Plant Cell Tiss Organ Cult (2015) 120:961–972
DOI 10.1007/s11240-014-0650-x

ORIGINAL PAPER

A *Scutellaria baicalensis* R2R3-MYB gene, *SbMYB8*, regulates flavonoid biosynthesis and improves drought stress tolerance in transgenic tobacco

Yuan Yuan · Linjie Qi · Jian Yang · Chong Wu · Yunjun Liu · Luqi Huang

Received: 19 August 2014 / Accepted: 20 October 2014 / Published online: 29 October 2014

Abstract R2R3-MYB proteins are involved in the primary and secondary metabolism, developmental processes and the responses to biotic and abiotic stresses. Little is known about the functions of R2R3-MYB proteins in *Scutellaria baicalensis* Georgi which is a traditional Chinese medicinal plants. In this study, the function of a *S. baicalensis* R2R3-MYB protein, SbMYB8, was investigated. *SbMYB8* had similar expression pattern with *SbC4H* and *SbCHS* in ABA-treated *S. baicalensis*, indicating that SbMYB8 might be involved in the flavonoid metabolism. SbMYB8 protein could bind to the GmMYB92 BS3 sequence of *SbCHS* promoter region, regulating the expression of *SbCHS*. The SbMYB8 protein was localized to the nucleus where it activated transcription. The transgenic tobacco plants over-expressing *SbMYB8* had higher caffeoylquinic acid contents, compared to that in wild type plants. Overexpression of *SbMYB*8 also changed the expression level of some flavonoid biosynthesis-related genes. It was found that overexpression of *SbMYB8* can improve stress tolerance of transgenic plants, and can alter the activity and expression levels of some antioxidant enzymes. These results indicate that SbMYB8 plays important roles in flavonoid biosynthesis and stress tolerance of plant.

Keywords R2R3-MYB protein · *SbMYB*8 · Medicinal plant · Flavonoid biosynthesis · Stress tolerance · Transgenic tobacco

Introduction

Environment stresses severely affect plant growth and lead to the loss of plants productivity (Mahajan and Tuteja 2005). Stress induces an extensive transcriptional reprogramming in plants, which involves ABA-dependent and ABA-independent pathways (Yamaguchi-Shinozaki and Shinozaki 2006). Plants could accumulate more secondary compounds to cope with environment stress (Bartels and Sunkar 2005). Flavonoids are this kinds of secondary compounds and play roles in stress protection. Flavonoids have the ultraviolet (UV) protection role due to their UV-absorbing characteristics (Booij-James et al. 2000). More evidence showed that flavonoids might play roles as antioxidants to scavenge reactive oxygen species (ROS) (Fini et al. 2011). Recently, it was showed that over-accumulation of flavonoid can enhance the tolerance of plants to oxidative and drought stress (Nakabayashi et al. 2014).

Plant flavonoids also exhibit several medicinal properties (Harborne and Williams 2000), and these flavonoids largely determine the quality of medicinal plants. *Scutellaria baicalensis* Georgi is a traditional Chinese medicinal plant and its roots are used to treat various diseases. The active compounds of *S. baicalensis* include baicalin, baicalein, wogonoside, wogonin, neobaicalein, visidulin I,

Yuan Yuan and Linjie Qi have contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s11240-014-0650-x) contains supplementary material, which is available to authorized users.

Y. Yuan · L. Qi · J. Yang · C. Wu · L. Huang (✉)
State Key Laboratory of Dao-di Herbs, National Resource Center for Chinese Materia Medica, Academy of Chinese Medical Sciences, Beijing 100700, China
e-mail: huangluqi@263.net

Y. Liu (✉)
Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing 100081, China
e-mail: liuyunjun@caas.cn

and oroxylin A, and these compounds exhibit anti-inflammatory, anti-tumor, and anti-HIV activities (Blach-Olszewska et al. 2008). These flavonoids, especially baicalin and baicalein, are regarded as the most important determinants of the quality of *S. baicalensis* (Yuan et al. 2013a).Water status also affects the flavonoid accumulation that in turn determine the quality of *S. baicalensis* (Yuan et al. 2010). It has been reported that water deficit affected the flavonoid accumulation and increased the expression of several baicalin and baicalein biosynthesis genes (Xu et al. 2010), including phenylalanine ammonia-lyase (PAL, EF501766), chalcone synthase (*CHS*, AB008748), UDP-glucuronate: baicalein 7-*O*-glucuronosyltransferase (*UBGAT*, EF512580) and β-glucuronidase (*GUS*, AB040072). β-glucuronidase (GUS) catalyzes baicalin to baicalein (Morimoto et al. 1998; Sasaki et al. 2000). Baicalein can be catalyzed back to baicalin by UDP-glucuronate: baicalein 7-*O*-glucuronosyltransferase (UBGAT) (Nagashima et al. 2000).

R2R3-MYB subfamily have been found to be involved in the primary and secondary metabolism, developmental processes and the responses to biotic and abiotic stresses (Meissner et al. 1999). 126 Arabidopsis R2R3-MYBs were categorized into 22 subgroups on the basis of the conserved amino acid sequence motifs (Kranz et al. 1998). Many MYB proteins play roles in plant response to diverse abiotic stresses. AtMYB2 protein up-regulated the expression of ABA-inducible genes in drought-treated Arabidopsis plants (Abe et al. 2003), and overexpression of *AtMYB15* enhanced the drought tolerance of transgenic Arabidopsis (Ding et al. 2009). Rice OsMYB2 and OsMYB4 proteins are shown to have functions in cold and dehydration tolerance (Soltesz et al. 2012; Yang et al. 2012). Several wheat R2R3-MYBs, including TaMYB1 (Lee et al. 2007), TaMYB2A (Mao et al. 2011), TaMYB33 (Qin et al. 2012), TaMYB73 (He et al. 2012), TaMYB30-B (Zhang et al. 2012a), were shown to improve stress tolerance in transgenic plants, respectively. MYB proteins were also reported to be involved in the flavonoid biosynthesis in many plant species (Cheng et al. 2013).

Eleven R2R3-MYBs were identified from *S. baicalensis* full-length cDNA library and were clustered into 5 subgroups (Yuan et al. 2013b). The protein level of a putative R2R3-MYB transcription factor in *S. baicalensis* roots was increased under water deficit condition, and this R2R3-MYB is also involved in flavonoid biosynthesis in *S. baicalensis* under stress (Yuan et al. 2012). GA_3 treatment increased the expression of *SbMYB8* which have the similar expression pattern with *SbCHS* and *SbC4H*, indicating that SbMYB8 might be involved in the flavonoid biosynthesis in *S. baicalensis* based on subgroup classification and co-expression analysis (Yuan et al. 2013b).

To further analyze the function of *SbMYB8* on flavonoid biosynthesis and drought tolerance, we generated transgenic tobacco plants over-expressing *SbMYB8*, and showed that *SbMYB8* can regulate flavonoid biosynthesis and improve drought stress tolerance in transgenic tobacco.

Materials and methods

Plant materials and growth condition

The seeds of *S. baicalensis* were obtained from Academy of Chinese Medical Sciences (Beijing, China), sterilized in 0.5 % NaOCl for 5 min, then washed three times with sterile water, and placed in petri dishes to germinate. The seedlings 2 weeks after germination were transferred to individual pots (ten seedlings per pot) containing 500 g dried soil in climate chamber at 25 °C with 16 h-light photoperiod under well-water condition. ABA (100 μM) were sprayed on leaves of plants 1 week after transplant of *S. baicalensis*. The leaves were sampled three times at 1, 2 and 3 h after spraying, respectively. The sample were rinsed three times in distilled water, and then stored at −80 °C for further experiments.

