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ORIGINAL ARTICLE

Overexpression of soybean *GmERF9* enhances the tolerance to drought and cold in the transgenic tobacco

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Abstract Ethylene response factors (ERFs) are widespread in plants, which are widely involved in plant response to biotic and abiotic stress. In this research, a soybean gene, *GmERF9*, was identified and the function was characterized. The results showed that GmERF9 contained a typical AP2/ERF binding domain and a putative nuclear localization signal sequence. The real-time fluorescence quantitative PCR (qPCR) revealed that the expression of *GmERF9* could be induced by ethylene (ET), abscisic acid (ABA), drought, salt and cold stresses. GmERF9 protein could specifically bind to the GCC-box and activate the expression of the reporter gene in the yeast cells and tobacco leaves. Overexpression of *GmERF9* enhanced the expression of pathogenesis-related (PR) genes, including *PR1, PR2, Osmotin* (*PR5*), and *SAR8.2*. Also, the overexpression of *GmERF9* increased the accumulation of proline and soluble carbohydrate, and decreased the accumulation of malondialdehyde under drought and cold stresses in the transgenic tobacco compared to the wild type (WT) tobacco, which indicated that *GmERF9* enhanced the tolerance to drought and cold stresses in the transgenic tobacco. In summary, the function of *GmERF9* is involved in the response to environmental stresses for plants, which can be used as a candidate gene for genetic engineering of crops.

Keywords Ethylene response factor · Expression pattern · Transcriptional activation · Multiple tolerances · Tobacco

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Introduction

The growth and productivity of plants are affected greatly by various biotic and abiotic stresses, such as pathogen attacks, drought, salinity and extreme temperatures (Manavalan et al. 2009). Since plants are nonmotile, they have evolved adaptive mechanisms to resist these unfavorable conditions, including modulating the expression of specific genes and producing a large number of stress-related proteins which play important roles in stress response and plant defense (Xiong et al. 1999; Demekamp and Smeekens 2003; Hazen et al. 2003). Transcription factors play pivotal functions in signal transduction by activation or suppression of defense response genes, especially when plants face stresses (Sharma et al. 2010). Several families of transcription factors are involved in regulating plant responses to various stresses (Singh et al. 2002; Rushton et al. 2010; Nakashima et al. 2012; Thirugnanasambantham et al. 2015), such as AP2/ERF, NAC, WRKY and bZIP, which were characterized according to their DNA-binding domain.

The plant-specific ethylene response factors (ERFs) are members of the AP2/ERF transcription factor superfamily. They contain a single AP2/ERF domain consisting of 58 or 59 amino acid residues, which has an alanine at position 14 and aspartic acid at position 19 (Nakano et al. 2006). ERFs are widely involved in plant cell development, hormone response, disease resistance and abiotic stress by specifically binding to stress-responsive *cis*-elements and regulating the transcription of target genes (Gutterson and Reuber 2004). ERFs are located in the downstream of signal transduction pathways of ethylene (ET), jasmine acid (JA), salicylic acid (SA) and abscisic acid (ABA), and are cross-talk among these signaling pathways (Glazebrook 2001; Lorenzo et al. 2003; Zhang et al. 2004). Evidences

accumulated over the years have proved that the *cis*-elements, including GCC-box, DRE/CRT, CE1, JERE, and CT-rich elements (Ohme-Takagi and Shinshi 1995; Menke et al. 1999; Xue and Loveridge 2004; Tang et al. 2005; Wu et al. 2008; Zhai et al. 2013a, b), were always located in the promoters of stress-related genes, and were able to bind to ERF proteins through the AP2/ERF domain. Additionally, ERF proteins often interact with other proteins directly or indirectly to regulate the expression of their target genes, including *NtbHLH* transcription factor proteins (De Boer et al. 2011), which indicates that the role of ERF proteins in the regulation of plant stress responses is complex.

The expression of ERF genes was induced by biotic and abiotic stress, and overexpression of ERF genes confer multiple tolerances to transgenic plants. *PsAP2* (a member of ERFs) could bind to the both GCC-box and DRE elements, and was response to wounding, dehydration, salt stress, ethylene, methyl jasmonate and ABA. The overexpressing *PsAP2* in transgenic plants exhibited the tolerance against multiple stresses (Mishra et al. 2015). The overexpression of *OsEREBP1*, another member of ERFs, in transgenic plants increased the expression of the genes related to lipid metabolism and pathogenesis-related (PR) proteins, attenuated disease caused by *Xanthomonas oryzae* and confered tolerance to drought and submergence in the transgenic rice (Jisha et al. 2015). Similarly, the submergence and drought tolerances were enhanced in the rice by overexpression of *SUB1A* via increasing the abundance of transcripts encoding ROS scavenging enzymes (Fukao et al. 2011).

Up to now, a large number of ERFs have been successively identified in different plants since the first group of ERF proteins were first isolated from tobacco (Ohme-Takagi and Shinshi 1995). But their biological functions and mechanisms are still not clear for most of them. Soybean is one of the most economically important crop species in the world. More than 90 ERF genes have been identified or deduced in soybean using the expressed sequence tags (ESTs) and phylogenetic approaches (Zhang et al. 2008), however, only six of them were characterized functionally. (Mazarei et al. 2002; Zhang et al. 2009, 2010; Zhai et al. 2013a, b; Dong et al. 2015). Because ERF genes function very differently, it is necessary to identify the functions for the key ERF genes that act in stress regulatory pathway. In present study, an ERF gene (*GmERF9*) with unknown function was identified in soybean and characterized in the transgenic tobacco. The functions, such as binding activity, transcriptional activation and stress-resistance, were investigated. The results showed that the expression of *GmERF9* could be induced by multiple stress conditions and correlated to the tolerances for drought and cold. These results would be helpful for understanding the regulation mechanism of ERF genes in soybean and are used to improve the abiotic tolerance for soybeans.

