Plant Molecular Biology **55:** 183–192, 2004.
© 2004 Kluwer Academic Publishers. Printed in the Netherlands.

Ectopic overexpression of tomato *JERF3* in tobacco activates downstream gene expression and enhances salt tolerance

Hui Wang†, Zejun Huang†, Qi Chen, Zhijin Zhang, Hongbo Zhang, Yanming Wu, Dafang Huang and Rongfeng Huang*

*The National Plant Gene Research Center (Beijing), Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, China (*author for correspondence; e-mail rongfeng@public3.bta.net.cn) †These authors contributed equally to this work.*

Received 14 January 2004; accepted in revised form 5 April 2004

Key words: DRE, ERF proteins, GCC box, JERF3, *PR* gene expression, salt tolerance

Abstract

The ethylene, jasmonic acid and osmotic signaling pathways respond to environmental stimuli and in order to understand how plants adapt to biotic and abiotic stresses it is important to understand how these pathways interact each other. In this paper, we report a novel ERF protein – jasmonate and ethylene-responsive factor 3 (JERF3) – that unites these pathways. JERF3, which functions as an *in vivo* transcription activator in yeast, binds to the GCC box, an element responsive to ethylene/JA signaling, as well as to DRE, a dehydration-responsive element that responds to dehydration, high salt and low-temperature. Expression of *JERF3* in tomato is mainly induced by ethylene, JA, cold, salt or ABA. Constitutive expression of *JERF3* in transgenic tobacco significantly activated expression of *pathogenesis-related* genes that contained the GCC box, resulting in enhanced tolerance to salt. These results indicate that JERF3 functions as a linker in ethylene- and osmotic stress-signaling pathways.

Introduction

Adverse environmental conditions such as drought, high salt, extreme temperatures and pathogen attack greatly impair crop productivity. Since plants cannot move to avoid stressful conditions, they must employ alternative ways to withstand stress by activating multiple defense mechanisms through expressing specific genes and synthesizing a large number of stress proteins that play distinct and important role in stress response and plant defense (Skriver and Mundy, 1990; Reymond and Farmer, 1998; Demekamp and Smeekens, 2003). Accumulating evidences demonstrated that stress responses of plants are regulated by multiple signaling pathways, for example, salicylic acid (SA), ethylene, jasmonic acid (JA) and abscisic acid (ABA) have been shown to be important components of defense response pathways (Dong, 1998; Reymond and Farmer, 1998; Dempsey *et al.*, 1999; Pieterse and van Loon, 1999; Xiong *et al.*, 2002), but the communication among these plant hormone signaling pathways is not well understood. Ethylene is an important endogenous plant hormone that affects virtually many aspects of plant growth and development and also participates in kinds of stress responses, such as seed germination, cell fate, fruit ripening, senescence and infection by pathogens. In response to stresses, ethylene induces the expression of certain defence genes. Exogenous application of ethylene induces accumulation of class I basic chitinases, class I basic β-glucanase and other basic-type pathogenesis-related (PR) proteins (Boller *et al.*, 1983; Felix and Meins, 1987). Through analysis of *PR* gene promoter, an 11 bp ethylene-responsive element TAAGAGCCGCC which has been referred to as the GCC box (Ohme-Takagi and

Shinshi, 1995) was confirmed to be necessary and sufficient for ethylene regulation in plants, and more recent research demonstrated that GCC box in *PDF1.2* of Arabidopsis also is responsive to JA signaling (Brown *et al.*, 2003). The ethylene-responsive factor (ERF) proteins which contain a highly conserved DNA binding domain (ERF domain) consisting of 58 or 59 amino acids (Hao *et al.*, 1998) were first isolated through the interaction with GCC box from tobacco (Ohme-Takagi and Shinshi, 1995). Current research evidenced that ERF protein also binds to dehydration-responsive element (DRE), which is responsive to dehydration, high salt and low-temperature (Park *et al.*, 2001; Hao *et al.*, 2002; Singh *et al.*, 2002). The ERF proteins Pti4/5/6 in tomato have been shown to bind to GCC box (Gu *et al.*, 2000, 2002) and activate *PR* gene expression (Zhou *et al.*, 1997; Gu *et al.*, 2000), while the Tsi1 protein can bind to both the GCC box and the DRE motifs, and overexpression of *Tsi1* enhances resistance against pathogen attack and osmotic stress in tobacco, demonstrating that these two different stress pathways can be linked by a single ERF protein (Park *et al.*, 2001). This cross-talk among different pathways provides a great regulatory potential for activating multiple resistance mechanisms in varying combinations and may help the plant to prioritize the activation of particular defense pathway over another, thereby providing an optimal defence against the abiotic and biotic stress. In this paper we report a novel member of ERF proteins in tomato, jasmonate and ethylene-responsive factor 3 (JERF3), which binds to ethylene/JA responsive GCC box and osmotic stress responsive DRE and mainly responds to jasmonate, ethylene, salt, cold, or ABA treatment. The function of the JERF3 protein as a trans-acting factor also was analyzed in yeast and in transgenic tobacco plants which activates expression of downstream genes thereby enhancing salt tolerance.

Materials and methods

Plant materials

All plants were grown in growth chambers at 25 °C with a 16-h-light/dark cycle (except mentioned in the text). Four-week-old tomato (*Lycopersicon esculentum* cv. Lichun) and 6-week-old tobacco (*Nicotiana tabacum* cv. NC89) were used for Northern blot. For ABA, NaCl treatment, the tomato plants were removed from soil, washed with water and put into a beaker containing 40 ml 0.1 mM ABA or 300 mM NaCl. 0.1 mM methyl jasmonic acid treatment was performed by spraying the plants. ABA and MeJA were dissolved in 0.01% ethanol, same concentration ethanol only was also used as control respectively in above treatments. Ethylene treatments were performed with 2 ml 40% ethephon; and 1 g $NaHCO_3$ dissolving in 200 ml H_2O, the control and treated tomato plants were placed in a sealed plexiglass chamber before leaf tissue was harvested. For low temperature treatment, tomato plants were put in 4 °C refrigerator. For detecting the expression of *JERF3* and downstream genes in transgenic tobacco plants, leaves from normally growing 4–5-week-old plants were used.

