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Original Article

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Molecular cloning and expression profile of an abiotic stress and hormone responsive MYB transcription factor gene from *Panax ginseng*

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Abstract

The v-myb avian myeloblastosis viral oncogene homolog (MYB) family constitutes one of the most abundant groups of transcription factors and plays vital roles in developmental processes and defense responses in plants. A ginseng (*Panax ginseng* C.A. Meyer) MYB gene was cloned and designated as *PgMYB1*. The cDNA of *PgMYB1* is 762 base pairs long and encodes the R2R3-type protein consisting 238 amino acids. Subcellular localization showed that PgMYB1-mGFP5 fusion protein was specifically localized in the nucleus. To understand the functional roles of *PgMYB1*, we investigated the expression patterns of *PgMYB1* in different tissues and under various conditions. Quantitative real-time polymerase chain reaction and western blot analysis showed that *PgMYB1* was expressed at higher level in roots, leaves, and lateral roots than in stems and seeds. The expression of *PgMYB1* was up-regulated by abscisic acid, salicylic acid, NaCl, and cold (chilling), and down-regulated by methyl jasmonate. These results suggest that *PgMYB1* might be involved in responding to environmental stresses and hormones.

Key words: abiotic stress, gene expression, hormone, MYB transcription factor, *Panax ginseng*

Introduction

Plant growth and productivity are actively influenced by various environmental conditions, including drought, high salt, and extreme temperature. Plants respond and adapt to these abiotic stresses with an array of biochemical and physiological changes [1,2]. Many adaptation processes are regulated by stress-responsive gene. Transcription factors (TFs) regulate the expression of genes that play important roles in plants to adapt to the environmental changes [3]. Numerous members of ethylene-responsive element-binding factors, basic-domain leucine-zipper, and v-myb avian myeloblastosis viral oncogene homolog (MYB) TF families are involved in regulating the stress responses [4]. MYB TFs are widely distributed in all eukaryotic organisms and constitute one of the largest TF families in the plant. MYB proteins are defined by a highly conserved MYB DNA binding domain (DBD) at the N-terminus [5]. MYB domain is usually composed of one to four imperfect repeats, each with ~50–53 amino acid residues that adopt a helix-turn-helix (HTH) conformation intercalating in the major groove of the DNA [6]. The first gene defined as encoding an MYB domain-containing protein was *v-myb* in the genome of avian myeloblastosis virus, therefore, called 'MYB' [7]. Thereafter, many MYB genes were recognized in animals, plants, fungi, and slime molds [5,6,8,9]. The structures and functions of MYB TFs in plants are conserved compared with those in animals and yeasts. MYB proteins in plants are classified into four subfamilies. MYB-related type has a single MYB repeat, R2R3-MYB has two repeats, 3R-MYB (R1R2R3-MYB) has three consecutive repeats, and 4R-MYB has four repeats [9]. Among these groups, R2R3-MYB TFs are the largest group of plant MYBs [10]. The first identified plant MYB gene *COLORED1* (*C1*) was isolated from *Zea mays* in 1987, which encoded a c-myb-like TF concerned in anthocyanin biosynthesis [11]. An increasing number of R2R3-MYB TFs have been identified subsequently and characterized in numerous plants [5].

The plant MYB TFs have been known to participate in diverse physiological and biological processes, including the regulation of secondary metabolism [12], controlling cell morphogenesis [13], regulation of meristem formation, floral and seed development [14], and the control of cell cycle [10]. Some are also involved in various defense and stress responses [15] and in hormone signaling pathways [16]. In *Arabidopsis thaliana*, the expression profiles of several MYB genes were tested for their responses to the phytohormones and stress conditions [6]. The MYB TF AhMYB6 from *Arachis hypogaea* is closely related to environmental stresses and induced by cold (chilling), salinity (NaCl), and abscisic acid (ABA) [17]. In *Arabidopsis*, AtMYB15 was demonstrated to be associated with cold stress tolerance [18]. TaMYB33 and TaMYB73 from *Triticum aestivum* exhibited enhanced salinity tolerance [19,20]. AtMYB13, AtMYB15, AtMYB33, and AtMYB101 of *Arabidopsis* were implicated in phytohormone ABA-mediated responses to environmental signals [21]. In *Nicotiana tabacum*, NtMYB1 and NtMYB2 were reported to be tightly regulated by elicitor treatments, such as salicylic acid (SA), suggesting its direct involvement in the pathogen resistance processes [22,23]. These observations suggest the involvement of MYBs in responses to abiotic stresses and hormones.

Panax ginseng C.A. Meyer is a perennial herbaceous plant belonging to the family Araliaceae. Ginseng is one of the most valuable traditional medicines in Asian countries for thousands of years. Ginsenosides are the pharmacologically active major ingredients of ginseng which play an important pharmacological role in human health [24], including stimulating or soothing the nervous system, balancing metabolic processes, decreasing blood sugar, improving muscle tone, and stimulating the endocrine system [25]. Moreover, ginseng has the advantage that it is free from harmful side effects. Nowadays, ginseng is consumed on a global scale. Markets for ginseng and related products are estimated to be 3.5 billion dollars worldwide and continue to expand with gradually elucidated pharmacological mechanisms of ginseng [26]. However, cultivation of ginseng has encountered difficulties because of its vulnerability to environmental stresses. Many of stress-related genes are influenced by environmental factors such as plant hormones, temperature, and salinity during the development of ginseng [27,28]. In order to understand the important roles of MYB TF in response to various stresses and hormones, cDNA of *PgMYB1* was isolated from 4-year-old *P. ginseng* roots. We also conducted the expression profiling of *PgMYB1* in response to abiotic stresses and hormones.

Materials and Methods

Plant materials

Ginseng (*P. ginseng* C.A. Meyer) was collected from Fusong County, Jinlin Province, China. Four-year-old fresh ginseng roots were used for gene cloning and hairy roots induction. Ginseng main roots, lateral roots, stems, leaves, and seeds were used for gene expression analysis.

RNA extraction and cDNA synthesis

Total RNA was isolated with E.Z.N.A.® Plant RNA kit (OMEGA, Norcross, USA). To remove residual DNA contamination, total RNA was digested with RNase-free DNase I (Promega, Madison, USA). RNA concentration, purity, and integrity were determined by spectrophotometric analysis (OD_{260}/OD_{280}) and examined by electrophoresis on 1.2% agarose gels. Reverse transcription was performed using RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, USA). cDNA was synthesized from total RNA following the manufacturer's instruction. The synthesized cDNA product was diluted appropriately and stored at −20°C until further analysis.

