Abstract

Phytophthora root and stem rot of soybean [Glycine max (L.) Merr.], caused by Phytophthora sojae Kaufmann and Gerdeman, is a destructive disease throughout the soybean planting regions in the world. Here, we report insights into the function and underlying mechanisms of a novel ethylene response factor (ERF) in soybean, namely GmERF5, in host responses to P. sojae and positively regulated the expression of the PR10, PR1-1, and PR10-1 genes. Sequence analysis suggested that GmERF5 contains an AP2/ERF domain of 58 aa and a conserved ERF-associated amphiphilic repression (EAR) motif in its C-terminal region. Following stress treatments, GmERF5 was significantly induced by P. sojae, ethylene (ET), abscisic acid (ABA), and salicylic acid (SA). The activity of the GmERF5 promoter (GmERF5P) was upregulated in tobacco leaves with ET, ABA, Phytophthora nicotianae, salt, and drought treatments, suggesting that GmERF5 could be involved not only in the induced defence response but also in the ABA-mediated pathway of salt and drought tolerance. GmERF5 could bind to the GCC-box element and act as a repressor of gene transcription. It was targeted to the nucleus when transiently expressed in Arabidopsis protoplasts. GmERF5 interacted with a basic helix-loop-helix transcription factor (GmbHHL) and eukaryotic translation initiation factor (GmEIF) both in yeast cells and in planta. To the best of our knowledge, GmERF5 is the first soybean EAR motif-containing ERF transcription factor demonstrated to be involved in the response to pathogen infection.

Key words: EAR, Glycine max, GmERF5, Phytophthora sojae, AP2/ERF, repressor.
They are characterized by a conserved DNA-binding domain, the AP2/ERF domain, which is 57–66 aa (Weigel, 1995; Okamuro et al., 1997). The AP2/ERF proteins are classified into five subfamilies, named AP2, RAV, DREB, ERF, and others, based on the number of AP2 domains and their sequence homology (Sakuma et al., 2002). The ERF subfamily proteins contain a single AP2/ERF domain, forming three β-sheets and an α-helix (Allen et al., 1998). The key residues for DNA-binding specificity in the β-sheet are those at positions 14 and 19, where alanine (A) and aspartic acid (D) are located (Sakuma et al., 2002). A phylogenetic tree based on an alignment of the AP2/ERF domains from ERF proteins in Arabidopsis divided members of the ERF subfamily into six groups termed B-1 to B-6 (Sakuma et al., 2002). Most ERF transcription factors function as transcription activators that positively regulate the transcript levels of their target genes (Fujimoto et al., 2000; Gu et al., 2000; Xu et al., 2007; Zhang et al., 2009; Zhai et al., 2013a). In contrast to the ERF activators, some B-1 ERF proteins have been shown to act as transcription repressors, which downregulated not only basal transcription levels of target genes but also the activity of certain other transcription factors (Fujimoto et al., 2000; Ohta et al., 2001; Song et al., 2005; Yang et al., 2005; Zhang et al., 2010a; Zhai et al., 2013b). This repression activity is believed to be related to a repression domain referred to as the ERF-associated amphiphilic repression (EAR) motif, which is located in the C-terminal region of these proteins with a conserved (L/F)DLN(L/F)αP sequence (Ohta et al., 2001).

ERF transcription factors have been identified in different plant species, such as Arabidopsis thaliana (Li et al., 2011), tobacco (Nicotiana tabacum) (Son et al., 2012), tomato (Solanum lycopersicum) (Pan et al., 2012), rice (Oryza sativa) (Quan et al., 2010; Zhang et al., 2013), wheat (Triticum aestivum) (Xu et al., 2007; Rong et al., 2014), and soybean (Glycine max) (Zhang et al., 2009). ERF transcription factors are involved mainly in the response to biotic stresses like pathogenesis by binding to the core GCCGCC sequence present in the promoter of ethylene-inducible pathogenesis-related (PR) genes (Ohme-Takagi and Shinshi, 1995). It has also been demonstrated that ERFs can recognize dehydrogenation-responsive elements (DREs/CRT) during abiotic stresses (Xu et al., 2007). There are some cases in which overexpression of ERF genes in transgenic tobacco or Arabidopsis plants induced expression of several PR genes, resulting in enhanced resistance to bacterial, fungal, or viral pathogens (Yi et al., 2004; Zuo et al., 2007). A recent study showed that the heterologous expression of Arabidopsis thaliana AtERF5 in tobacco aided the defence against the fungal pathogen Alternaria brassicicola and the bacterial pathogen Pseudomonas syringae pv. tomato DC3000 (Son et al., 2012). In addition, the overexpression of NtERF5 in tobacco plants enhanced resistance to tobacco mosaic virus (Fischer and Droge-Laser, 2004). Some ERF proteins also respond to plant hormones and abiotic stresses in plants (McGrath et al., 2005; Zhang et al., 2010a; Zhai et al., 2013a). For instance, GmERF3 in soybean was induced by salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA). Moreover, overexpression of the soybean GmERF3 in transgenic tobacco showed improved tolerance to both salinity and drought (Zhang et al., 2009). Thus, ERF proteins play dual roles in response to biotic and abiotic stresses in plants.

Many ERF transcription factors require interaction with other proteins to regulate the expression of their target genes (Xu et al., 1998; Cheong et al., 2003; Koyama et al., 2003). It has been reported that the resistance gene product Pto, a Ser/Thr protein kinase in tomato plants, phosphorylated and thereby activated Pt4, an ERF transcription factor that bound to the GCC-box element of PR genes (Gu et al., 2000). Moreover, AtERF7 was phosphorylated by the Ser/Thr protein kinase PKS3 in vitro, suggesting that AtERF7 might be a substrate of PKS3 (Song et al., 2005). Xu et al. (2007) found that TaERF1 was to be responsible for the interaction with TaMAPK1, and the interaction strengthened the activation activity of TaERF1. Furthermore, it was reported that ORC1 and a basic helix–loop–helix (bHLH) factor in tobacco cooperatively mediated JA-elicited nicotine biosynthesis (De Boer et al., 2011), and that ERF factor W17 in wheat interacted with HSP90 and PPR protein in vivo (Qiu et al., 2011). It seems that the role of ERF transcription factors in the regulation of plant stress responses is complex. However, the biological function of these factors and the ERF-mediated signal transduction pathway is not, as yet, very clear.

