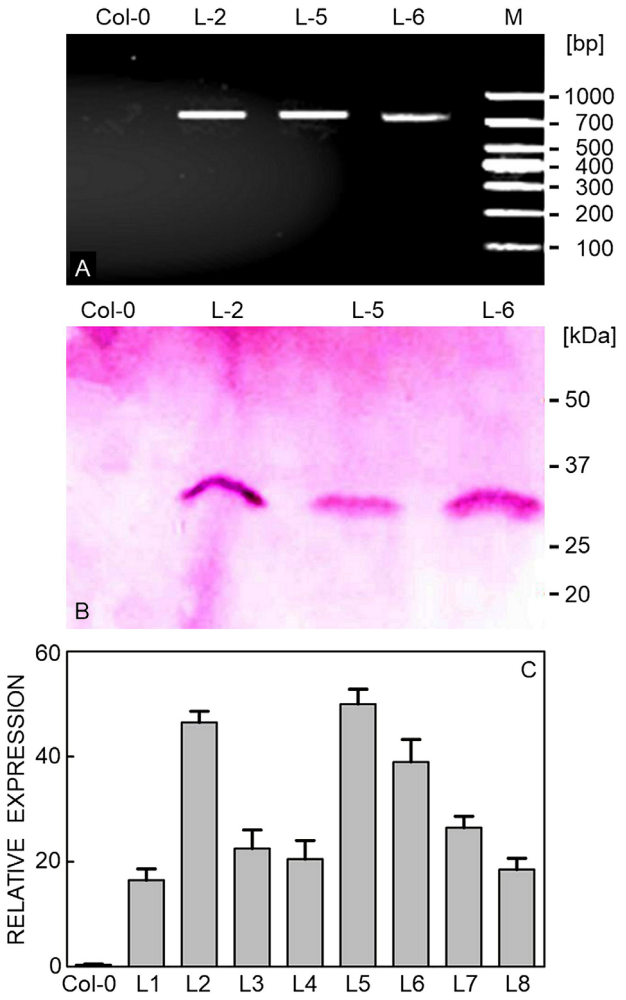

Fig. 1. Analysis by PCR (*A*) and Western-blot detection (*B*) of transgenic *Arabidopsis* plants. The expression of *Panax ginseng myeloblastosis 4* (*PgMYB4*) in different transgenic *Arabidopsis* lines (*C*). Col-0 - wild-type *Arabidopsis*, L1 to L8 - transgenic *Arabidopsis* lines, M - DL 1000TM DNA marker.

approximately 730-bp fragment in the T3 generation plants resistant to hygromycin (Fig. 1*A*). The same fragment was not amplified in the wild type *Arabidopsis* (control). Consistent with the PCR results, Western blot involving anti-*PgMYB4* polyclonal antibodies confirmed that the transgenic *Arabidopsis* plants, but not the wild-type plants, produced a protein between 25 and 37 kDa (Fig. 1*B*). The expression of *PgMYB4* in transgenic *Arabidopsis* was detected in all *PgMYB4* transgenic lines but not in the wild type (Fig. 1*C*). Three independent transgenic lines (L2, L5, and L6) showed significantly higher expression than others, and we selected them for subsequent experiments.

The *NCBI* database comparative analysis showed that *PgMYB4* contained two highly conserved MYB domains, which were located at 14 - 63 AA and 67 - 114 AA, respectively (Fig. 2 Suppl.). The PgMYB4 protein is consistent with the conserved domain of the R2R3-MYB subfamily. For further analysis, we used the *NCBI BLAST* tool to conduct the multiple sequence alignment of PgMYB4 with the MYBs from other plants (Fig. 2*A*). The *PgMYB4* gene exhibits a high homology with the *Cucumis sativus MYB4* (XP_008447428.1), *Fragaria vesca MYB4* (XP_004151985.1), and *Gossypium hirsutum MYB4* (XP_016709539.1). Like other *Panax ginseng* MYB proteins, PgMYB4 contains two domains. Each domain has three tryptophan residues that are spaced by 18 or 19 AAs. However, in the R3 MYB4 motif, the first tryptophan residue is replaced by phenylalanine (Afrin *et al.* 2015, Choi *et al.* 2017). The result shows that PgMYB4 belongs to the R2R3-MYB subfamily.

To further identify *PgMYB4* gene in *P. ginseng*, we constructed a phylogenetic tree (Fig. 2*B*). The phylogenetic analysis involved PgMYB4 with 11 MYB AA sequences from other species. Our results showed that PgMYB4 was most closely related to *Cucumis sativus* and *Fragaria vesca*, followed by *Gossypium hirsutum*. Interestingly, the majority of R2R3-MYB proteins, similar to PgMYB4, seems to be involved in response to various biotic and abiotic stresses (He *et al.* 2016). Therefore, we can speculate that PgMYB4 protein might be involved in abiotic stress responses.

