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Isolation and characterization of a novel transcriptional repressor *GmERF6* from soybean

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Abstract

A new ethylene response factor (ERF), *GmERF6,* was isolated from soybean. Protein sequence alignment of GmERF6 revealed an AP2/ERF domain, two putative nuclear localization signals (NLSs), and an ERF-associated amphiphilic repression (EAR) motif. Real-time quantitative PCR analysis revealed that the expression of *GmERF6* was differentially induced in soybean seedlings by drought, salt, cold, salicylic acid, ethylene, abscisic acid, and methyl jasmonate. Transient expression experiments demonstrated that *GmERF6* functions as a transcriptional repressor to downregulate the transcriptional levels of the reporter gene and repress the activated ability of other transcriptional activator. Transgenic *Arabidopsis* lines constitutively expressing *GmERF6* showed an increased tolerance to drought compared to wild-type plants.

Additional key words: *Arabidopsis thaliana*, drought tolerance, ethylene response factor, expression pattern, *Glycine max*, transcriptional regulation.

Introduction

Plants are often exposed to a wide range of adverse environmental conditions, such as drought, extreme temperatures, salinity, UV irradiation, wounding, and pathogen attacks which may severely affect their growth and development. In response to stress, plants modulate the expression of specific sets of genes and produce a large number of stress-related proteins which play important roles in stress response and plant defense (Ishitani *et al*. 1997, Reymond and Farmer 1998, Xiong *et al*. 1999, Demekamp and Smeekens 2003). The stress response of plants is regulated by multiple signalling pathways in which ethylene (ET), salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA) have important roles (Glazebrook 1999, Knight and Knight 2001). There is a significant overlap among the patterns of gene expression that is induced in plants in response to different stresses (Durrant *et al*. 2000, Seki *et al*. 2001) but the cross talk among these signalling pathways is not well understood (Dong 1998, Reymond and Farmer 1998, Dempsey *et al*. 1999). The regulation of plant defence in response to stress is very complicated because a number of transcription factor families are involved (Rushton and Somssich 1998, Singh *et al*. 2002). Transcriptional factors can be a valuable resource in transgenic technology attributing novel traits to the transgenic plants (Hussain *et al*. 2011). For this reason, it will be interesting to identify key transcription factors in plant defence and, subsequently, to use them to increase crop resistance to various stresses.

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Abbreviations: ABA - abscisic acid; DRE/CRT - dehydration-responsive element/C-repeat; EAR - ERF-associated amphiphilic repression; ERF - ethylene response factor; ET - ethylene; GUS - β-glucuronidase; JA - jasmonic acid; MeJA - methyl jasmonate; NLS - nuclear localization signal; PEG - polyethylene glycol; PR - pathogenesis-related; SA - salicylic acid; TT - transgenic type; WT - wild type.
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Ethylene-responsive factors (ERFs) are a subfamily of the AP2/ERF transcription factor superfamily which is present only in the plant kingdom. In *Arabidopsis thaliana*, there are approximately 147 members of the AP2/ERF transcription factor family, all of which have diverse functions throughout the plant life cycle including regulation of development and responses to abiotic and biotic stresses (Feng *et al*. 2005, Nakano *et al*. 2006). ERFs contain a highly conserved DNA binding domain (AP2/ERF domain) consisting of 58 or 59 amino acid residues, which binds to the GCC box found in some PR (pathogenesis-related) gene promoters and to the dehydration-responsive/C-repeat (DRE/CRT) element found in some dehydration and low-temperature-responsive gene promoters (Ohme-Takagi and Shinshi 1995, Park *et al*. 2001). Most ERFs identified so far are transcription activators as well as transcription repressors containing the ERF-associated amphiphilic repression (EAR) motif with the conserved sequence L/FDLNL/F(X)P (Ohta *et al*. 2001). In *Arabidopsis*, there are 14 ERFs that encode transcription repressors (Nakano *et al*. 2006) and all have different expression patterns suggesting that they may have different functions (Yang *et al*. 2005). Among them, AtERF4 and AtERF7 repress the expression of downstream genes resulting in decreased sensitivity to ABA when they are over-expressed in *Arabidopsis* (Song *et al*. 2005, Yang *et al*. 2005). Ohta *et al*. (2000) reported that *ERF3* reduced the transcription of a reporter gene in tobacco protoplasts suggesting that *ERF3* functions as a repressor. The first identified ERF repressor in soybean, *GmERF4*, is induced by multiple abiotic and biotic stresses and transgenic tobacco plants constitutively expressing *GmERF4* showed an increased tolerance to salt and drought stresses (Zhang *et al*. 2010).