In a previous study, a cDNA library enriched for mRNAs encoding expressed sequence tags (ESTs) that increased in abundance during infection with Phytophthora sojae was constructed by suppression subtractive hybridization coupled with cDNA microarrays from leaf tissues of high-resistance soybean ‘Suinong 10’, and an EST homologous to an AP2/ERF transcription factor NtERF5 (GenBank accession no. AY655738) showed differential abundance in response to P. sojae infection (Xu et al., 2012). In the present work, we isolated this AP2/ERF transcription factor, a new member of the soybean ERF family containing an EAR motif, designated GmERF5 (GenBank accession no. HQ896930, NCBI protein no. AEX25891.1), from soybean ‘Suinong 10’. The full-length GmERF5 protein could bind to the GCC-box element and its expression patterns were also induced by P. sojae and phytohormones. GmERF5 could not activate transcription in yeast cells and could interact with a bHLH transcription factor (GmbHLH) and eukaryotic translation initiation factor (GmEIF) both in yeast cells and in planta. The promoter activity and functions of GmERF5 in transgenic soybean plants were also investigated.

Materials and methods

Plant materials and stress treatments

‘Suinong 10’, a popular soybean cultivar with gene-for-gene resistance against the predominant race 1 of P. sojae in Heilongjiang, China (Zhang et al., 2010b), was used in this study. The seeds were grown in a growth chamber maintained at 22 °C and 70% relative humidity with a 16 h light/8 h dark cycle. Fourteen days after planting, seedlings at the first-node stage (Fehr et al., 1971) were used for various treatments.

For chemical treatments, soybean leaves were each sprayed with 200 μM ABA, 100 μM methyl jasmonate (MeJA), or 2 mM SA. ET
treatment was performed in a sealed Plexiglass chamber by dissolving 2 ml of 40 % ethephon and 1 g of NaHCO₃ in 200 ml of H₂O. The unifoliolate leaves were harvested for RNA isolation at 0, 3, 6, 12, 24, 36, and 72 h after the treatments and immediately frozen in liquid nitrogen and stored at −80 °C until required for quantitative real-time PCR (qRT-PCR) analysis. ABA, MeJA, SA, ethephon, and NaHCO₃ were purchased from Sigma (USA). For P. sojae treatment, the soybean plants were infected with P. sojae zoospores following the method described by Ward et al. (1979) and Morris et al. (1991) with minor modifications. Zoospores were developed using the procedure of Ward et al. (1979), and the concentration was estimated using haemocytometer to approximately 1 × 10⁵ spores ml⁻¹. The unifoliolate leaves were also harvested at 0, 3, 6, 12, 24, 36, and 72 h after the treatment and immediately frozen in liquid nitrogen and kept at −80 °C until used for quantitative RT-PCR analysis.

Isolation of the GmERF5 gene

A suppression subtractive hybridization library coupled with cDNA microarrays was queried using a soybean EST encoding a putative AP2/ERF transcription factor, previously shown to be differentially expressed in ‘Suinong 10’ infected with P. sojae (Xu et al., 2012). Here, the full-length cDNA (termed GmERF5, GenBank accession no. HQ896930, NCBI protein no. AEX25891.1) of the EST was amplified by RT-PCR from cDNA of ‘Suinong 10’ using the primer pair GmERF5F and GmERF5R (see Supplementary Table S1) at JXB online for primer sequences). The primer pair was designed based on the flanking sequences by searching the Phytozome database (http://www.phytozome.net/). PCR was performed as follows: 94 °C for 5 min, followed 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 90 s, with a final extension at 72 °C for 10 min. The amplification product was gel purified and cloned into the pMD18-T vector (TaKaRa, Dalian, China). An analysis of protein structure was performed using SMART (http://smart.embl-heidelberg.de/). Sequence alignments were performed using DNAMAN software (http://www.lynnon.com/). A phylogenetic analysis of GmERF5 and various heterologous AP2/ERF members was performed using MEGA4 software (Tamura et al., 2007).

qRT-PCR analysis

qRT-PCR analysis was performed to determine the transcript abundance of GmERF5. Total RNA was isolated from soybean leaves using TRIzol reagent (Invitrogen, Shanghai, China) according to the manufacturer’s protocol. Total RNA (1 μg) was used for the first-strand cDNA synthesis using a Moloney murine leukemia virus reverse transcriptase kit (TaKaRa, Dalian, China). qRT-PCR analysis was performed using a real-time RT-PCR kit (Takara, Japan) on a CFX96 Touch™ Real-Time PCR Detection System (BioRad, USA). First-strand cDNA was diluted and 1 μl of diluted cDNA was used as template in a 20 μl quantitative PCR. DNA accumulation was measured using SYBR Green as the reference dye. The soybean housekeeping gene GmActin4 (GenBank accession no. AF049106) was used as the internal control. Each qRT-PCR was run in three technical replicates.

Subcellular localization

To determine the subcellular localization of GmERF5, the full-length GmERF5 was inserted into the NcoI/SpeI site of PCAMBIA1302 vector to generate GmERF5-GFP. The transient expression of green fluorescent protein (GFP)-fused proteins in Arabidopsis

Expression and purification of fusion proteins

The full-length cDNA of GmERF5 was inserted at the Ndel/I SacI site of pET29b (+) vector (Novagen, Germany), generating pET29b (+)-GmERF5. The recombinant fusion plasmid was transformed into Escherichia coli BL21 (DE3) cells. His-tagged proteins were induced with 0.5 mM isopropyl-β-D-thiogalactoside at 37 °C for 5 h. The fusion protein was purified at room temperature and quantified according to the pET System Manual (Novagen).