We evaluated the osmotic stress tolerance of the PgMYB4-overexpressing transgenic *Arabidopsis* plants by placing T_3 transgenic seeds in half-strength MS medium containing various mannitol concentrations. The germination rate of the transgenic plants (L2, L5, and L6) was significantly higher than that of the wild-type plants for all three tested concentrations. The differences between the transgenic and wild-type plants were more apparent as the mannitol concentration increased. At 300 mM mannitol, the germination rates of the transgenic plants were 51.78, 49.13, and 47.72 %, respectively, whereas the germination rates of wild-type plants were only 25 % (Fig. 3*A*). Conversely, the germination rate for both plant types was 100 % in the normal culture medium.

The root length of transgenic and wild-type *Arabidopsis* plants was also carried on seedlings grown in the mannitol-containing medium for 11 d. The roots of the transgenic *Arabidopsis* were longer than those of the wild-type *Arabidopsis*. The difference in the root lengths increased with increasing mannitol concentrations. Specifically, the root lengths of the transgenic *Arabidopsis* plants were about 1.3-fold and 2.1-fold greater than those of the wild-type plants at mannitol concentrations of 200 mM and 300 mM, respectively (Fig. 3*B*,*C*). There were no differences in the primary root length between transgenic and wild-type plants in the control culture medium.

The overexpression of *PgMYB4* improved the survival rate of whole plants exposed to severe water shortage. We did not supply water to the T_3 generation plants for 14 d. No obvious phenotypic differences were detected between the transgenic and wild-type plants on day 5. However, on day 14, the wild-type plants were yellowish and wilted, and some plants died, whereas the transgenic plants exhibited less severe dehydration stress symptoms (*e.g.*, fewer yellow leaves) (Fig. 4*A*). After re-watering for 7 d, the survival rates of the transgenic and wild-type plants were 80, 75, 78, and 30 %, respectively (Fig. 4*B*). The much higher recovery rate for the *PgMYB4*-overexpressing

