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Expression profiles and transactivation analysis of a novel ethylene-responsive transcription factor gene *GhERF5* from cotton

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Abstract

Plant AP2/EREBP transcription factors play important roles in plant development and in plant responses to biotic and abiotic stresses. A novel gene for ethylene-responsive element binding protein (EREBP), designated *GhERF5,* which encodes a protein of 255 amino acids, was isolated by RACE-PCR from cotton (*Gossypium hirsutum*) seedlings. Sequence alignment revealed that GhERF5 contains a typical AP2/ERF domain, and belongs to the B3 subgroup of the ERF subfamily. Particle bombardment assay showed that GhERF5 functions as an *in vivo* transcription activator in tobacco cells, and it is located in the nuclei of onion epidermis cells. Semi-quantitative RT-PCR revealed that the expression of *GhERF5* was highly and rapidly induced when plants were treated with exogenous ethylene, abscisic acid (ABA), salt, cold and drought. Promoter analysis indicated that there are conserved *cis*-acting elements induced by these stresses in the 5′-upstream region of the *GhERF5* gene. These results suggest that the *GhERF5* gene might play an important role in cotton response to ethylene, ABA and environmental stresses.

Keywords: Ethylene-responsive element binding factor (ERF); Abiotic stresses; Nuclear localization; Transactivation; *Gossypium hirsutum*

1. Introduction

The plant hormone ethylene influences many aspects of plant growth and development, including seed germination, cell elongation, cell fate, fruit ripening, leaf abscission and senescence. Ethylene also regulates plant responses to biotic stresses induced by pathogenic infection, and to abiotic stresses induced by flooding or drought [1–3]. Ethylene-responsive element binding factors (ERFs) contain a highly conserved DNA-binding domain consisting of 58–59 amino acids, and have been shown to specifically bind to a GCC box, a DNA sequence involved in the ethylene-responsive transcription of genes [4–6]. In addition, ERF proteins have also been found to bind to non-GCC box *cis*-elements [7,8]. So far, only a few members of this family have been characterized, such as Pti4, Pti5, Pti6 [9] and JERF1, JERF3 from tomato [6,10], NtERF1, NtERF2 and NtERF3 from tobacco [11], and AtERF1 to AtERF5 from *Arabidopsis* [12]. Among these members, some have been shown to participate not only in plant hormone signal transduction, but also in plant responses to biotic pathogens and environmental stresses [13]. Although many ERF genes have been isolated, the regulatory functions of most of them in plants, especially these ERF proteins functioning in multi-stress responses, are not well explored.

Cotton (*Gossypium hirsutum* L.) is an economically important plant. However, the growth and productivity of cotton is adversely affected by abiotic and biotic stresses. Therefore, efforts to investigate the molecular mechanisms of stress adaptation and to strengthen stress tolerance in

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this plant are of fundamental importance in cotton production. As AP2/ERF transcription factors are relatively useful for generating transgenic plants tolerant to high salt, low temperature and drought stresses as well as pathogen attack [7,14–17], we focused our attention on the isolation and functional characterization of such transcription factors in cotton. In our previous papers, we reported the characterization and the functional analyses of two cotton dehydration responsive element binding proteins, GhDBP1 and GhDBP3 [18,19]. Our results have demonstrated that GhDBP1 is a transcriptional repressor [18], while GhDBP3 is an ABA-induced transcriptional activator [19]. Here, we report the isolation and the functional analysis of a new cotton ERF subfamily gene, *GhERF5*.

2. Materials and methods

2.1. Plant materials and stress treatments

Cotton (*Gossypium hirsutum* cv. Zhongmian 12) plants were grown in potting soil in a culture room at 25 °C with a 16 h light and 8 h dark cycle. Abiotic stress treatments were performed in 2-week old seedlings for 24 h or for the specific time when indicated. Low-temperature treatments were performed by transferring plants to a growth chamber set to 4 °C for different periods of time under the light and photoperiodic conditions described above. Dehydration was induced by removing the plants from the pots and placing them on a dry filter paper. The relative water content of dry-treated seedlings was reduced to 50%. Salinity and ABA treatments were performed by submerging the roots of the plants in an aqueous solution of 400 mM NaCl or 100 μM ABA. Ethephon, which emits ethylene when dissolved in water, was used as a substitute for ethylene. During the treatments, the Petri dishes were sealed with a parafilm, and then the whole plants were harvested 1, 3, 6, 12 and 24 h after treatments. All tissues harvested for nucleic acid extraction were weighed, immediately frozen in liquid nitrogen, and stored frozen at −70 °C until use.

2.2. Isolation of the GhERF5 cDNA by RACE-PCR

Total RNA was isolated from seedlings and various organs using the RNeasy plant mini kit (Qiagen, CA, USA) according to the manufacturer's instructions. Rapid amplification of cDNA ends (RACE) was performed using a RACE kit (TaKaRa, Dalian, China) to isolate the cotton ERF cDNAs. The gene-specific primer P1 (5′-ACG GAGACAGCGGTGAAAGA-3′) and the oligonucleotide included in the 3-RACE kit (3 sites Adaptor Primer) were used as the antisense primer to isolate 3′ ends. The 5-RACE was performed using a 5-Full RACE Core Set following the manufacturer's instructions. Briefly, 5-phosphorylated RT-primer (5′-(P)AACCGGCTTGAGCTA-3′) was used to synthesize the single-stranded cDNA, which was then ligated to be circularized. The ligation products were amplified by inverse PCR using the primers S1 (5′-GATCA CGTCAAAAAGGGCAAC-3′) and A1 (5′-GAACCTCTT TCACCGCTGTCTC-3′). The PCR-amplified products were cloned into pMD18-T vector (TaKaRa, Dalian, China) and sequenced. DNA sequence data were assembled and analyzed using the DNAman analysis program (Lynnon Biosoft, USA). Database searches were performed at the National Center for Biotechnology Information (Bethesda, MD) BLAST search program. Alignment of the cotton ERF protein with other structurally related AP2/ERF proteins was performed using the Clustal X program [20], and further adjusted by the GenDoc program [21].