Gene expression analysis in *S. baicalensis*

Total RNA was extracted from plant tissues using Trizol reagent (Invitrogen, USA) and pretreated with RNase-Free DNase (Promega, USA) to eliminate genomic DNA contamination. RNA integrity was analyzed on 1 % agarose gel. RNA quantity was determined using a NanoDrop 2000C spectrophotometer (Thermo Scientific, USA).

Semi-quantitative RT-PCR was carried out for *SbPAL1* (HM062775), *SbPAL2* (HM062776), *SbPAL3* (HM062777), *SbC4H* (HM062778), *Sb4CL* (HM166767), *SbCHS* (AB008748), *SbUBGAT* (EF512580), *SbGUS* (AB040072), *SbMYB8* (KF008657) and *Sb18S* (FJ527609) using the One-Step RT-PCR kit (TakaRa) with specific primers (Table S1). The *Sb18S* gene was chosen as a loading control. The one-step RT-PCR was done as follows: 94 °C for 3 min, 31 cycles of 94 °C for 30 s, annealing temperature for 40 s, and 72 °C for 40 s, and 72 °C for 10 min.

Subcellular localization

The whole coding sequence of *SbMYB8* was ligated into pE3025 vector (Li et al. 2011) digested with *Eco*RI and *Kpn*I to generate plasmids pGEM-SbMYB8. In the plasmids, *SbMYB-GFP* fusion genes are under the control of CaMV 35S promoter. The construct was confirmed by sequencing and used for transient transformation of onion

(*Allium cepa*) epidermis via a gene gun (Bio-Rad). After 24 h of incubation, GFP fluorescence in transformed onion cells was observed under a confocal microscope (Zeiss, Germany).

Transactivation assay

To determine the transactivation activity, the open reading frames of *SbMYB8* were generated by PCR amplification, cloned into vector pBD-GAL4 which was digested with *Eco*RI and *Sal*I, to construct pBD-SbMYB8. The constructs were transformed into YGR2 cells by the lithium acetate-mediated method. The transformed yeast strains were placed on SD/–Trp medium at 28 °C for 2 days. Yeast transformants from SD medium lacking Trp were then transferred and streaked onto solid SD agar lacking Trp/His/Ade (SD/–Trp/–His/–Ade) to score the growth response after 3 days. For the colony-lift filter assay (X-gal assay), the yeast was transferred to Whatman filter paper plus X-gal for transcription activation activity analysis within 8 h. pGAL4 and pBD-GAL4 was used as a positive control and negative control, respectively.

Expression of SbMYB8 protein in *E. coli*

The open reading frame (ORF) of *SbMYB*8 was cloned into the expression vector pGEX-4T-1 and transformed into Transetta (DE3) chemically competent cells (Beijing TransGen Biotech Co., Ltd, China), respectively. The vector pGEX-4T-1 (+) allows in-frame cloning of PCR products resulting in a GST-tag attached at the N-terminal end of the recombinant protein. Expression of the recombinant protein was induced by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG) and cells were harvested at 9 h.

Electrophoretic mobility shift assay

MYB RS and MRE-core elements in the promoter of *SbC4H* and box-L3 and GmMYB92 BS3 elements in *SbCHS* promoter were synthesized and labeled with biotin [Sangon Biotech (Shanghai) Co., Ltd., China] for chemiluminescence using a light shift chemiluminescent electrophoretic mobility shift assay kit (Pierce). After labeling, complementary labeled strands were mixed together in an equimolar ratio and annealed at room temperature after denaturation at 90 °C. Gel mobility shift assays were performed by incubating 0.5 ng of labeled probe with *SbMYB*8 protein and competing oligonucleotides in binding buffer (10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5 % glycerol, and 1 μg/μl poly (dIdC) at room temperature for 30 min. Mixtures were size-fractionated on a non-denaturing 46 % polyacrylamide gel followed by drying and transfer to nitrocellulose membranes and detection by streptavidin-HRP/chemiluminescence for biotin-labeled probes.

Tobacco transformation

SbMYB8 fragments were inserted into binary vectors pCambia1305 to produce p35Spro-SbMYB8. The constructs was then transformed into *Agrobacterium tumefaciens* EHA105. Tobacco (*Nicotiana tabacum*) leaf discs were transformed via an *A. tumefaciens* mediated leaf disc procedure (Horsch et al. 1985) and selected using 50 mg L^{-1} hygromycin B and 200 mg L^{-1} carbenicillin. After rooting and acclimatization, regenerated plants were grown in a greenhouse to set seeds by self-pollination. T_1 transgenic plants were used for further analyses.

Chemical analysis

The leaves of 2-month-old plants were sampled to determine flavonoid content. 100 mg powdered tobacco leaf was extracted for 1 h in 1 mL ethyl alcohol. The solution was filtered through a membrane filter (0.2 μm), and flavonoid concentrations were determined using an UPLC-Q-Tof system with a 1.0 mL/min flow rate. UPLC was performed on a diamonsil C_{18} column (4.6 mm × 250 mm, 5 μm). The detection wavelength was set at 354 nm and the column temperature was maintained at 30 °C. The mobile phase consisted of acetonitrile-methanoic acid (A; 99.9:0.1, v/v) and deionized water-trifluoroacetic acid (B; 99.9:0.1, v/v). The initial condition was A–B (5:95, v/v) for 20 min, and this was linearly changed to A–B (10:90, v/v) at 20 min, to A–B (20:80, v/v) at 40 min, and to A–B (40:60, v/v) at 60 min. UPLC grade acetonitrile (E. Merck, Darmstadt, Germany) was used for the UPLC analysis. Caffeoylquinic acid dimer and caffeoylquinic acid were identified using LC–MS and LC–MS/MS. The injection volume of the sample solution was 20 μL, and the experiment was repeated six times.

Abiotic stress treatments

Tobacco seeds were germinated and seedlings were grown for 20 days at 25 °C in a 16 h: 8 h, dark: light photoperiod on Petri dishes containing MS medium with 1 % sucrose substrate and 10 mg/L hygromycin. For stress treatments, the seedlings were transferred on Petri dishes supplemented with 150 mM NaCl, 0.2 M mannitol or 100 μM ABA for 3, 6, and 9 days. Control plants were grown on Petri dishes only containing MS medium. 8–10 seedlings per treatment were collected and subjected to total RNA isolation using the Trizol reagent (Invitrogen, USA) and subjected to real-time PCR as described above. The experiments were repeated three times.

Quantitative real-time PCR

Total RNA was reverse-transcribed using Reverse Transcriptase MMLV (Takara, China). Real-time PCR was performed using SYBR Premix Ex Taq kits (TaKaRa, China) following the manufacturer's instructions and conducted in triplicate using an ABI7500 Real-Time PCR System (ABI, USA). Gene-specific primers of *NtPAL1* (M84466), *NtPAL2* (D17467), *NtC4H* (AJ937847), *NtCHI* (AB213651), *NtCHS* (AF311783), *NtUFGT* (GQ395697), *NtGT4* (AB176522), *NtAT1* (JN390826), *NtCCoAMT* (NTU62736), *NtHCT* (NTU62736), *NtPOX2* (AB178954), *NtSOD* (EU342358), *NtCAT* (U93244) and *SbMYB8* were designed using Primer3 (http://frodo.wi.mit.edu/primer3/). The primer sequences are listed in Table S1. The lengths of PCR products ranged from 100 to 250 bp. *Ntactin* was chosen as an endogenous control in studying gene expressions in various samples of transgenic tobacco. The specificity of amplification was assessed by melting curve analysis, and the relative abundance of genes was determined using the comparative Ct method as suggested in ABI 7500 Software v2.0.1 (ABI).

Enzyme activity analysis

Fresh tissue (100 mg) was ground in liquid nitrogen and extracted with 50 mM Na_3PO_4 buffer [pH 7.0 for CAT and pH 7.8 for SOD and POD (peroxidase)] containing 1 % (w/v) polyvinyl pyrrolidone and 0.1 mM Na_2EDTA. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15,000×*g* for 20 min. The supernatant was used to determine the enzyme activities and protein concentration. The extractions and enzyme activity assays were done at 4 and 25 °C, respectively.