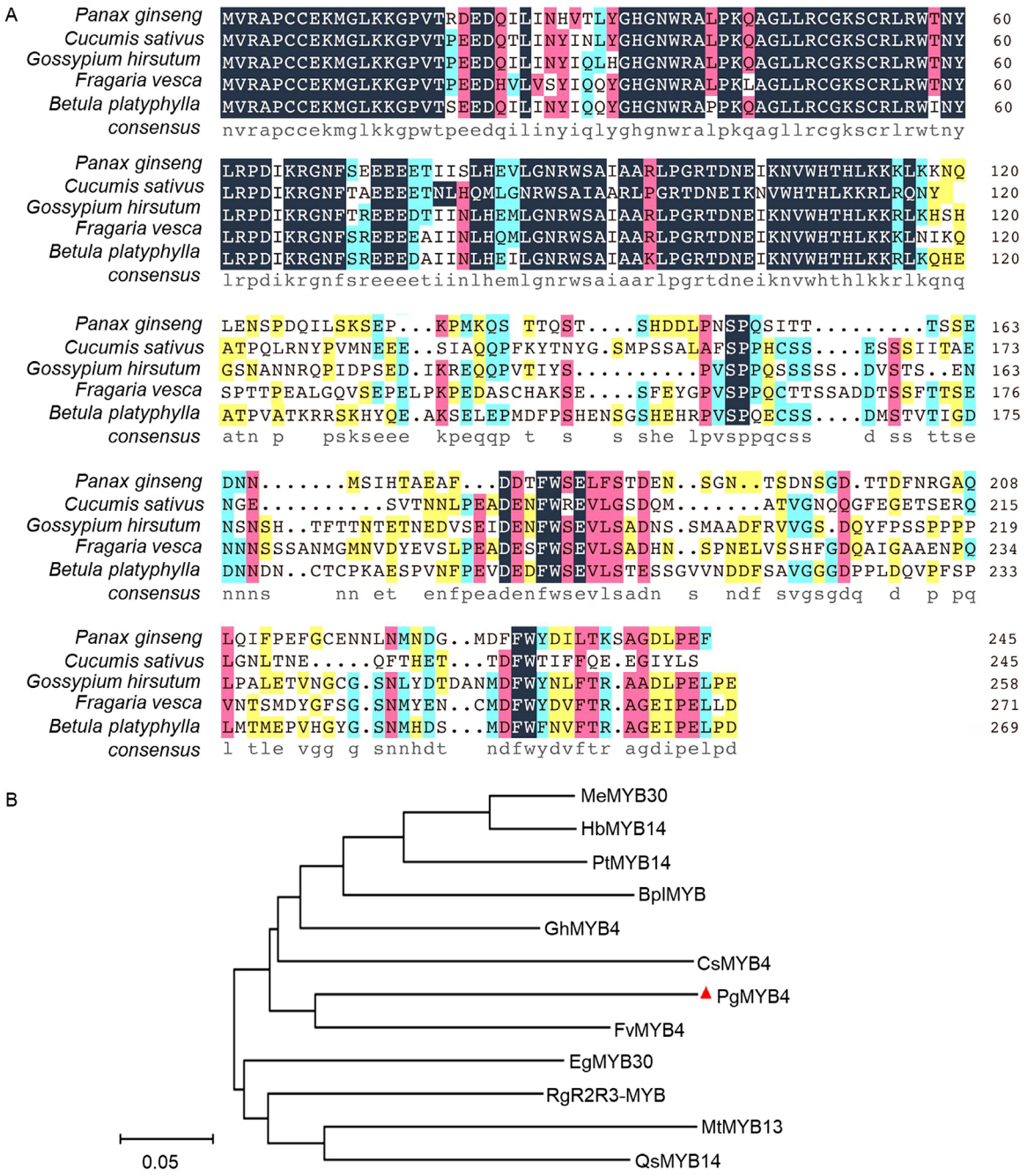

Fig. 2. Sequence analysis of *myeloblastosis 4* from *Panax ginseng* (PgMYB4). *A* - Sequence alignment of PgMYB4 with other homolog proteins, including CsMYB4 (*Cucumis sativus*, XP_004151985.1), GhMYB4 (*Gossypium hirsutum*, XP_016709539.1), FvMYB4 (*Fragaria vesca subsp. vesca*, XP_004289939.1), BplMYB (*Betula platyphylla*, QEK22814.1). *B* - a phylogenetic tree of PgMYB4. The sequences used are from: MeMYB30 (*Manihot esculenta*, XP_021629490.1), HbMYB14 (*Hevea brasiliensis*, XP_021656703.1), PtMYB14 (*Populus trichocarpa*, XP_002302045.1), BplMYB (*Betula platyphylla*, QEK22814.1), GhMYB4 (*Gossypium hirsutum*, XP_016709539.1), CsMYB4 (*Cucumis sativus*, XP_004151985.1), FvMYB4 (*Fragaria vesca subsp. vesca*, XP_004289939.1), EgMYB30 (*Elaeis guineensis*, XP_010920477.1), RgR2R3-MYB (*Rehmannia glutinosa*, AKV71950.1), MtMYB13 (*Medicago truncatula*, XP_013468731.1), and QsMYB14 (*Quercus suber*, XP_023879142.1).

transgenic plants implied that *PgMYB4* increased the drought tolerance of the transgenic *Arabidopsis*.

The overexpression of *PgMYB4* increased the plant survival rate under drought conditions. Consequently, we examined three physiological indices (*i.e.*, chlorophyll content, proline content, and water loss rate) of the drought-stressed transgenic and wild type *Arabidopsis* plants. Under normal conditions, the leaf chlorophyll content of the transgenic and wild-type *Arabidopsis* plants was similar. After a 5-d drought treatment, the leaf chlorophyll