2.3. Isolation of the GhERF5 promoter by genome walking

Total genomic DNA was extracted from leaf tissue as described by Paterson [22], and was digested with the proper restriction endonucleases. Cloning of the promoter region was performed with an improved PCR-based genomic walking method that was described previously [23]. Major PCR band(s) were isolated from the gel using the Qiagen Gel extraction III kit (Qiagen, Germany), and the isolated fragments were then cloned into a pMD18-T vector (TaKaRa, Dalian, China). Recombinant plasmid DNA that was used for sequencing was prepared using the QIAprep spin mini prep kit (Qiagen, Germany), and the inserts were sequenced using a Big-Dye™ terminator cycle sequencing ready reaction kit (Perkin Elmer, USA) on an ABI Prism™ 377 DNA Sequencer. The promoter sequences were analyzed with the PLACE database (http://www.dna.affrc.go.jp/htdocs/PLACE/) [24].

2.4. Semi-quantitative RT-PCR analysis

Total RNA was isolated from different parts of the mature plants or seedlings using the RNeasy plant mini kit (Qiagen, CA, USA) according to the manufacturer's instructions. The RNA concentration was estimated based on the absorbance at 260 and 280 nm. Semi-quantitative RT-PCR analysis was carried out as described previously [19]. The gene-specific primers were RS5 (5′-GATCAC GTCAAAAAGGGCAAC-3′) and RA5 (5′-GGGATGGA GGTTGTCCATAAC-3′) for *GhERF5*; SSU1 (5′-AACTT AAAGGAATTGACGGAAG-3′) and SSU2 (5′-GCATCA CAGACCTGTTATTGCC-3′) for cotton small-subunit (SSU) rRNA. The cotton SSU rRNA was used as the control to show the normalization of the amount of templates in PCR. In order to ensure the gene specificity of these primers, we sequenced the PCR fragments obtained with these primers and found that they were indeed derived from the *GhERF5* transcript or cotton SSU rRNA. All RT-PCR expression assays were performed and analyzed at least three times in the independent experiments.

2.5. Subcellular localization of GhERF5

The coding region of GhERF5 was amplified by PCR, and the amplified fragment was inserted into the NcoI and BamHI sites of the pCK-GFP vector to generate an in-frame fusion construct with a green fluorescent protein (*GhERF5*-GFP). The fusion construct and control GFP vector were introduced separately into the onion epidermal cells by particle bombardment using a Bio-Rad Biolistic PDS 1000/He system. Plasmid DNA (1 μg) was used to coat 0.5 mg of 1 μm gold particles as described by Huang [19]. The onion epidermal cells had previously been incubated on MS agar plates in the light at 22 °C for 24 h. The initial pressure of bombardment was 1100 psi, and the traveling distance of the particles to the plant tissues was 6 cm. Bombarded tissues were placed on the same agar plates and incubated at 22 °C for 24 h in darkness, followed by monitoring of the localization of GFP with a confocal microscope (Olympus, FluoView™ FV300).

2.6. Transactivation assay in yeast

Transactivation by GhERF5 was examined in a strain of *Saccharomyces cerevisiae*, AH109, in which *lacZ* and *His3* reporter genes were driven by *GAL1* and *MEL1* promoters, respectively, and two transformation markers *trp1-901* and *leu2-3* were present [25]. Effector plasmids were constructed by ligating the entire coding region of GhERF5 into the BamHI/PstI sites of the pGBKT7 vector (Clontech, USA) to produce the fusion protein with GAL4 DNA-binding domain (BD). The resulting construct (pGBKT7-GhERF5) and the empty control vector (pGBKT7, as a negative control) were transformed into AH109 yeast cells according to the manufacturer's instructions (Clontech, USA). The vector pCL1 encoding the full length, wild-type GAL4 protein was used as a positive control. The transformants were selected by growth on Trp-synthetic dropout medium at 30 °C for 3 days. The β-galactosidase activity was assayed on a synthetic dextrose (SD) plate without His and with 5 mM 3-AT (3-aminotriazole), which suppresses leaky histidine expression, according to the method of Duttweiller [26].

2.7. Transactivation activity of the 4×GCC-LUC reporter genes in tobacco cells

Transactivation activity of the 4×GCC was performed on the tobacco cells using the particle bombardment