The SOD activity was measured spectrophotometrically as previously described (Beyer and Fridovich 1987). The CAT activity was assayed according to Sinha (1972). The POD activity was determined as described (Chance and Maehly 1955). The protein concentration in the extracts was measured using the described method (Bradford 1976).

Results

The expression pattern of *SbMYB8* and flavonoid biosynthesis-related genes

We have cloned full-length *SbMYB8* cDNA from a full-length cDNA library of *S. baicalensis*, and showed that the expression of *SbMYB8* was increased after spraying exogenous GA_3 (Yuan et al. 2013b). In this study, *S. baicalensis* leaves were sprayed with ABA and the expression levels of *SbMYB8* and the flavonoid biosynthesis-related genes were investigated. The result showed that exogenous ABA decreased the expression of *SbMYB8*. The expression of some flavonoid biosynthesis-related genes, *SbPAL3*, *SbC4H*, *SbCHS* and *SbGUS* were also decreased by exogenous ABA treatment (Fig. 1). *SbMYB8* had similar expression pattern with *SbC4H* and *SbCHS*, indicating that SbMYB8 might be involved in the flavonoid metabolism.

Subcellular localization of SbMYB8

To investigate the potential role of SbMYB8, its subcellular localization was determined. The full-length cDNA sequence of *SbMYB8* was fused in front of the 5′ terminus of *GFP* reporter gene under the control of CaMV 35S promoter with the correct reading frame. The recombinant constructs of the *SbMYB8-GFP* fusion gene and *GFP* alone were transformed into onion (*Allium cepa*) epidermal cells by particle bombardment, respectively. GFP alone localized throughout the whole cell. SbMYB8-GFP fusion protein accumulated mainly in the nucleus, suggesting that SbMYB8 is a nucleus-localized protein (Fig. 2). The result was consistent with the predicted localization results.

Transactivation assay of SbMYB8

Most nucleus-localized proteins have functions as transcriptional factors. To investigate whether SbMYB8 has transcriptional activity, we performed transcription activity analysis on SbMYB8 using a yeast GAL4 system. The full-length cDNA of *SbMYB8* was fused to the GAL4 DNA-binding domain of the pGBKT7 vector to construct plasmid pBD-SbMYB8 which was then transformed into the yeast strain YGR2. The yeasts transformed with pBD-SbMYB8 could grow on the selection mediums lacking tryptophan and adenine and on the medium lacking tryptophan, adenine and histidine (Fig. 3). Where as the yeast transformants containing empty vector pGBKT7 could not grow on the selection medium. These results suggest that SbMYB8 protein can function as transcriptional activator.

SbMYB8 could bind to the GmMYB92 BS3 sequence of the *SbCHS* promoter

Due to that SbMYB8 might play a role as transcriptional activator (Fig. 3) and there was a co-expression between *SbMYB8* and *SbCHS*, *SbC4H* (Fig. 1), it is assumed that SbMYB8 may regulate the expression of *SbCHS* and *SbC4H* by interacting with their promoter. To confirm this hypothesis, the interaction between SbMYB8 with *SbCHS and SbC4H* promoter sequence was assayed with electrophoretic mobility shift assay (EMSA) experiments.

The promoter sequences of *SbCHS* and *SbC4H* were first analyzed (Table S2). A upstream region of *SbCHS* and

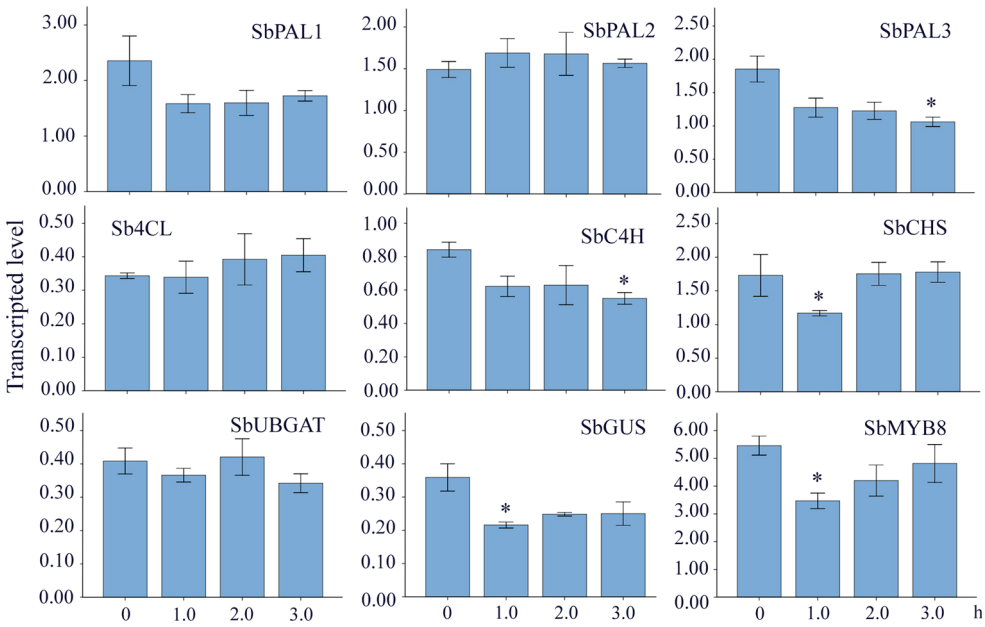

Fig. 1 Effects of ABA treatment on the expression of flavonoid biosynthesis related genes in *S. baicalensis*. RT-PCR analysis of expression of *SbPAL1*, *SbPAL2*, *SbPAL3*, *SbC4H*, *Sb4CL*, *SbCHS*, *SbGUS*, *SbUBGAT* and *SbMYB8* in leaves of *S. baicalensis* after spraying 100 μM ABA. *Vertical bars* indicate the standard deviation of three biological replicates. *Asterisks* indicate a significant difference at the $P < 0.05$ level

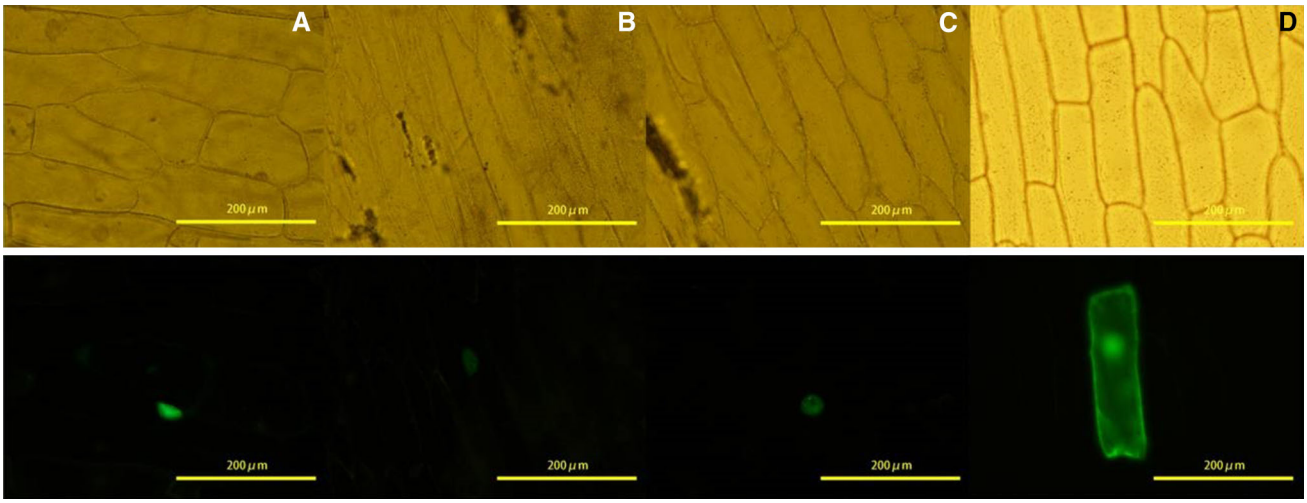

Fig. 2 Subcellular localization of SbMYB8. The recombinant constructs of the *SbMYB8-GFP* fusion gene and *GFP* alone were transformed into onion (*Allium cepa*) epidermal cells by particle bombardment. **a**, **b**, **c** vector pGEM-SbMYB8; **d** empty vector pE3025