Materials and methods

Plant materials and treatments

The seedlings of Hefeng 46 soybean were grown in a light growth chamber at 25 °C up to they develpoed four leaves, and then were subjected to different stress treatments. For salt and drought treatments, the seedlings were put into MS solution with 200 mM NaCl and 20% polyethylene glycol (PEG) 8000, respectively. For cold treatment, the seedlings were put into a 4 °C growth chamber with natural light. For ethylene treatment, the seedlings were put into a sealed plexiglass chamber by dissolving 2 mL of 40% ethephon and 1 g of $NaHCO_3$ in 200 mL of H_2O (Zhang et al. 2009; Zhai et al. 2013b). For ABA treatment, the seedlings were sprayed with 200 μM ABA. For all treatments, the leaves were collected at different time points and immediately frozen in liquid nitrogen for the preparation of total RNAs.

Clone and sequence analysis of *GmERF9*

A BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using ERF conserved DNA binding domain was carried in NCBI database, 15 ERF sequences were found with unknown functions. The results of quantitative real-time PCR (qPCR), which was performed as described in "Expression analysis of *GmERF9*" section, revealed that one of them could be induced by stresses, therefore, the cDNA sequence encoded this protein was cloned by reverse transcription PCR (RT-PCR) from Hefeng 46 soybean leaf using the following primer: F, 5′-ATGCAAAGTTCAATCTCACAATC-3′; and R, 5′-TCAAACAACCATTAGTGGAGATAG-3′. The polymerase chain reaction (PCR) products were cloned into pMD18-T vector (TaKaRa, China) and sequenced for confirmation. The isoelectric point and molecular weight were predicted by ExPASy (http://www.expasy.org/), and nuclear localization signal was predicted by WoLF PSORT (http://www.genscript.com/wolf-psort.html).

Expression analysis of *GmERF9*

Total RNA of soybean was extracted using Plant RNAzol kit (Dingguo, China) according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed using M-MLV reverse transcriptase (TaKaRa, China). qPCR was performed with SYBR Green I dye (TaKaRa, China) on a BIO-RAD CFX96 Real-Time PCR system (BIO-RAD, America) in 20 μL reaction mixture. Soybean *β-tubulin* gene was chosen as internal control (Xu et al. 2010). Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). qPCR reaction parameters were: 95 °C (10 s), then 40 cycles of 95 °C

(20 s), 58 °C (20 s) and 72 °C (30 s). Each assay was carried out for three biological replications and three technological replications. The gene-specific primer pairs used for qPCR were listed in Table 1.

Electrophoretic mobility shift assay (EMSA)

The *GmERF9* coding region was cloned into the *Eco*RI–*Sal* I sites of pET-28a vector (Novagen, Germany) to produce 6×his-tagged recombinant protein in *Escherichia coli* BL21 (DE3). The purification of recombinant protein was conducted using a Ni column (Boxin, China) according to the manufacturer's procedures. Wild-type and mutated (m) forms of the GCC-box (Ohme-Takagi and Shinshi 1995) and DRE/CRT (Xu et al. 2007) elements were synthesized as probes for EMSA, and their nucleotide sequences were as follows: GCC-box forward, 5′-AATTCATAAGAGCCGCCACTCATAAGAGCCGCCACTCCC-3′; and mGCC-box forward, 5′-AATTCATAAGATCCTCCACTCATAAGATCCTCCACTCCC-3′; DRE/CRT forward, 5′-ATTTCATGGCCGACCTGCTTTCATGGCCGACCTGCTT-3′; and mDRE/CRT forward, 5′-ATTTCATGAAAAAACTGCTTTCATGAAAAAACTGCTT-3′. EMSA was performed according to the labelled Steptavidin-biotin EMSA User Manual (Boxin, China).

Transcriptional activation assay in yeast

The coding region of *GmERF9* was cloned into the *Sma* I–*Sal* I sites of the pGBKT7 vector (Clontech, America). The constructs pGBKT7-GmERF9 and pGBKT7 (negative control) were transformed into yeast AH109 strain (Tianenze, China), following the protocol. Selection media for the transformants were SD medium (Clontech, America) without Trp and SD medium without Trp, His and Ade.

Table 1 Primer pairs used for qPCR and semiquantitative RT-PCR

Gene name (GenBank accession number)	Forward/reverse primers (positions 5′–3′)
GmERF9 (XM003555644)	CATACCAACCTTCAAATGCCTC TTTCTATTAGGGTCACGGATTTC
β-Tubulin (GMU12286)	GGAAGGCT TTCTTGCATTGGTA AGTGGCATCCTGGTACTGC
α-Tubulin (AB052822)	ATGAGAGAGTGCATATCGAT TTCACTGAAGAAGGTGTTGAA
PR1 (X12737)	TTGCCTTCATTTCTTCTTGTC AATCGCCACTTCCCTCA
PR2 (M60460)	CTTAGCGAATACCAACCCG TCAGAAGGCCAGCCACT
Osmotin (*PR5*) (M29279)	CTCCTTGCCTTGGTGACTT ACCGCCTATGGGTGTCG
SAR8.2 (M97194)	TTTCTTTGCCTTTCTTTGG GTATCTTACGGGTGATTCCTG

After 3 days of growing at 30 °C on selection media, transformants were analyzed to determine the transcription activation of GmERF9 protein according to the expression of the *LacZ* gene on the colony lift filter using 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (x-gal) as a substrate.

Transcriptional activation assay in vivo

For construction of the reporter plasmid, a 346 bp DNA fragment containing the GCC-box originating from the *AtPDF1.2* (GenBank accession number: NM123809) promoter (Zarei et al. 2011) was amplified (F, 5′-GGGAATTCATTATTTTCTTGAGTC-3′; and R, 5′-GGCCATGGGATGATTATTACTATT-3′) and cloned into the *EcoR* I–*Nco* I sites of the pCMBIA1301 vector to replace the CaMV 35S promoter. For construction of the effector plasmid, the coding region of *GmERF9* was cloned into the *Nde* I–*EcoR* I sites of the pRI101 vector (TaKaRa, China) under the control of the CaMV 35S promoter. Both the reporter plasmid and the effector plasmid were transformed into *Agrobacterium* EHA105 strain for further analysis. The transient expression assay was performed by introducing various plasmids into the tobacco (*Nicotiana tabacum* cv. NC89) leaf cells of 8-week-old seedlings using *Agrobacterium*-mediated transformation as described by Yang et al. (2000). GUS histochemical staining and fluorometric GUS analysis were performed approximately 40 h later as described by Jefferson (1987).