Electrophoretic mobility shift assay (EMSA)

The DNA-binding activity of GmERF5 was examined using a digoxigenin-dUTP-labelled double-stranded oligonucleotide GCC-box probe as described previously (Zhang et al., 2009). The probe was 5′-ATCCATAAGACGCGCCTCAATATAAGACCGATCAA-3′ (forward) and 5′-TTGATGTTCTTATTATTGTTGCGCCGCTTATTAGGT-3′ (reverse). The probe of mutant GCC-box (mGCC-box) was 5′-ATCCATAAGACTCCACTAAATAATGACCGATCAA-3′ (forward) and 5′-TTGATGTTCTTATTATTGTTGGAAGTCTTATTAGGT-3′ (reverse). The EMSA was performed as described by Kass et al. (2000).

Transactivation assays

For transactivation assay, the β-glucuronidase (GUS) gene in pCAMBIA3301 (http://www.cambia.org) was replaced by GmERF5 or GmERF5–EAR as the effector plasmids. The GCC-box and sequence from the RD29A gene promoter was multimerized four times and placed upstream of the cauliflower mosaic virus (CaMV) 35S promoter (−42 to +8), containing a TATA box. This construct was inserted into pXGUS-P (Chen et al., 2009), and fused to the GUS gene as the reporter plasmid. The transactivation assay was performed by polyethylene glycol transfection of Arabidopsis protoplasts as described by Yoo et al. (2007). Twenty micrograms of reporter plasmid and 20 μg of effector plasmid or control plasmid (pXGUS-P-35Smini) were co-transfected into 4 × 10⁴ protoplasts. The transfected cells were incubated at 22 °C in light for 18–20 h. GUS activity was determined as described elsewhere (Lu et al., 1998).

Transcriptional activation assay of GmERF5

The GmERF5 full-length cDNA was amplified by RT-PCR with the gene-specific primers GmERF5–YF and GmERF5–YR (Supplementary Table S1). PCR was carried out using KOD-Plus Neo DNA polymerase (Toyobo), with an initial denaturation step of 94 °C for 5 min, followed by 30 cycles at 94 °C for 15 s, 60 °C for 50 s, and 68 °C for 90 s, and a final extension at 68 °C for 10 min. Purified PCR products were inserted into the EcoRI/PstI site of pGBK7 vector (Clontech, USA). Fusion plasmids and pGADT7 vector were transformed into yeast strain Y2HGold (Clontech). After selection of the yeast transformants carrying the GmERF5 gene on SD(-Trp, -Leu) medium, they were transferred to SD(-Trp, -His, -Ade, X-α-Gal) medium to identify the transcriptional activation. Yeast cells carrying the pGBK7-53 and pGADT7-SV40 plasmids were used as positive controls, and yeast cells harbouring the pGBK7-Lam and pGADT7-SV40 plasmids were used as negative controls.

cDNA library construction

The ‘Suinong 10’ seedlings at the first-node stage (Fehr et al., 1979) and Morris et al. (1991) with minor modifications. The unifoliolate leaves were harvested at 12, 24, 36,

Downloaded from http://jxb.oxfordjournals.org/ at Pennsylvania State University on March 4, 2016
and 48 h after treatment. Total RNA was isolated from the equally mixed soybean leaves using TRIZol reagent (Invitrogen). mRNA was purified using an Oligotex mRNA Mini kit (Qiagen, Germany). The cDNA library was constructed by reverse transcription of mRNA using the SMART 

and linear vector (pgADT7-Rec) were co-transformed into yeast strain Y187, generating a yeast two-hybrid library. The transformants were selected on SD(-Leu) medium according to the manufacturer’s instructions. The transformation efficiency was approximately 1.2 × 10^6 colony-forming units per µg of pgADT7-Rec.

Library screening
Screening of interaction clones was carried out following the manufacturer’s protocols (Clontech). A total of 1 × 10^6 transformants from the cDNA library were screened for growth on the SD(-Leu, -Trp, -His, -Ade) medium for 3–5 d at 30 °C until the colonies reached a diameter larger than 2 mm, and they were then transferred to selective medium containing X-a-Gal (20 µg ml^-1) and aureobasidin A (125 µg ml^-1). The blue colonies were further characterized. The interaction between mammalian p53 and simian virus 40 (SV40) served as a positive control, whereas co-expression of Lam and SV40 served as a negative control.

Plasmid construction for protein–protein interactions in yeast
The GmHLH, GmA2, GmSYT1L, and GmEIF cDNAs were amplified by PCR and inserted into pgADT7. Fusion plasmids and pgGBK7-GmERF5 were transformed into yeast strain Y2HGold (Clontech). After selection of transformants on SD (-Trp, -Leu, -His, -Ade) medium, they were transferred to SD (-Trp, -His, -Ade) medium to identify the protein–protein interaction. Yeast cells carrying the pgGBK7-53 and pgADT7-SV40 plasmids were used as the positive control; yeast cells harbouring the pgGBK7-Lam and pgADT7-SV40 plasmids were used as the negative control.

Bimolecular fluorescence complementation (BiFC) assays
For transfection into Arabidopsis protoplasts, the full-length sequence of GmERF5 was PCR amplified and then subcloned into the pSATE-nEYFP-N1 vector, and the full-length coding sequences of GmHLH, GmA2, GmSYT1L, and GmEIF cDNA were also amplified by PCR and then subcloned into the pSATE-nEYFP-N1 vector. The resulting constructs were used for transient assays by polyethylene glycol transfection of Arabidopsis protoplasts as described by Yoo et al. (2007). Transfected cells were imaged using a TCS SP2 confocal spectral microscope imaging system (Leica).