SbC4H were obtained from genome database of *S. baicalensis* (Yuan et al. unpublished). Transcription factor binding sites and regulatory elements were predicted using Softberry (linux1.softberry.com). Regulatory elements (MYB RS and MRE-core related with MYB) were found in the promoter of *SbC4H*, and box-L3 and GmMYB92 BS3 elements were found in the promoter of *SbCHS*. The MYB RS, MRE-core, box-L3 and GmMYB92 BS3 sequences were used as probes for EMSA analysis, respectively. No binding bands were detected with crude proteins of *E. coli* without or with empty vector (Fig. 4 lane 1 and lane 7). SbMYB8 could only specifically bind to the GmMYB92 BS3 sequence, and unlabeled probes inhibit the binding (Fig. 4). These results confirmed that SbMYB8 proteins could bind to the GmMYB92 BS3 sequence of *SbCHS* promoter region, regulating the expression of *SbCHS*.

Overexpression of *SbMYB8* changed the synthesis of caffeoylquinic acid in transgenic tobacco

To further analyze the in vivo role of SbMYB8 in plants, *SbMYB8* was transformed into tobacco plants, and the integration of *SbMYB8* was confirmed using PCR analysis (data not shown). The real-time RT-PCR analysis results showed that the expression of *SbMYB8* was markedly increased in the transgenic plants (Table S3). Three

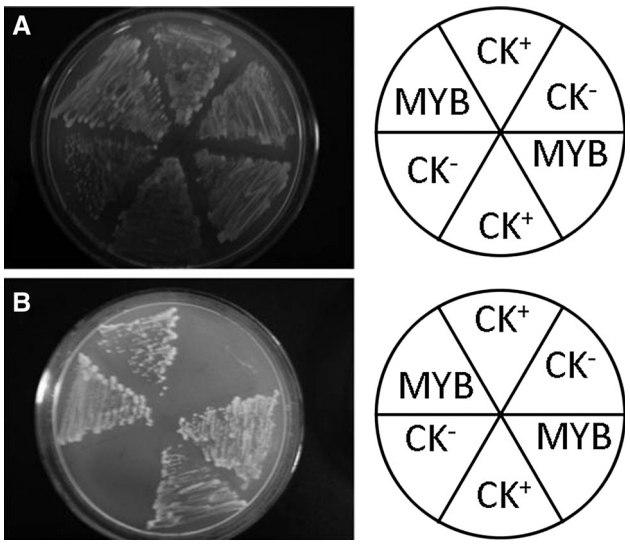

Fig. 3 Transactivation assay of SbMYB8. Vector pBD-SbMYB8 was transformed into the yeast strain YGR2. pGAL4 and pBD-GAL4 was used as a positive control and negative control, respectively. **a** The medium lacking tryptophan and adenine (SD/–Trp/–Ade); **b** the medium lacking tryptophan, adenine and histidine (SD/–Trp/–Ade/–His)

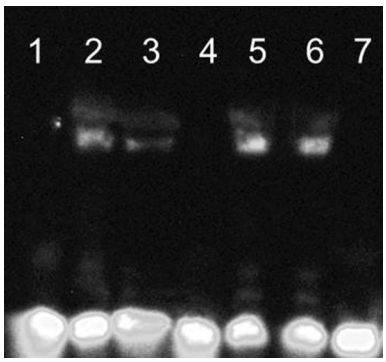

Fig. 4 Electrophoretic gel mobility shift assays for box-L of the *NtPAL* promoter. *1*, *4* the crude protein purified from *E. coli* strains containing empty vector pGEX-4T-1; *2*, *3*, *5*, *6* the purified SbMYB8 protein; *2* biotin labeled GmMYB92 BS3 probe; *3* biotin labeled and unlabeled GmMYB92 BS3 probes. *5* biotin labeled MYB RS probe; *6* biotin labeled and unlabeled MYB RS probes; *7 E. coli*

independent transgenic lines (126–193, 126–194 and 126–93) overexpressing *SbMYB8* were selected for further analysis.

To investigate whether the overexpression of *SbMYB8* in transgenic tobacco plants affected the accumulation of flavonoid, we performed HPLC analysis of the transgenic leaf samples. Two peaks were significantly increased in transgenic plants overexpressing *SbMYB8*, whereas these two peaks could not be detected in WT plants and transgenic plants transformed with empty vector pCambia1305 (Fig. 5). These two peaks were identified as caffeoylquinic acid dimer (Fig. 5b) and caffeoylquinic acid (Fig. 5c) by LC–MS analysis.

SbMYB8 regulates the expression of flavonoid biosynthesis-related genes

Due to overexpression of *SbMYB8* increased the accumulation of caffeoylquinic acid, we further investigate whether overexpression of *SbMYB8* affected the expression of flavonoid biosynthesis-related genes including *NtPAL1*, *NtPAL2*, *NtC4H*, *NtCHS*, *NtCHI*, *NtCCOAMT*, *NtUFGT* and *NtGT4*. In transgenic plants, the expression levels of *NtPAL1*, *NtPAL2*, *NtC4H*, *NtCHS*, *NtCHI*, *NtCCOAMT* and *NtUFGT* were increased, compared with WT tobacco plants (Fig. 6, Table S4), indicating that SbMYB8 might regulate the caffeoylquinic acid synthesis by affecting the expression of these genes.

To further investigate how SbMYB8 affects flavonoid biosynthesis-related genes in transgenic plant under stress condition, transgenic plants were treated with ABA, NaCl and mannitol, respectively, and the expression of *NtPAL1*, *NtPAL2*, *NtC4H*, *NtCHS*, *NtCHI*, *NtCCOAMT*, *NtUFGT*, *NtHCT*, *NtAT1* were measured. The results showed that NaCl treatment increased the expression of *NtPAL2*, *NtC4H*, *NtCHI*, *NtCCOAMT* and *NtHCT*, but decreased the expression of *NtPAL1*, *NtUFGT* and *NtAT*. When transgenic plants were treated with ABA, the expression levels of *NtPAL1*, *NtPAL2*, *NtCHS*, *NtCHI*, *NtCCOAMT*, *NtUFGT*, *NtHCT* and *NtAT* were increased. Mannitol treatment increased the expression of *NtPAL1*, *NtC4H*, *NtCHI*, *NtCCOAMT*, but decreased the expression of *NtUFGT*. ABA, NaCl and mannitol treatment also increased the expression of *NtPAL1*, *NtPAL2*, *NtC4H*, *NtCHS*, *NtCHI*, *NtCCOAMT*, *NtUFGT*, *NtHCT* and *NtAT* in WT plants, indicating that stress could stimulate the expression of flavonoid related gene.