Screening with yeast one-hybrid

The reporter plasmids were constructed as described by Liu *et al.* (1998). The GCC box (AGTGCCAAAAGCCGCCACACCCCT) present in the promoter (−74 to −51) of tomato *NP24* was synthesized into four tandemly repeated copies and then inserted into EcoRI-XbaI sites of the MCS upstream of *HIS3* minimal promoter in pHISi-1 vector (Clontech, Palo Alto, CA). The same fragment was cloned into EcoRI-SmaI sites of the MCS upstream of the *LacZ* minimal promoter in pLacZi vector (Clontech). These two plasmids were transformed simultaneously into yeast YM4271. Yeast transformants containing *HIS3* and *LacZ* reporter genes that could not grow in selective medium under 10 mM 3-aminotriazole (3-AT, a competitive inhibitor of the *HIS3* gene product) were used for further analysis.

The tomato cDNAs from tomato leaves treated with methyl jasmonic acid and ethylene were fused into downstream of transcriptional activation domain of the yeast expression vector pB42AD (Clontech), and introduced into yeast strains carrying the above dual reporters. Transformants were first selected using the selective medium containing 30 mM 3-AT. After β-galactosidase activity assay, plasmids from yeast positive clones were transformed into *E. coli*, and subsequently sequenced.

Binding assay in vitro

JERF3 full encoding area was cloned in frame into NdeI-HindIII sites of the pET28a vector (Novagen). The resulting construct was transformed into *E. coli* strain BL21. Expression and purification of recombinant JERF3 were conducted with a commercial kit following the manufacturer's introduction (Invitrogen). The oligonucleotides of the wild-type GCC box sequence AGTGCCAAAA GCCGCCACACCCCT and mutant GCC box sequence AGTGCCAAAATCCACTACACCCCT, or wild-type DRE (ATTTCATGGCCGACCT GCTTTT) and mutant DRE (ATTTCATA ATCAACCTGCTTTT) were end labeled with ^{32}P-dATP as probe. The assay mixtures contained 1.2 μg recombinant JERF3, 2 ng binding probe (8×10^4 cpm), 2 μg of Salmon DNA, 20 mM Hepes-KOH, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 10% glycerol, and 0.5 mM DTT in a 10 μl reaction volume. The mixtures were incubated at room temperature for 15 min and separated on a 4% polyacrylamide gel in $0.5 \times$ TBE buffer. Subsequently, the gel was exposed to Imaging Screening-K and visualized with the Molecular imager® FX (Bio-Rad).

Transactivation assays in yeast

Coding region of *JERF3* was fused in frame downstream of DNA binding domain in pLexA. The plasmids were transformed into EGY48 containing p8op-lacZ as described by the manufacturer (Clontech). The transformants were selected by growth on selective medium plates at 30 °C for 3 days. The colony lift filter assay using o-nitrophenyl β-D-galactopyranoside as a substrate was performed subsequently to determine the ability of transcription activation.

Subcecullar localization of JERF3

The coding region of *JERF3* and *DNLS* which deleted the sequence containing the predicted nuclear localization signal (NLS) were amplified by polymerase chain reaction (PCR) to introduce XbaI at the 5′ end and SmaI at the 3′ end. The resulting fragments were digested with XbaI and SmaI and cloned into the expression vector pROK2 (derived from pBI121), yielding the plasmids pJERF3, pDNLS, respectively. Then the coding region of green fluorescent protein (GFP) was amplified from pBIN 35S mGFP4 by PCR to introduce a SmaI site at 5′ end and a SacI site at 3′ end. The PCR products were digested with SmaI and SacI, and fused in frame into pJERF3 or pDNLS, resulting in JERF3-GFP or, DNLS-GFP. Then, the above constructs were introduced into Agrobacterium LBA4404 for further analysis. Agrobacterium culture for transient expression in onion epidermal cells was prepared as described by Yang *et al.* (2000). The onion epidermal cells were dipped in the prepared Agrobacterium solution for 40 min, transferred to Murashige and Skoog (MS) plates, and incubated at 25 °C under light for 24–48 h. The localization of the fusion protein was observed by confocal microscope (Bio-Rad).

RNA gel analyses

Total RNA was extracted from leaves. Twenty micrograms of total RNA was separated on a 1.5% formaldehyde-agarose gel, and transferred onto nylon membranes. Using ^{32}P-dCTP labeled DNA as probe, the membrane was hybridized as the standard procedure of Northern blot. The hybridized membrane was exposed to Imaging Screening-K and visualized with the Molecular imager® FX (Bio-Rad).

The probe for *JERF3* expression was chose the JERF3 3′ flanking sequence. And the probes of downstream gene expression were cloned by reverse transcription-PCR using the following specific primers: 5′-CTATTCCTTGTTTCTCACC-3′ and 5′-CCTTAACACATGACAAAGC-3 for *prb-1b* (*PR1*); 5′-GGGAATGAAATCAGCCCTG-3′ and 5′-GTCCCAAACTCCACCAGAG-3′ for *GLA* (*PR2*); 5′-CTCTCCTACTCCTCTCTGC-3′ and 5′-CACCAGGACTAACACCAAG-3′ for *CHN50* (*PR3*); and 5′-CTTCCTCCTTGCCTTT GTG-3′ and 5′-GCCACTTCATCACTTCCAG-3′ for *osmotin* (*PR5*).

Generation of transgenic JERF3 tobacco

Two plant expression vectors pJERF3 and pDNLS were transformed into tobacco (NC89) separately via Agrobacterium-mediated transformation.