Isolation and activity analysis of the GmERF5 promoter
The promoter fragment of GmERF5, designated GmERF5P, was isolated from the soybean genome by searching the Phytozone database (http://www.phytozone.net/) using the primer pair GmERF5Pf and GmERF5Pr (Supplementary Table S1). The putative motif sequences were analysed using plant cis-acting element databases at Plant CARE (http://bioinformatics.psb.ugent. be/webtools/plantcare/html/). GmERF5P was inserted into the ClaI/BamHI site of the pCAMBI121 vector (Clontech) to replace the CaMV 35S promoter, which was upstream of the GUS reporter gene. This vector was transformed into Agrobacterium tumefaciens strain LBA4404 via tri-parental mating. Transient expression of tobacco was performed using Agrobacterium tumefaciens-mediated transformation as described by Horsch et al. (1985). After transformation for 48 h, the transformed tobacco leaves were treated with 10% PEG 8000, 200 mM NaCl, 100 µM ABA, a temperature of 4 °C, ET, or P. nicotianae for 10 h. Fluorometric GUS analysis of the treated tobacco leaves was performed as described by Jefferson (1987).

Plasmid construction for protein–protein interactions in yeast
The GmHLH, GmA2, GmSYT1L, and GmEIF cDNAs were amplified by PCR and inserted into pgADT7. Fusion plasmids and pgGBK7-GmERF5 were transformed into yeast strain Y2HGold (Clontech). After selection of transformants on SD(-Leu, -Trp, -His, -Ade) medium for 3-5 d at 30 °C until the colonies reached a diameter larger than 2 mm, and they were then transferred to selective medium containing X-a-Gal (20 µg ml^-1) and aureobasidin A (125 µg ml^-1). The blue colonies were further characterized. The interaction between mammalian p53 and simian virus 40 (SV40) served as a positive control, whereas co-expression of Lam and SV40 served as a negative control.

Pathogen response assays of transgenic tobacco and soybean plants
To investigate whether the GmERF5-transformed plants resisted pathogen infection, artificial inoculation procedures were performed according to the methods described by Dou et al. (2003) with some modifications. The leaves of three T2 transgenic tobacco plants (identified by PCR) were infected with a P. nicotianae inoculum, and three T1 transgenic soybean plants, of which the T1 plants were tested through PCR and Southern blot hybridization, were treated with a P. sojae inoculum. The leaves were incubated in a mist chamber at 25 °C with 90 % relative humidity under a 14-h photoperiod at a light intensity of 350 µmol m^-2 s^-1 for investigation. The non-transformed leaves were used as controls. Disease symptoms on each leaf were observed and photographed after inoculation using a Canon IXUS 860IS camera.

Results
Sequence analysis of the GmERF5 transcription factor gene
The full-length cDNA sequence of GmERF5 was cloned from ‘Suinong 10’ total RNA by RT-PCR. The nucleotide sequence of GmERF5 was submitted to the DNA Data Bank of Japan (DDBJ) under accession number HQ896930. Sequence analysis showed that it comprised 1040 bp, containing a 636 bp open reading frame encoding a polypeptide of 211 aa with predicted molecular mass of 23.17 kDa and an isoelectric point of 5.37. The predicted structure of GmERF5 included a conserved 58-residue AP2/ERF DNA-binding domain. Since residue 14 of the domain was alanine and residue 19 was aspartic acid, the gene was deemed to be a member of the AP2/ERF transcription factor ERF subfamily (Supplementary Fig. S1 at JXB online). The GmERF5 N terminus contained a putative basic amino acid region (R27KRP) that might act as nuclear localization signal, and the C terminus possessed the core sequence of an EAR motif (FDLNLP) (Supplementary Fig. S1). Alignment and phylogenetic tree analysis revealed that GmERF5 was a B-1 gene within the ERF subfamily (Sakuma et al., 2002), and was closely related to A. thaliana B-1 genes AtERF3, -4, and -7,
as well as to soybean genes \textit{GmERF4} and \textit{-6}, tobacco gene \textit{NiERF3}, cotton gene \textit{GhERF4}, tomato gene \textit{LeERF3}, and wheat gene \textit{TaERF4} (Fig. 1A). These results suggested that \textit{GmERF5} may have a similar function to the B-1 group members in plants. Analysis of the conserved AP2/ERF domain of 58 aa showed that it shared 81.0–91.4\% amino acid identity with other members of the B-1 group (Fig. 1B). The prediction of the three-dimensional structure of \textit{GmERF5}, based on the data from Phyre (http://www.sbg.bio.ic.ac.uk/phyre/), showed that this protein had a long C-terminal α-helix (α) wrapped in a three-stranded anti-parallel β-sheet (from β1 to β3) (Fig. 1C), and that its AP2/ERF domain was divided into conserved segments (YRG and RAYD) (Fig. 1B) (Mazarel et al., 2002). The analysis of homologues of \textit{GmERF5} in the soybean genome, based on data obtained from the Phytozome database (http://www.phytozome.net/soybean), indicated that the two genes were clustered on two linkage groups, namely one each on Gm 02 and Gm 14, and had no introns.

Response of \textit{GmERF5} transcript levels to stress treatments

To evaluate \textit{GmERF5} responsiveness to biotic and abiotic qRT-PCR was used to determine the expression patterns of \textit{GmERF5} in ‘Suinong10’ plants. The examination of tissue-specific transcript abundance in ‘Suinong10’ showed that \textit{GmERF5} was constitutively and highly expressed in the roots, followed by the stems and leaves (Fig. 2A). Under \textit{P. sojae} treatment, \textit{GmERF5} mRNA rapidly increased and reached a maximum level at 36 h after the treatment, followed by a decline (Fig. 2B). \textit{GmERF5} expression was also induced with ET, ABA, SA, and MeJA treatments. A significant induction of \textit{GmERF5} was detected in the leaves at 3 h after the treatments, and the transcripts remained at their highest between 9 and 12 h (Fig. 2C).

Subcellular localization of the \textit{GmERF5} protein

To investigate the subcellular localization of \textit{GmERF5}, its localization was analysed by expressing a gene encoding a \textit{GmERF5–GFP} fusion protein under the control of the 35S promoter in \textit{Arabidopsis} protoplasts. As shown in Fig. 3, confocal microscopic observations demonstrated that GFP fluorescence was dispersed throughout the entire cells bombarded with the control plasmid 35S–GFP. By contrast, the \textit{GmERF5–GFP} fusion protein was localized exclusively to the nucleus of the \textit{Arabidopsis} cells. The results clearly indicated that \textit{GmERF5} is a nuclear-localized protein.