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1     ATTTTCAGTTTTACCTGAAATTTCAGCTTACGAACTTGTTTTACCTGAAAATTTGAATTTTTAGCATCGGAAAAG
76    CTTTCAATTTTCGTTATGGAAATGTACCCAAGTACGATTGATTCCGATTTAATGATGCTGGACTCAATTCGGAGA
                  M  E  M  Y  P  S  T  I  D  S  D  L  M  M  L  D  S  I  R  R     20
151   CACTTACTCGGCGAATCATCCGACTTGCGGTTCACTTCTTCAAATGACTGTACCAACGTGGGCGCGGCTCCCCCT
       H  L  L  G  E  S  S  D  L  R  F  T  S  S  N  D  C  T  N  V  G  A  A  P  P     45
226   ATGTTTTGCAGGAGCTCCAGCTTTAGCCGCTTGTACCCTTGTTTGACTGACACCTGGGGTGACCCCCCACTCAAA
       M  F  C  R  S  S  S  F  S  R  L  Y  P  C  L  T  D  T  W  G  D  P  P  L  K     70
301   GAGAACGATTCTGAAGACATGCTAGTTTTCGGGTACCTTAGAGATGCTTTAACCGTCGGTTGGGCCCCTCCGAT
       E  N  D  S  E  D  M  L  V  F  G  Y  L  R  D  A  L  T  V  G  W  A  P  S  D     95
376   CACTCTTCCCCGACTTTCCCGCCAATTAAACCGGAACCTCAGGAGATTCCGACGGAGACAGCGGTGAAAGAGGTT
       H  S  S  P  T  F  P  P  I  K  P  E  P  Q  E  I  P  T  E  T  A  V  K  E  V    120
451   CCTACGGTGGCGAATGCGGCGGTTCCCACGGTGGTTCCTGCCAAAGGGAAGCATTACAGAGGAGTAAGGCAAAGG
       P  T  V  A  N  A  A  V  P  T  V  V  P  A  K  G  K  H  Y  R  G  V  R  Q  R    145
526   CCGTGGGGAAAGTTCGCGGCAGAGATTAGGGATCCGGCTAAAAACGGGGCTCGAGTTTGGCTAGGCACTTTCGAG
       P  W  G  K  F  A  A  E  I  R  D  P  A  K  N  G  A  R  V  W  L  G  T  F  E    170
601   ACAGCTGAGGATGCGGCTTTGGCTTATGATAAGGCAGCTTATAGGATGCGTGGCTCAAGGGCTTTGTTGAATTTC
       T  A  E  D  A  A  L  A  Y  D  K  A  A  Y  R  M  R  G  S  R  A  L  L  N  F    195
676   CCATTGAGGGTGAATTCCGGGGAACCCGACCCAGTTAGGATCACGTCAAAAAGGGCAACACCGGAGCCATCTACT
       P  L  R  V  N  S  G  E  P  D  P  V  R  I  T  S  K  R  A  T  P  E  P  S  T    220
751   TCTTCGTCCTCGGGTTCAGAAAACGGGTCACCAAAAAGGAGGAGAAAAGTAGGTAGCGCGGCTCCGGTTGTAGCT
       S  S  S  S  G  S  E  N  G  S  P  K  R  R  R  K  V  G  S  A  A  P  V  V  A    245
826   CAAGCCGGTTTAAATATGGGTTTTGGAGCGTAAAGTATTGAGGTGGGACCTTGTACATGTGGTGAACAGCTATTA
       Q  A  G  L  N  M  G  F  G  A  *                                              255
901   GTCAGTGAAATGCTGCTGAGGTGTTAATAGTGTAATGTAAGCAGAAGTAAGCTTATAGTTATGGACAACCTCCAT
976   CCCATAGGAATTTTGTAGAATCGGAAAAAAAGTTATTTTTGTTCTTAAAAATAGTATTAACAGCGTGGTTAAAAC
1051  TAAAAAAAAAAAAAAAAAA
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Fig. 1. Nucleotide and deduced amino acid sequences of *GhERF5* gene. The amino acid sequence is displayed in a one-letter code under the coding sequence, and the AP2/ERF domain is underlined. Nucleotides are numbered on the left side, and amino acids are numbered on the right. The dotted lines indicate the nuclear localization signal sequence (PKRRRK).

method. The reporter 4×GCC-LUC plasmid [11] was kindly provided by Dr. Masaru Ohme-Takagi (National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan). The effector plasmid 35S-*GhERF5* was constructed by replacing the GUS gene in PBE2113-GUS [27] with the coding region of the *GhERF5* gene. A blank plasmid of pBE2113 without the GUS gene was used as the control. The plasmid pRTL-NLUC (CaMV35S-Renilla LUC-NOS), kindly provided by Dr. Gregory B. Martin (Cornell University, USA), was used as a reference plasmid. Transient assays were performed using the particle bombardment method [18]. After bombardment, the samples were incubated on filter paper moistened with 50 mM phosphate buffer (pH 7.0) in a dark chamber at 22 °C for 20 h. The samples were then frozen in liquid nitrogen for luciferase (LUC) assays. LUC assays were performed with the dual-luciferase reporter assay system (Promega, USA) using a luminescence reader (TD-20/20, Turner, USA). To normalize values after each assay, the ratio of LUC activity (Firefly LUC/Renilla LUC) was calculated. The data were collected from triplicate samples and from three independent experiments.

3. Results

3.1. Sequence analysis of the GhERF5 gene and its promoter region

To clone the full length cDNA of the novel cotton *ERF* gene, a cloning strategy combining the bioinformatics analysis and the RACE-PCR technique was used. The isolated cDNA, designated *GhERF5* (*Gossypium hirsutum* ERF-binding protein 5) (GenBank Accession No. AY781118), has a single open reading frame (ORF) of 768 bp with a 5′-untranslated region of 90 bp and a 3′-untranslated region of 211 bp. The predicted translation product has 255 amino acids, and codes for a protein with a molecular mass of 27.7 kDa and pI of 8.41 (Fig. 1). Sequence alignment revealed that the deduced protein has a central 58-amino acid AP2/ERF domain with two conserved amino acid residues, alanine in position 14 and aspartic acid in position 19 (Fig. 2), which is a typical characteristic of the ERF-binding domain [28], suggesting that GhERF5 is a member of the ERF proteins. GhERF5 shares a very high similarity to the ERF domains of *Arabidopsis* AtERF1 (96.61%) and AtERF2 (96.61%), tomato LeERF1 (88.14%) and Pti4 (91.53%), but a low similarity to the entire sequences of these ERF proteins (AtERF1, 55.60; AtERF2, 55.14; LeERF1, 48.55 and Pti4, 48.53%).