Analysis of salt tolerance in transgenic JERF3 plants

Leaf discs of 1 cm diameter were cut from healthy and fully expanded leaves of wild-type and transgenic *JERF3* plants, and floated in MS medium containing 300 mM NaCl for 48 or 72 h (Veena *et al*., 1999) under white light at 25 °C. The contents of chlorophyll were determined as Aono *et al*. (1993), and the data were shown as percentage relative to controls.

Results

Isolation and sequence analysis of JERF3 full length cDNA

In order to clone the regulatory proteins that interact with ethylene/JA responsive element GCC box (Ohme-Takagi and Shinshi, 1995; Brown *et al*., 2003), 1.2×10^6 yeast transformants were screened with the method of yeast one hybrid from tomato expression cDNA library with a four time repeated GCC box present in the promoter (−74 to −51 bp) of tomato *NP24* as bait, and 14 positive clones were isolated. Of one positive clone with full length cDNA, designed *jasmonate and ethylene-responsive factor 3* (*JERF3*), encodes a putative protein containing 327 amino acids with a predicted molecular mass of 36.45 ku. NCBI blast revealed that the deduced amino acid sequence contains a conserved ERF DNA binding domain, suggesting that JERF3 is a member of ERF proteins. Data analysis indicated that JERF3 contains a basic region in its N-terminal region that might function as a nuclear localization signal (NLS) and an acidic C-terminal region that might act as an activation domain for transcription (Figure 1). More interesting is that JERF3 shows a very high similarity with ERF proteins of tobacco Tsi1 (63.2%), tomato Pti4 (73.7%) and *Arabidopsis* ERF1 (63.2%) in ERF DNA binding domain (Figure 2A), but very low similarity for the whole sequences among JERF3 and ERF1 (24.6%), Pti5 (28.8%), Pti4 (23.4%), and Tsi1 (23.2%). Phylogenetic analysis based on the sequence alignment shows that the JERF3 protein is a novel member of ERF proteins (Figure 2B).

```
1     GACAATTATACATTTTCCGTCAAAACATAAATCATGTGTGGTGGTTCTATAATCTCCGAT
1                                       M  C  G  G  S  I  I  S  D
61    TACATAGACCCTAGCCGGACTTCTCGCCGGCTCACCGCCGAGTTTCTATGGGGTCGTTTC
10     Y  I  D  P  S  R  T  S  R  R  L  T  A  E  F  L  W  G  R  F
121   GATCTCGGTAAGAAGCAAAAAAATCCCAACAATTATCACTCTAAAGCTAAGCATTTGCGA
30     D  L  G  K  K  Q  K  N  P  N  N  Y  H  S  K  A  K  H  L  R
181   TCTGAAGTTGTTGACGACTTTGAAGCCGATTTTCAGGACTTCAAAGAGTTATCCGATGAT
50     S  E  V  V  D  D  F  E  A  D  F  Q  D  F  K  E  L  S  D  D
241   GAGGATGTTCAAGTCGATGTCAAGCCATTTGCCTTCTCTGCTTCCAAACACTCTACTGGT
70     E  D  V  Q  V  D  V  K  P  F  A  F  S  A  S  K  H  S  T  G
301   TCCAAATCTTTGAAAACTGTTGATTCAGACAAGGATGCTGCTGCTGATAAATCCTCTAAG
90     S  K  S  L  K  T  V  D  S  D  K  D  A  A  A  D  K  S  S  K
361   AGAAAGAGGAAGAATCAATATAGAGGGATCAGACAGAGACCTTGGGGTAAGTGGGCAGCT
110    R  K  R  K  N  Q  Y  R  G  I  R  Q  R  P  W  G  K  W  A  A
421   GAAATACGTGACCCAAGGAAAGGGGTTCGGGTCTGGCTGGGAACCTTCAATACTGCAGAA
130    E  I  R  D  P  R  K  G  V  R  V  W  L  G  T  F  N  T  A  E
481   GAAGCTGCCAAAGCTTATGATATTGAGGCGAGGAGGATCAGAGGCAAGAAGGCTAAGGTA
150    E  A  A  K  A  Y  D  I  E  A  R  R  I  R  G  K  K  A  K  V
541   AACTTTCCTGATGAAGCTCCCGCCCCTGCATCAAGACACACTGTTAAGGTGAATCCTCAG
170    N  F  P  D  E  A  P  A  P  A  S  R  H  T  V  K  V  N  P  Q
601   AAGGTCCTTCCTGAGGAGAGCCTGTATTCACTTCAGTCCGACTCAGCAATCATGAACAGC
190    K  V  L  P  E  E  S  L  Y  S  L  Q  S  D  S  A  I  M  N  S
661   GTGGAGGATGACCATTATGATTCTTTTGGATTTTTTGAAGAGAAACCCATGACAAAACAG
210    V  E  D  D  H  Y  D  S  F  G  F  F  E  E  K  P  M  T  K  Q
721   TATGGATATGAGAATGGGAGCAGTGCTTCTGCAGATACGGGATTTGGTTCGTTCGTCCCT
230    Y  G  Y  E  N  G  S  S  A  S  A  D  T  G  F  G  S  F  V  P
781   TCAGCTGGCGGTGATATCTACTTCAACTCTGATGTAGGAAGCAACTCTTTTGAATGCTCT
250    S  A  G  G  D  I  Y  F  N  S  D  V  G  S  N  S  F  E  C  S
841   GATTTTGGTTGGGGAGAGCCATGCTCCAGGACTCCAGAGATATCATCTGTTCTGTCAGCT
270    D  F  G  W  G  E  P  C  S  R  T  P  E  I  S  S  V  L  S  A
901   GCTATTGAATGTAATGAAGCTCAATTTGTTGAAGATGCCAATTCTCAGAAAAAGTTGAAA
290    A  I  E  C  N  E  A  Q  F  V  E  D  A  N  S  Q  K  K  L  K
961   TCATGCACCAACAACCCCGTAGCTGATGATGGAAACCCCCGTTACTATGGTACCTGAAGA
310    S  C  T  N  N  P  V  A  D  D  G  N  P  R  Y  Y  G  T  *
1021  GCTTCCAGCTTTTGAACCTCAGATGAATTTCTTTCATCTCCCATATATGGAGGGAAATTG
1081  GGATGCATCAGGTGGTAACTTCCTCAACACAAGTGCAACTCAAAATGGTGGTGAAAATGC
1141  TATGGACCTGTGGTCCTTTGATGATGTTCCTTCTTTAATGGGAGGTATCTTTTAAGTCAA
1201  CATGCCTTGAGTTTTGTAAATAAGGCTTCATGTGAGTGATTTTTTGCTGTTGTATAATGT
1261  ACTTAGTGCAAATATTTGATATGTTAATTTGATCTCCGTTAACTTGTTCTTTTAGGTGTG
1321  TCTGGTGGTGGAAGAAGAATGATCCACAGAGAACCATGATTAAGCCATGGATAATGCACT
1381  AAGTAGAGCAGTGCATAGGTAATTAGGTTTAATTAACTACTAGTAGAGATGTTAAATTCG
1441  AGTTTTACTTAAAAACAATTGGGCTGTATGGATTATGAGAATTGATGAGACCTCGATGCT
1501  TGCTATAACGTTACCTTTTTAGTGTTGCTTTCACAAAAAAAAAAAAAAAAAA
```