\textit{GmERF5} specifically binds to the GCC-box element in vitro

To test whether \textit{GmERF5} binds to the \textit{cis}-acting element of the GCC-box in \textit{vivo}, His-tagged \textit{GmERF5} was purified and EMSA with a digoxigenin–ddUTP-labelled double-stranded oligonucleotide GCC-box probe was performed. The sequences of the GCC-box and mGCC-box are shown in Fig. 4A. The results showed that \textit{GmERF5} specifically recognized and bound to GCC-box (Fig. 4B, lane 2) but not mutated GCC-box (mGCC-box) (Fig. 4B, lane 4). Competition experiments were conducted to examine the specificity of the mobility shift. When the ratio of unlabelled to labelled GCC-box probe was 100:1, almost no labelled probe was bound (Fig. 4B, lane 3); when the 100-fold unlabelled mGCC probe was used as the competitor, no binding competition was observed (Fig. 4B, lane 5).

To investigate whether \textit{GmERF5} is a transcriptional repressor, we performed a transactivation assay in \textit{Arabidopsis} protoplasts using a reporter gene that had four tandem copies
of the GCC-box and effector plasmids with GmERF5 or GmERF5–EAR (Fig. 4C). As shown in Fig. 4D, GmERF5 appeared to repress reporter gene expression since in its presence GUS expression was reduced to 56% of the control level, whereas GmERF5–EAR led to a 1.66-fold higher transactivation activity as compared with the control. These data indicated that GmERF5 acts as a repressor of GCC-box-mediated transcription and that the EAR motif is responsible for the transcriptional repression of GmERF5.

Yeast two-hybrid screening of GmERF5 interacting protein

To determine whether or not GmERF5 showed transcription activation activity in yeast cells, a yeast two-hybrid analysis was performed. The results indicated that GmERF5 could not activate transcription in yeast, which meant that it could be used to screen the library (Supplementary Fig. S2 at JXB online). Screening of interaction clones was carried out following the manufacturer’s protocols (Clontech). A total of
ERF5 improves Phytophthora sojae resistance in soybean

1 × 10^7 transformants from the cDNA library were screened for growth on the SD(-Leu, -Trp, -His, -Ade) medium for 3–5 d at 30 °C, and 211 colonies with a diameter larger than 2 mm were transferred to selective medium containing X-α-Gal (20 µg ml–1) and aureobasidin A (125 µg ml–1). Among them, 66 blue colonies were characterized and sequence analysed by homology analysis using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sixteen ESTs of candidate genes that might interact with GmERF5 are listed in Table 1. Homology analysis showed that these candidate proteins were related to energy metabolism, stress and defence, transcription and translation, signal transduction, and growth and development. Function prediction of the candidate proteins suggested that GmERF5 was possibly involved in several stress signal transduction pathways and may play an important role in regulation of resistance to stresses.

GmERF5 interacts with candidate proteins in yeast and in planta

To determine which protein is responsible for the interaction with GmERF5, five correct fusion genes comprising SYT1L, bHLH, AL2, EIF, and MAPK2L from the 16 candidate genes were chosen to be verified. Full-length cDNAs of these genes were cloned (named GmSYT1L, GmbHLH, GmAL2, GmEIF, and GmMAPK2L) and constructed in pGADT7. Analysis of whether or not these proteins had transcription activation and interaction with GmERF5 in yeast cells was performed. Our results showed that the five proteins could not activate transcription in yeast cells, and four of the proteins, GmSYT1L, GmbHLH, GmAL2, and GmEIF, could interact with GmERF5 in the yeast colony growth assay (Fig. 5A).

To further confirm the occurrence of these interactions in planta, a BiFC assay was performed using an Arabidopsis protoplast transient expression system. Co-expression of both N-terminal yellow fluorescent protein (YFP^N)-tagged GmERF5 and C-terminal YFP (YFP^C)-tagged GmbHLH, or YFP^N-tagged GmERF5 and YFP^C-tagged GmEIF resulted in significant fluorescence in the chloroplasts of Arabidopsis protoplast cells (Fig. 5B). However, no fluorescence was detected in Arabidopsis protoplast cells co-transformed with YFP^N-GmERF5 and YFP^C-GmSYT1L or YFP^N-GmERF5 and YFP^C-GmAL2 (data not shown). These results suggested that the GmERF5 protein interacts with the GmbHLH and GmEIF proteins in planta.

Isolation and activity analysis of the GmERF5 promoter

The promoter sequence of the GmERF5 gene was isolated from ‘Suinong 10’ genomic DNA by RT-PCR. The cloned GmERF5 promoter sequence was determined to be 1731 bp (Supplementary Fig. S3 at JXB online). By searching the putative cis-acting elements in the GmERF5 promoter region using Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), we found a number of putative stress-related cis-elements in GmERF5P. These motifs included 10 pathogen- and salt-induced GT-1 elements, one ET-response element, two wounding-responsive W-box elements, one GA-response element, one ABA-responsive transcriptional factor DBP5-binding site, eight MYB-binding sites and five MYC-binding sites (Supplementary Table S2 at JXB online).
To elucidate the regulation mechanism of GmERF5 under drought, salt, cold, ET, ABA, and P. nicotianae stresses, the promoter activities in the tobacco leaves were measured 10 h after the treatments (Fig. 6). The GUS activity driven by GmERF5P was weak under normal conditions but was induced by all the treatments described above. The level of GUS activity was the highest with ET, ABA, and P. nicotianae treatments, followed by salt and drought treatments, but the level was relatively low under cold stress.