PgMYB1 cDNA cloning

PgMYB1 cDNA was obtained by reverse transcription-polymerase chain reaction (RT-PCR). The primer pair (5′-ATGGGTAGAGCTCCTTGTTGTG-3′ as forward and 5′-GCACACTCCCCACTTGTATAAC-3′ as reverse) was designed based on the information of transcriptome analysis of *P. ginseng*, using the PRIMER 5.0 program (http://www.premierbiosoft.com/). PCR reaction was performed in a 50 μl reaction volume containing 5 μl of 10× PCR buffer (Mg^{2+} Plus), 4 μl of dNTP (2.5 mM each), 2 μl of each primer (10 μM), 34.25 μl of ddH_2O, 0.25 μl (5 U/μl) of *Ex* Taq DNA polymerase (TaKaRa, Dalian, China), and 2.5 μl of cDNA template. The PCR reactions were as follows: 2 min initial denaturation at 94°C for 1 cycle, then 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and elongation at 72°C for 1 min, followed by a 7 min extension at 72°C. Specific PCR products were selected by 1% agarose gels electrophoresis and cloned into pGEM-T Easy vector (Promega) for sequencing.

Analysis of *PgMYB1* gene

The open reading frame (ORF) analysis and amino acid sequence comparison were carried out using ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.htm) and ClustalX program, respectively. The physicochemical properties of protein [molecular weight and isoelectric point (p*I*)] were analyses by ExPASy bioinformatics resource portal website of protparam tools (http://web.expasy.org/protparam/). Prediction of transmembrane domains was generated using the TMHMM 2.0 (http:/www.cbs.dtu.dk/services/TMHMM-2.0). The structure of PgMYB1 protein was predicted by protein homology/analogy recognition engine (http://www.sbg.bio.ic.ac.uk/phyre). Phylogenetic analysis of deduced amino acid alignments was carried out using the neighbor-joining method with the MEGA 4.0 program [29]. Bootstrap analysis with 1000 replicates was used to assess the strength of nodes in the tree.

Subcellular localization analysis

To confirm the subcellular localization of the PgMYB1 protein, the ORF of the *PgMYB1* cDNA was amplified with primer1 (5′-GAAGATCTATGGGTAGAGCTCCTTGTTGTG-3′) and primer2 (5′-GACTAGTAAATTCCGGTAATTCTGGTAATTCC-3′) using In-Fusion HD Cloning Kit (Clontech, Mountain View, USA) according to the instructions. The PCR product was cloned into the N terminus of the green fluorescent protein (GFP) gene (PgMYB1-mGFP5) under the control of the CaMV35S promoter of pCAMBIA1302. The recombinant vector (pCAMBIA1302-PgMYB1) was transformed into the epithelial cells of tobacco (*N. tabacum*) leaves and inner epidermis of onion (*Allium cepa*) using *Agrobacterium tumefaciens* GV3101. After the transformed tobacco leaves and onion epidermis cells were incubated for 18 h at 26°C in the dark, GFP was detected under a fluorescence microscope (Nikon Eclipse 80i, Nikon, Tokyo, Japan) with the excitation wave length in blue light at 498 nm and the emission wave length at 510 nm. The epithelial cells of tobacco leaves and onion epidermis cells transformed with the empty pCAMBIA1302 vector were assayed in parallel as the negative control.

Protein extraction and western blot analysis

Proteins were extracted from *P. ginseng* using protein extraction buffer as described previously [30]. The total protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's protocol. The extraction buffer contained 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)–HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Nonidet P-40, 4 M urea, and 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail Complete Mini tablets (Roche, Penzberg, Germany). Proteins (100 µg) were separated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (8% polyacrylamide) and transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, USA) using the semi-dry Bio-Rad system in 50 mM Tris, 40 mM glycine, 0.0375% SDS, and 10% methanol. A peptide corresponding to an internal sequence of PgMYB1 was synthesized and this peptide was linked to keyhole limpet hemocyanin and injected into two rabbits. The peptide and rabbit polyclonal antibodies were prepared by CW Biotechnology (Shanghai, China). Antibodies were diluted as follows: anti-PgMYB1 antibody, 1:400; anti-Actin antibody (AbMART, Shanghai, China), 1:800; horseradish peroxidase-labeled goat anti-rabbit and goat anti-mouse IgG antibodies (AbMART), 1:5000.

Stress and hormone treatments

To investigate the response of *PgMYB1* gene to various stress and hormone treatments, 2 weeks cultured hairy roots (transformed by *Rhizobium rhizogenes* A4 without any foreign DNA) were maintained in MS media under the following conditions: 20 µM ABA, 200 µM SA, 100 µM methyl jasmonate (MeJA), 50 mM NaCl, and 4°C for 0, 1, 3, 6, 12, 24, 36, and 48 h. The hairy root samples after post-treatment were collected immediately and frozen in liquid nitrogen and finally stored at −70°C until use.

Expression analysis by quantitative real-time PCR

The gene transcript levels in different tissues and hairy roots of ginseng were quantified by quantitative real-time PCR (qRT-PCR) using the SYBR Green stains on the Mastercycler ep realplex^2 detection system (Eppendorf, Hamburg, Germany). Total RNA was extracted from ginseng samples using E.Z.N.A. Plant RNA kit. Reverse transcription reactions were performed using RevertAid™ First Strand cDNA Synthesis Kit (Thermo). A 50 µl reaction mixture contained 25 µl of 2× SYBR Premix Ex Taq II (TaKaRa), 1 µl each of (10 µM) forward and reverse primer, 1.5 µl cDNA, and 21.5 µl ddH_2O. The PCR conditions were: 95°C for 10 min; 35 cycles of 95°C for 15 s, 58°C for 30 s, 72°C for 30 s; 95°C for 15 s; 60°C for 1 min and 95°C for 15 s. At the end of the amplification cycle, a melting analysis was conducted to verify the specificity of the reaction. The *PgMYB1* relative expression level for each sample was normalized with the *β-actin* internal control (GenBank accession number AY907207) and was calculated using the formula $2^{-\Delta\Delta Ct}$. All qRT-PCR reactions were performed in triplicate. Specific primers for *PgMYB1* were 5′-TCCGGAGATCGACGAGAGTT-3′ as forward, 5′-TTTGCAC ACTCCCCACTTGT-3′ as reverse; and *β-actin* were 5′-TGCCCC AGAAGAGCACCCTGT-3′ as forward, 5′-AGCATACAGGGAAA GATCGGCTTGA-3′ as reverse.

Statistical analysis

All results were reported as the mean ± standard error of three independent experiments. The data were analyzed statistically by analysis of variance, and the difference between the means of samples was analyzed by the least significant difference at probability level of 0.05.