Figure 1. Nucleotide and deduced amino acid sequence of JERF3. The ERF domain is underlined and a basic region acted as a nuclear localization signal is shown in italics with underlined, and an acidic C-terminal region acted as a transcriptional activator is shown in italics. The JERF3 is accessible in GenBank databases under accession number AAQ91334.

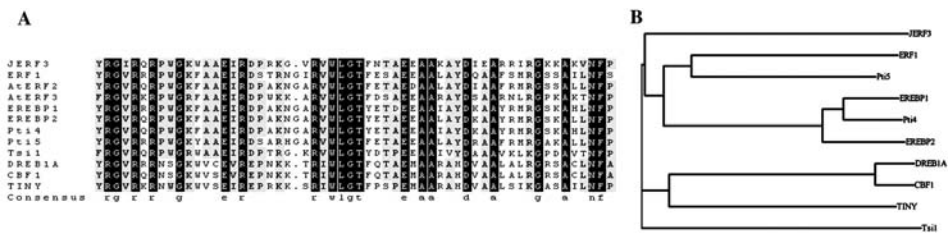

Figure 2. Amino acid alignment of ERF domain region and dendrogram of ERF proteins. (A) Comparison of the deduced amino acid sequences of JERF3 and ERF proteins from tobacco, tomato and *Arabidopsis*. Boxes in black represent 100% similarity, gray for 75% conserved amino acids. Protein names are indicated at the left. (B) Phylogenetic tree produced after ERF protein sequences were aligned using DNAMAN software.

Characterizations of JERF3 as transcription factor

To test whether tomato JERF3 can interact with ethylene GCC box, we tested the binding affinity of JERF3 to GCC box *in vitro*. Firstly, JERF3 fusion protein was expressed in pET28a and purified with the His-affinity column. The binding ability of recombinant JERF3 to synthesized GCC box and the mutant GCC box was tested by using an electrophoresis mobility shift assay. As shown in Figure 3A, the recombinant JERF3 protein could bind to GCC box, but not to mGCC-box suggesting specific interaction of JERF3 with GCC box *in vitro*.

Evidences have shown that ERF proteins, such as CBF1, Tsi1 and DREB proteins, can bind to

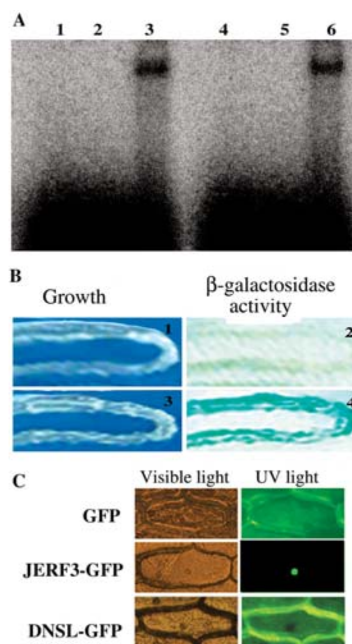

Figure 3. Characterizations of JERF3. (A) Electrophoretic mobility shift assay showing sequence-specific binding of JERF3 protein to GCC box, DRE, mutant GCC box (mGCC box), or mutant DRE (mDRE), respectively. Lane 1 and lane 4 contained only the free probes of GCC box and DRE, respectively, while lane 2 and lane 5 contained 1.2 μg of purified recombinant JERF3 plus labeled mGCC box or mDRE, respectively. The lane 3 and lane 6 contained 1.2 μg of purified recombinant JERF3 plus labeled GCC box and DRE, respectively. (B) Transcriptional activation of LexA DNA binding domain fusion protein with deletion of JERF3 activation domain (JERF3DAD) (panel 1 and 2) or full length JERF3 (panel 3 and 4). Left panels show the growth of yeast cells on selective medium, right panels for the assay of reporter activity. (C) Subcellular localization of the JERF3 protein.

DRE (Stockinger *et al*., 1997; Park *et al*., 2001; Sakuma *et al*., 2002). In order to test whether JERF3 interacts with DRE, we tested the binding affinity of JERF3 with DRE by using an electrophoresis mobility shift assay. Our result indicated that the recombinant JERF3 protein interacted with DRE, but not with the mutant DRE (Figure 3A), further demonstrating that the JERF3 protein not only takes part in GCC box-mediated signaling pathway, but also DRE-mediated signaling.