Generation of transgenic tobacco plant

The pRI101-GmERF9 vector which was constructed in "Transcriptional activation assay in vivo" section was transformed into the tobacco (*Nicotiana tabacum* cv. NC89) via *Agrobacterium*-mediated transformation as described by Hoekema et al. (1983). T_1 generation of regenerated kanamycin-resistant plants were identified by semiquantitative RT-PCR and used for further analysis. Tobacco *α-tubulin* gene (Sahoo et al. 2014) was chosen as internal control for semiquantitative RT-PCR and its PCR reaction parameters were: 94 °C (8 min), and 28 cycles of 94 °C (40 s), 54 °C (40 s) and 72 °C (1 min), then 72 °C (8 min). The gene-specific primer pairs used for semiquantitative RT-PCR were listed in Table 1.

Expression analysis on putative downstream genes of *GmERF9*

Total RNAs were isolated from the leaves of 6-week-old wild-type (WT) and *GmERF9* T_1 transgenic tobacco plants with Plant RNAzol kit (Dingguo, China). One microgram of total RNA was reverse transcribed using M-MLV reverse

transcriptase (TaKaRa, China). qPCR was performed as described in "Expression analysis of *GmERF9*" section excepting the annealing temperature was replaced by 55 °C. The gene-specific primer pairs used for expression analysis of stress-responsive genes were listed in Table 1.

Measurement of proline, soluble carbohydrate and malondialdehyde contents

The seedlings of WT and T_1 transgenic tobacco were grown in the pot with nutrient media (soil:humus soil = 2:1) under standard growth conditions in greenhouse. About 6 weeks after sowing, the plants of four-leaf stage were chosen for stress treatments. For drought treatment, the seedlings were stopped watering for 7, 10, and 15 days. And for cold treatment, the seedlings were put into a 4 °C growth chamber for 12, 24 and 48 h. The leaf samples were collected at different time points after drought and cold treatments, respectively. Proline contents were determined by the ninhydrin reaction as described by Bates et al. (1973). Soluble carbohydrate contents were determined by anthrone method as described by Zhai et al. (2013b). Malondialdehyde contents were determined by the thiobarbituric reaction as described by Shao et al. (2006).

Statistical analysis

The data were analyzed by Student's *t* test to assess the deviation. The values were expressed as mean ± standard deviation via three parallel measurements.

Results

Identification and sequence analysis of *GmERF9*

Based on the NCBI blast search, 15 ERF sequences were obtained for soybean, but none of them was studied for their function. The further analyses on the expression showed that only one of them could be induced by the stresses in Hefeng 46 cultivar (the data were shown in Fig. 2), all of the rest had no such effects. This gene was termed as *GmERF9* and shared 100% sequence homology with the sequence XM003555644 (GenBank accession number: XM003555644). *GmERF9* had only one open reading frame (ORF) with a length of 801 bp, which encoded 266 amino acids (29.83 kD) with an isoelectric point of 9.03. The predicted GmERF9 protein contained a typical AP2/ERF binding domain consisting of 59 amino acids (Fig. 1), in which there were two key amino acid residues, the 14th alanine and the 19th aspartate acid, suggesting that *GmERF9* belongs to the ERF subfamily of the AP2/ERF family (Hao et al. 1998). In the AP2/ERF domain, there was a putative nuclear localization signal (NLS) sequence (R122RRP).

Expression patterns of *GmERF9* in response to the stress conditions

qPCR analyses were performed to investigate whether the expressions of *GmERF9* were regulated by phytohormones and abiotic stresses. The results showed that the expression levels of *GmERF9* were increased clearly, and reached the maximum at 5, 24, 2, 1, and 2 h, respectively, under the treatments of ET, ABA, drought, salt and cold (Fig. 2).

GmERF9 protein specifically binds to the GCC-box in vitro

To explore whether GmERF9 protein is able to bind to the GCC-box or DRE/CRT elements specifically in vitro, the GmERF9 protein was expressed in *E. coli* and purified for EMSA. As shown in Fig. 3, the protein-probe complex migrated more slowly than the free probes did, which indicated that GmERF9 protein did bind to the GCC-box, and it was also confirmed by the competition experiments by adding of 200-fold excess of unlabeled wild GCC-box oligonucleotide and unlabeled mGCC-box oligonucleotide. However, the binding of GmERF9 protein to DRE/CRT element was not observed. These observations suggested that *GmERF9* might be involved in regulating the expression of stress-relative genes by interacting with the GCC-box in the promoters.

Transcriptional activation of *GmERF9*

To determine whether *GmERF9* could act as a transcriptional activator in the yeast, the yeast two-hybrid analysis was used. The transformants harboring pGBKT7-GmERF9 and pGBKT7 (positive control) could grow on SD media lacking Trp, whereas only the transformant harboring pGBKT7-GmERF9 could grow on SD media lacking Trp, His and Ade (Fig. 4a). The results, together with the β-galactosidase activity assay (Fig. 4b), indicated that *GmERF9* possessed the transcriptional activity in the yeast cells.

The further analyses on the transcriptional activation was performed by transient expression in the tobacco leaves. A reporter plasmid containing the *AtPDF1.2* promoter with a GCC-box to promote *GUS* expression and an effector plasmid containing *GmERF9* driven by the CaMV 35S promoter (Fig. 5a) were delivered into the tobacco leaves by *Agrobacterium*-mediated transformation. As shown in Fig. 5b, c, the *GUS* gene slightly expressed when the tobacco leaves were transformed with the reporter plasmid alone, while the expression level of *GUS* was