Overexpression of *SbMYB8* increased the stress tolerance of transgenic plants

The physiological performance of transgenic tobacco plants overexpressing *SbMYB8* was investigated (Figure S1). When WT tobacco plants treated with ABA, NaCl and mannitol, respectively, the fresh weights of plants were significantly decreased. Whereas, stress treatments did not affected the growth of transgenic plants except that a significant decrease of fresh weight was observed when treated with mannitol for 6 h. Significant decreases of main root length were observed for WT plants which were treated with ABA, NaCl or mannitol, and for transgenic plants treated with ABA or NaCl. When treated with mannitol, transgenic plants had significantly longer roots

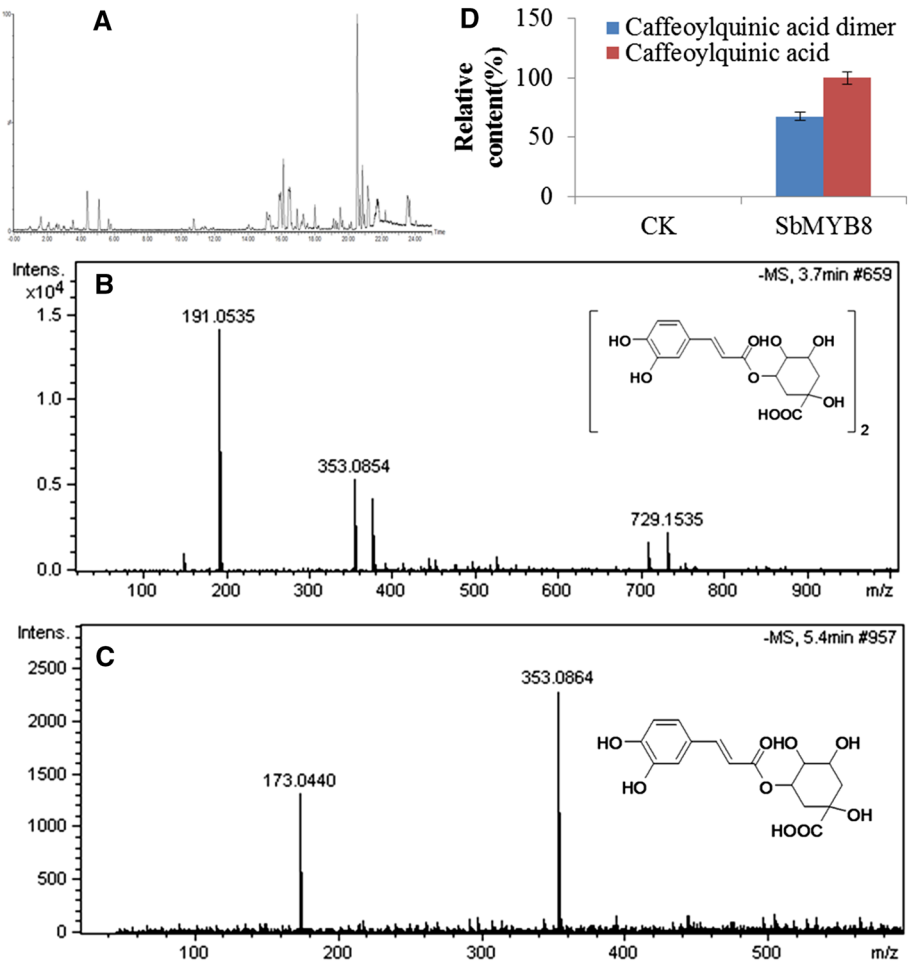

Fig. 5 Chemical analysis of *SbMYB8*-overexpressing transgenic tobacco plants. **a** UPLC analysis; **b**, **c** caffeoylquinic acid dimer and caffeoylquinic acid were identified using LC–MS and LC–MS/MS; **d** content of caffeoylquinic acid dimer and caffeoylquinic acid in transgenic tobacco

than WT plants. Taken together, these results clearly showed that overexpression of *SbMYB8* significantly increased the stress tolerance of transgenic plants, especially increased the drought tolerance (Fig. 7).

The activities of several antioxidant enzymes were analyzed. NaCl, ABA or mannitol treatment significantly increased the activities of GR, POD, APX, SOD, and CAT in WT plants. For transgenic plants, GR activities were not affected by stress treatment. ABA treatment increased POD activity at 3 day, and the activities of APX and SOD at 6 day. The activities of CAT and SOD were significantly increased by ABA treatment for 9 days. NaCl treatment increased the activities of CAT, APX and SOD in transgenic plants (Fig. 8).

Discussion

MYB proteins play key roles in regulatory networks controlling development, metabolism and responses to biotic and abiotic stresses. It was known that subgroup 7 R2R3-MYBs regulated chalcone synthese (CHS), chalcone isomerase (CHI), flavonol 3-hydroxylase (F3H), flavonol 3′-hydroxylase (F3′H) and flavonol synthase (FLS), and subgroup 6 R2R3-MYBs activated dihydroflavonol-4-reductase (DFR), leucoanthocyanidin dioxygenase (LDOX) and anthocyanidin reductase (ANR) (Preston et al. 2004). Anthocyanin biosynthesis in vegetative tissues was regulated by AtMYB75/PAP1, AtMYB90/PAP2, AtMYB113 and AtMYB114 (subgroup 6) (Gonzalez et al. 2008). Eleven R2R3-SbMYBs were identified from *S. baicalensis* full-length cDNA library and were clustered into 5 subgroups (Yuan et al. 2013b). GA_3 treatment increased the expression of *SbMYB8*, and *SbCHS* and *SbC4H* have the similar expression pattern with *SbMYB8*, indicating that SbMYB8 might be involved in the flavonoid biosynthesis in *S. baicalensis* based on subgroup classification and co-expression analysis (Yuan et al. 2013b).

Subgroup 6 R2R3-MYBs have been shown to be involved in the flavonoid pathway regulation (Espley et al. 2007; Huang et al. 2013; Niu et al. 2010). SbMYB8 belongs to subgroup 6 (Yuan et al. 2013b). Exogenous ABA decreased the expression of *SbMYB8*, *SbPAL3*, *SbC4H*, *SbCHS* and *SbGUS* in *S. baicalensis* leaves