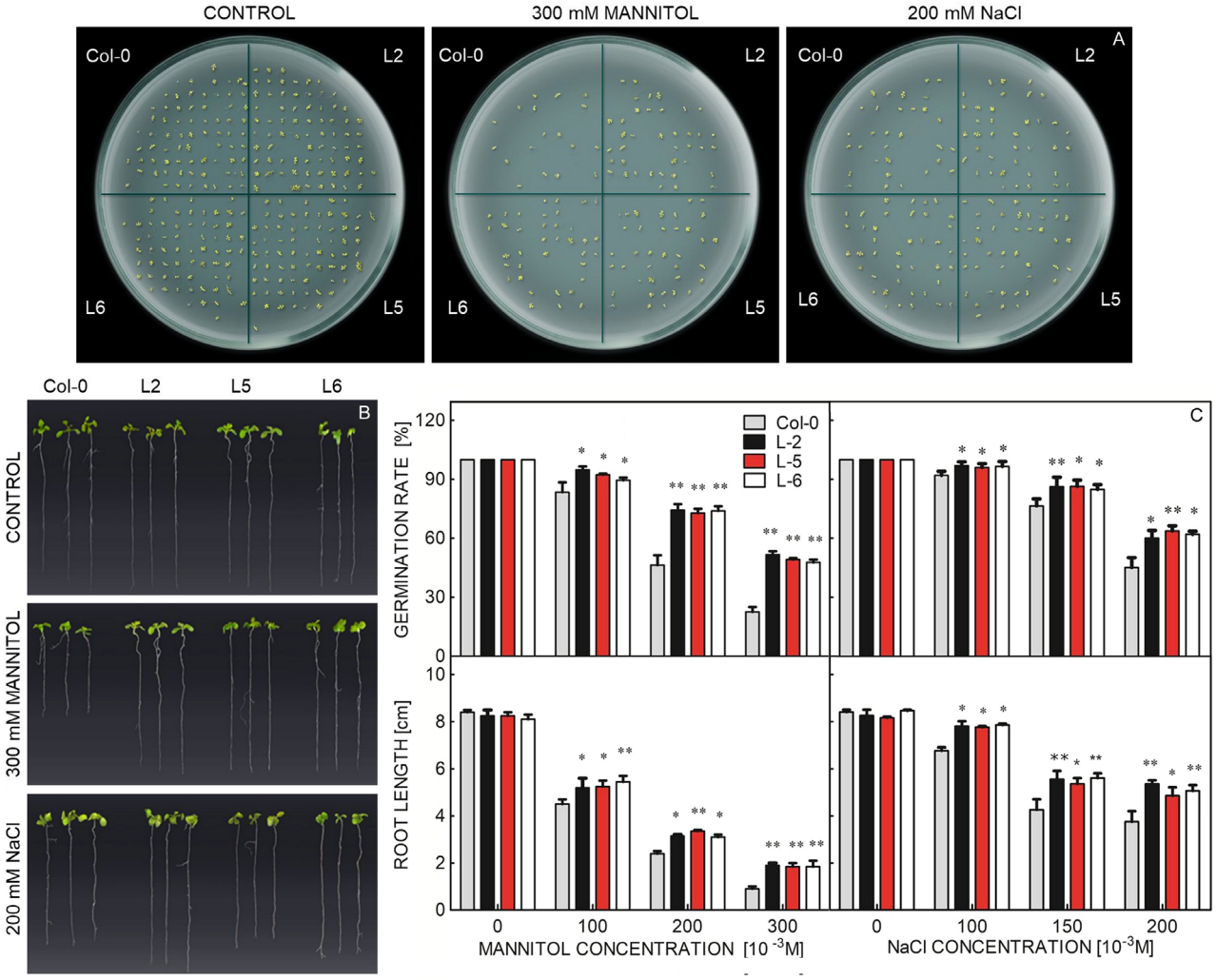

Fig. 3. Effects of abiotic stresses on seed germination and root growth of wild-type *Arabidopsis* (Col-0) and transgenic *Arabidopsis* lines (L2, L5, and L6). Seed germination and root growth (*A,B*), and germination rate and root length (*C*) under mannitol and NaCl stresses. Means ± SDs, $n = 3$; ** and * indicate statistically significant differences compared to the control at $P < 0.01$ and $P < 0.05$, respectively.

content in transgenic *Arabidopsis* decreased by only 2.80, 4.81, and 3.78 %, which was much less than the 13.85 % decrease in the wild-type *Arabidopsis* (Fig. 5*A*). The proline content increased by about 3.3-fold in transgenic plants, but only 2.2-fold in wild-type plants (Fig. 5*B*). The water loss rates from the shoots of the transgenic and wild-type plants were about 50.13, 51.1, 51.11, and 64.73 % at the 3-h time-point (Fig. 5*C*).

To better understand the possible regulatory mechanisms of *PgMYB4* in response to drought stress, the expressions of drought stress-related genes, including *RD19A*, *RD22*, *RD29A*, *COR15A*, *COR47*, and *P5CS1* (Guo *et al.* 2014, Qin *et al.* 2015), was detected in transgenic and wild-type lines by RT-qPCR. Before drought treatment, most of the gene expressions showed no obvious differences between the WT and transgenic plants. However, under drought stress, the expression of five genes was significantly induced (Fig. 6). Specifically, the overexpression of *PgMYB4* increased the expression of *RD19A* by 2.7- to 3-folds, *RD22* by 2.2 to 3.8-folds, *RD29A* by 1.9- to 3.6-folds, *COR15A* by 5.2- to 6.6-folds, *COR47* by 2.6- to 3.6-folds, and *P5CS1* 2.5- to 4.3-folds.

Overexpression of *PgMYB4* conferred salt stress tolerance to transgenic *Arabidopsis* plants as the germination rate was higher for the T_3 transgenic seeds than for the wild-type seeds in half-strength MS medium supplemented with NaCl. The differences were more obvious at higher NaCl concentrations. At 200 mM NaCl, the germination rates of the transgenic and wild-type seeds were 61 to 63 % and 45 %, respectively (Fig. 3*C*). The overexpression of *PgMYB4* improved the development of the main root under high salt stress, as determined by the root length. *Arabidopsis* seedlings were exposed to high salt stress for 11 d. The roots of transgenic plants were longer than those of wild-type, with more substantial differences under higher NaCl concentrations. In medium containing 200 mM NaCl, the transgenic *Arabidopsis* roots were 4.8 - 5.6 cm long, which were 1.3- to 1.5-times longer than the wild-type plant roots (Fig. 3*C*).