Soybean (*Glycine max* L.) is one of the most economically important crops in the world. Ninety-eight unigenes containing a complete AP2/ERF domain have been identified in a soybean database (Zhang *et al*. 2008). However, only three members of the ERF family have been functionally characterized so far (Mazarei *et al*. 2002, Zhang *et al*. 2009, 2010). The functions of other members of this family remain to be determined. Here, we report the isolation and characterization of a new transcriptional repressor gene, *GmERF6*, in soybean. Its expression patterns induced by different stresses, transcriptional repressive activity, and function in transgenic *Arabidopsis* lines of *GmERF6* were also investigated.

Materials and methods

Seedlings of soybean [*Glycine max* (L.) Merr. cv. Jilin 32], grown hydroponically in a greenhouse at 25 °C for 7 d, were subjected to various stress treatments. Salt and drought stresses were carried out by supplementing Hoagland solution with NaCl and polyethylene glycol (PEG) 8000 to final concentrations of 200 mM and 20 %, respectively. For cold treatment, seedlings were put into a 4 °C growth chamber. For ABA, SA, and methyl-jasmonate (MeJA) treatments, seedlings were sprayed with 200 μM ABA, 2 mM SA and 100 μM MeJA, respectively, dissolved in Hoagland solution. Ethylene treatment was performed in a sealed plexiglass chamber by dissolving 2 cm^3 of 40 % ethephon and 1 g of $NaHCO_3$ in 200 cm^3 of H_2O. After each treatment, soybean seedling leaves were harvested at the indicated times and quickly frozen in liquid nitrogen, stored at -80 °C for the preparation of total RNA.

An *NCBI BLAST* search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of gene expression profiles of cDNA sequences from immature soybean embryo uncovered a cDNA sequence that is highly homologous to other plant ERF transcription factors. This cDNA was designated as *GmERF6*. Using the primer pair F: 5′-CTTCCTACT CCTCCCTTTCAC-3′ and R: 5′-CGTAGTAGTGTT CCCAGATGC-3′ polymerase chain reaction (PCR) was performed as follows: 94 °C for 8 min and 30 cycles of 94 °C for 40 s, 52 °C for 40 s and 72 °C for 8 min. The amplified fragments were cloned into the pMD-18T cloning vector (*TaKaRa*, Changchun, China) and sequenced for confirmation.

To construct a reporter plasmid, a 346-bp region of the upstream sequence from the *Arabidopsis thaliana* L. cv. Columbia gene *AtPDF1.2* containing the GCC box was amplified (F: 5′-GGGAATTCATTATTTTCT TGAGTC-3′; R: 5′-GGCCATGGGATGATTATTACT ATT-3′) and cloned into the pCMBIA1301 plasmid to replace the *CaMV 35S* promoter. To construct the effector plasmids, the *GUS* gene in pCMBIA1301 was replaced with the full coding regions of *GmERF6* or *GmERF7* (encoding a transcriptional activator in soybean, GenBank acc. No. JN416602). Both the reporter plasmid and the effector plasmid were introduced into the *Agrobacterium tumefaciens* strain EHA105 for *Agrobacterium*-mediated transformation of soybean callus. GUS activity was measured approximately 24 h later as described by Jefferson (1987).