Enhanced resistance to P. nicotianae and P. sojae in transgenic plants

To investigate whether overexpression of GmERF5 enhanced resistance in transgenic plants, three T2 transgenic tobacco plants (numbered T1–T3, as described above) were investigated after inoculation with P. nicotianae, and three T2 transgenic soybean plants (numbered G1–G3), of which the T1 transgenic soybean plant was confirmed through PCR and

Table 1. Part of library screening results by yeast two-hybrid

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank ID</th>
<th>Number of clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synaptotagmin-1-like (SYT1L)</td>
<td>LOC100778906</td>
<td>10</td>
</tr>
<tr>
<td>Mitogen-activated protein kinase 2-like (MAPK2L)</td>
<td>LOC100807109</td>
<td>1</td>
</tr>
<tr>
<td>Zinc ribbon protein (AL2)</td>
<td>LOC100777123</td>
<td>1</td>
</tr>
<tr>
<td>Basic helix–loop–helix transcription factor (bHLH)</td>
<td>LOC100806368</td>
<td>1</td>
</tr>
<tr>
<td>Eukaryotic translation initiation factor (eIF)</td>
<td>LOC100791863</td>
<td>1</td>
</tr>
<tr>
<td>Ras-related protein RAEB1c-like (RAEB1c)</td>
<td>LOC100779203</td>
<td>1</td>
</tr>
<tr>
<td>Pathogen-related protein-like (PRP)</td>
<td>LOC100805630</td>
<td>1</td>
</tr>
<tr>
<td>DNA mismatch repair protein mutS (MutS)</td>
<td>LOC100791619</td>
<td>1</td>
</tr>
<tr>
<td>Chlorophyll a-b binding protein P4</td>
<td>LOC100802922</td>
<td>2</td>
</tr>
<tr>
<td>Granule-bound starch synthase</td>
<td>LOC100037476</td>
<td>2</td>
</tr>
<tr>
<td>Cucumin-like</td>
<td>LOC100793805</td>
<td>3</td>
</tr>
<tr>
<td>Ankyrin repeat-containing protein (ANKP)</td>
<td>LOC100799055</td>
<td>1</td>
</tr>
<tr>
<td>Ribonucleoprotein chloroplastic-like</td>
<td>LOC100801829</td>
<td>2</td>
</tr>
<tr>
<td>DNA-directed RNA polymerases (RPAC2)</td>
<td>LOC1005000695</td>
<td>1</td>
</tr>
<tr>
<td>Early light-induced protein (ELIF)</td>
<td>GMU82810</td>
<td>1</td>
</tr>
<tr>
<td>Peroxiredoxin Q (PrxQ)</td>
<td>AK285279.1</td>
<td>1</td>
</tr>
</tbody>
</table>

Fig. 5. Interaction of GmERF5 with candidate proteins in yeast cells and in planta. (A) GmERF5 interacts with candidate proteins in yeast cells. Yeast Y2HGold cells harboured the Gal4 DNA-binding domain (BD) fusion construction and the Gal4 activation domain (AD) fusion constructs. The yeast cells were selected on SD medium lacking Leu and Trp (DDO) and interaction was assessed according to their ability to grow on selective SD media lacking Leu, Trp, His, and Ade (QDO) for 5 d. The combination of pGBK7-T53/pGADT7-SV40 was used as a positive control and pGBK7-Lam/pGADT7-SV40 as a negative control. (B) BiFC analyses of GmERF5 interactions with GmbHLH and GmEIF. GmERF5-YFPN+GmbHLH–YFPC and GmERF5–YFPN+GmbHLH–YFPC were co-transfected into Arabidopsis protoplasts. The bright-field, YFP fluorescence (yellow), chlorophyll autofluorescence (red), and combined images were visualized with a confocal microscope 16 h after transfection. Bars, 10 μm. (This figure is available in colour at JXB online.)
Southern hybridization, were developed to screen for resistance to \textit{P. sojae}. After 5 d of incubation with \textit{P. nicotianae}, severe symptoms (necrosis and chlorosis) around the infection areas were observed in non-transgenic tobacco plants, but the transgenic \textit{GmERF5} tobacco plants showed much less severe lesions (Fig. 7). After 6 d incubation with \textit{P. sojae}, the leaves of the non-transgenic soybean plants exhibited clear and large lesions compared with those of the transgenic plants (Fig. 8). These results indicated that overexpression of \textit{GmERF5} gene in tobacco and soybean plants improves resistance to \textit{P. nicotianae} and \textit{P. sojae}, respectively.

\textbf{GmERF5 transgenic soybean exhibits increased PR gene expression}

To test whether the GmERF5 protein could regulate PR gene expression, the expression of \textit{GmERF5} and three PR genes was analysed in 35S:GmERF5 transgenic soybean plants and wild-type plants using qRT-PCR. As shown in Fig. 9, there were three plants (G1, G2, and G3) whose expression was seven times more than that of the control, and the highest number (G2) was 26 times. Transcripts of \textit{GmPR10} were increased significantly in all the transgenic lines. In addition, expression of the \textit{GmPR1-1} and \textit{GmPR10-1} genes, which contained a GCC-box in their promoters, was greatly increased in \textit{GmERF5} transgenic soybean but barely detected in wild-type plants grown under regular conditions. These data indicated that \textit{GmPR10}, \textit{GmPR1-1}, and \textit{GmPR10-1} were upregulated in \textit{GmERF5} transgenic soybean.