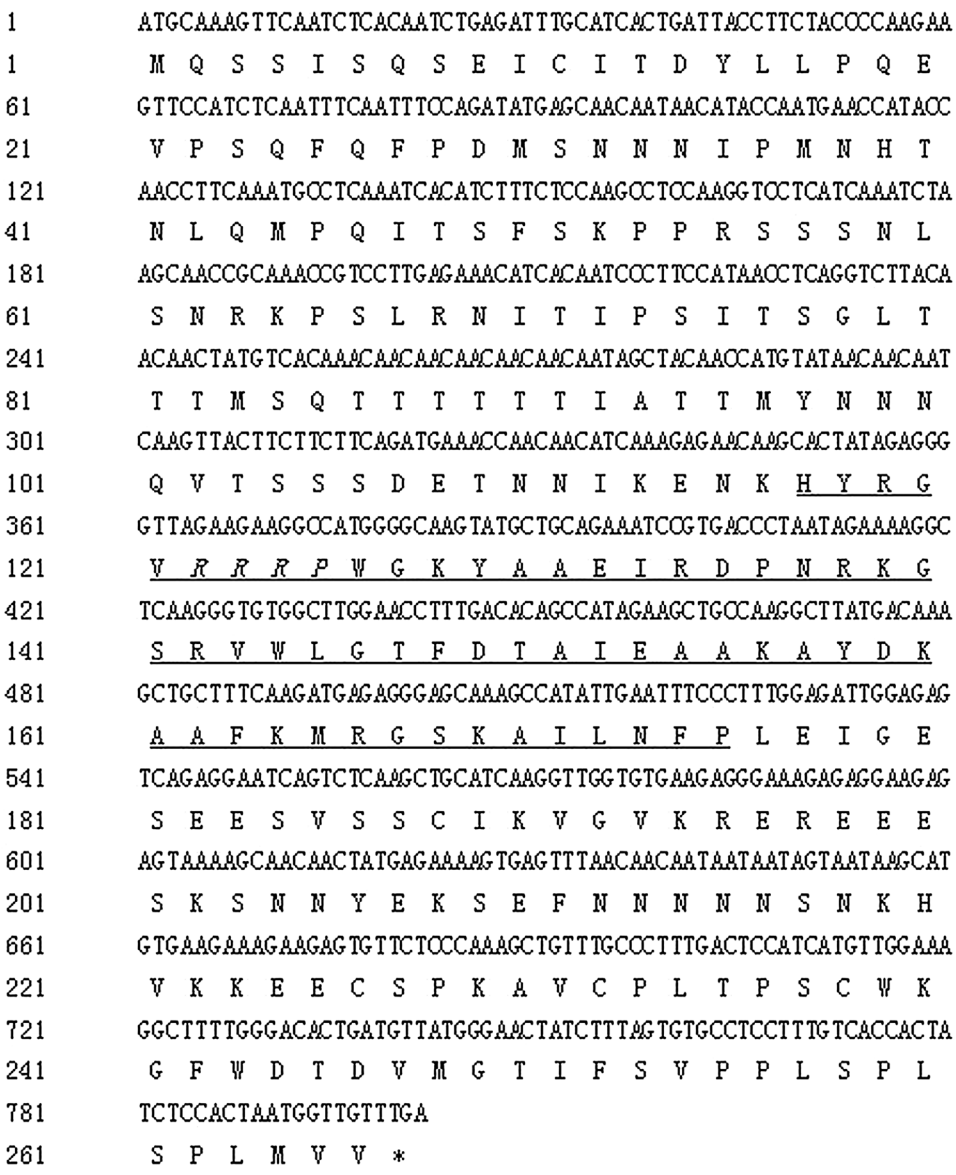

Fig. 1 Nucleotide and deduced amino acid sequence from GmERF9. The AP2/ERF domain was *underline* and the putative NLS sequence was shown in *italics*

upregulated when the tobacco leaves were co-transformed with both the reporter plasmid and effector plasmid. These results indicated that *GmERF9* could activate the expression of the genes, which contained the GCC-box in their promoters.

Overexpression of *GmERF9* enhanced the expression of PR genes in the transgenic tobacco

To further determine whether *GmERF9* activates the expression of PR genes, which contained GCC-box in their promoters, three independent transgenic tobacco lines that constitutively express *GmERF*9 (designated OE) were developed. The expression of *GmERF9* were analysed by semiquantitative RT-PCR in three transgenic tobacco lines and WT tobacco. As shown in Fig. 6a, there was no transcript of *GmERF9* in WT tobacco. The transgenic lines OE2, which had the highest expression of *GmERF9*, was chosen for detecting the expression of possible target genes by qPCR. The gene expression analysis (Fig. 6b) showed that the transcript levels of *PR1, PR2, Osmotin* (*PR5*) and *SAR8.2* were increased in OE2 tobacco compared to the WT tobacco, of which the expressions of *PR1* and *Osmotin* were thousands of times higher in OE2 tobacco than the ones in WT tobacco, which suggests that the overexpression of *GmERF9* in OE2 tobacco activates the transcripts of downstream genes under normal conditions.