Results

Cloning and sequencing of *PgMYB1*

Using a pair of primers corresponding to conserved plant MYB genes, one MYB cDNA was isolated from ginseng hairy roots by RT-PCR method. The gene was named *PgMYB1* (*P. ginseng* MYB). The *PgMYB1* cDNA is 762 base pairs (bp) long (**Fig. 1**) that has ORF region 717 bp and 3′-UTR (45 bp) sequences. The ORF encodes a protein with 238 amino acid residues with a predicted molecular weight of 27.3 kDa and p*I* of 7.01 (http://web.expasy.org/protparam/). The NCBI database comparative analysis predicted that PgMYB1 protein contains two highly conserved MYB domains (**Fig. 2A**). For further analysis, PgMYB1 is alignment with other plant MYBs by using NCBI BLAST tool site. PgMYB1 exhibits high homology with the potato StMYB4 (XP_006354484.1), carrot DcMYB1 (BAE54312.1), alfalfa MtMYB50 (ABR28338.1), and grape VlMYBB1-2 (BAC07544.1). Like other plant MYB proteins, PgMYB1 also contains two domains. Each domain contains three conserved tryptophan residues (W), spaced 18 or 19 amino acid residues, wherein the first type of tryptophan residues R3 MYB1 motif is replaced by phenylalanyl acid residues (F). The alignment in the conserved regions of the MYB TF is shown in **Fig. 2B**. The results show that the PgMYB1 contains perfectly conserved domains with other R2R3-MYB subfamilies.

The location of the putative transmembrane helices predicted by TMHMM 2.0 is shown in **Fig. 3**. Hydropathy analysis shows that PgMYB1 has no transmembrane domain, indicating PgMYB1 is not a transmembrane protein. Secondary structure was predicted by using GOR ExPASy website, indicating that PgMYB1 protein contains 36.97% of alpha helix, 7.98% of extending chain, and 55.04% of random coil (**Fig. 4A**). The predicated three-dimensional structure of PgMYB1 protein has been analyzed using protein homology/analogy recognition engine (Phyre) as shown in **Fig. 4B**,

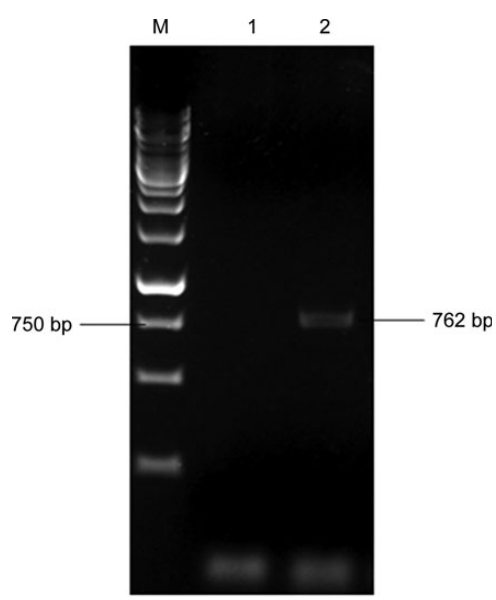

Figure 1. The full length cDNA of *PgMYB1* M: 1 kb DNA maker; 1: blank; 2: MYB fragment.

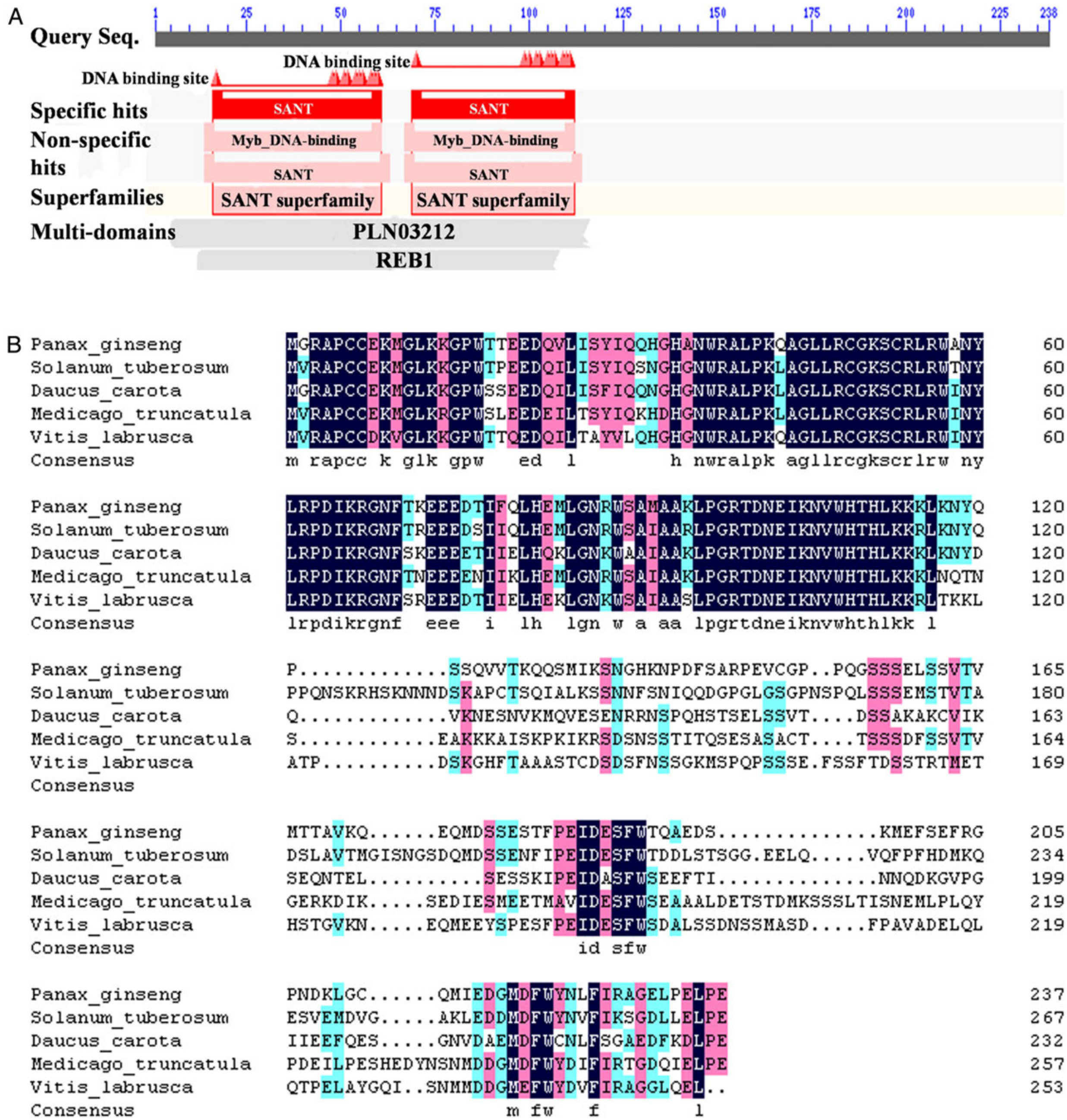

Figure 2. Amino acid alignment of the plant MYB proteins (A) Conserved domains of *PgMYB1* from *P. ginseng.* (B) MYBs included in this comparison are StMYB4 from *Solanum tuberosum* (XP_006354484.1), DcMYB1 from *Daucus carota* (BAE54312 0.1), MtMYB50 from *Medicago truncatula* (ABR28338.1), and VlMYBB1-2 from *Vitis labrusca* (BAC07544.1).

which clearly shows that it has two MYB domains and each domain contains three α-helices forming HTH structure, indicating that PgMYB1 protein belongs to the R2R3-MYB family.