To investigate the transcriptional activation activity of JERF3, we fused the coding regions for *JERF3* or deletion of the predicted activation domain with the LexA DNA binding domain in pLex A vector. The expression vectors were introduced into yeast strain EGY48 separately, which transformed with the autonomously replicating reporter plasmid p8op-lacZ. By colony-lift filter assay of β-galactosidase, it was found that JERF3 proteins fused to LexA DNA binding domain activated transcription of the *LacZ* reporter, while the fusion of deleted putative activation domain with LexA DNA binding domain did not activate transcription of the *LacZ* reporter (Figure 3B), indicating that JERF3 protein functions as transcription activator in yeast.

Inspection of the amino acid sequence of JERF3 revealed that it has a typical NLS, a stretch of basic amino acids, KKRR, in N-terminal region. To confirm the above analysis, subcellular localization of JERF3-GFP, DNLS-GFP, or GFP was investigated by Agrobacterium-mediated transient expression in onion epidermis cell. Using a fluorescence microscope, the localization of JERF3-GFP fusion protein was localized to the nucleus of the onion epidermal cell. Because of JERF3 NLS deletion, the DNLS-GFP distributed throughout the cell, except for the nucleus, whereas the control GFP was uniformly distributed throughout the cell (Figure 3C), suggesting that JERF3 has the characterization of nuclear localization of transcription factor.

Expression of JERF3 responsive to various stresses and hormones

Plant responses to stresses are regulated by multiple signaling pathways and show significant overlap between the patterns of gene expression in response to different stresses (Singh *et al*., 2002).

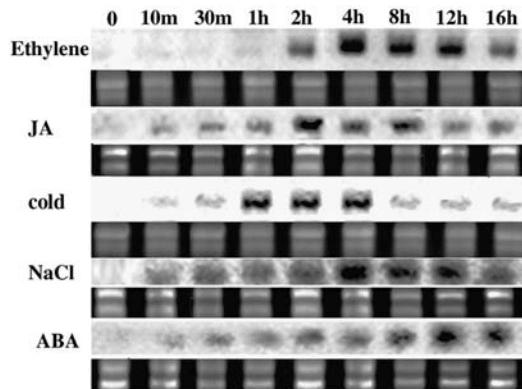

Figure 4. Inducible expression of *JERF3* gene in tomato. Equal loading was verified by visualizing rRNA on a gel stained with ethidium bromide.

To investigate time-dependent induction patterns, the expression of *JERF3* in response to these was further analyzed (Figure 4). *JERF3* transcript was obviously observed after 2 h exposure to ethylene, and accumulated to peak after 4 h treatment. While *JERF3* gene was induced by MeJA treatment within 10 min, and was strongly expressed after 2 h induction. Under low temperature treatment, the *JERF*3 mRNA was accumulated within 10 min and peaked within 1 h. Under high-salt treatment *JERF3* mRNA was accumulated within 10 min and strongly expressed after 4 h. Also the expression of *JERF3* was obviously observed after 30 min, and dramatically decreased after 12 h treatment of abscisic acid (Figure 4), suggesting that JERF3 is involved in multiple signaling pathways responsive to biotic and abiotic stresses.

Constitutive expression of JERF3 and GCC box-containing genes in transgenic JERF3 tobacco

Many *PR* genes, such as *PR1*, *PR2*, *PR3*, and *PR*5, have been characterized to have the GCC box sequence in their promoters, and to increase plant resistance to pathogen attack (Ohme-Takagi and Shinshi, 1995; Gu *et al*., 2000; Park *et al*., 2001). To determine whether JERF3 activates expression of downstream *PR* genes, tobacco transgenic plants were developed that constitutively express JERF3 from the 35S promoter. After antibiotic selection and Northern blot analysis, nine individual overexpressing *JERF3* tobacco lines (designated OE) and five individual expressing *JERF3-DNLS* tobacco lines were obtained. Of four lines overexpressing *JERF3* in tobacco (OE) and three lines expressing *JERF3-DNLS* were confirmed to show 3:1 segregation in T1 seed germination for kanamycin selection. And these one copy insertion transgenic plants were further used in the following experiments. We first tested the expression of downstream genes in transgenic plants. Our result showed that some GCC box-containing *PR* genes such as *osmotin*, *Prb-1b*, *GLA1*, *CHN50* were expressed in unstressed transgenic tobacco plants that constitutively expressed *JERF3* gene, but did not be detect in unstressed wild-type tobacco (WT) and transgenic tobacco plants expressing JERF3-DNLS (NLS was deleted) (Figure 5), suggesting that JERF3 could activate the expression of downstream genes.

Ectopic expression of JERF3 enhances salt tolerance

When ERF gene, *Tsi1*, was overexpressed, the tobacco plants showed enhanced resistance to pathogen attack and osmotic stress (Park *et al*., 2001). This finding correlates with the ability of the Tsi1 protein binding to both GCC box and DRE motifs, demonstrating that these two different stress pathways can be linked by a single ERF protein (Singh *et al*., 2002). The expression of *JERF3* gene in tobacco led to the constitutive expression of several *PR* genes. The *PR* genes, for example, the *osmotin*, are normally upstream

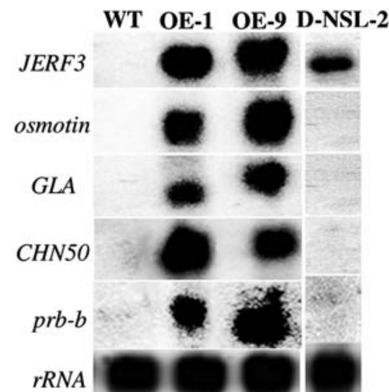

Figure 5. OE plants overexpressing *JERF3* activates constitutively expression of *PR* genes, but DNSL did not. RNA gel blots were hybridized with the probes indicated beside of the panel. Equal loading was verified by 18S rRNA as probe.