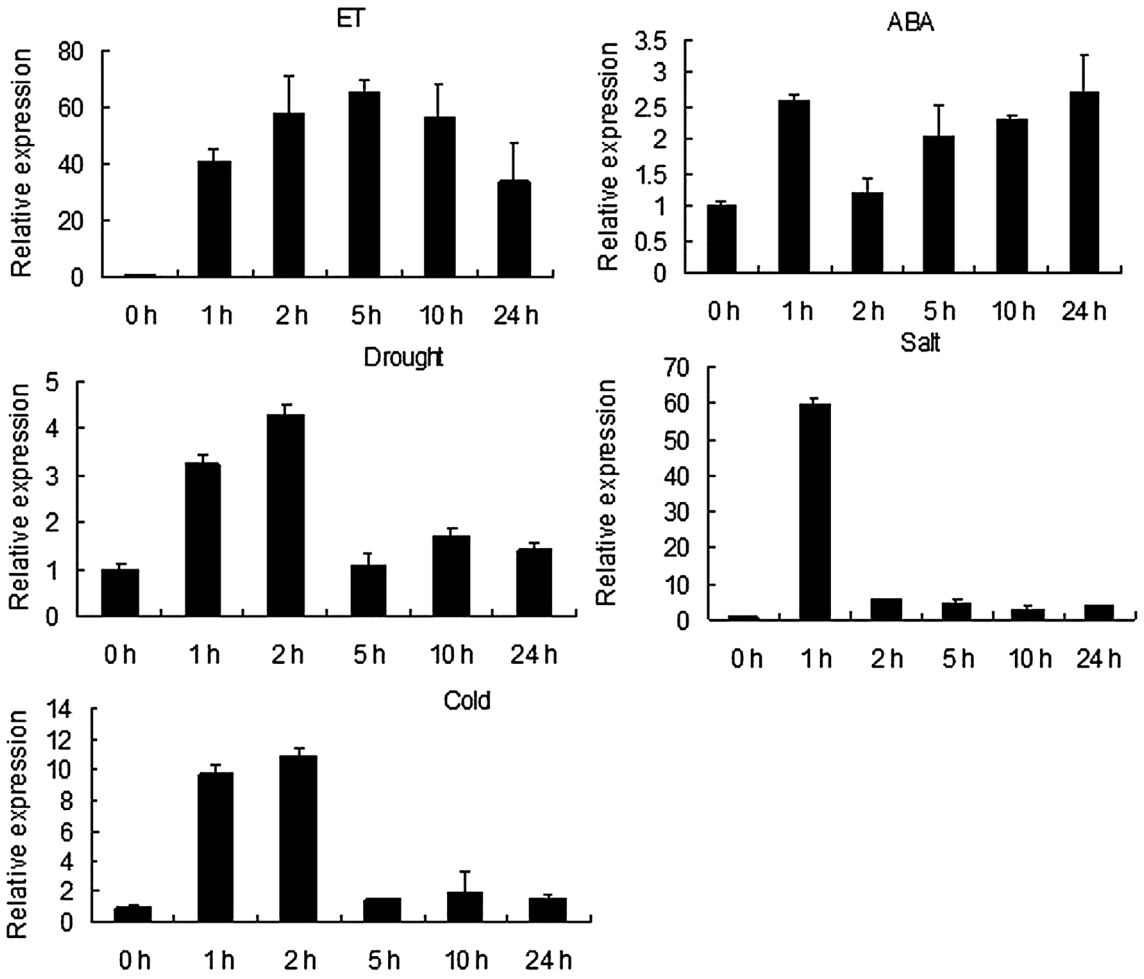

Fig. 2 qPCR analyses of *GmERF9* transcripts. The samples were collected at 0–2, 5, 10 and 24 h after the treatments of ET, ABA, drought, salt and cold

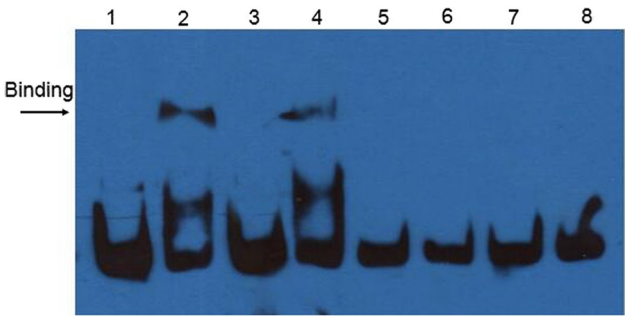

Fig. 3 Sequence-specific binding activity of GmERF9 to the GCC-box or DRE/CRT elements. The *lanes* represented the following conditions: *lane 1*, free labeled GCC-box probe; *lane 2*, GmERF9 protein and labeled GCC-box probe; *lane 3*, GmERF9 protein, labeled GCC-box probe and 200×unlabeled GCC-box probe; *lane 4*, GmERF9 protein, labeled GCC-box probe and 200×unlabeled mGCC-box probe; *lane 5*, free labeled DRE/CRT probe; *lane 6*, GmERF9 protein and labeled DRE/CRT probe; *lane 7*, GmERF9 protein, labeled DRE/CRT probe and 200×unlabeled DRE/CRT probe; *lane 8*, GmERF9 protein, labeled DRE/CRT probe and 200×unlabeled mDRE/CRT probe

Overexpression of *GmERF9* enhanced drought and cold tolerances in the transgenic tobacco

Some PR genes, for example, the *osmotin*, are normally upregulated in response to not only pathogen but also abiotic stresses, such as NaCl and ABA treatments (Xu et al. 1994; Raghothama et al. 1997; Wang et al. 2004). To investigate whether the accumulation of these PR proteins in the transgenic tobacco enhances abiotic tolerance, the contents of proline, soluble carbohydrate and malondialdehyde in *GmERF9* transgenic tobacco were measured under drought and cold stresses. Under normal growth conditions, proline contents in OE2 tobacco and WT tobacco had no obvious differences (Fig. 7). During drought and cold treatments, the proline contents in both OE2 tobacco and WT tobacco rose up continuously, but the contents in OE2 tobacco were always higher than the ones in WT tobacco. This effect was much significant at 7 and 15 days under drought treatment ($P < 0.05$) and at 24 h under cold treatment ($P < 0.05$).

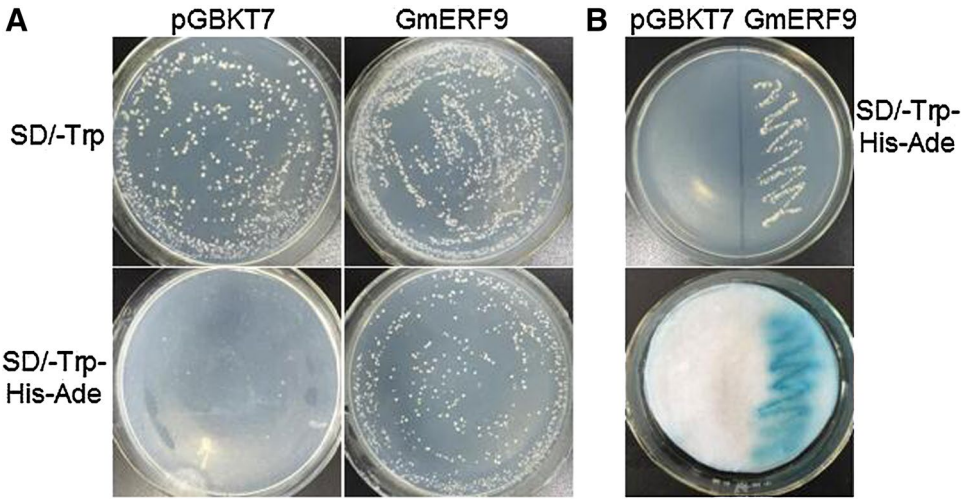

Fig. 4 Transcriptional activation of *GmERF9* in the yeast expression system. **a** The growing of transformant harboring pGBKT7-GmERF9 on SD media lacking Trp and SD media lacking Trp, His and Ade. **b** β-galactosidase activity assay