Homology analysis

In order to evaluate the structural characteristics of MYB genes in *P. ginseng*, the amino acids were aligned with the MYB proteins from other plants by using MAGE 4.0 analysis. In this study, phylogenetic tree was constructed using the highest homology of *PgMYB1* with 35 MYB TFs nucleic acid and amino acid sequences of full length mRNA information (**Fig.** 5). Phylogenetic analysis revealed that, the closest PgMYB1 homologs are tobacco NtMYB1 (AAB41101.1), potato StMYB4 (XP_006354484.1), tomato SlMYB4 (XP_004247733.1), carrot DcMYB1 (BAE54312.1), and peanut AhMYB6 (AHB59594.1) with 58%, 60%, 59%, 58%, and 51% similarity, respectively. Interestingly, the majority of R2R3-MYB proteins identical to PgMYB1 have been shown to be involved in response to various environmental stresses, and some of these genes are constitutively expressed in certain tissues, such as leaves and roots involved in plant defense [31,32]. Expression analysis has indicated that NtMYB1 and NtMYB2 proteins play a role in the extrusion of defensive compounds in the leaves [22,23]. The expression of AhMYB6 exhibits protective functions in roots and leaves of peanut [17]. We can therefore speculate that PgMYB1 protein might be related to the abiotic stress response in special tissues of ginseng.

Subcellular localization of PgMYB1 protein

The subcellular location of PgMYB1 was investigated by the expression of fusion protein at the N terminus of GFP under the control of the cauliflower mosaic virus (CaMV) 35S promoter. PgMYB1-mGFP5 was expressed in the epithelial cells of tobacco leaves and epidermal cells of onion by infiltration with *Agrobacterium*. Subcellular localization of PgMYB1-mGFP5 was observed using a fluorescence microscope. Empty 35S::mGFP5 was used as a control. The results of both tobacco and onion showed that the PgMYB1-mGFP5 fusion protein was specifically localized in the nucleus, whereas GFP alone showed ubiquitous distribution in the whole cell (**Fig. 6**). These results suggest that PgMYB1 is a nucleus-localized protein.

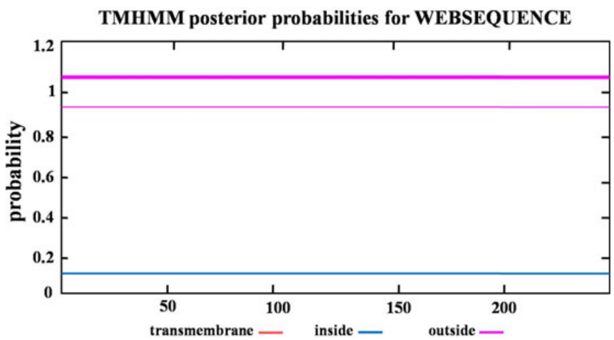

Figure 3. PgMYB1 transmembrane helices prediction by TMHMM Server (version 2.0) Blue line indicates intracellular loop and pink lines indicate extracellular loops.

Tissue-specific expression patterns of *PgMYB1*

The RT-PCR and qRT-PCR analyses were performed to determine the patterns of *PgMYB1* gene expression in different tissues of *P. ginseng*. As shown in **Fig. 7A,B**, the *PgMYB1* transcript was expressed constitutively in stems, main roots, lateral roots, leaves, and seeds with the highest level in main roots, leaves, and lateral roots. While lower expression was observed in stems and seeds. In plants, MYB TFs have various expression patterns in different tissues. For example, NtMYB1 and NtMYB2 were expressed in the leaves [22,23], whereas AhMYB6 transcripts were found both in the roots and leaves [17]. As is well-known, roots and leaves are the most important tissues in plants that face various environment stresses from the below ground and above ground. Leaves remain in the direct contact with airborne pathogens, herbivores, and adverse environment. Likewise, the soil is affluent in pathogenic organisms and herbivores. Root herbivores can do harm to plants as much, or sometimes even more, as shoot feeding herbivores and roots need protection after wounding [33].