In order to determine the relationship between GhERF5 and other ERF-binding proteins, phylogenetic analysis was carried out, and a phylogenetic tree was drawn using the TreeView program [29]. As shown in Fig. 3, GhERF5 is positioned in the B-3a ERF group. Data analysis also indicated that GhERF5, as well as AtERF1, AtERF2, Pti4 and LeERF1, contains a basic region (PKRRRK) in its C-terminal region that might function as a nuclear localization signal (NLS), and an acidic N-terminal region that might act as an activation domain for transcription [12,30].

The previous study on ERF genes demonstrated that the transcription of the ERF gene was differentially induced by

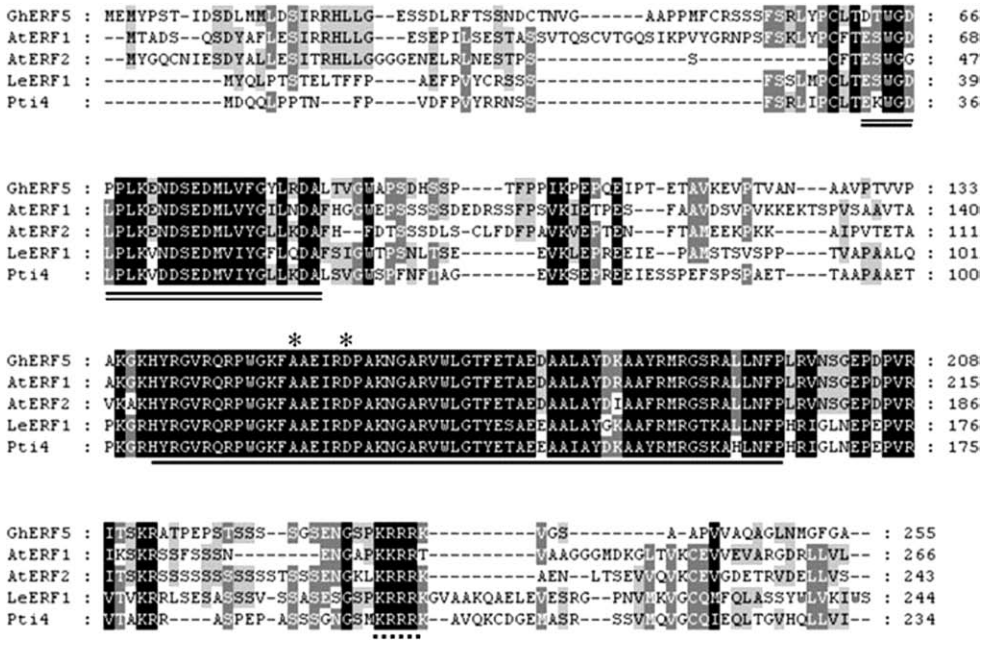

Fig. 2. Comparison of the derived amino acid sequence of cotton GhERF5 with other ERFs from *Arabidopsis* (AtERF1 and AtERF2) and tomato (LeERF1 and Pti4). Amino acid residues conserved in at least three of the five sequences are shaded, while amino acids identical in all five proteins are shown in dark gray. The AP2/ERF domain is underlined, and the putative acidic domain is double-underlined. The dotted lines indicate the putative nuclear localization signal sequence. The alanine and aspartic acid residues at positions 14 and 19 in the AP2/ERF domain are marked by asterisks. Dashes show gaps in the amino acid sequences introduced to optimize alignment.