\section*{Discussion}

In this study, we identified \textit{GmERF5}, a new member of the soybean ERF transcription factor family containing an EAR motif, that enhanced soybean resistance to \textit{P. sojae} when overexpressed. To date, 98 unigenes of the ERF family containing a complete AP2/ERF domain have been identified in the soybean genome (Zhang et al., 2008). However, only five members of the ERF family have been functionally characterized so far in this species (Mazarell et al., 2002; Zhang et al., 2009, 2010; Zhai et al., 2013a, b). \textit{GmERF5} is the first soybean EAR motif-containing ERF transcription factor shown to be expressed in response to pathogen infection. Many ERF genes have been shown to act as activators or repressors of transcription. \textit{GmERF3/7} (Zhang et al., 2009; Zhai et al., 2013a), \textit{AtERF12/15} (Fujimoto et al., 2000), and \textit{NtERF2/4} (Ohta et al., 2000) have been reported to function as activators of transcription, whereas \textit{GmERF4/6} (Zhang et al., 2010a; Zhai et al., 2013b), \textit{AtERF3/4} (Fujimoto et al., 2000; Yang et al., 2005), \textit{AtERF7-12} (Song et al., 2005), and \textit{NtERF3/5} (Fischer and Droge-Laser, 2004; Ohta et al., 2001) containing a conserved C-terminal EAR repressor domain were shown to be active repressors. The sequence of the EAR motif was first reported as (L/F)DLN(L/F)xP in tobacco \textit{NtERF3} and \textit{Arabidopsis AtERF3} and \textit{AtERF4} (Ohta et al., 2001). Recent research showed that overexpression of \textit{AtERF6} in \textit{Arabidopsis} enhanced resistance to the fungal pathogen \textit{Botrytis cinerea} (Molliat et al., 2012). By contrast, expression of ERF6–EAR, in which \textit{AtERF6} was fused to the EAR motif, strongly suppressed \textit{B. cinerea}-induced defence gene expression, leading to hypersusceptibility of the \textit{AtERF6–EAR} transgenic \textit{Arabidopsis} plants to \textit{B. cinerea} (Meng et al., 2013). In addition, McGrath et al. (2005) reported that an EAR motif containing transcriptional repressor \textit{AtERF4} functioned as a negative regulator in the expression of JA-responsive gene \textit{PDF1.2}. In contrast, a truncated-type of \textit{AtERF4} without the EAR motif acted as a positive regulator of \textit{PDF1.2}. These results showed that the EAR motif may play crucial roles in ERF transcriptional repressor.
GmERF5 is very similar to GmERF4 and AtERF6, both of which have been shown to be transcription repressors (Zhang et al., 2010a; Zhai et al., 2013b). Sequence analysis revealed that GmERF5 possessed an EAR motif (FDLNLPP) and a basic amino acid domain (R27KRP), which may function as potential nuclear localization signals (Dingwall and Laskey, 1991). Our results showed that recombinant GmERF5 protein was localized to the nucleus and bound specifically to the GCC-box in vitro; however, it was unknown if GmERF5 had other binding specificities. We showed that GmERF5 can indeed repress the basal transcription level of a reporter gene in Arabidopsis cells. This finding suggested that GmERF5 may act as a GCC-mediated transcriptional repressor. There have been several reports about the functions of the EAR domain-containing ERF protein in plant responsiveness to abiotic stresses (Fujimoto et al., 2000; Ohta et al., 2001; Song et al., 2005; Yang et al., 2005; Zhang et al., 2013). As examples, rice OsERF4a is a positive regulator of shoot growth and water-stress tolerance during early growth stages (Joo et al., 2013), wheat TaERF4 enhances the level of sensitivity to salinity stress in Arabidopsis, possibly via the repression of tonoplast Na+/H+ antiporter activity (Dong et al., 2012), and soybean GmERF4 and GmERF6 increases the tolerance to salt and drought stress in tobacco plants (Zhang et al., 2010a; Zhai et al., 2013b). In the present study, we demonstrated that overexpression of the GmERF5 gene in tobacco and soybean plants improved resistance to P. nicotianae and P. sojae, respectively. It has been reported that some ERF proteins containing the EAR motif usually suppress expression of PR genes contained a GCC-box in their promoters (Zhai et al., 2013b). It was shown that GmERF4 repressed the expression of NtPR1, NtPR2, and NtPR4 in transgenic tobacco plants (Zhang et al., 2010a). Consistent with this, we found that

![Fig. 8. Response of GmERF5 transgenic soybean plants to P. sojae.](https://example.com)

Detached leaves of three GmERF5 transgenic soybean lines (G1–G3) and non-transgenic soybean lines (CK) were infected with P. sojae. (This figure is available in colour at JXB online.)

![Fig. 9. Expression analysis of GmERF5 and three PR genes in wild-type and 35S::GmERF5 transgenic soybean plants.](https://example.com)

The relative transcript abundance of GmERF5, GmPR10 (FJ960440), GmPR1-1 (XM003545722), and GmPR10-1 (NM001251335) in three transgenic lines (G1–G3) was compared with that in wild-type soybean plant. The amplification of the soybean Actin (GmAclntn4) gene was used as an internal control to normalize all the data. Statistically significant differences were performed between the overexpression transgenic lines and wild type. Three technical replicates were averaged and statistically analysed using Student’s t-test (**P<0.01). Bars indicate standard error of the mean.
overexpression of GmERF5 in tobacco inhibited the expression of NtPR1 and NtPR4 (Supplementary Fig. S4 at JXB online). In contrast, we found that GmPR1-1 and GmPR10-1 genes, which contain a GCC-box in their promoters, were upregulated in GmERF5 transgenic soybean plants. Here, we provide the first report that ERF proteins containing the EAR motif improve the transcript level of PR genes in soybean. These results suggest that the two proteins that interacted with GmERF5 may commonly regulate the expression of PR genes in soybean. There are two reasons that may explain this result. One is that the regulation mechanism of GmERF5 may be different between soybean and tobacco, and the other is that GmERF5 probably did not bind directly to the PR genes in soybean. It has been demonstrated that GmPR10 overexpressing transgenic tobacco and soybean plants could improve resistance to P. nicotianae and P. sojae (Xu et al., 2014). Moreover, we also found that the transcript level of GmPR10 was significantly increased in GmERF5 transgenic soybean plants. Taken together, we suggest that GmERF5 may positively regulate the expression of PR genes and enhance resistance to P. sojae in soybean. McGrath et al. (2005) reported that transcriptional repressor AtERF4-overexpressing transgenic plants showed decreased resistance to a necrotrophic fungal pathogen, Fusarium oxysporum, compared with the wild-type plants. By contrast, the overexpression of NtERF3 in transgenic tobacco plants enhanced resistance to tobacco mosaic virus by hypersensitive response-like cell death (Ogata et al., 2012). Thus, ERFs containing the EAR motif may play a crucial role in the plant defence response to disease. However, the molecular mechanism of ERFs containing the EAR motif remains unknown and expression of ERF-associated EAR motif-mediated resistance is less characterized at the protein level.