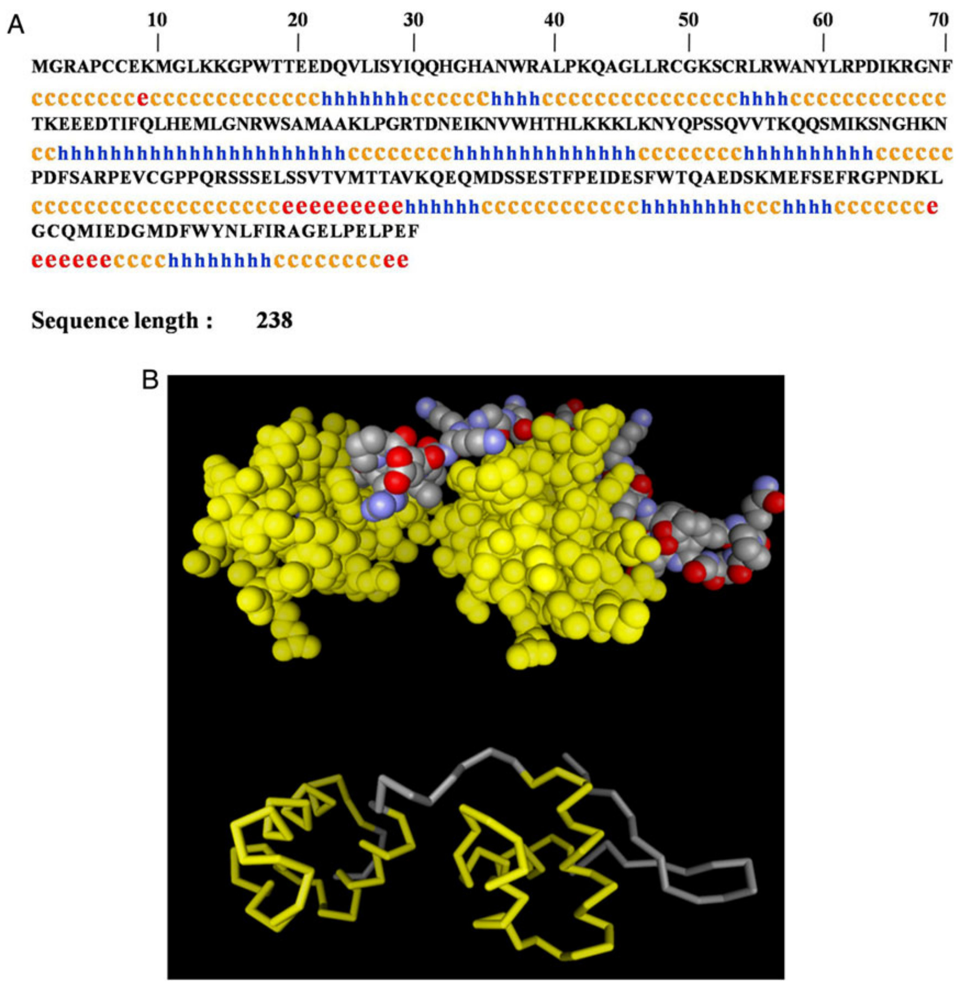

Figure 4. Schematic illustration of the structure of the PgMYB1 protein (A) Secondary structure prediction by GOR ExPASy website. (B) The predicted three-dimensional structure was constructed by Phyre based on template-based homology modeling. The yellow regions of MYB domain represent DBDs, *PgMYB1* contains two DBDs and each domain has three α-helix formation HTH structure.

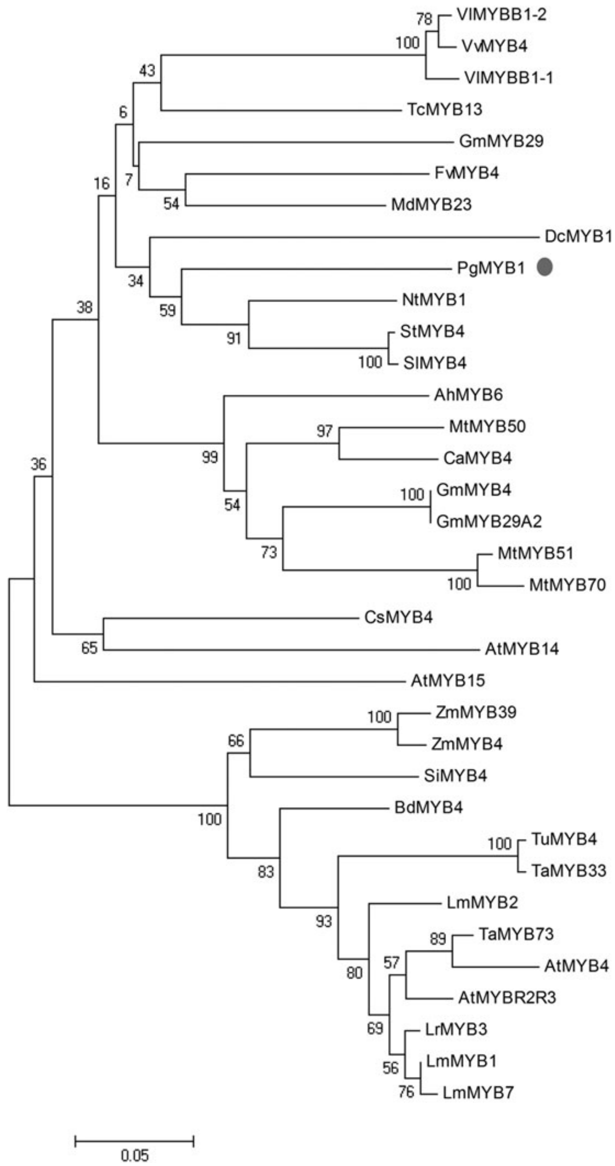

Figure 5. Phylogenetic analysis of the amino acid sequences with other plant MYB proteins calculated with the program MEGA 4.0 using the neighbor-joining method The sequences used are from: VlMYBB1-2 (*V. labrusca*, BAC07544.1); VvMYB4 (*Vitis vinifera*, XP_002285193.1); VlMYBB1-1 (*V. labrusca*, BAC07543.1); TcMYB13 (*Theobroma cacao*, XP_007035114.1); GmMYB29 (*Glycine max*, BAA81732.1); FvMYB4 (*Fragaria vesca*, XP_004296369.1); MdMYB23 (*Malus domestica*, AAZ20439.1); DcMYB1 (*D. carota*, AE54312.1); PgMYB1 (*P. ginseng*, FJ475127.1); NtMYB1 (*N. tabacum*, AAB41101.1); StMYB4 (*S. tuberosum*, XP_006354484.1); SlMYB4 (*Solanum lycopersicum* XP_004247733.1); AhMYB6 (*A. hypogaea*, AHB59594.1); MtMYB50 (*M. truncatula*, ABR28338.1); CaMYB4 (*Cicer arietinum*, XP_004495379.1); GmMYB4 (*G. max*, XP_003518022.1); GmMYB29A2 (*G. max*, BAA81732.1); MtMYB51 (*M. truncatula*, ABR28339.1); MtMYB70 (*M. truncatula*, ABR28344.1); CsMYB4 (*Cucumis sativus*, XP_004147145.1); AtMYB14 (*A. thaliana*, NP_180676.1); AtMYB15 (*Arabidopsis lyrata* subsp. *lyrata* XP_002883414.1); ZmMYB39 (*Z. mays*, NP_001106008.1); ZmMYB4 (*Z. mays*, NP_001152064.1); SiMYB4 (*Setaria italic*, XP_004953149.1); BdMYB4 (*Brachypodium distachyon*, XP_003575392.1); TuMYB4 *Triticum urartu*, EMS62386.1); TaMYB33 (*T. aestivum*, AEO21928.1); LmMYB2 (*Leymus multicaulis*, ADN96005.1); TaMYB73 (*T. aestivum*, AEW23186.1); AtMYB4 (*Aegilops tauschii*, EMT29840.1); AtMYB2R3 (*A. tauschii*, AEV91155.1); LrMYB3 (*Leymus racemosus*, ADI24681.1); LmMYB1 (*L. multicaulis*, ADN96004.1); and LmMYB7 (*L. multicaulis*, ADI24680.1).

Therefore, the expression of PgMYB1 protein predominately in roots (main and lateral) and leaves might indicate that it usually plays roles in the extrusion of defensive compounds in these organs. In order to further understand the expression profile of *PgMYB1* in protein level, an antibody was generated against amino acid sequence in a region unique to PgMYB1. Immunoblotting analysis of various plant tissues showed that *PgMYB1* expression was consistent with transcript level at different tissues of *P. ginseng* (**Fig.** 7C).