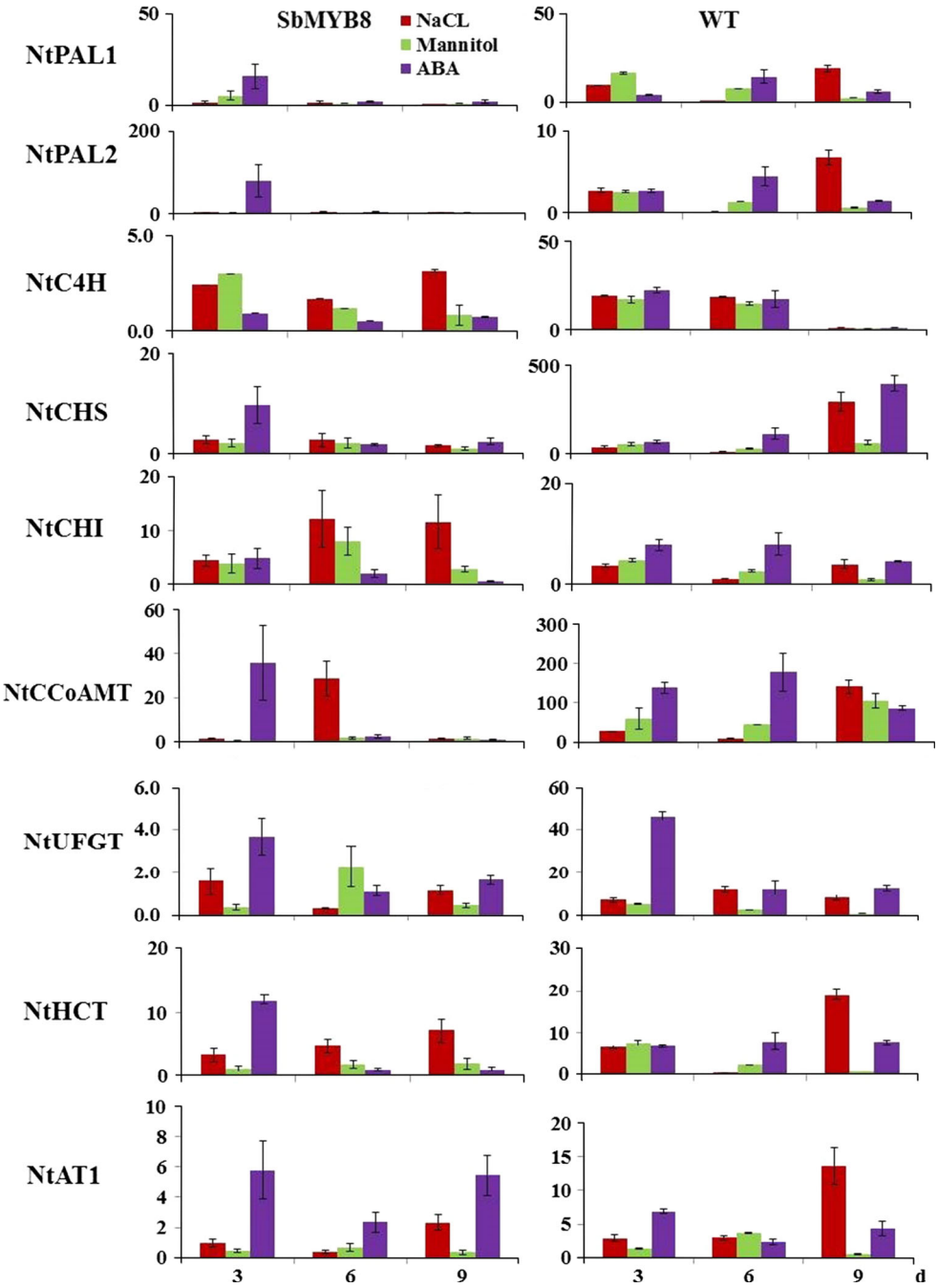

Fig. 6 Transcriptional level of flavonoid biosynthesis related genes in T_1 transgenic tobacco after stress treatment. qRT-PCR analysis of transcriptional level of *NtPAL1*, *NtPAL2*, *NtC4H*, *NtCHS*, *NtCHI*, *NtGT4*, *NtUFGT*, *NtAT1*, *NtCCoAMT1* and *NtHCT* in leaves of *SbMYB8*-overexpressing tobacco plants at 3, 6, 9 days after NaCl, ABA and mannitol treatment, respectively. *Vertical bars* indicate the standard deviation of three biological replicates. *Asterisks* indicate a significant difference at the $P < 0.05$ level

(Fig. 1), and *SbMYB8* had similar expression pattern with *SbC4H* and *SbCHS*. Transcription activity analysis results showed that SbMYB8 can function as transcriptional activator (Fig. 3). SbMYB8 can bind to the promoter region of *SbCHS* gene which is an important gene involved in the flavonoid biosynthesis in *S. baicalensis* (Fig. 4). These results indicate that SbMYB8 might be involved in the flavonoid metabolism. To confirm this hypothesis, *SbMYB8* was transformed into tobacco plants and it was observed that overexpression of *SbMYB8* increased the synthesis of caffeoylquinic acid in transgenic tobacco plants (Fig. 5).

Caffeoylquinic acid is an important group of phenylpropanoid compound and plays role as antioxidants with health-promoting activity. It has been shown that Arabidopsis MYB12 protein could also activate the caffeoylquinic acid biosynthesis in transgenic tomato overexpressing *AtMYB12* (Luo et al. 2008). The tuber-specific expression of a potato MYB gene *StMtf1* up-regulated the biosynthesis of caffeoylquinates, and these changes were associated with the

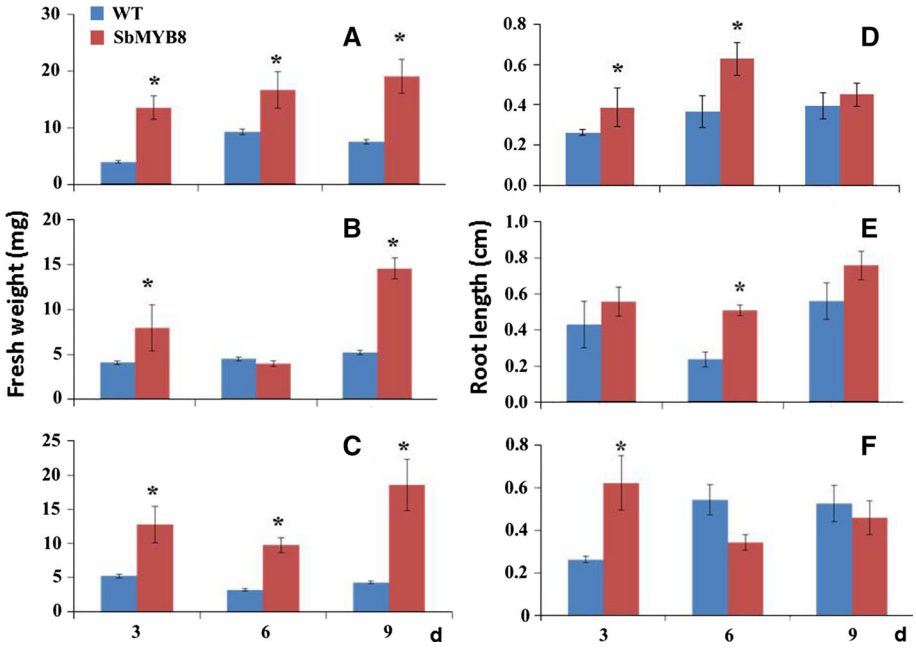

Fig. 7 Fresh weight and root length of T_1 transgenic tobacco after stress treatment. Fresh weight and root length of *SbMYB8*-overexpressing tobacco plants at 3, 6, 9 days after NaCl(A), mannitol(B) and ABA(C) treatment, respectively. *Vertical bars* indicate the standard deviation of ten biological replicates. *Asterisks* indicate a significant difference at the $P < 0.05$ level

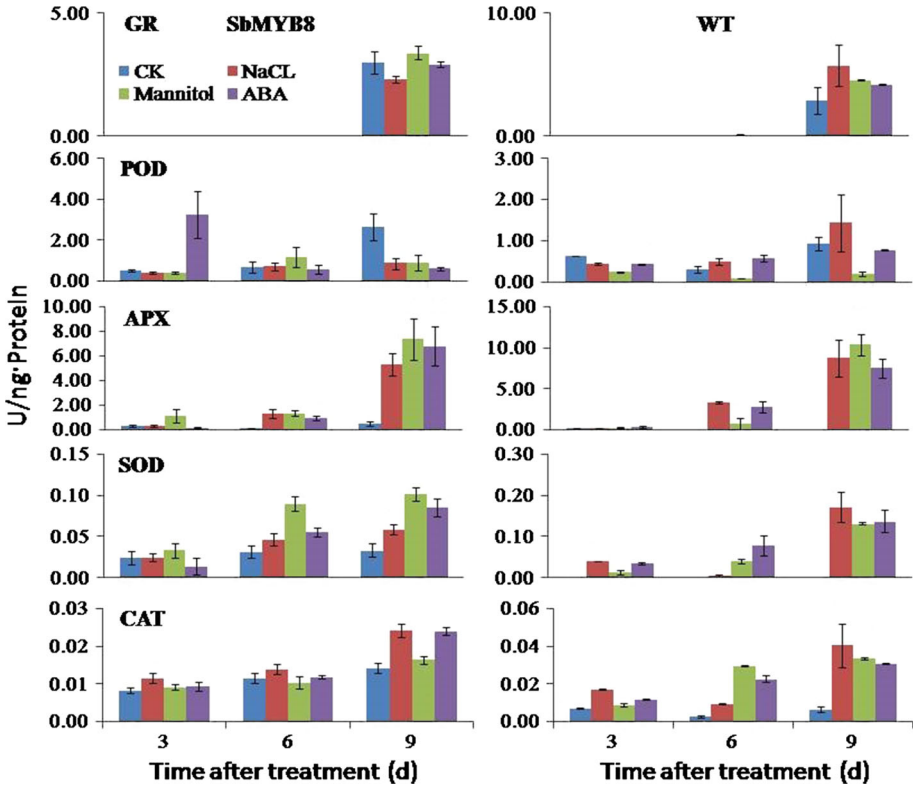

Fig. 8 The activity of antioxidant enzymes. Tobacco seeds were germinated and seedlings were grown for 20 days on MS medium with 10 mg/L hygromycin. The alive seedlings were transferred on Petri dishes supplemented with 150 mM NaCl, 200 mM mannitol or 100 μM ABA. At 3, 6 and 9 days, the samples were collected for the enzyme activities analysis. *Vertical bars* indicate the standard deviation of three biological replicates. *Asterisks* indicate a significant difference at the $P < 0.05$ level