Under high salt stress, the whole-plant survival rates

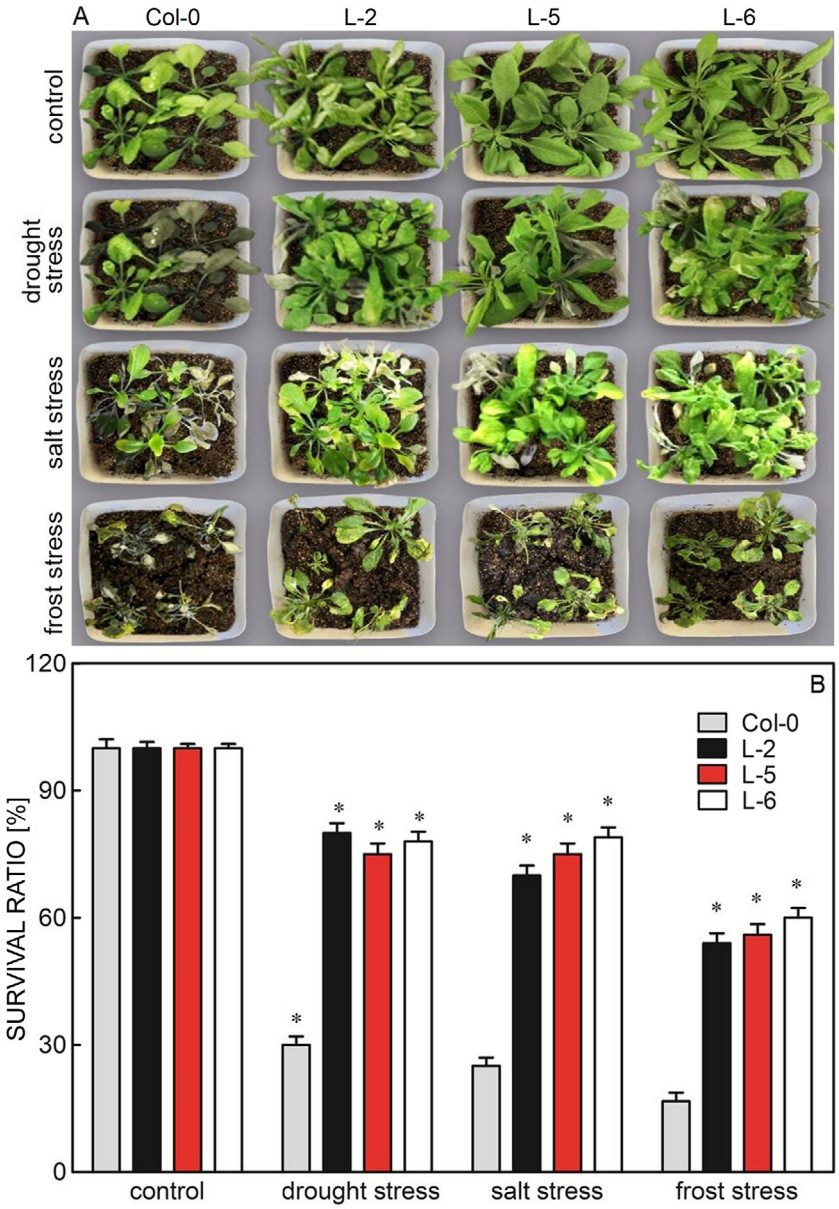

Fig. 4. Responses of transgenic and wild-type *Arabidopsis* plants to drought, high salinity, and frost stresses. *A* - phenotypic analysis of transgenic *Arabidopsis* (L-2, L-5, and L-6) and wild-type plants (Col-0). *B* - survival rates of transgenic and wild-type plants. Col-0 - wild-type *Arabidopsis*; L2, L5, L6 - transgenic *Arabidopsis* lines. The total number of surviving plants / total number of plants employed in the experiment for each line: drought stress: L2 (40/50), L5 (39/52), L6 (43/55), Col-0 (16/53); salt stress: L2 (36/50), L5 (39/52), L6 (43/55), Col-0 (13/53); frost stress: L2 (23/50), L5 (25/52), L6 (28/55), Col-0 (8/53). Means ± SDs, $n = 3$; ** and * indicate statistically significant differences compared to the control at $P < 0.01$ and $P < 0.05$, respectively.

were higher for the PgMYB4-overexpressing plants than for the wild-type controls. When plants were treated with 300 mM NaCl, the leaves of both the transgenic and wild-type plants turned yellow, but to varying degrees. The transgenic *Arabidopsis* plants grew much better than the wild-type plants. Specifically, the wild-type plants had many yellow leaves and some died. After allowing plants to recover by watering for one week, the survival rate (72, 75, and 78 %) of transgenic *Arabidopsis* was about 3-times higher than that (25 %) of the wild-type control (Fig. 4*B*).

To investigate the possible mechanism of the *PgMYB4*-mediated response to salt stress, we analyzed the expressions of salt stress-related genes. Before salt treatment, the genes showed no differences or slightly higher expressions in the transgenic plants. Among all analyzed stress-related genes, the expressions of most genes were not induced significantly under salt-stress, except *KIN1* (Fig. 7 and Fig. 3 Suppl.). Its expression increased by 20-fold in wild-type plants exposed to high salinity. The *KIN1* expression in the *PgMYB4*-overexpressing transgenic plants was 7.8-fold greater than that in the wild-type plants (Fig. 7). These results suggested that *PgMYB4* might be involved in the regulation of salt stress response.