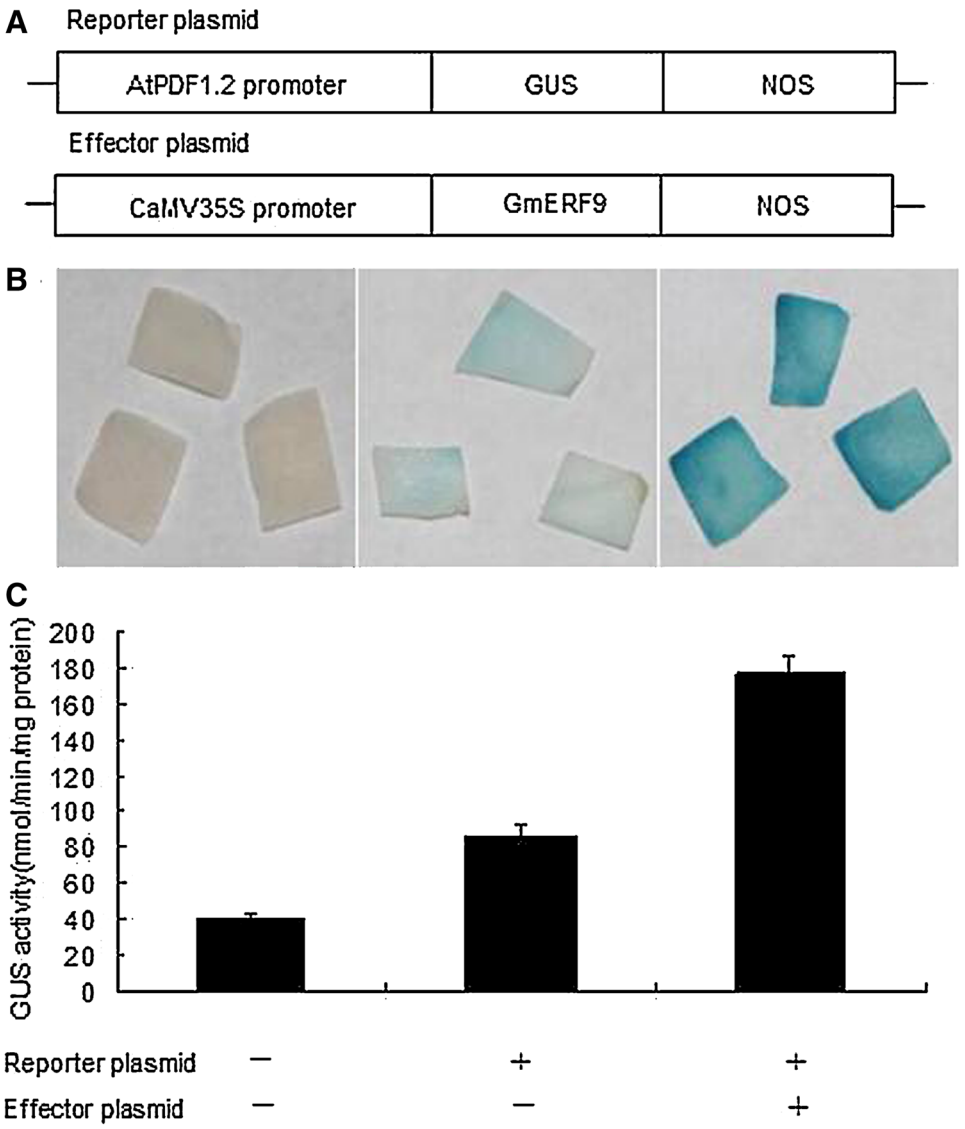

Fig. 5 Transcriptional activation of *GmERF9* in the tobacco leaves. **a** *Schematic diagram* of the reporter and effector plasmids used in the transient expression assays. **b** The histochemical staining analysis as the *GUS* reporter gene activated by *GmERF9*. **c** Fluorometric analysis as the *GUS* reporter gene activated by *GmERF9*