There have been reports that some ERFs are able to regulate gene expression directly or indirectly by interacting physically with other proteins or by activating transcription factors (Gu et al., 2000; Cheong et al., 2003; Xu et al., 2007; Xu et al., 2011). For instance, the interaction between NtERF3 and NtUBC2 is likely to increase the stability of NtERF3 and suppress the transactivation activity of ERF activators (Koyama et al., 2003). Here, the proteins that might interact with GmERF5 were screened in a yeast two-hybrid system, and four proteins, GmbHLH, GmAL2, GmEIF, and GmSYT1L were tested to verify the interaction with GmERF5 in yeast cells. However, no interaction between the GmERF5 protein and the GmAL2 and GmSYT1L proteins in planta were observed. A recent study suggested that the AP2/ERF factor NtORC1 interacts with NtbHLH and commonly regulates the expression of genes containing the G-box and the GCC motif in its promoter region (De Boer et al., 2011). Similarly, we speculated that GmERF5 and GmbHLH proteins might cooperatively regulate the resistant to P. sojae infection. EIF might be involved in the regulation of GmERF5 transcription. These results suggest that the two proteins that interacted with GmERF5 may commonly regulate the expression of its target genes.

In plants, the phytohormones SA, JA, and ET are known to play crucial roles in regulating the induced defence response (Pieterse et al., 2009, 2012; Robert-Seilaniantz et al., 2011; Sugano et al., 2013). Cross-talk between induced ET, SA, and JA defence-signalling pathways is thought to contribute to induction of a powerful defence response in plants (Koornneef and Pieterse, 2008). For example, ERF1 was reported to integrate defence signals from ET and JA pathways and induce downstream defence-related genes including plant defensin1.2 (PDF1.2) (Lorenzo et al., 2003). GmERF3 might act as a connector linking the ET, JA, and SA signalling pathways that mediate biotic and abiotic stresses (Zhang et al., 2009). In this study, our results indicated that mRNA transcripts of GmERF5 were remarkably increased by ET, SA, and P. sojae stresses but were relatively low under MeJA stress. Therefore, we speculate that GmERF5 might play an important role in soybean plant resistance to P. sojae depending mainly on ET and SA signalling. In addition, the expression of GmERF5 was also strongly induced by ABA. It has been reported that ABA regulates interacting signalling pathways involved in plant responses to several abiotic stresses including salt and drought (Shinozaki et al., 2003). To further explore the potential function of GmERF5, the activity of GmERF5P under various stresses was also examined in this study. The presence of putative cis-elements indicated that there were several pathogen- and salt-induced binding sites (GT-1, MYB, MYC, ERELEE4, and W-box) in GmERF5P (Supplementary Table S2). Our results suggested that the activities of GmERF5P were high with salt and drought treatments as well as with ET, P. nicotianae, and ABA. Thus, GmERF5 might also involve in ABA-mediated pathways of salt and drought tolerance.

Our results suggest a model in which the GmERF5 gene is activated by P. sojae infection, and exogenous ABA, SA, ET, and MeJA also induce moderate GmERF5 mRNA accumulation. We demonstrated that the GmERF5 protein could bind to the GCC-box present in vitro. We also demonstrated that GmEIF and GmbHLH could interact with GmERF5 both in yeast cells and in planta. Therefore, we speculate that GmERF5 and GmbHLH cooperatively regulate the response to P. sojae resistance. Mitogen-activated protein kinase (MAPK) cascades are important signalling modules in defence responses (Ichimura et al., 2002; Pedley and Martin, 2005; Pitzschke et al., 2009; Andreasen and Ellis, 2010; Rodriguez et al., 2010; Tena et al., 2011). Recent research suggests that TaMAPK1 phosphorylates TaERF1, which might enhance the activity of the TaERF1 protein (Xu et al., 2007). Phosphorylation of AtERF6 by AtMPK3/AtMPK6 in either the gain-of-function transgenic plants or in response to B. cinerea infection increased AtERF6 protein stability in vivo (Meng et al., 2013). Similarly, future studies are needed to identify the MAPK-dependent phosphorylation cascade, which might directly impinge on the conformation and properties of the GmERF5 protein. In addition, we need to determine how the complex may be regulated by SA, ET, and MeJA signalling.

**Supplementary data**

Supplementary data are available at JXB online.

**Supplementary Table S1.** Oligonucleotide primers used in this study.
Supplementary Table S2. Location and putative function of cis-elements in the GmERF5 promoter.

Supplementary Fig. S1. The open reading frame sequence and deduced polypeptide sequence of GmERF5.

Supplementary Fig. S2. Transcription activation assay of GmERF5 was performed in the yeast strain Y2HGold, which contains the HIS3 and ADE2 reporter genes under distinct GAL4-responsive promoter elements.

Supplementary Fig. S3. The promoter region of the GmERF5 gene predicted cis-acting elements are indicated by shadow or boldface type, and the names are shown above the elements.

Supplementary Fig. S4. GmERF5 overexpression tobacco plants reduces the expression of NtPR1 and NtPR4 containing the GCC-box in their promoter regions.

Acknowledgements

The research was supported through funding from the Heilongjiang Province outstanding youth fund (JC201308), NSFC Projects (31071839, 31171577, 31101167), the Specialized Research Fund for the Doctoral Program of Higher Education (20112325120005), the Science and Technology Innovation Project in Harbin (2012RFQXN011, 2012RFXXN019), and the Research Fund for Young Teachers through NEAU (2012 RCB 08).

References


Li ZF, Zhang LX, Yu YW, Guan RD, Zhang ZJ, Zhang HW, Huang RF. 2011. The ethylene response factor AtERF11 that is transcriptionally modulated by the bZIP transcription factor HY5 is a crucial repressor for ethylene biosynthesis in Arabidopsis. The Plant Journal 68, 88–96.