increased expression of phenylpropanoid synthesis related genes (Rommens et al. 2008). We further analyzed whether the increased caffeoylquinic acid contents in transgenic plants overexpressing *SbMYB8* was due to the changed expression of flavonoid biosynthesis-related genes. Compared with WT plants, the expression levels of *NtPAL1*, *NtPAL2*, *NtC4H*, *NtCHS*, *NtCHI*, *NtCCOAMT* and *NtUFGT* were increased in transgenic plants overexpressing *SbMYB8* (Fig. 6), indicating that SbMYB8 regulates the caffeoylquinic acid synthesis by affecting the expression of these genes. A few reports have showed that manipulating the expression of flavonoid biosynthesis related genes can alter

the flavonoid accumulation (Butelli et al. 2008). Suppression of *Nicotiana attenuate MYB8* could induce specific alterations in the accumulation of coumaroyl-containing metabolites, and result in a strong suppression of dicaffeoylspermidine in transgenic tobacco plants (Kaur et al. 2010). For *S. baicalensis*, two MYBs (SbMYB2 and SbMYB7) have also been shown to regulate the flavonoid biosynthesis in transgenic tobacco plants by affecting the transcriptional level of some flavonoid-related genes (Yuan et al. 2013b).

Biotic and abiotic stresses can increased the accumulation of ROS in plants. Flavonoids, as important non-enzymatic antioxidants, could enhance the stress tolerance of plants by scavenging ROS, even if there are debates about the antioxidant function of flavonoids (Fini et al. 2011). Under stress condition, transgenic tobacco plants overexpressing *SbMYB8* had higher fresh weight than non-transgenic plants, indicating that overexpression of *SbMYB8* enhanced the stress tolerance of transgenic plants. However, the above results only showed the stress tolerance of transgenic plants at seedling stage, further experiments will be performed to investigate the stress tolerance of transgenic plants at different growth stage. We speculate that the enhancement of stress tolerance might be due to the over-accumulation of caffeoylquinic acid in transgenic plants. A few reports have showed the relationship between flavonoid accumulation and the stress tolerance of plants (Winkel-Shirley 2002). It was reported that in *MYB*-overexpressing transgenic Arabidopsis plants, the over-accumulation of anthocyanin was directly associated with oxidative and drought tolerance (Nakabayashi et al. 2014). Transgenic potato plants overexpressing *IbMYB1* showed high amounts of secondary metabolites, and had higher salt tolerance than non-transgenic plants (Cheng et al. 2013). A few R2R3-MYB proteins, i.e. Chrysanthemum CmCYB2 (Shan et al. 2012), wheat TaPIMP1 (Zhang et al. 2012b), TaMYB30-B (Zhang et al. 2012a), Arabidopsis AtMYB20 (Cui et al. 2013), were also shown to have the functions of improving tolerance to biotic and abiotic stresses when they were overexpressed in transgenic plants. However, some R2R3-MYBs improved the stress tolerance of transgenic plants not by the accumulation of flavonoids. For example, AtMYB20 negatively regulated type 2C serine/threonine protein phosphatases to enhance the salt tolerance of transgenic plants (Cui et al. 2013).

When plants are exposed to stress condition, cellular enzymatic antioxidants play important roles for the defense of plants against ROS (Miller et al. 2010). Stress treatments enhanced the activities of antioxidant enzymes, i.e. GR, POD, APX and CAT in WT plants (Fig. 8). There were higher activities of these enzymes in transgenic plants than those in WT plants, indicating that overexpression of *SbMYB8* alleviated ROS accumulation by maintaining high antioxidant enzyme activities. The increased flavonoid content and antioxidant enzyme activities in transgenic tobacco plants overexpressing *SbMYB8* could confer transgenic plants with high stress tolerance.

As a traditional Chinese medicine, the qualities of *S. baicalensis* grown in different environment conditions were different. This difference might be due to the different levels of active ingredients. In this study, we showed that SbMYB8 protein can play a role in the regulation of the flavonoid accumulation. So, it might be possible to regulate the quality of *S. baicalensis* by modifying the expression of *SbMYB8* gene or other genes to increase the flavonoid content in *S. baicalensis*. The work on transformation of some *MYB* genes into *S. baicalensis* plant is under way.

Acknowledgments This work was supported by National Science Fund for Distinguished Young Scholars (81325023), Beijing Nova Program (2008B82), China Postdoctoral Funding (201003228).

References

Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell 15:63–78

Bartels D, Sunkar R (2005) Drought and salt tolerance in plants. Crit Rev Plant Sci 24:23–58

Beyer W, Fridovich I (1987) Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Anal Biochem 161:559–566

Blach-Olszewska Z, Jatczak B, Rak A, Lorenc M, Gulanowski B, Drobna A, Lamer-Zarawska E (2008) Production of cytokines and stimulation of resistance to viral infection in human leukocytes by *Scutellaria baicalensis* flavones. J Interferon Cytokine Res 28:571–581

Booij-James IS, Dube SK, Jansen MA, Edelman M, Mattoo AK (2000) Ultraviolet-B radiation impacts light-mediated turnover of the photosystem II reaction center heterodimer in Arabidopsis mutants altered in phenolic metabolism. Plant Physiol 124:1275–1284

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Butelli E, Titta L, Giorgio M, Mock HP, Matros A, Peterek S, Schijlen EG, Hall RD, Bovy AG, Luo J (2008) Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. Nat Biotechnol 26:1301–1308

Chance B, Maehly A (1955) Assay of catalases and peroxidases. Methods Enzymol 2:764–775

Cheng YJ, Kim MD, Deng XP, Kwak SS, Chen W (2013) Enhanced salt stress tolerance in transgenic potato plants expressing IbMYB1, a sweet potato transcription factor. J Microbiol Biotechnol 23:1737–1746

Cui MH, Yoo KS, Hyoung S, Nguyen HT, Kim YY, Kim HJ, Ok SH, Yoo SD, Shin JS (2013) An Arabidopsis R2R3-MYB transcription factor, AtMYB20, negatively regulates type 2C serine/threonine protein phosphatases to enhance salt tolerance. FEBS Lett 587:1773–1778

Ding Z, Li S, An X, Liu X, Qin H, Wang D (2009) Transgenic expression of MYB15 confers enhanced sensitivity to abscisic acid and improved drought tolerance in *Arabidopsis thaliana*. J Genet Genomics 36:17–29

Espley RV, Hellens RP, Putterill J, Stevenson DE, Kutty-Amma S, Allan AC (2007) Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. Plant J 49:414–427

Fini A, Brunetti C, Di Ferdinando M, Ferrini F, Tattini M (2011) Stress-induced flavonoid biosynthesis and the antioxidant machinery of plants. Plant Signal Behav 6:709–711

Gonzalez A, Zhao M, Leavitt JM, Lloyd AM (2008) Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in Arabidopsis seedlings. Plant J 53:814–827

Harborne JB, Williams CA (2000) Advances in flavonoid research since 1992. Phytochemistry 55:481–504

He Y, Li W, Lv J, Jia Y, Wang M, Xia G (2012) Ectopic expression of a wheat MYB transcription factor gene, TaMYB73, improves salinity stress tolerance in *Arabidopsis thaliana*. J Exp Bot 63:1511–1522