Expression profiles of *PgMYB1* in response to abiotic stresses and hormones

Increasing evidences suggest that MYB family proteins participate in a multitude of physiological processes that allow the plant to adapt to the external environments and cope with biotic and abiotic stresses [10,34]. To determine the expression profile of *PgMYB1* at different time points after various treatments, the expression levels of *PgMYB1* were analyzed by qRT-PCR. Hormones such as ABA, SA,

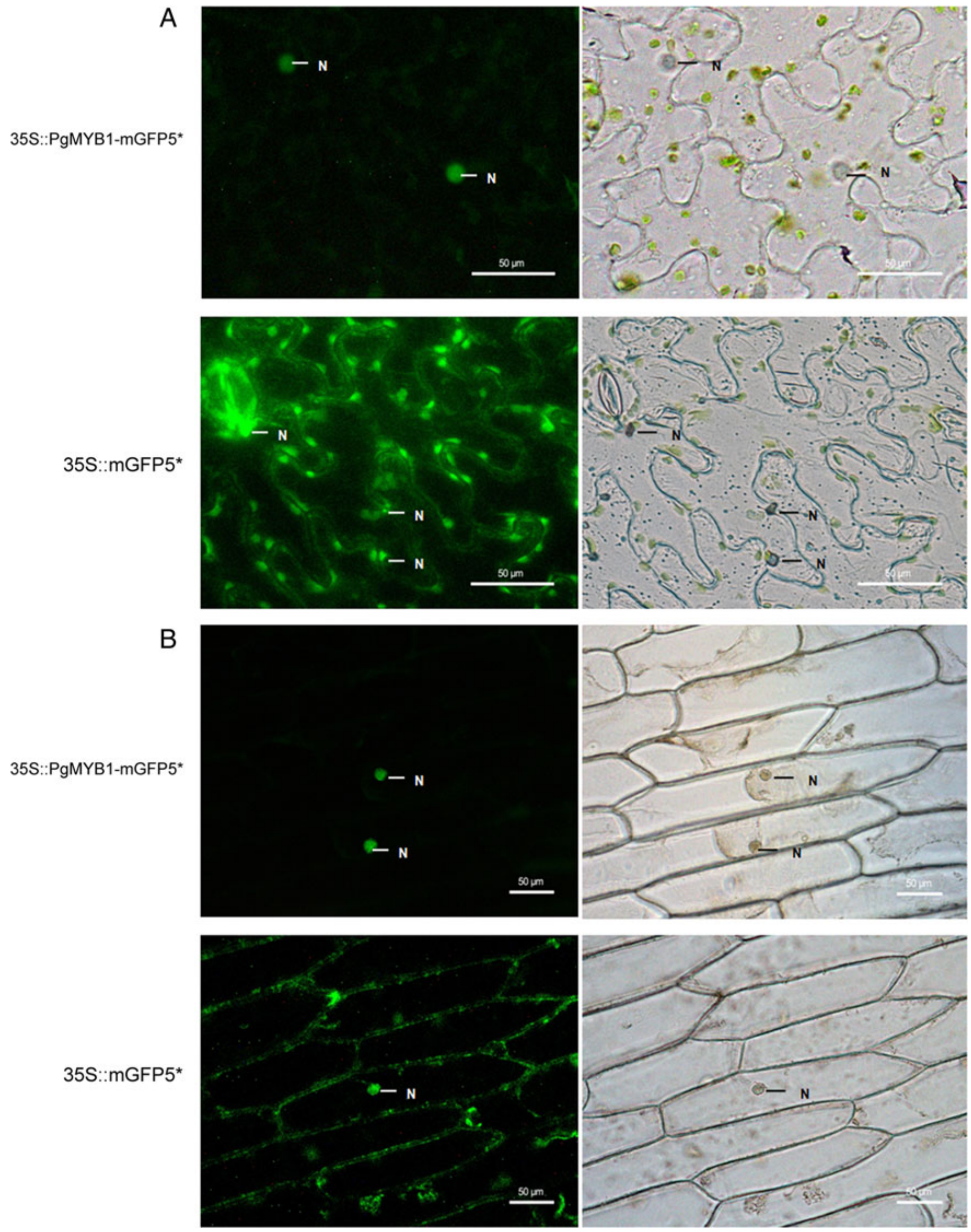

Figure 6. Subcellular localization of PgMYB1 protein (A) GFP fluorescence in tobacco (*N. tabacum*) epithelial cell transfected with plasmid 35S::PgMYB1-mGFP5**Agrobacterium* containing the recombinant vector, and 35S::mGFP5**Agrobacterium* containing the empty vector. Dark- and bright-field images are shown. (B) GFP fluorescence in onion (*A. cepa*) epidermal cell transfected with plasmid 35S::PgMYB1-mGFP5**Agrobacterium* containing the recombinant vector and 35S::mGFP5**Agrobacterium* containing the empty vector. Dark- and bright-field images are shown.

or MeJA differentially activate stress-related gene expressions [28]. It is well-known that ABA plays a vital role in adaptation to abiotic environmental stresses including desiccation, salinity, and low temperature [35,36]. For example, in *A. thaliana*, AtMYB102 was demonstrated to be up-regulated in roots, leaves, and young flowers, but down-regulated in stems when treated with ABA. Furthermore, AtMYB13, AtMYB15, AtMYB33, and AtMYB101 were also involved in ABA-mediated responses to environmental signals [34]. **Figure** 8A shows the expression levels of *PgMYB1* under ABA. From 1 to 48 h post-treatments, the expression of *PgMYB1* increased gradually and reached maximum point at 48 h, indicating that *PgMYB1* in *P. ginseng* might participate in ABA-mediated response to stress condition.

Wounding is a serious stress condition that can be caused by both biotic and/or abiotic stress factors and often closely related to water stress. SA and MeJA, two antagonists in the wounding signal pathway, were used to investigate whether *PgMYB1* responded to wounding treatment. In the case of SA treatment, *PgMYB1* expression increased immediately and peaked at 24 h, and then declined continually to 48 h (**Fig.** 8**B**). The statistical analysis showed that SA obviously induced *PgMYB1* expression after treatment ($P < 0.05$). SA is a key compound in the plant defense response network. In most cases, SA controls the local apoptotic hypersensitivity response and establishes systemic acquired resistance during plant defense to necrotrophic pathogens [37]. SA also triggers responses to some abiotic stresses. In *N. Tabacum*, NtMYB1 and NtMYB2 proteins are induced rapidly