Further, the coding region of *GmERF6* was cloned into the binary vector pBI121 under the control of the *CaMV 35S* promoter. The pBI121-GmERF6 plasmid was introduced into *Arabidopsis* using *Agrobacterium*-mediated transformation following the floral dip method (Clough and Bent 1998). *Arabidopsis* plants were grown in a growth chamber at temperature of 22 °C, a 16-h photoperiod, irradiance of 100 μmol m^{-2} s^{-1}) and air

humidity of 80 %. The seeds from transformed plants (T_0) were harvested and sowed on Murashige and Skoog (MS) medium containing 50 mg cm^{-3} kanamycin. The T_1 and T_2 transformants were screened and verified by PCR. Positive T_3 transformants were used for further analyses. Rosette leaves of wild type (WT) and transformed (TT) *Arabidopsis* plants were detached, placed in weighing dishes and incubated at room temperature. The water loss rate was monitored at the indicated times.

Total RNA from soybean and *Arabidopsis* was extracted using *RNAplant plus* reagent (*Tiangen*, Changchun, China) according to the manufacturer's protocol and reversely transcribed using the *M-MLV* reverse transcriptase (*TaKaRa*). The real-time quantitative RT-PCR analysis was performed using *SYBR Green I* dye (*TaKaRa*) on real-time PCR machine (*Applied Biosystems 7500*, Foster City, USA). The soybean *β-tubulin* gene (GenBank acc. No. GMU12286) was chosen as an internal control. For semiquantitative RT-PCR analyses, all PCR reactions were performed with 2× *Taq PCR MasterMix* (*Tiangen*, Changchun, China), a pair of primers (0.2 μM each) and cDNA in a final volume of 0.025 cm^3. The PCR reaction for each gene fits within the following parameters: 94 °C for 8 min and 25 - 30 cycles of 94 °C for 40 s, 51 - 57 °C for 40 s and 72 °C for 8 min. The *Arabidopsis Atactin* gene (GenBank acc. No. AK230311) was chosen as an internal control. The gene-specific primer pairs used for RT-PCR are listed in Table 1.

Table 1. The gene-specific primer pairs used for RT-PCR.

Gene	Forward	Reverse
GmERF6	5′-CAACAACATTCGCAGTCCCA-3′	5′-AGTCGTTACGGCGGAAATC-3′
β-tubulin	5′-GGAAGGCTTTCTTGCATTGGTA-3′	5′-AGTGGCATCCTGGTACTGC-3′
Atactin	5′-CCTTGAAGTATCCTATTGAGC-3′	5′-GGTCTTTGAGGTTTCCATCT-3′
AtKin1	5′-CATCATCACTAACCAAAACACAC-3′	5′-GATACACTCTTTCCCGCCT-3′
AtSOS1	5′-CATCCTCACAATGGCTCTAA-3′	5′-CTCCTTCCTTTTCACTTTCA-3′
AtPR3	5′-CACTTACAACGCCTTTATCACC-3′	5′-AGTCAACTCCTATTGCTCTACCG-3′
AtRD22	5′-CCAAACACTCCCATTCCC-3′	5′-TGCCTCCGTAACCATCCT-3′
AtPDF1.2	5′-AGAAGCCAAGTGGGACAT-3′	5′-CGATTTAGCACCAAAGATT-3′
AtPR4	5′-CTTTTATCATACACAGTGGCTACG-3′	5′-CATCCAAATCCAAGCCTCC-3′
AtERF7	5′-TCATCAGCGAGGAGACAAG-3′	5′-CGAAACAGGAAAAGCGA-3′
AtERF4:	5′-AGGTGGGATGGAGAAGAGA-3′	5′-GAAAGCCAATAGAAGGAGC-3′
AtNIMIN1	5′-ATCTAACGGCGGAGAAAGG-3′	5′-CACAACGCTAACAAATGAAAC-3′