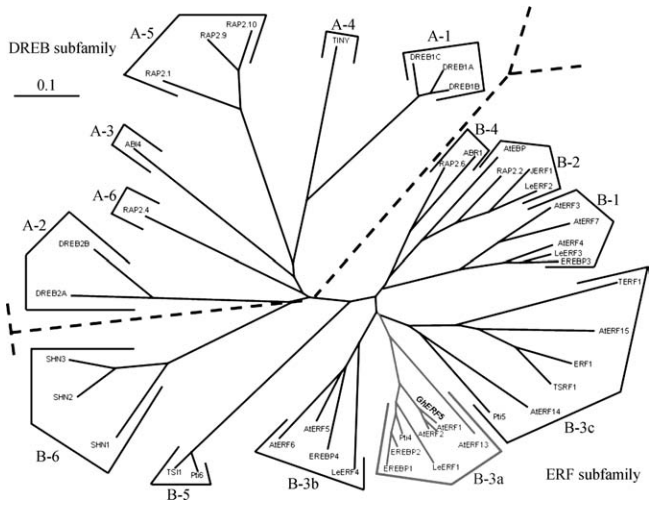

Fig. 3. Phylogenetic analysis of GhERF5 with other AP2/ERF transcription factors. AP2/ERF proteins were aligned using the CLUSTALX program, and the phylogenetic tree was drawn by the TreeView program. The GenBank Accession Nos. are as follows: AtEBP (Y09942), AtERF1 (AB008103), AtERF2 (AB008104), AtERF3 (AB008105), AtERF4 (AB008106), AtERF5 (AB008107), AtERF6 (AB013301), AtERF7 (AB032201), AtERF13 (NM_130048), AtERF14 (NM_100317), AtERF15 (NM_179831), SHN1 (NM_101405), SHN2 (NM_122448), SHN3 (NM_121157), DREB1A (AB007787), DREB1B (AB007788), DREB1C (AB007789), DREB2A (AB007790), DREB2B (AB007791), TINY (NM_122482), RAP2.1 (NM_103607), RAP2.2 (NM_112281), RAP2.3 (NM_112550), RAP2.9 (NM_179009), RAP2.10 (NM_119854), EREBP1 (D38123), EREBP2 (D38126), EREBP3 (D38124), EREBP4 (D38125), LeERF1 (AY192367), LeERF2 (AY192368), LeERF3 (AY192369), LeERF4 (AY192370), JERF1 (AY044235), Pti4 (U89255), Pti5 (U89256), Pti6 (U89257); ERF1 (EU395634), TSRF1 (AF494201), Tsi1 (AF058827), ABI4 (NM_129580).

pathogen infection and exogenous ethylene treatment [10,12,31]. To better understand the organization of the regulatory region of the *GhERF5* gene, a 713 bp fragment, designated pGhERF5, was cloned by the genome walking method (Fig. 4). Sequencing analysis showed that this sequence is localized upstream of the *GhERF5* gene, as the first 90 nucleotides of the cDNA were present at the 3′ end of the fragment. The first base of the cDNA isolated using 5′-RACE was designated +1 as the putative transcription initiation site (Fig. 4). A promoter motif search of the pGhERF5 sequence was performed to reveal the putative *cis*-elements using the PLACE database (http://www.dna.affrc.go.jp/PLACE/). A number of conserved regulatory motifs present in most eukaryotic promoters were found to exist in the pGhERF5 sequence (Fig. 4). These motifs include ABRE (etiolation-induced expression of erd1 in *Arabidopsis*, from −680 to −676), ASF-1 (involved in the transcriptional activation of many genes by auxin and/or salicylic acid, from −304 to −300), box-L-like (involved in the phenylpropanoid biosynthetic pathway, from −183 to −177), Dof (signal-responsive and/or tissue-specific gene expression, from −653 to −650), DC3 (DPBF binding core sequence, from −312 to −318), ElRE (elicitor responsive element, from −456 to −461), ERE (ethylene-responsive element, from −395 to −402), GT-1 motif (involved in pathogen- and salt-induced SCaM-4 gene expression, from −198 to −193), LTRE (core of low-temperature responsive element, from −330 to −326), MYB1AT and MYC (recognition site in the promoter of the dehydration responsive gene rd22, from −257 to −252 and from −271 to −266), SEBF (binding site of the potato silencing element factor SEBF in the PR-10a promoter, from −152 to 146), T/G box (found in jasmonate induction genes from −681 to −676), W box (a binding site of the WRKY transcription, from −456 to −460), TATA box (TATA box elements are critical for accurate initiation, TATATAA, from −88 to −82). The presence of these putative *cis*-acting elements indicates that the *GhERF5* gene might be regulated by various *cis*-elements in a complicated manner via the interaction between the promoter and the corresponding *trans*-acting factors. However, the functional significance of these putative regula-

Fig. 4. Nucleotide sequence of the 5′-flanking promoter region of the *GhERF5* gene. The putative transcription start site is designated as +1 and indicated by an arrow. The deduced *cis*-elements with a significant similarity to the previously identified *cis*-acting elements are underlined, and the name is given under its elements. The ATG translation start codon is marked in bold type.

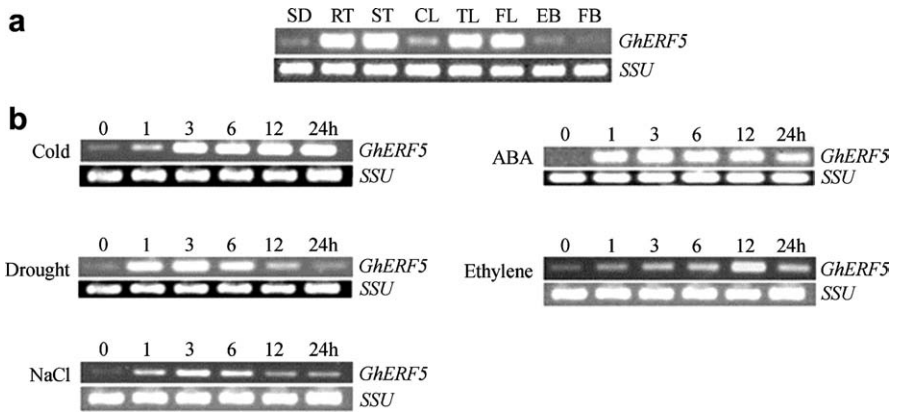

Fig. 5. RT-PCR analysis of the *GhERF5* gene expression. Cotton small-subunit rRNA (*SSU*) was used as a control to show the normalization of the amount of templates in PCR. a, Tissue-specific expression of *GhERF5*. Total RNA was isolated from true leaves (TLs), cotyledons (CLs), stems (STs), roots (RTs), flowers (FLs), young embryos (YEs), old embryos (OEs) and fibers (FBs). b, Inducible expression of *GhERF5*. Total RNA was isolated from 2-week old cotton seedlings treated by cold, drought, NaCl, ABA and ethylene.

tory elements for the expression of the *GhERF5* gene in cotton remains to be elucidated.