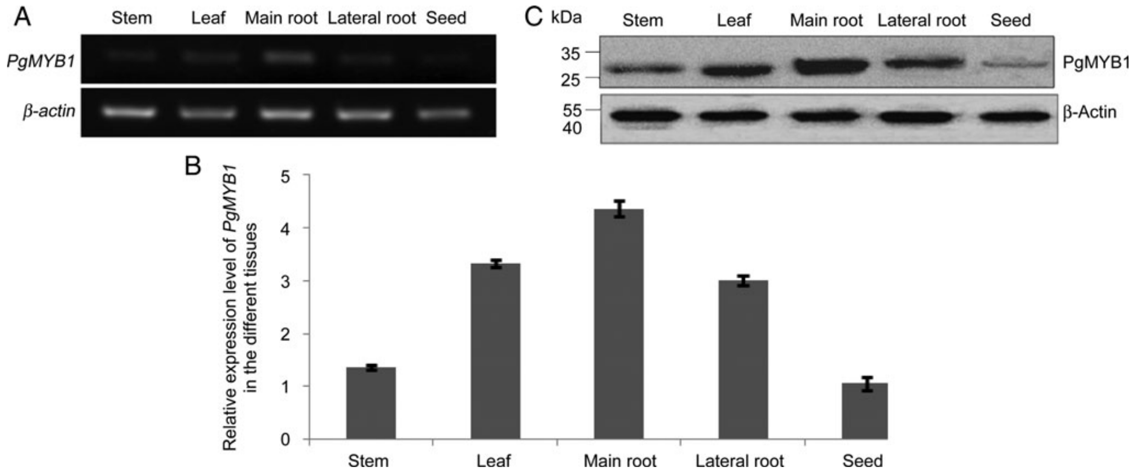

Figure 7. Expression levels of PgMYB1 in different tissues of 4-year-old ginseng (A) RT-PCR. (B) qRT-PCR. Relative expression is quantified based on the corresponding gene expression in the stem using the $2^{-\Delta\Delta Ct}$ method. The ginseng *β-actin* gene was used as an internal control. (C) Western blot analysis. The total proteins (100 μg) were detected using anti-PgMYB1 antibodies and anti-Actin antibody as a loading control.

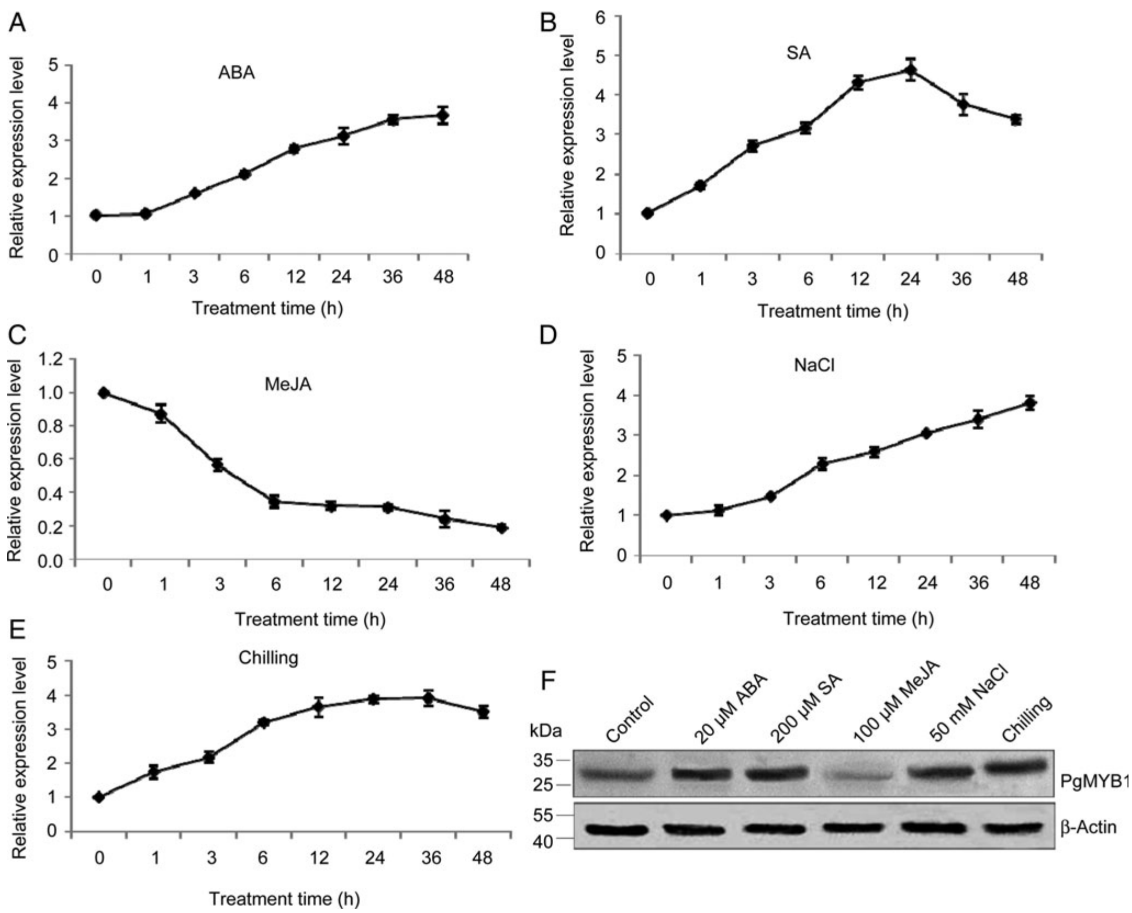

Figure 8. Expression of *PgMYB1* in hairy roots of ginseng at different hormone treatments (20 μM ABA, 200 μM SA, and 100 μM MeJA) and stress conditions (50 mM NaCl and 4°C) (A–E) qRT-PCR analysis of *PgMYB1* at different time points. The error bars represent the standard error of means of three independent replicates. All data were normalized to the *β-actin* expression level. Relative expression is quantified based on the corresponding gene expression at 0 h using the $2^{-\Delta\Delta Ct}$ method. (F) Western blot analysis of PgMYB1 at 12 h. The total proteins (100 μg) were detected using anti-PgMYB1 antibodies and anti-actin antibody as a loading control.

by SA and exhibit a transient expression pattern after treatment with the signaling compounds [22]. In *A. thaliana*, AtMYB30 are also induced after SA treatment [38]. These results imply that *PgMYB1* may mediate the defense responses in an SA-dependent way in ginseng. The expression level of *PgMYB1* decreased gradually after MeJA treatment and to ~20% at 48 h (**Fig.** 8C). As is well-known, MeJA is a lipid-based hormone signal that regulates a wide range of processes in plants, ranging from controlling plant insect defense, wounding responses and development to secondary metabolite synthesis [31]. In this study, MeJA is not a good elicitor for *PgMYB1* expression. These data suggest that *PgMYB1* may be involved in a phytohormone-mediated response to plants by sharing different signaling pathways.