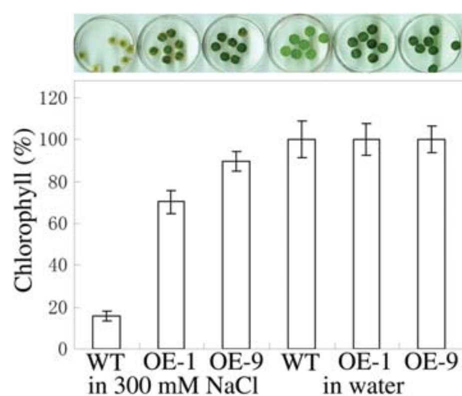

Figure 6. OE plants overexpressing *JERF3* in tobacco enhances salt resistance. Phenotypic differences were observed after 72 h treatment with 300 mM NaCl and chlorophyll contents were measured after 48 h NaCl-treatment. Data are shown as percentage relative to controls from three independent experiments ±SE.

regulated in response to not only pathogen but also osmotic stress. To investigate whether the accumulation of these *PR* genes in plants enhances salt tolerance, we incubated the leaf discs of transgenic tobacco and wilt type plants in NaCl solution. The phenotypic difference between wild-type and transgenic plants is significantly different after 72 h treatment with 300 mM NaCl. The leaf discs from wild type were bleached, while leaf discs from transgenic *JERF3* kept green (upper panel of Figure 6). And the chlorophyll content in these plants after 48 h salt treatment further confirmed the above observed phenotypic differences in the treated leaf discs (lower panel of Figure 6), indicating that ectopic expression of *JERF3* in tobacco confers salt tolerance.

Discussion

Genomic research indicated that there are 124 ERF proteins in *Arabidopsis*, one of the largest transcriptional factor family constituting of up to ~9% of the estimated total transcription factors (Riechmann *et al*., 2000), suggesting that ERF family proteins are important plant specific transcriptional factors. In tomato, though it is not available for the genomic information now, the cloning and identification of ERF proteins has made great progresses (Zhou *et al*., 1997; Tournier *et al*., 2003). Based on the strategy of protein–DNA interaction, we successfully isolated ERF regulatory proteins from tomato that interact with ethylene responsive element GCC box. Data analysis indicated that one of the isolated ERF proteins, JERF3, shows high homologue to the reported ERF proteins only in ERF binding domain, but very low similarity outside the ERF domain, suggesting JERF3 is a novel member of ERF proteins. In tobacco, at least four different ERF proteins, ERF1~4, have been identified (Ohme-Takagi and Shishi, 1995). ERF2 and ERF4 enhance the GCC box-mediated transcription of reporter gene in tobacco protoplasts, suggesting that they act as transcriptional activators (Ohta *et al*., 2000). In contrast to ERF2 and ERF4, ERF3 reduces the transcription of the reporter gene, indicating that ERF3 functions as a repressor. Based on the analysis of predicted protein sequence, JERF3 protein contains an acidic activation domain in C-terminal that act as transcription activation. Actually, JERF3 activates the expression of downstream genes, such as *LacZ* in yeast and *PR* genes in transgenic *JERF3* tobacco, possibly mediating the interaction with GCC box which is very similar to the characterization of tobacco Tsi1 (Park *et al*., 2001). As transcription factor, another important characterization is its nuclear localization. Sequence analysis indicates that JERF3 protein contains a nuclear localization signal in its N-terminal region. And transient expression using JERF3-GFP demonstrated that JERF3 specifically targeted to nucleus of onion epidermal cells. Biochemical analysis further demonstrated that JERF3 not only interacts with ethylene responsive GCC box, but also dehydrate responsive DRE motif, which is similar to the characterization of Tsi1 in tobacco (Park *et al*., 2001), but their protein sequences have only 23.2% similarity. Therefore the isolation and characterization of *JERF3* in this paper will provide more clues to understand the regulation of ERF proteins in various physiological responses.

Many studies showed that ERF overexpression enhanced resistance to specific stress and growth defects through downstream regulation of GCC box or DRE-motif-containing genes (He *et al*., 2001; Park *et al*., 2001; Berrocal-Lobo *et al*., 2002; Wu *et al*., 2002). For example, expression of Pti4 enhances resistance to bacterial growth through activating expression of GCC box-containing genes (Gu, *et al*., 2002). Ectopic expression of rice OsDREB1A in *Arabidopsis* results tolerance to

drought, high-salt, and freezing, via the interaction with DRE (Dubouzet *et al.*, 2003).The JERF3 protein contains a highly conserved ERF domain, which could bind to both the GCC box and DRE, indicating that the JERF3 could induce the expression of both GCC box and DRE-containing genes. In fact, constitutive expression of *JERF3* in transgenic tobacco positively activates GCC box-containing gene expression. And this activation greatly enhances tolerance to high-salt stress, strongly suggesting that the JERF3 protein activates expression of osmotic stress responsive genes. This enhancement of salt tolerance may be resulted by the contribution of osmotin and other osmotic stress-inducible DRE-containing genes. Also we are checking the resistance to diseases. Preliminary research indicated that JERF3 is involved in response to fungi disease attack (RF Huang *et al.*, not published data).

Accumulated evidence has revealed that ERF proteins are an important component in ethylene signaling (Solano *et al.*, 1998; Alonso *et al.*, 2003; Guo and Ecker, 2003; Potuschak *et al.*, 2003), which is up-regulated by EIN3 and EILs (Guo and Ecker, 2004). Binding of ethylene inactivates the receptors, resulting in deactivation of CTR1 (Hua and Meyerowitz, 1998), which allows EIN2 to function as a positive regulator of the ethylene pathway. In this cascade, the EIN2 protein activates EIN3 and, subsequently activating ERF proteins, such as ERF1 (Solano *et al.*, 1998), thereby regulating the expression of genes involved in the response to ethylene (Chakravarthy *et al.*, 2003). JERF3 belongs to the ERF proteins showing distinct regulation in salt resistance through activation of the downstream expression of ethylene responsive GCC box-containing genes, indicating that our novel identified JERF3 might also position downstream of EIN3 in ethylene signaling pathway.