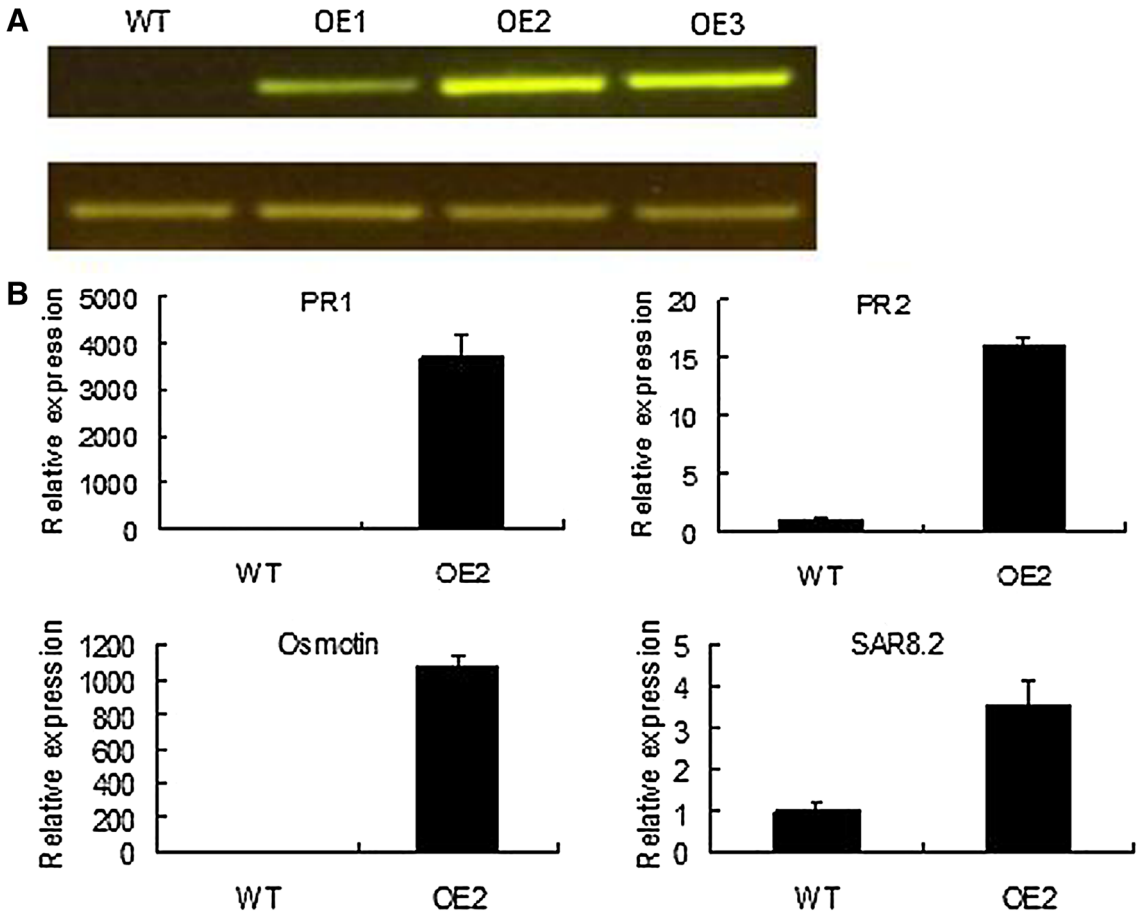

Fig. 6 Expression of putative downstream genes of *GmERF9*. **a** RT-PCR analysis of *GmERF9* expression in transgenic tobacco lines. **b** Expression analysis of PR genes on WT and OE2 tobacco under normal conditions

Comparison studies on soluble carbohydrate contents between OE2 tobacco and WT tobacco showed that the levels of soluble carbohydrate were remarkably higher in OE2 tobacco than that in WT tobacco during both drought and cold treatments. Furthermore, malondialdehyde contents in OE2 tobacco were always lower than the ones in WT tobacco at different time points during drought and cold treatments. These results indicated that the *GmERF9* transgenic tobacco were able to adapt to drought and cold stresses. On the other hand, *GmERF9* transgenic tobacco did not exhibit obviously tolerance against salt stress (data not shown).

Discussion

In this study, a ERF transcription factor with unknown function (*GmERF9*) was identified in soybean and characterized in transgenic tobacco. Based on the protein sequence derived from cDNA sequence (Fig. 1), this gene belonged to ERF subfamily, but not DREB subfamily. According to the study from Sakuma et al. (2002), the most significant difference on the AP2/ERF domains between ERF and DREB subfamilies was the two conserved amino acid residues. In the AP2/ERF domain of DREB subfamily, the 14th residue is valine and 19th residue is glutamic acid. However, in the AP2/ERF domain of ERF subfamily, the 14th residue is alanine and 19th residue is aspartic acid, which were believed to contribute binding activity to GCC-box in many ERFs (Sakuma et al. 2002). The differences of 14th residue and 19th residue may imply their divergence on the functions between the two subfamilies (Rehman and Mahmood 2015). Many studies support the view point that DREB and ERF subfamilies have established in the early stages of land plant evolution and shared a common ancestor (Mizoi et al. 2012; Rehman and Mahmood 2015). The analysis shows that GmERF9 contains a putative NLS sequence, which is consistent with the fact that ERF proteins identified so far were all located in nucleus (Park et al. 2001; Zhang et al. 2009, 2010; Zhai et al. 2013b).

The expression of ERF genes can be activated by various stresses, such as dehydration, high salinity, low or