3.2. Expression patterns of the GhERF5 gene in different organs and under various abiotic stresses

Expression pattern analysis can help to reveal the possible biological functions of the target gene. To examine the expression patterns of the *GhERF5* gene, we first monitored the mRNA transcript level of the *GhERF5* gene in different organs by semi-quantitative RT-PCR. As shown in Fig. 5a, a high level of *GhERF5* transcripts was detected in roots, stems, true leaves and flowers, and a slightly low level in cotyledons, and a very low level in seeds, mature embryos and fibers, demonstrating a tissue-specific expression profile of the *GhERF5* gene under non-stress conditions. The previous reports on *Arabidopsis* ERF expression study indicated that *Arabidopsis AtERF1* had the highest level of mRNA expression in flowers and rosette leaves compared with other tissues, such as roots, siliques and stems [32]. Tomato *LeERF1* transcripts were detected only in stems. These results documented different expression patterns and suggested the respective functions of these ERFs.

To further investigate whether *GhERF5* expression is induced by abiotic stresses and exogenous hormones, we examined the expression levels of *GhERF5* in 2-week-old seedlings under various treatments. The results in Fig. 5b show that all these treatments significantly increased the transcription of *GhERF5*. When exposed to cold, the expression of *GhERF5* was induced rapidly and reached a peak after 3 h, then remained unchanged for up to 24 h. Upon exposure to drought, the expression of *GhERF5* was significantly increased at 1 h and peaked at 3 h, then declined obviously. When treated with NaCl, the expression of *GhERF5* increased slightly, maintained a high level from 3 to 6 h, and then declined. ABA and ethylene treatments could also induce *GhERF5* transcripts. The expression levels of *GhERF5* were observed to increase markedly after treatment with ABA for 1 h, and reached a maximal level at 3 h, and then maintained a high level for the duration of the treatment. Upon exposure to ethephon (1 mM), the transcripts of *GhERF5* increased gradually and reached a peak at 12 h, then declined. These divergent responses of *GhERF5* to stresses are similar to the previous reports on other plant *ERF* genes. For example, the levels of *AtERF1*, *AtERF2* and *AtERF5* transcripts in *Arabidopsis* increased 2- to 3-fold after 12 h of ethylene treatment, while *AtERF4* was also induced by cold, high NaCl and drought stress [12]. The observation that both ethylene and ABA could induce the expression of *GhERF5* suggests that *GhERF5* might have a role in the cross-talking of the two hormones (Fig. 5b).

3.3. Subcellular localization of transiently expressed fusion proteins

Analysis of the amino acid sequences of GhERF5 revealed that it contains one nuclear localization sequence PKRRRK (Fig. 1). To clarify its nuclear location, the coding region of the *GhERF5* gene was fused in-frame to the *GFP* gene, and the resulting construct was introduced into onion epidermis cells by particle bombardment. As shown in Fig. 6b, the fluorescence of *GhERF5-GFP* was localized exclusively in the nucleus, whereas the fluorescence of GFP alone was observed in the whole cell (Fig. 6a). This clearly demonstrates that GhERF5 is a nuclear-localized protein.

3.4. GhERF5 transactivation in the yeast GAL4 system

To characterize the transcriptional activity of GhERF5, we performed a transient expression assay using a GAL4-responsive reporter system. As shown in Fig. 7a, the effector plasmid contains translational fusions between the GAL4-binding domain and the *GhERF5* gene. Two reporter constructs were transformed into the yeast strain