After incubation at -6 °C for 50 min, the leaves of both

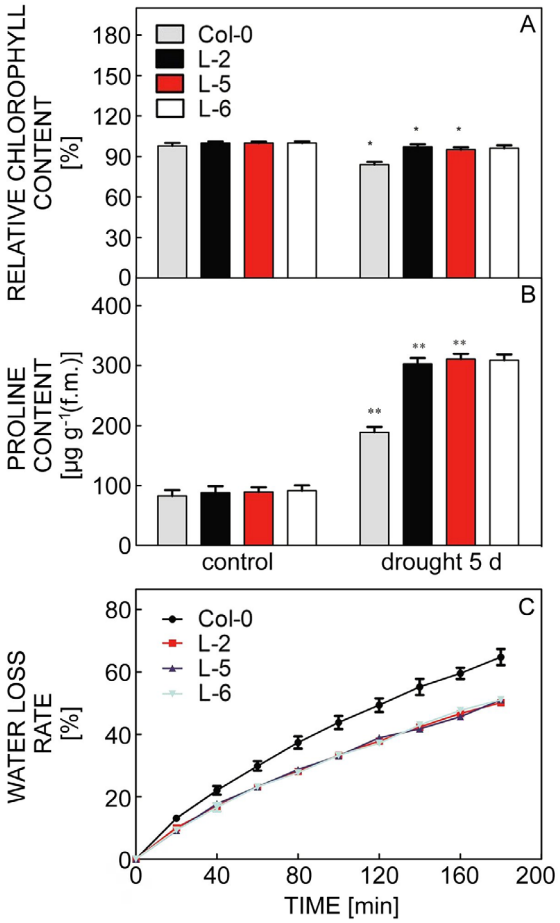

Fig. 5. Relative chlorophyll content (*A*), proline content (*B*), and water loss (*C*) of wild-type and transgenic *Arabidopsis* under drought stress for 5 d. Col-0 - wild-type *Arabidopsis*; L2, L5, L6 - transgenic *Arabidopsis*. Means ± SDs, $n = 3$; ** and * indicate statistically significant differences compared to the control at $P < 0.01$ and $P < 0.05$, respectively.

wild-type and transgenic *Arabidopsis* plants were dark green (*i.e.*, freezing damaged). After a 1-week recovery, the wild-type leaves were more damaged (*i.e.*, dark green and dehydrated) than the transgenic leaves. The survival rates of the wild-type and transgenic *Arabidopsis* plants were about 16.7 % and 54 to 60 %, respectively (Fig. 4*B*).

To further clarify the *PgMYB4*-mediated increase in the frost tolerance of transgenic *Arabidopsis*, we analyzed the expressions of stress-related genes in response to low temperature conditions. Obvious expression changes were detected for *DREB2A* and *KIN1*. The expressions of other genes decreased. After the freezing treatment, the *DREB2A* and *KIN1* expression were 3.4- to 3.8-times and 2.9- to 3.4-times higher, respectively, in the transgenic plants than in the wild-type *Arabidopsis* plants (Fig. 7). Thus, the increased resistance of *PgMYB4*-overexpressing plants to frost stress may result, in part, from the enhanced expression of these two genes.

Discussion

Stress affects plant growth and development, even though plants generally have a mechanism to overcome various biotic and abiotic stresses. *Arabidopsis thaliana* is a model plant, which is often used along with transgenic techniques to investigate gene functions related to stress responses (Wu *et al.* 2018, Yao *et al.* 2018). In the current study, we constructed an expression vector with the ginseng *MYB4* gene for the subsequent transformation of *Arabidopsis*. We studied if the overexpression of *PgMYB4* can increase the abiotic stress tolerance of the transgenic plants.

To investigate whether *PgMYB4* influences stress responses, we assessed the stress tolerance of transgenic *Arabidopsis* during the seedling stages. Seed germination is dependent on environmental factors, such as water, temperature, and oxygen (Hoang *et al.* 2014). Abiotic stress restricts germination by altering physiological or biochemical events.

Mannitol and NaCl can decrease the water absorption by plants by decreasing the substrate osmotic potential (Jakab *et al.* 2005). Treating the seeds of wild-type and *PgMYB4*-overexpressing transgenic plants with different mannitol and NaCl concentrations resulted in decreased germination rates and root lengths. The differences between the wild-type and transgenic plants were greater under higher mannitol or NaCl concentrations. These observations imply that the transgenic *Arabidopsis* seeds were able to better absorb or retain water than the wild-type seeds, which likely contributed to their greater germination rate and root growth. These results also indicate that the overexpression of *PgMYB4* might significantly increase plant tolerance to drought or high salt stress.

Chlorophyll is critical for photosynthesis in plants and its content directly influences photosynthesis and metabolism. To some extent, the chlorophyll content influences stress tolerance. A severe lack of water damages the structure of chloroplast lamellae and lowers the chlorophyll content of plant leaves. Accordingly, the chlorophyll content may be a useful indicator of plant sensitivity to drought stress (Dong *et al.* 2019, Carvalho *et al.* 2019). In this study, we observed that drought stress decreased the chlorophyll content considerably more in the wild type than in the *PgMYB4*-overexpressing *Arabidopsis*, which indicates a more substantial drought tolerance of the transgenic plants. Proline is one of the components of plant proteins. Proline accumulates in highly drought-tolerant plants (Vannini *et al.* 2007). Similar to chlorophyll, proline content can act as a physiological indicator of drought tolerance (Szabados *et al.* 2010). Our data revealed a greater accumulation of proline in *PgMYB4*-overexpressing *Arabidopsis* than in the wild-type controls, indicating that the transgenic plants are more drought-tolerant than the wild-type plants. The water loss rate from the shoots of the transgenic *Arabidopsis* was slower than from shoots of the wild-type, suggesting that the introduction of *PgMYB4* might promote stomatal closure in leaves to minimize water loss, thereby enhancing drought tolerance.