Ethylene, MeJA and ABA play key roles in regulating plant response to various stresses (Dong, 1998; Reymond and Farmer, 1998; Dempsey *et al.*, 1999; Pieterse and van Loon, 1999; Xiong *et al.*, 2002). Multiple signal-transduction pathways seem to converge on ERF proteins and GCC box or DRE through a variety of protein-protein and protein-DNA interactions, suggesting important roles of ERF proteins in control of transcription of genes via targeting *cis*-element for acclimation to environmental changes (Ohme-Takagi *et al.*, 2000). For instance, the transcription factor ERF1 is a downstream component of ethylene and integrates ethylene and jasmonate pathways. *ERF1* expression requires both signaling pathways simultaneously and ERF1 is responsible for the transcription activation of ethylene/jasmonate-dependent defense-related genes. So the ERF1 is a key integrator of ethylene and jasmonate signals in the regulation of ethylene/jasmonate-dependent defenses (Lorenzo *et al.*, 2003), while NPR1 can modulate cross-talk between salicylate- and jasmonate-dependent defense pathways (Splel *et al.*, 2003). Moreover, *Tsi1* gene expression was rapidly induced after salt, wounding, SA, ethylene or methyl jasmonate treatment, suggesting that the Tsi1 protein have a role in the activating expression of GCC box-containing genes in salt and pathogen signal pathways (Park *et al.*, 2001). In this study the expression of *JERF3* was induced by NaCl, ethylene, or JA, which is similar to the expression of *Tsi1*, but it also can be induced by cold and ABA, which is distinct from *Tsi1* (Park *et al.*, 2001). Although these different pathways are usually considered to function independently from each other, it is certainly possible that some cross-talk exists among these pathways. Cross-talk between different pathways provides a 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 (Park *et al.*, 2001). Based on the above discussion we speculate that JERF3 likely plays an important common component in multiple signaling pathways responsive to biotic and abiotic stresses.

Accession numbers

The gene bank accession numbers for the sequences mentioned in this article are AY383630 (*JERF3*), X66942 (*prb-1b*), M60402 (*GLA*), X51599 (*CHN50*), X95308 (*osmotin*).

Acknowledgements

The authors greatly appreciate Dr. Daowen Wang for his discussion about the results, and Dr. Daoxin Xie and Dr. Qiang Liu for their help

for the GCC box bait and tomato cDNA library construction. This work was supported by the Major State Basic Research program of China (G1999011704), Special Foundation of Transgenic Plants in China (JY03A23-02), the Foundation of Third World Academy of Sciences and the National Grand Scientific Engineering Program of China.

References

Alonso, J.M., Stepanova, A.N., Solano, R., Wisman, E., Ferrar, S., Ausubel, F.M. and Ecker, H.R. 2003. Five components of the ethylene-response pathway identified in a screen for week ethylene-insensitive mutants in Arabidopsis. Proc. Natl. Acad. Sci. USA 100: 2992–2997.

Aono, M., Kubo, A., Saji, H., Tanaka, K. and Kondo, N. 1993. Enhanced tolerance to photooxidative stress of transgenic *Nicotiana tabacum* with high chloroplastic glutathione reductase activity. Plant Cell Physiol. 34: 129–136.

Berrocal-Lobo, M., Molina, A. and Solano, R. 2002. Constitutive expression of ethylene-response-factor1 in *Arabidopsis* confers resistance to several necrotrophic fungi. Plant J. 29: 23–32.

Boller, T., Gehri, A., Mauch, F. and Voegeli, U. 1983. Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. Planta 157: 22–31.

Brown, R.L., Kazan, K., McGrath, K.C., Maclean, D.J. and Manners, J.M. 2003. A role for the GCC-box in jasmonate-mediated activation of the *PDF1.2* gene of Arabidopsis. Plant Physiol. 132: 1020–1032.

Chakravarthy, S., Tuori, R.P., D'Ascenzo, M.D., Fobert, P.R., Despres, C. and Martin, G.B. 2003. The tomato transcription factor Pti4 regulates defense-related gene expression via GCC box and non-GCC box cis elements. Plant Cell 15: 3033–3050.

Demekamp, M. and Smeekens, S.C. 2003. Integration of wounding and osmotic stress signals determines the expression of the AtMYB102 transcription factor gene. Plant Physiol. 132: 1415–1423.

Dempsey, D.A., Shah, J. and Klessig, D.F. 1999. Salicylic acid and disease resistance in plants. Crit. Rev. Plant Sci. 18: 547–575.

Dong, X. 1998. SA, JA, ethylene and disease resistance in plants. Curr. Opin. Plant Biol. 1: 316–322.

Dubouzet, J.G., Sakuma, Y., Ito, Y., Kasuga, M., Dubouzet, E.G., Miura, S., Seki, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. 2003. OsDREB genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt-and cold-responsive gene expression. Plant J. 33: 751–763.

Felix, G. and Meins, F. Jr. 1987. Ethylene regulation of β-1,3-glucanase in tobacco. Planta 172: 386–392.

Gu, Y., Yang, C., Thara, Y.K., Zhou, J. and Martin, G.B. 2000. Pti4 is induced by ethylene and salicylic acid, and its product is phosphorylated by the Pto kinase. Plant Cell 12: 771–786.

Gu, Y.Q., Wildermuth, M.C., Chakravarthy, S., Loh, Y.T., Yang, C., He, X., Han, Y. and Martin, G.B. 2002. Tomato transcription factors pti4, pti5, pti6 activate defense responses when expressed in Arabidopsis. Plant Cell 14: 817–831.