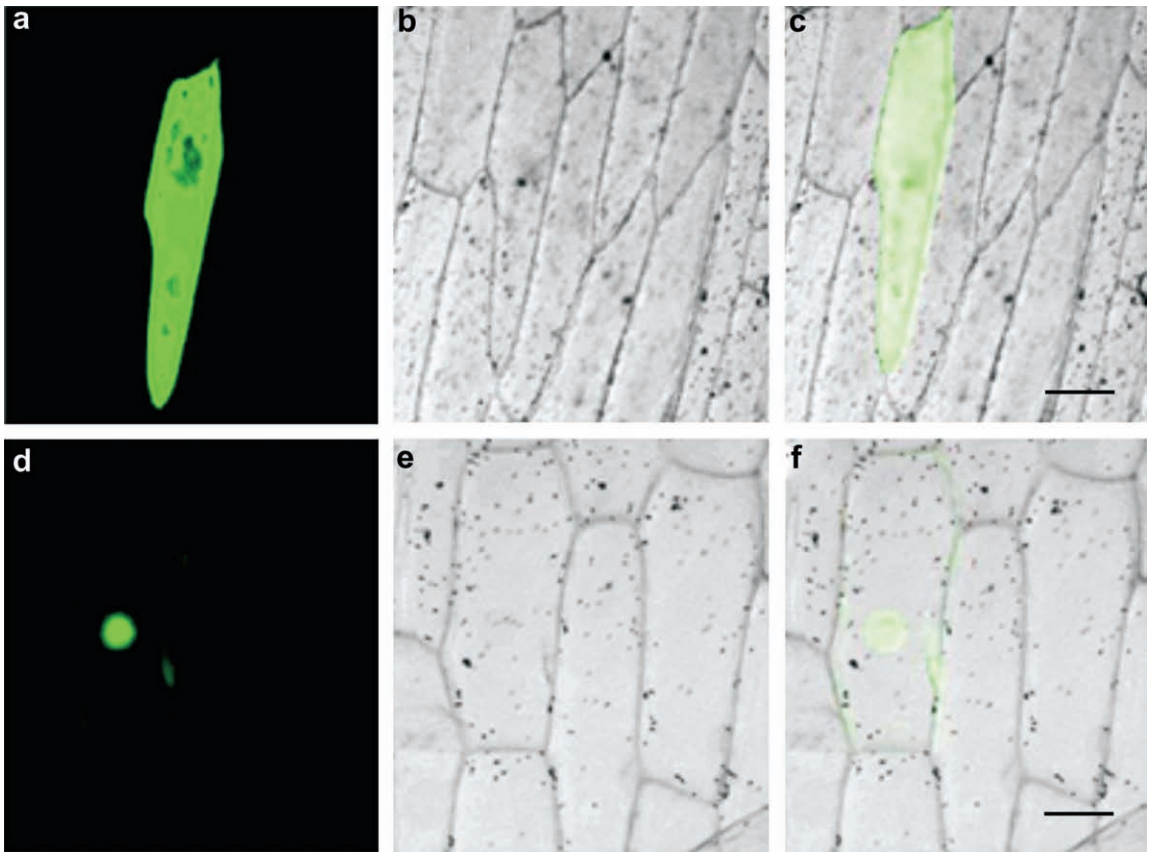

Fig. 6. Subcellular localization of the GhERF5 protein in onion epidermis cells. Onion epidermis cells were transformed with 35S-GFP control (a–c) and *GhERF5-GFP* fusion box (d–f). After incubation for 24 h, the transformed cells were observed under a confocal microscope. The photographs were taken in a dark field for green fluorescence (a and d), in bright light for the morphology of the cells (b and e) and in combination (c and f). Bar = 10 μm.

AH109. One GAL4 upstream activating sequence (GAL1 UAS) was fused to a minimal promoter (GAL1 TATA) to control the expression of the *His3* reporter gene; the other GAL4 upstream activating sequence (MEL1 UAS) was fused to a minimal promoter (MEL1 TATA) to control the expression of the *lacZ* reporter gene. If the GhERF5 protein possesses transcriptional activation ability, it will cooperate with the BD of GAL4 to promote the expression of the *His3* and *lacZ* genes, resulting in the growth of the transformed yeast cells on SD/-His plate supplemented with 5 mM 3-AT and *lacZ* (Fig. 7b). Meanwhile, the yeast colony will display a blue color. Fig. 7b shows that the transformed yeast cells harboring pBD-GhERF5 or pCL1 (positive control) could grow on SD/-His X-Gal plates supplemented with 5 mM 3-AT and display a blue color. However, the yeast cells containing

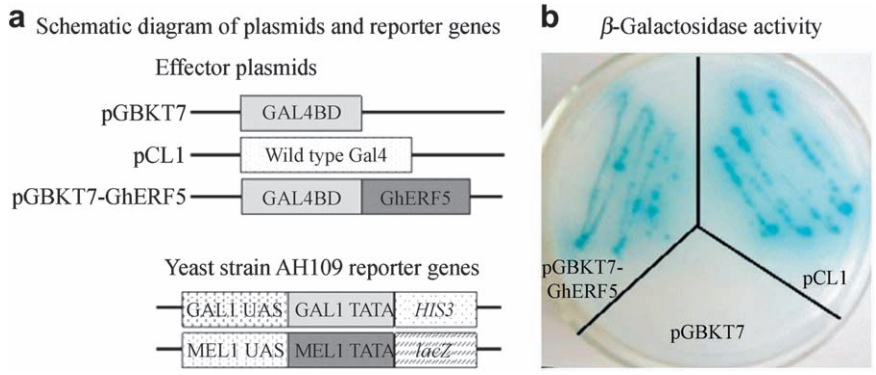

Fig. 7. Transactivation activity of GhERF5 in yeast cells. (a) Schematic diagram of the effector and reporter plasmids used in these experiments. The effector plasmid (pGBKT7-GhERF5) was constructed by fusing the full length of GhERF5 cDNA to the GAL4 DNA-binding domain (GAL4BD) coding region. The reporter genes were the *LacZ* gene or *HIS3* gene. (b) The transformants were selected by growth on Trp^- synthetic medium at 30 °C for 3 days. The existence of transcriptional activation activity was confirmed by the β-galactosidase assay on SD/-His plate supplemented with 5 mM 3-AT.

pGBKT7 (negative control) could not grow on the plate. This result indicates that the GhERF5 protein is capable of functioning as a transcriptional activator in yeast.

3.5. Transactivation of 4×GCC-LUC by GhERF5 in tobacco cells

The ERF proteins modulate the expression of many pathogenesis-related (PR) genes through interaction with the GCC box present in their promoter regions. The ability of GhERF5 to activate GCC-dependent transcription was assessed in tobacco cells using the transient expression particle bombardment method. The GCC elements were multimerized four times in tandem, and were fused to the minimal TATA box and the *LUC* gene. These constructions were then used as reporter plasmids (Fig. 8a). The effector construct comprised the full length cDNA of GhERF5 fused to the CaMV 35S promoter. This effector plasmid, along with a 4×GCC-LUC reporter construct, was transferred to mature tobacco leaf cells by particle bombardment. When the 4×GCC-LUC construct was used as a reporter gene, the GhERF5 produced an approximately 8.2-fold increase in LUC activity over the control (Fig. 8b). This clearly demonstrates that GhERF5 is able to bind specifically to GCC upstream promoter elements and activate *LUC* gene expression.