The overexpression of *PgMYB4* increased the tolerance of transgenic *Arabidopsis* to drought, high salinity, and

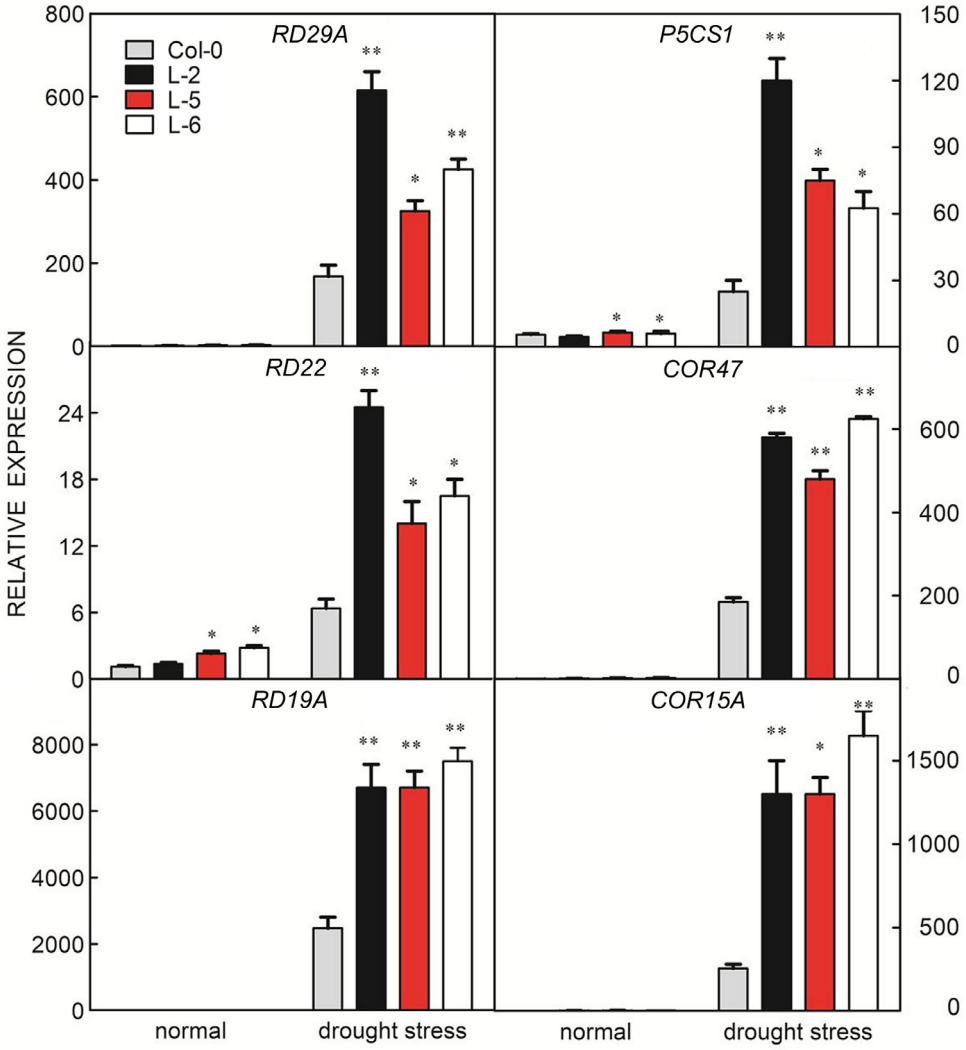

Fig. 6. Expression of drought stress related genes in wild-type and transgenic *Arabidopsis* under normal conditions and drought stress. Col-0 - wild-type *Arabidopsis*; L2, L5, L6 - transgenic *Arabidopsis*. *RD29A - responsive to desiccation 29A*, *P5CS1 - pyrroline-5-carboxylate synthase 1*, *RD22 - responsive to dehydration 22*, *COR47 - cold-regulated 47*, *RD19A - responsive to dehydration 19A*, *COR15A - cold-regulated 15A*. Means ± SDs, $n = 3$; ** and * indicate statistically significant differences compared to the control at $P < 0.01$ and $P < 0.05$, respectively.

low-temperature stresses. Under all three stress conditions, the survival rates of the transgenic *Arabidopsis* were at least threefold more elevated than the survival rates of the wild-type plants. These findings are consistent with those of previous studies on different MYB TFs. For example, the ectopic expression of maize *CmMYB2* in *Arabidopsis* enhances the tolerance to drought and salt stresses (Shan *et al*. 2012). Additionally, wheat *TaMYB33*-overexpressing transgenic *Arabidopsis* plants are reportedly sensitive to ABA and highly tolerant of drought and saline conditions (Qin *et al*. 2012). Thus, *PgMYB4* influences the responses of transgenic *Arabidopsis* to some abiotic stresses.