In ginseng, the *PgMYB1* mRNA level was changed considerably and increased progressively at salinity stress. Comparing with the control hairy roots, *PgMYB1* transcripts (under salinity stress) slowly accumulated up to 3 h and then rapidly reached a high level at 48 h. Salinity stress in soil or water is one of the major abiotic stresses especially in arid and semi-arid regions and can severely limit plant growth and yield. Moreover, the salinity stresses are closely related to the ABA signal pathway. However, the expression of *PgMYB1* was induced gradually by salinity stress, which is closely related to the results of ABA treatment (**Fig. 8D**). In *Arabidopsis*, AtMYB2 was induced by high-salt conditions [39].

When ginseng hairy roots were exposed to chilling stress at 4°C, the *PgMYB1* transcript level was slightly induced from 1 to 36 h, compared with the control hairy roots. The induction peaked at 36 h with 3.90-fold induction and decreased to 3.5-fold at 48 h (**Fig. 8E**). Cold stress has been reported to increase the transcript level of MYB in other plants such as AtMYB15 from *Arabidopsis*, which is involved in cold stress tolerance [18].

In the present study, we demonstrated that *PgMYB1* was up-regulated by ABA and salinity, up-regulated up to 24 h by SA and chilling, but down-regulated by MeJA. In order to further explore the expression pattern of *PgMYB1* in protein level, western blot analysis was carried out with anti-PgMYB1 antibody and a single band of ~27 kDa was detected. The expression pattern of *PgMYB1* at protein level at 12 h after various treatments is shown in **Fig. 8F**. Immunoblotting analysis of various stress and hormonal treatment showed that *PgMYB1* expression was consistent with transcription level at different stress conditions. Our results, therefore, suggested that the *PgMYB1* might participate in the functional processes of phytohormones and abiotic stresses responses.

Discussion

The MYB superfamily has been described as the largest TF family in plants, with at least 204 and 218 members in *Arabidopsis* and rice, respectively [6]. However, no related information has been reported in the *P. ginseng*. In this study, an MYB gene was identified by RT-PCR. Bioinformatics analyses revealed that *PgMYB1* contained R2 and R3 MYB domain repeats and was a typical R2R3-type MYB protein. Phylogenetic analysis of the MYB proteins in *Arabidopsis*, rice, wheat, and grape, and the evolutionary relationships of this gene family within and among the different species have been systematically studied [6,40,41]. As the PgMYB1 is a new member of MYB protein family, we searched the NCBI non-redundant protein database using the blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). It was found that most of the R2R3-MYB proteins related to PgMYB1 are involved in abiotic stress responses, which thus prompts us to explore the functions of MYB protein in ginseng abiotic stress regulation.

Plants experience various environmental stresses such as drought, salt, and cold. During the response and adaptation to abiotic stress, there are many changes in biochemical and physiological processes, and many genes are activated, leading to the accumulation of numerous proteins involved in resistance to abiotic stress, such as late embryogenesis abundant proteins [42]. The expression of stress-induced genes is largely regulated by specific TFs [43]. Previous studies have indicated that the MYB TFs play essential roles in the regulation of gene expression to cope with environmental changes [9,44]. In recent years, a number of MYB proteins from *Arabidopsis* and rice were characterized as key factors in the signaling pathways for plant resistance to abiotic stresses [9,45]. Nevertheless, functional study of this protein family in *P. ginseng* is still lacking. Cold stress that includes chilling (<20°C) and/or freezing (<0°C) temperatures, adversely affects the growth and development of plants [46]. Expression data from different plant species have indicated that the members of the MYB family such as OsMYB4 and AtMYB15 participate in plant responses to cold stresses [18,35]. Similarly, in this study, the *PgMYB1* in *P. ginseng* was induced by cold stress treatment, which indicates that *PgMYB1* could also participate in cold stress tolerance. Excessive salt is another major stress that causes ion imbalance and water deficiency. High salinity affect plants in two main ways: high concentration of salts in the soil disturbs the capacity of roots to extract water and high concentration of salts within the plant itself can be toxic [47]. Some MYB proteins have effects on salt responses, such as TaMYB1, OsMYB3R-2, GmMYB76, GmMYB92, GmMYB177, and AtMYB41 [32,48–50]. Furthermore, AhMYB6 and TaMYB33 also exhibit enhanced salinity tolerance in peanut and wheat, respectively [19]. In the present study, phylogenetic analysis reveals that PgMYB1 protein is closely related to AhMYB6 and TaMYB33. Moreover, RT-PCR and qRT-PCR analysis also showed that the *PgMYB1* transcript level was enhanced gradually, and its expression remained high for salt stress, suggesting that *PgMYB1* might participate in salt stress regulation.

In *Arabidopsis*, the expression profiles of 163 MYB genes were identified for their responses to the phytohormones and stress conditions [6]. In order to understand the potential roles of *PgMYB1* in *P. ginseng*, the expression pattern of *PgMYB1* in response to different phytohormones was studied. SA plays an important role in signaling the activation of plant defense and also triggers responses to some abiotic stresses. An increased transcriptional expression of *PgMYB*1 was found after SA treatment. But the expression level of *PgMYB1* decreased gradually after MeJA treatment, indicating that MeJA is not a good elicitor for *PgMYB*1 expression. ABA plays a critical role in the adaptive response to abiotic stresses in plants. The pathways leading to adaptation to stress can be divided into two major categories: ABA-dependent and ABA-independent pathways [51]. Several *R2R3-MYB* genes have been reported as important mediators of ABA-mediated gene expression under environmental stress conditions. For example, MYB41, MYB44, and MYB102 were all responsive to ABA and at least one kind of abiotic stress in *Arabidopsis* [15,49,52]. Recently, an MYB-related gene *LcMYB1* has also been proved to be induced by high salt and ABA stress in Sheep grass. According to our results, the *PgMYB1* gene was obviously inducible in *P.* ginseng roots, suggesting that it might play roles in ABA-dependent signal transduction pathways under the abiotic stress conditions. It should be noted that previous investigations also indicated that MYB TFs might function as key mediators of stress responses through complex activities spanning ABA and other multiple stress signaling pathways. For example, AtMYB102 had been shown to respond to ABA, JA, salt stress, and wounding [15]. AtMYB44 mRNA accumulation was induced in most tissues by a variety of hormone treatments (ABA, IAA, ET, JA, and GA), environmental conditions (drought, high salinity, and low temperature), and pathogen infections [6,52]. These results suggested that MYB family proteins may regulate the stress response through complicated mechanisms in plants. However, more studies are needed to clarify the functions of this protein in ginseng.

In summary, we have provided evidence that the *PgMYB1* in ginseng is up-regulated by ABA, SA, chilling, and salinity, but down-regulated by MeJA. Ginseng is a useful resource for biomedical industry. Further information on MYB sequence, stress or hormone-responsive expression patterns would be useful for guiding ginseng breeding and studying the regulation of stress tolerance and ginsenosides accumulation in *P. ginseng*. Future studies should focus on exploring the biological function of MYB protein in ginseng.

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