Results and discussion

Among the cDNA sequences from soybean immature embryo gene expression profiles, a cDNA clone was identified whose predicted translation product has an AP2/ERF domain. This clone, designated *GmERF6* (GenBank acc. No. JN416601), was chosen for further functional analysis. The full-length cDNA sequence of *GmERF6* has an open reading frame of 582 bp encoding a polypeptide of 193 amino acids with a predicted molecular mass of 21.24 kDa (pI 9.65). The deduced protein product has a central 58 amino acids AP2/ERF domain, two putative nuclear localization signal (NLS) sequences (R31KRP and P45GKKTRV) and a conserved repression-associated EAR motif in the C-terminal region (Fig. 1). The results from database searching (http://www.plantgdb.org/GmGDB/) revealed that the *GmERF6* gene did not contain any introns and was located on chromosome 12. To investigate the identity between *GmERF6* and other *ERF* genes, we performed amino acid sequence alignments using *DNAman* software. This analysis indicated that GmERF6 protein was grouped into Class II of the four classes of ERF proteins in plant (Fujimoto *et al*. 2000, Tournier *et al*. 2003). Among these proteins, the predicted amino acid sequence of GmERF6 was highly homologous to GmERF4, with 49.12 % identity for the full-length sequences and 91.38 % identity for the AP2/ERF domains (Fig. 1). These findings suggest that *GmERF6* is a novel member of the ERF family and thus may be involved in the modulation of stress-related genes expression in the nucleus acting as a transcription repressor.

To further elucidate the function of *GmERF6* in plant response to various stresses, we investigated the expression profile of *GmERF6* in soybean seedling leaves that experienced various stresses. The results showed that the expression of *GmERF6* was clearly increased upon drought, salt, cold, SA, ETH, ABA, and MeJA treatments (Table 2). *GmERF6* mRNA increased rapidly under drought, salt and ABA treatments, reaching a maximum at 1 h after treatments and thereafter declined. With cold, SA, and MeJA treatments, *GmERF6* expression exhibited a similar pattern with expression reaching a maximum level at 5 h after cold treatment and 2 h after SA and

Fig. 1. Amino acid sequences alignment of GmERF6 protein and other ERF proteins. The AP2/ERF domain is *underlined* and the EAR motif is *double underlined*. *Asterisks* denote NLS and an *inverted triangle* denotes the conserved Ala and Asp residues.

Table 2. *GmERF6* expression profile induced by various treatments. The relative expressions of *GmERF6* under various treatments were relative to that under normal growing conditions in seedling leaves. Means ± SE, $n = 3$.

Time [h]	Drought	Salt	Cold	SA	ETH	ABA	MeJA
0	1.00 ± 0.37	1.00 ± 0.19	1.00 ± 0.52	1.00 ± 0.24	1.00 ± 0.05	1.00 ± 0.10	1.00 ± 0.40
1	3.68 ± 0.21	3.41 ± 0.56	1.92 ± 0.25	3.32 ± 0.46	0.79 ± 0.10	1.64 ± 0.28	1.65 ± 0.50
2	2.83 ± 0.40	2.03 ± 0.21	2.91 ± 0.23	6.29 ± 0.38	1.53 ± 0.12	0.49 ± 0.11	2.98 ± 0.63
5	2.52 ± 0.21	1.62 ± 0.37	3.13 ± 0.31	2.13 ± 0.45	1.56 ± 0.20	0.56 ± 0.21	0.95 ± 0.35
10	2.43 ± 0.57	1.10 ± 0.27	2.47 ± 0.56	2.25 ± 0.42	1.68 ± 0.21	1.17 ± 0.43	1.34 ± 0.57

Table 3. Transcriptional repression by *GmERF6* was analyzed by assessing GUS activity [pmol(MU) mg^{-1}(protein) min^{-1}] driven by a *AtPDF1.2* promoter, under this repressor, effector *GmERF7*, or their combination. Means ± SE, $n = 3$. Different letters indicate significant differences at $P < 0.05$ according to one-way *ANOVA*.