Guo, H. and Ecker, J.R. 2003. Plant responses to ethylene gas are mediated by $SCF^{EBF1/EBF2}$-dependent proteolysis of EIN3 transcription factor. Cell 115: 667–677.

Guo, H. and Ecker, J.R. 2004. The ethylene signaling pathway: new insights. Curr. Opin. Plant Biol. 7: 40–49.

Hao, D., Ohme-Takagi, M. and Sarai, A. 1998. Unique mode of GCC box recognition by the DNA-binding factor (ERF domain) in plant. J. Biol. Chem. 273: 26857–26861.

Hao, D., Yamasaki, K., Saraai, A. and Ohme-Takagi, M. 2002. Determinants in the sequence specific binding of two plant transcription factors, CBF1 and NtERF2, to the DRE and GCC motifs. Biochemistry 41: 4202–4208.

He, P., Warren, R.F, Zhao, T., Shan, L., Zhu, L., Tang, X. and Zhou, J.M. 2001. Overexpression of Pti5 in tomato potentiates pathogen-induced defense gene expression and enhances disease resistance to *Pseudomonas syringae* pv. tomato. Mol. Plant Microbe Interact. 14: 1453–1457.

Hua, J. and Meyerowitz, E.M. 1998. Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. Cell 94: 261–271.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. 1998. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell 10: 1391–1406.

Lorenzo, O., Piqueras, R., Sanchez-Serrano, J.J. and Solano, R. 2003. ETHYLENE RESPONSE FACTOR 1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell 15: 165–178.

Ohme-Takagi, M. and Shinshi, H. 1995. Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. Plant Cell 7: 173–182.

Ohme-Takagi, M., Suzuki, K. and Shinshi, H. 2000. Regulation of ethylene-induced transcription of defense genes. Plant Cell Physiol. 41: 1187–1192.

Ohta, M., Ohta-Takagi, M. and Shinshi, H. 2000. Three ethylene-responsive transcription factors in tobacco with distinct transactivation functions. Plant J. 22: 29–38.

Park, J.M., Park, C.J., Lee, S.B., Ham, B.K., Shin, R. and Paek, K.H. 2001. Overexpression of the tobacco *Tsi1* gene encoding an EREBP/AP2-type transcription factor enhances resistance against pathogen attack and osmotic stress in tobacco. Plant Cell 13: 1035–1046.

Pieterse, C.M.J. and van Loon, L.C. 1999. Salicylic acid-independent plant defence pathways. Curr. Rev. Plant Biol. 4: 52–58.

Potuschak, T., Lechner, E., Parmentier, Y., Yanagisawa, S., Grava, S., Koncz, C. and Genschik, P. 2000. EIN3-dependent regulation of plant ethylene hormone signaling by two Arabidopsis F box proteins: EBF1 and EBF2. Cell 115: 679–689.

Reymond, P. and Farmer, E.E. 1998. Jasmonate and salicylate as global signals for defense gene expression. Curr. Opin. Plant Biol. 1: 404–411.

Riechmann, J.L., Heard, J., Martin, G., Reuber, L., Jiang, C.Z., Keddie, J. Adam, L., Pineda, O., Ratcliffe, O.J., Samaha, R.R., Creelman, R., Pilgrin, M., Broun, P., Zhang, J.Z., Ghandehari, D., Sherman, B.K. and Yu, G.-L. 2000. Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. Science 29: 2105–2110.

Sakuma, Y., Liu, Q., Dubouzet, J.G., Abe, H., Shinozaki, K. and Yamaguchi-Shinozaki, K. 2002. DNA-binding specificity of the ERF/AP2 domain of Arabidopsis DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. Biochem. Biophys. Res. Commun. 290: 998–1009.

Singh, K.B., Foley, R.C. and Onate-Sanchez, L. 2002. Transcription factors in plant defense and stress responses. Cur. Opin. Plant Biol. 5: 430–436.

Skriver, K. and Mundy, J. 1990. Gene expression in response to abscisic acid and osmotic stress. Plant Cell 2: 503–512.

Solano, R., Stepanova, A., Chao, Q. and Ecker, J.R. 1998. Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Gen. Dev. 12: 3703–3714.

Splel, S.H., Koornneef, A., Claessens, S.M., Korzelius, J.P., van Pelt, J.A., Mueller, M.J., Buchala, A.J., Metraux, J.P., Brown, R., Kazan, K., van Loo, L.C., Dong, X. and Pieterse, C.M. 2003. NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell 15: 760–770.

Stockinger, E.J., Gilmour, S.J. and Thomashow, M.F. 1997. *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc. Natl. Acad. Sci. USA 94: 1035–1040.

Tournier, B., Sanchez-Ballesta, M.T., Jones, B., Pesquet, E., Regad, F., Latche, A., Pech, J.C. and Bouzayen, M. 2003. New members of the tomato ERF family show specific expression pattern and diverse DNA-binding capacity to the GCC box element. FEBS Lett. 550: 149–154.

Veena, Reddy, V.S. and Sopory, S.K. 1999. Glyoxylase I from *Brassica juncea*: molecular cloning, regulation and its over-expression confer tolerance in transgenic tobacco under stress. Plant J. 17: 385–395.

Wu, K., Tian, L., Hollingworth, J., Brown, D.C. and Miki, B. 2002. Functional analysis of tomato pti4 in *Arabidopsis*. Plant physiol. 128: 30–37.

Xiong L., Schumaker, K.S. and Zhu, J.K. 2002. Cell signaling during cold, drought, and salt stress. Plant Cell 14: S165–S183.

Yang, Y., Li, R. and Qi, M. 2000. *In vivo* analysis of plant promoters and transcription factors by agroinfiltration of tobacco leaves. Plant J. 22: 543–551.

Zhou, J., Tang, X. and Martin, G.B. 1997. The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a *cis*-element of pathogenesis-related genes. EMBO J. 16: 3207–3218.