Horsch RB, Rogers SG, Fraley RT (1985) Transgenic plants. Cold Spring Harb Symp Quant Biol 50:433–437

Huang W, Sun W, Lv H, Luo M, Zeng S, Pattanaik S, Yuan L, Wang Y (2013) A R2R3-MYB transcription factor from *Epimedium sagittatum* regulates the flavonoid biosynthetic pathway. PLoS One 8:e70778

Kaur H, Heinzel N, Schottner M, Baldwin IT, Galis I (2010) R2R3-NaMYB8 regulates the accumulation of phenylpropanoid-polyamine conjugates, which are essential for local and systemic defense against insect herbivores in Nicotiana attenuata. Plant Physiol 152:1731–1747

Kranz HD, Denekamp M, Greco R, Jin H, Leyva A, Meissner RC, Petroni K, Urzainqui A, Bevan M, Martin C (1998) Towards functional characterisation of the members of the R2R3-MYB gene family from *Arabidopsis thaliana*. Plant J 16:263–276

Lee TG, Jang CS, Kim JY, Kim DS, Park JH, Kim DY, Seo YW (2007) A Myb transcription factor (TaMyb1) from wheat roots is expressed during hypoxia: roles in response to the oxygen concentration in root environment and abiotic stresses. Physiol Plant 129:375–385

Li D, Zhang Y, Hu X, Shen X, Ma L, Su Z, Wang T, Dong J (2011) Transcriptional profiling of Medicago truncatula under salt stress identified a novel CBF transcription factor MtCBF4 that plays an important role in abiotic stress responses. BMC Plant Biol 11:109

Luo J, Butelli E, Hill L, Parr A, Niggeweg R, Bailey P, Weisshaar B, Martin C (2008) AtMYB12 regulates caffeoyl quinic acid and flavonol synthesis in tomato: expression in fruit results in very high levels of both types of polyphenol. Plant J 56:316–326

Mahajan S, Tuteja N (2005) Cold, salinity and drought stresses: an overview. Arch Biochem Biophys 444:139–158

Mao X, Jia D, Li A, Zhang H, Tian S, Zhang X, Jia J, Jing R (2011) Transgenic expression of TaMYB2A confers enhanced tolerance to multiple abiotic stresses in *Arabidopsis*. Funct Integr Genomics 11:445–465

Meissner RC, Jin H, Cominelli E, Denekamp M, Fuertes A, Greco R, Kranz HD, Penfield S, Petroni K, Urzainqui A (1999) Function search in a large transcription factor gene family in *Arabidopsis*: assessing the potential of reverse genetics to identify insertional mutations in R2R3 MYB genes. Plant Cell 11:1827–1840

Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R (2010) Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ 33:453–467

Morimoto S, Tateishi N, Matsuda T, Tanaka H, Taura F, Furuya N, Matsuyama N, Shoyama Y (1998) Novel hydrogen peroxide metabolism in suspension cells of *Scutellaria baicalensis* Georgi. J Biol Chem 273:12606–12611

Nagashima S, Hirotani M, Yoshikawa T (2000) Purification and characterization of UDP-glucuronate: baicalein 7-O-glucuronosyltransferase from *Scutellaria baicalensis* Georgi. cell suspension cultures. Phytochemistry 53:533–538

Nakabayashi R, Yonekura-Sakakibara K, Urano K, Suzuki M, Yamada Y, Nishizawa T, Matsuda F, Kojima M, Sakakibara H, Shinozaki K (2014) Enhancement of oxidative and drought tolerance in *Arabidopsis* by overaccumulation of antioxidant flavonoids. Plant J 77:367–379

Niu SS, Xu CJ, Zhang WS, Zhang B, Li X, Lin-Wang K, Ferguson IB, Allan AC, Chen KS (2010) Coordinated regulation of anthocyanin biosynthesis in Chinese bayberry (*Myrica rubra*) fruit by a R2R3 MYB transcription factor. Planta 231:887–899

Preston J, Wheeler J, Heazlewood J, Li SF, Parish RW (2004) AtMYB32 is required for normal pollen development in *Arabidopsis thaliana*. Plant J 40:979–995

Qin Y, Wang M, Tian Y, He W, Han L, Xia G (2012) Over-expression of TaMYB33 encoding a novel wheat MYB transcription factor increases salt and drought tolerance in *Arabidopsis*. Mol Biol Rep 39:7183–7192

Rommens CM, Richael CM, Yan H, Navarre DA, Ye J, Krucker M, Swords K (2008) Engineered native pathways for high kaempferol and caffeoylquinate production in potato. Plant Biotechnol J 6:870–886

Sasaki K, Taura F, Shoyama Y, Morimoto S (2000) Molecular characterization of a novel beta-glucuronidase from *Scutellaria baicalensis* georgi. J Biol Chem 275:27466–27472

Shan H, Chen S, Jiang J, Chen F, Chen Y, Gu C, Li P, Song A, Zhu X, Gao H (2012) Heterologous expression of the chrysanthemum R2R3-MYB transcription factor CmMYB2 enhances drought and salinity tolerance, increases hypersensitivity to ABA and delays flowering in *Arabidopsis thaliana*. Mol Biotechnol 51:160–173

Sinha AK (1972) Colorimetric assay of catalase. Anal Biochem 47:389–394

Soltesz A, Vagujfalvi A, Rizza F, Kerepesi I, Galiba G, Cattivelli L, Coraggio I, Crosatti C (2012) The rice Osmyb4 gene enhances tolerance to frost and improves germination under unfavourable conditions in transgenic barley plants. J Appl Genet 53:133–143

Winkel-Shirley B (2002) Biosynthesis of flavonoids and effects of stress. Curr Opin Plant Biol 5:218–223

Xu H, Park NI, Li X, Kim YK, Lee SY, Park SU (2010) Molecular cloning and characterization of phenylalanine ammonia-lyase, cinnamate 4-hydroxylase and genes involved in flavone biosynthesis in *Scutellaria baicalensis*. Bioresour Technol 101:9715–9722

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Plant Biol 57:781–803

Yang A, Dai X, Zhang WH (2012) A R2R3-type MYB gene, OsMYB2, is involved in salt, cold, and dehydration tolerance in rice. J Exp Bot 63:2541–2556

Yuan Y, Hao J, Yang B, Li H, Li Z (2010) Climate change affected the best producing area of Chinese herbal medicine *Scutellaria baicalensis* Georgi. J Tradit Med (Russia) 3 s: 241–248

Yuan Y, Liu Y, Wu C, Chen S, Wang Z, Yang Z, Qin S, Huang L (2012) Water deficit affected flavonoid accumulation by regulating hormone metabolism in *Scutellaria baicalensis* Georgi roots. PLoS One 7:e42946

Yuan Y, Liu Y, Luo Y, Huang L, Chen S, Yang Z, Qin S (2013a) High temperature effects on flavones accumulation and antioxidant system in *Scutellaria baicalensis* Georgi cells. Afr J Biotechnol 10:5182–5192

Yuan Y, Wu C, Liu Y, Yang J, Huang L (2013b) The Scutellaria baicalensis R2R3-MYB transcription factors modulates flavonoid biosynthesis by regulating GA metabolism in transgenic tobacco plants. PLoS One 8:e77275

Zhang L, Zhao G, Xia C, Jia J, Liu X, Kong X (2012a) A wheat R2R3-MYB gene, TaMYB30-B, improves drought stress tolerance in transgenic *Arabidopsis*. J Exp Bot 63:5873–5885

Zhang Z, Liu X, Wang X, Zhou M, Zhou X, Ye X, Wei X (2012b) An R2R3 MYB transcription factor in wheat, TaPIMP1, mediates host resistance to *Bipolaris sorokiniana* and drought stresses through regulation of defense- and stress-related genes. New Phytol 196:1155–1170