WT	*AtPDF1.2*	*AtPDF1.2* + *GmERF6*	*AtPDF1.2* + *GmERF7*	*AtPDF1.2* + *GmERF6* + *GmERF7*
0.066 ± 0.006c	0.090 ± 0.011b	0.067 ± 0.004c	0.128 ± 0.004a	0.071 ± 0.002c

MeJA treatments. ETH treatment induced *GmERF6* expression slower than other treatments and the maximum number of transcripts reached at 10 h after treatment.

Fujimoto *et al*. (2000) reported that *Arabidopsis AtERF3* and *AtERF4* acted as repressors to downregulate basal transcription levels of reporter genes as well as the transactivation activity of other transcription factors. To determine whether *GmERF6* has repressive activity *in vivo*, a reporter plasmid containing the *Arabidopsis AtPDF1.2* gene promoter with a GCC box to promote *GUS* expression and an effector plasmid containing either *GmERF6* or *GmERF7* driven by the *CaMV 35S* promoter were delivered to soybean callus by *Agrobacterium*-mediated transformation. As shown in Table 3, GUS activity was repressed when co-expressed with *GmERF6*. Furthermore, co-expression of *GmERF6*, *GmERF7* and the reporter gene resulted in a level of GUS activity that was similar to the levels obtained when only the reporter gene was expressed, indicating that *GmERF6* repressed not only the activity of the reporter gene but also the activity of transcriptional activator *GmERF7*.

Transgenic *Arabidopsis* overexpressing *GmERF6* was generated and expression of putative downstream stress-responsive genes was analyzed using semiquantitative RT-PCR. *GmERF6* was expressed only in TT lines but not in WT plants. The expression levels of *AtKin1*, *AtSOS1*, *AtPR3*, and *AtRD22* were decreased in TT lines

compared to WT plants. In contrast, the expression levels of *AtPDF1.2* and *AtPR4* were increased in TT lines. To address this, we examined the expression of several transcriptional repressors in *Arabidopsis*. As expected, the decrease in expression of *AtERF4*, *AtERF7*, and *AtNIMIN1* resulted in an increase in the expression of several stress-related genes (Fig. 2).

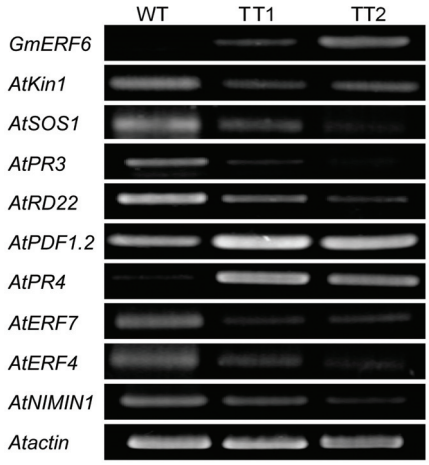

Fig. 2. Expression patterns of stress-related genes in WT and *GmERF6* TT *Arabidopsis* under normal growing conditions. TT1 and TT2 indicate *GmERF6* transgenic independent *Arabidopsis* lines.

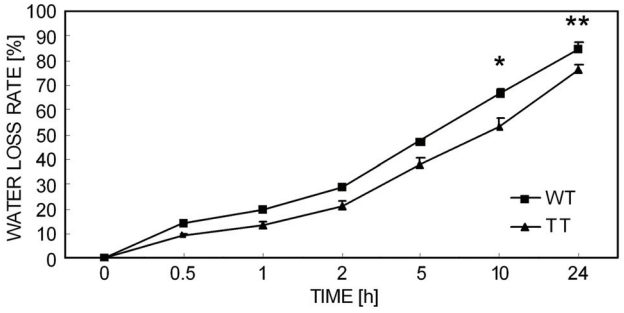

Fig. 3. Water loss rate of detached leaves of WT and TT *Arabidopsis* plants. Means ± SE, $n = 3$. ** and * indicate significant difference at $P < 0.01$ and $P < 0.05$ between the TT and WT *Arabidopsis* plants, respectively, according to Student's *t*-test.