To confirm the mechanism underlying this enhanced tolerance to drought, salt, and low-temperature due to *PgMYB4* overexpression, we quantified the expressions of several marker genes associated with stress responses. It is known that *RD19A*, *RD22*, *RD29A*, *COR15A*, *COR47*, and *P5CS1* are inducible by cold, salt, or drought stress (Guo *et al.* 2014, Wang *et al.* 2016). *KIN1* is an important cold-inducible gene in *Arabidopsis*. DREBs have been involved in cold stress through regulating the transcription of the low temperature responsive genes (Nakashima *et al.* 2009).

Under non-stress conditions, *RD19A, RD29A, COR15A, COR47*, *P5CS1* and *KIN1* showed similarly low expressions in *PgMYB4*-overexpressing and wild type plants. However, under drought or salt stress, the expressions of these genes in the transgenic plants were higher than in the wild type plants (Fig. 6 and 7). *PgMYB4*-overexpression did not enhance the expressions of stress-responsive genes under normal growth condition. We speculate that *PgMYB4* may mediate the activation of these stress-responsive genes accompanied by other stress-responsive regulators. Under drought stress, *RD19A*, *RD22*, *RD29A*, *COR15A*, *COR47*, and *P5CS1* were more highly expressed in the transgenic *Arabidopsis* than in the

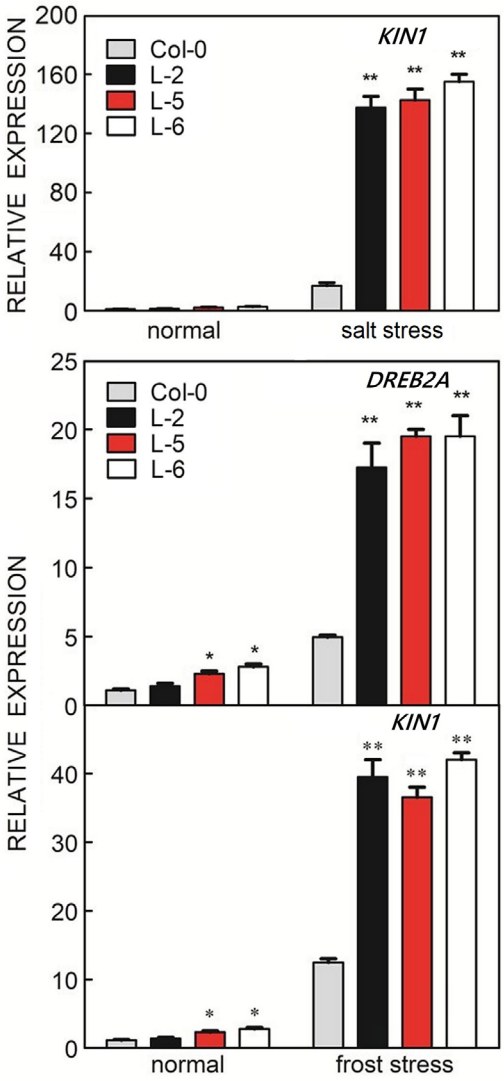

Fig. 7. Expressions of high salt stress and frost stress related genes in transgenic *Arabidopsis* under normal conditions and under salt or frost stress. Col-0 - wild-type *Arabidopsis*. L2, L5, L6 - transgenic *Arabidopsis*. Means ± SD, $n = 3$; ** and * indicate statistically significant differences compared to the control at $P < 0.01$ and $P < 0.05$, respectively.

wild-type. Drought, which is one of the most common stresses plants are exposed to, affected the expression of more genes compared to the other tested abiotic stresses. In response to the high salt and cold stress, the expression of *KIN1* in transgenic plants was up-regulated compared with that in wild type plants. In addition, *DREB2A* expression is substantially higher in transgenic plants than in wild-type under low temperature conditions (Guo *et al*. 2014). We observed that the *P5CS1* gene was significantly up-regulated in transgenic plants under drought conditions. Pyrroline-5-carboxylate synthetase (*P5CS*) is the crucial enzyme for proline biosynthesis in plants (De Ronde *et al*. 2004). The increased expression of *P5CS1* in transgenic plants resulted in increased proline accumulation. Our findings were consistent with the previous reports that overexpression of *P5CS1* gene in transgenic plants resulted in accumulation of free proline, and increased tolerance to abiotic stress (Hong *et al.* 2016).

Our studies suggest that overexpression of *PgMYB4* resulted in drastically increased tolerance to abiotic stresses probably by elevated stress-responsive gene expression and inhibited stomata opening and water loss. These findings imply that *PgMYB4* probably functions as a positive regulatory factor that improves stress tolerance by up regulating the expression of multiple stress-related genes, and involved in the mediation of stoma function.

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