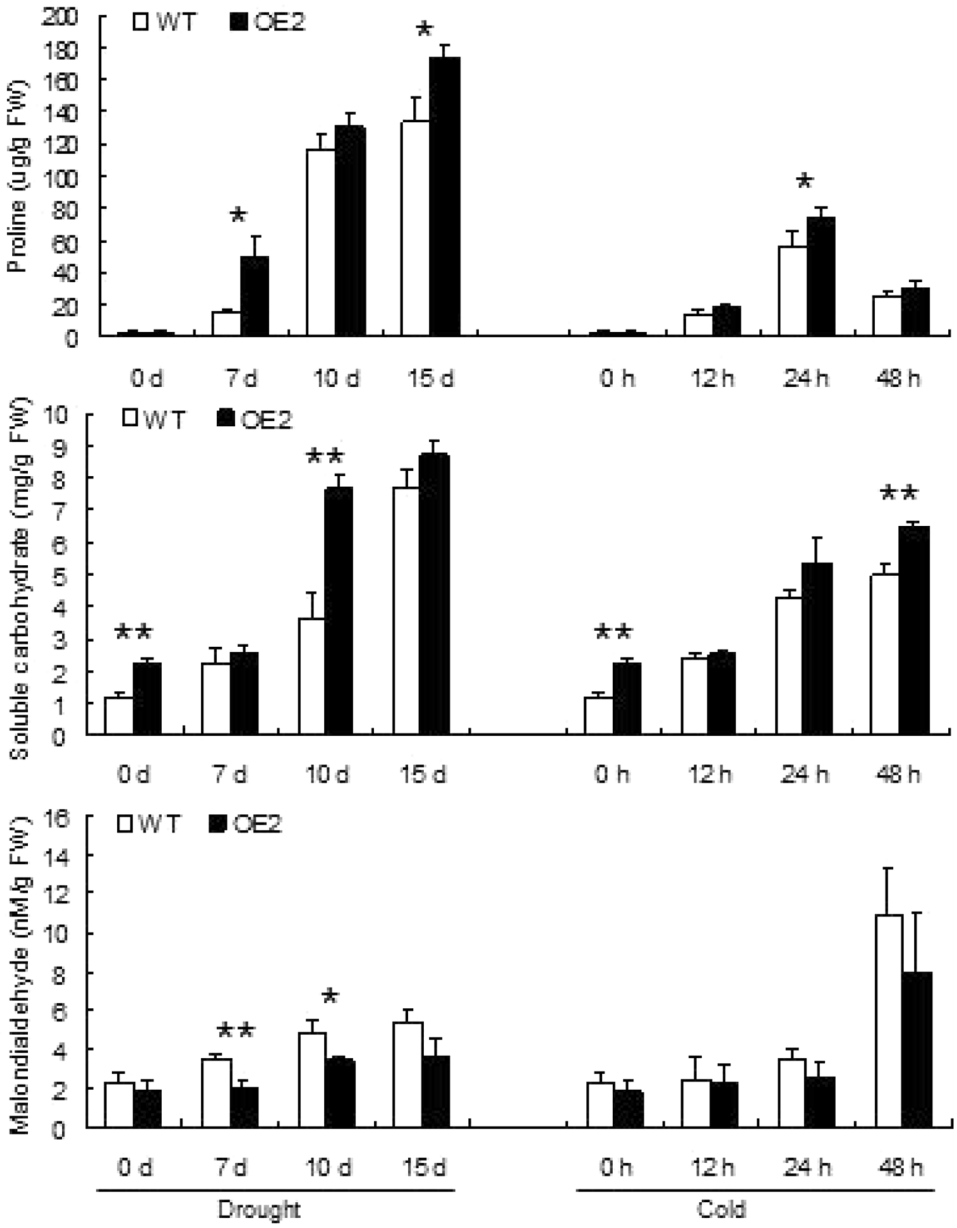

Fig. 7 OE2 tobacco overexpressing *GmERF9* enhances drought and cold tolerances. Data represented the average of three replicates ± SE. *,**Indicated significant difference in comparison to the WT plants at $P < 0.05$ and $P < 0.01$, respectively (Student's *t* test)

high temperature in popcorn (Shi et al. 2015), tea plant (Wu et al. 2015) and potato (Bouaziz et al. 2015). In this research, *GmERF9* was strongly induced by ET, salt, drought, cold and ABA (Fig. 2), which indicate that both biotic and abiotic signal pathways may be involved in and co-regulate the expression of tolerance-resistance genes as the plants response to the stimuli. During the processes, cross talking may occur between the two pathways as reported in rice and tomato, where the expressions of both *TERF1* and *TSRF1* were induced by the cross talking between biotic and abiotic stresses (Gao et al. 2008). Therefore, it suggests that *GmERF9* may contain some *cis*-elements in the promoter region that are regulated by salt, drought, cold stresses and exogenous ET and ABA treatments. In addition, ERF proteins may also affect other gene expression by either regulating the expression of other transcriptional factors or interacting physically with other transcriptional factors (Buttner and Singh 1997; Chakravarthy et al. 2003).

Some ERF proteins, such as JERF1 (Zhang et al. 2004), AtERF53 (Cheng et al. 2012) and PsAP2 (Mishra et al. 2015), bound specifically to the DRE/CRT element with the core sequence of C/GCCGAC. However, the binding activity of GmERF9 protein is different from these ERF proteins, it can not bind to the DRE/CTR element, but to the GCC-box (Fig. 3), which is more close to GmERF7 (Zhai et al. 2013a, b) and OPBP1(Guo et al. 2004). In the case of GmERF9, further studies are needed to clarify

the differences between GmERF9 and other ERF proteins regarding the binding activity to the various element, such as GCC-box, DRE/CRT, CE1, JERE, and CT-rich. Each transcription factor responses to the stresses differently, which may be due to the difference of DNA binding activities, or the presence of post-translation modification, or an interaction with different proteins (Oñate-Sánchez and Singh 2002).

ERF proteins can act as either transcriptional activators or repressors in transcriptional regulation in plants (Ohta et al. 2001). Most ERF proteins identified are transcription activators, such as *Arabidopsis AtERF1, AtERF2* and *AtERF5* (Fujimoto et al. 2000), soybean *GmERF3* and *GmERF7* (Zhang et al. 2009; Zhai et al. 2013b), wheat *TaERF1* (Xu et al. 2007), and popcorn *ZmERF1* (Shi et al. 2015). Just a few of them are transcription repressors with a conserved associated amphiphilic repression (EAR) (Fujimoto et al. 2000; Ohta et al. 2001). Our findings show that *GmERF9* acts also as a transcriptional activator (Figs. 4, 5), which can activate the expressions of the reporter genes, such as *LacZ* in the yeast cells and *GUS* in the tobacco leaves.

Many studies show that the overexpression of ERFs enhances the resistance to multiple stresses through the regulation on the genes with GCC-box or DRE/CRT motif in their promoters. For instance, the tomato *JERF3* mediated the osmotic, salinity and dehydration stress tolerances when it was overexpressed in transgenic tobacco (Wu et al. 2008). In this study, the transgenic approach were used to elucidate the role of *GmERF9* in various stress responses. Earlier studies showed that the expression of some transcription factors with constitutive promoters often caused growth retardation and abnormality for plants (Nakashima et al. 2007; Hu et al. 2008). In this study, the constitutive expression of *GmERF9* driven by CaMV 35S promoter did not cause any undesirable growth on the phenotypes (data was not shown). The constitutive expression of *GmERF9* in transgenic tobacco positively activated the expression ofPR genes, including *PR1, PR2, Osmotin* (*PR5*), and *SAR8.2* (Fig. 6). It should be emphasized that all of these genes contain GCC-box in their promoters and are related to the systemic acquired resistance in tobacco (Ward et al. 1991).

To evaluate the physiological changes on *GmERF9* transgenic tobacco under abiotic stress, the contents of proline and soluble carbohydrate as osmotic regulators, and the content of malondialdehyde as indicator for membrane integrity were measured in the WT and transgenic tobacco. The contents of proline and soluble carbohydrate were increased, but the content of malondialdehyde was decreased in *GmERF9* transgenic tobacco compared to WT tobacco under drought and cold stresses (Fig. 7). Similar results were also obtained in *GmERF3* and *GmERF7* transgenic tobacco (Zhang et al. 2009; Zhai et al. 2013b). These results further demonstrated that the tolerances are increased after *GmERF9* was transfered into the tobacco. However, the *GmERF9* transgenic tobacco did not exhibit any detectable changes against salt stress, which indicated that the up regulation of some genes expression, such as the expression of *GmERF9* under salt stress, may not be sufficient to increase the resistance to stress (Kim et al. 2003). These results support the idea that a large number of ERF genes in the plant genome show not only morphology on the sequences, but also reflect function diversities for dealing with different stresses (Fischer and Dröge-Laser 2004; Zhai et al. 2013b).

In summary, our study reveals that overexpression of *GmERF9* confers the drought and cold tolerances to the transgenic tobacco. It could be a good candidate gene for engineering plants for multiple stress tolerances without any growth defects.

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