To determine whether overexpression of *GmERF6* could enhance plant tolerance to drought stress, the water loss rate of detached leaves of WT and TT *Arabidopsis* plants was investigated (Fig. 3). The water loss rate of TT plants was lower than that of WT plants. These results together with observation of growth under drought (data not shown) indicated that *GmERF6* TT *Arabidopsis* displayed higher tolerance against drought stress compared to WT *Arabidopsis*. On the contrary, the *GmERF6* TT *Arabidopsis* did not exhibit any detectable tolerance against cold or salt stress (data not shown).

A large number of studies have revealed the important roles of transcriptional repressors in modulating plant defense and stress responses (Song *et al*. 2005, Yang *et al*. 2005, Ciftci-Yilmaz *et al*. 2007). Stress-associated EAR-repressors primarily fulfill two roles in plant cells: *1*) they repress defence- and stress-related genes in the absence of any stress, *2*) they control the activation of stress-related genes, preventing damage to the plant that could result from inappropriate activation of the stress response (Kazan 2006). The repression-associated EAR motif has primarily been found in Class II ERFs and plant zinc finger proteins (Ohta *et al*. 2001), however, the mechanism by which the EAR motif inhibits transcription is currently unknown. Zhang *et al*. (2008) reported, there are four ERF repressors in soybean that contain a common EAR motif. In this work, we report the cloning and functional analysis of a new transcriptional repressor, *GmERF6*, from soybean in efforts to better elucidate the repressive mechanism played by ERF repressors.

Amino acid sequence analysis revealed that GmERF6 was most closely related to the GmERF4, a repressor in soybean, implying that they may have evolved from a common ancestor and retained a similar function. Previous studies showed that members of the ERF repressor family exhibit differential expression under abiotic and biotic stresses (Fujimoto *et al*. 2000). Our data indicated that *GmERF6* was differentially regulated by abiotic stresses and stress-responsive phytohormones that mimic biotic stresses. It suggests that *GmERF6* is likely regulated by multiple stress-signalling pathways in response to various stresses. Using transient expression experiments, we have shown that *GmERF6* can function as a transcriptional repressor through binding to a GCC box promoter, leading to a decrease in the transcription levels of the reporter gene and a decrease in the activity of a transcriptional activator.

As a transcriptional repressor, *GmERF6* represses the expression of *AtKin1*, *AtSOS1*, *AtPR3*, and *AtRD22* in transgenic *Arabidopsis*. Surprisingly, *GmERF6* over-expression also leads to an upregulation of *AtPR4* and *AtPDF1.2* which seems a bit contradictory with the fact that *GUS* activity was repressed in soybean callus cells when *GmERF6* under *CaMV 35S* promoter was co-transformed with *GUS* gene under *AtPDF1.2* promoter. Zhang *et al*. (2010) speculated that the enhanced tolerance of transgenic plants expressing EAR-repressors occurs as a result of the suppression of other repressors of the defence response by EAR-repressors. We suppose that some stress-related repressors were repressed preferentially by *GmERF6* which resulted in removing the suppression of the downstream gene such as *AtPDF1.2* and *AtPR4* in transgenic *Arabidopsis*. To investigate this hypothesis, we examined the expression of six transcriptional repressors in *Arabidopsis* plants in which the expression of *AtERF4*, *AtERF7*, and *AtNIMIN1* were strongly decreased. The decreased expression of *AtERF4*, *AtERF7*, and *AtNIMIN1* may lead to an activation of the defence response and an enhanced

tolerance to stresses. Consistent with this, transgenic *Arabidopsis* overexpressing a transcriptional repressor *GmERF6* demonstrated improved stress tolerance to drought stress.

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