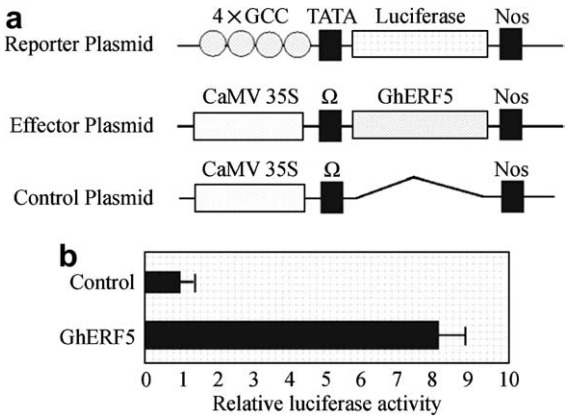

Fig. 8. Transient expression assays of the *GhERF5* gene in tobacco cells. (a) Schematic overview of the effector, reporter and reference plasmid constructs used in the particle bombardment experiments. The effector plasmids contain the CaMV 35S promoter fused to the full length ChERF5 cDNA. The reporter constructs possess the GCC element tandemly repeated four times, which were fused to the reporter *luciferase* gene. The reference plasmid is composed of the CaMV 35S promoter and the *luciferase* gene. "Nos" indicates the polyadenosyl signal of the *nopaline synthase* genes. (b) Transactivation of the 4×GCC-LUC fusion genes by the GhERF5 protein in tobacco cells. Reporter genes containing GCC upstream *cis*-acting elements were transfected by bombardment with the effector plasmid or vector control in wild-type tobacco leaf cells. To normalize transfection efficiency, the plasmid carrying the CaMV 35S promoter-*luciferase* gene was co-bombarded in each experiment. LUC activity was expressed in arbitrary units relative to luciferase activity. This assay was repeated three times with similar results. The values are means ± SD ($n = 3$).

4. Discussion

Accumulated evidence indicates that ERF proteins in plants belong to one of the largest transcription factor families and suggests that ERF proteins might play crucial roles in regulating plant development and in response to environmental stresses [33]. In this study, *GhERF5* gene was isolated and shown to encode an ERF protein. Based on the analysis of the predicted protein sequence, GhERF5 contains an acidic activation domain in the N-terminal region, which might play a role in transactivation. *In vivo* assay demonstrated that GhERF5 could activate the expression of downstream genes, such as *lacZ* and *LUC* in yeast and tobacco cells. As a transcription factor, another important regulatory mechanism is its nuclear localization [34]. Some ERF activators including the tomato Pti4/5/6 and the tobacco Tsi1 were shown to be localized in the nucleus [16,35]. Sequence analysis indicated that the GhERF5 protein contains a nuclear localization signal in its C-terminal region, and transient expression using *GhERF5-GFP* demonstrated that GhERF5 is specifically targeted to the nucleus of onion epidermal cells. Based on the conserved alanine at position 14 and aspartic acid residue at position 19 in the AP2/ERF domain, GhERF5 was classified as a member of the B-3a ERF subgroup. *In vivo* assay showed that GhERF5 activated LUC activity of 4×GCC-LUC in tobacco cells, which indicates that GhERF5 can bind to the GCC box. This is in accordance with the reports that this group member plays an important role in ethylene- and pathogen-responsive gene expression, and has multiple functions in the regulation of GCC-mediated gene expression in plants [9,16].

The observation of the accumulation of *GhERF5* transcripts at different times with ethylene treatment in the present study agrees with the demonstrated fact that the *A. thaliana* ERF1 is rapidly induced by ethylene, and its role is to regulate ethylene-inducible genes [14]. ABA mediates the responsiveness of plants to drought, cold or salt stress [36], and ethylene has been shown to enhance ABA action in seeds [37], but may counteract ABA effects in vegetative tissues under drought stress [38]. Plant responses to stresses are regulated by multiple signaling pathways and exhibit a significant overlap between the patterns of gene expression in response to different stresses [39] and different plant hormones, including ethylene and ABA [40,41]. The expression of several ERF genes can be induced by ethylene, ABA and environmental stress [10,12,31], suggesting that these proteins could play important roles in the processes regulated by these hormones. Therefore, ERF proteins may play a key role in the integration of ethylene and ABA signals for the regulation of stress response genes. Our results showed that ABA could induce the expression of *GhERF5* in cotton, which might suggest that *GhERF5* is involved in both ethylene and ABA signaling pathways. Cross-talk between different pathways provides great regulatory potential for activating multiple defense and stress responses, and different stress pathways can be

linked by certain transcription factors, such as Tsi1 in ethylene- and osmotic stress-signaling pathways [35].

Our promoter analysis indicated that the 5′-upstream region of *GhERF5* possesses some elements induced by physiological and environmental factors (e.g., pathogens, hormones, dehydration and low temperature induced factors). These elements may contribute to the defense-related function. Analyses of the promoter regions of these ABA-dependent genes may reveal some essential *cis*-acting elements, and may give some clues to the question of how these genes respond to the ABA signal. In fact, we actually found several ABA responsive *cis*-elements among the promoter region of the *GhERF5* gene. For instance, in DC3 and LTRE (Fig. 4), Dc3 expression is normally embryo specific, and can also be induced by ABA.

In conclusion, based on the results presented in this paper, it is reasonable to expect that *GhERF5* most likely acts as an important common component in multiple signaling pathways responsive to biotic and abiotic stresses. To further explore *GhERF5* and its function, development of transgenic plants with overexpression of the *GhERF5* gene is underway.

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