Ectopic expression of an EAR motif deletion mutant of \textit{SlERF3} enhances tolerance to salt stress and \textit{Ralstonia solanacearum} in tomato

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Abstract Ethylene-responsive transcription factors (ERFs) bind specifically to \textit{cis}-acting DNA regulatory elements such as GCC boxes and play an important role in the regulation of defense- and stress-related genes in plants. In contrast to other ERFs, class II ERFs contain an ERF-associated amphiphilic repression (EAR) domain and act as GCC-mediated transcriptional repressors. In this study, \textit{SlERF3}, a class II ERF was isolated from tomato and characterized. To examine whether the EAR motif of class II ERF proteins participates in ERF-mediated functions in plants, the EAR domain was deleted to generate \textit{SlERF3RD}. We show that \textit{SlERF3RD} protein retains the character of a transcription factor and becomes a GCC-mediated transcriptional activator. Constitutive expression of full-length \textit{SlERF3} in tomato severely suppressed growth and, as a result, no transgenic plants were obtained. However, no apparent effects on growth and development of \textit{SlERF3RD} transgenic plants were observed. Overexpression of \textit{SlERF3ARD} in transgenic tomato induced expression of pathogenesis-related protein genes such as \textit{PR1}, \textit{PR2} and \textit{PR5}, and enhanced tolerance to \textit{Ralstonia solanacearum}. Furthermore, transgenic \textit{Arabidopsis} and tomatoes constitutively expressing \textit{SlERF3ARD} exhibited reduced levels of membrane lipid peroxidation and enhanced tolerance to salt stress. In comparison with wild-type plants grown under stress conditions, transgenic \textit{SlERF3ARD} tomatoes produced more flowers, fruits, and seeds. This study illustrates a gene-enhancing tolerance to both biotic and abiotic stresses in transgenic plants with the deletion of a repressor domain. Our findings suggest that class II ERF proteins may find important use in crop improvement or genetic engineering to increase stress tolerance in plants.

Keywords AP2/ERF · EAR · Pathogen resistance · Repression domain · Salt tolerance

Abbreviations

ERF Ethylene-responsive factor
EAR ERF-associated amphiphilic repression
JA Jasmonic acid
SA Salicylic acid
Introduction

The ethylene-responsive factor (ERF) family, a large transcription factor gene family, belongs to the AP2/ERF superfamily, which is defined by the highly conserved AP2 DNA-binding domain consisting of 60–70 amino acid residues (Jofuku et al. 1994; Sakuma et al. 2002). According to the number of AP2/ERF domains, the AP2/ERF superfamily is divided into ERF, AP2, and RAV families (Sakuma et al. 2002; Nakano et al. 2006). The ERF family is further classified into two subfamilies: dehydration-responsive element-binding protein (DREB) and ERF subfamilies. The former is involved in hormonal signal transduction and plant responses to abiotic stresses (Hsieh et al. 2002b; Narusaka et al. 2003; Qin et al. 2008), and the latter is involved in both plant defense- and stress-signaling pathways (Yang et al. 2005; Onate-Sanchez et al. 2007; Pre et al. 2008).

Previous studies have reported that members of the AP2/ERF superfamily involved in the transcription of downstream genes via binding to cis-acting promoter elements such as GCC, CRT/DRE, JERE, or VWRE (Ohme-Takagi and Shinshi 1995; van der Fits and Memelink 2001; Gu et al. 2002; Sasaki et al. 2007). Based on the amino acid sequence analysis, Fujimoto et al. (2000) and Tournier et al. (2003) categorized ERF proteins into four classes. Among them, class II ethylene-responsive transcription factors (ERFs) contain a conserved repressor domain, L/FDLNL/F(x)P, termed ERF-associated amphiphilic repression (EAR) motif or CMVIII-1 motif, at the C terminus. This group of ERF proteins containing the EAR motif was later classified as B1-1a group (Nakano et al. 2006).

In contrast to other ERFs acting as transcriptional activators, EAR-containing ERFs act as a GCC-mediated transcriptional repressor (Fujimoto et al. 2000; Ohta et al. 2001). Several class II ERFs have been isolated and proved to be transcriptional repressors such as AtERF4, AtERF7, AtERF10, AtERF11, AtERF12, and NtERF3 (Ohta et al. 2001; McGrath et al. 2005). Furthermore, the fusion of different activation domains of various transcription factors with EAR could also repress the transcription of specific target genes (Ohta et al. 2001; Yang et al. 2005), and even result in loss-of-function phenotypes in transgenic plants (Hiratsu et al. 2003). Recently, the EAR motif has been found to convert a transcriptional complex into a transrepressor (Matsui and Ohme-Takagi 2009).

Similar to other AP2/ERF transcription factors, EAR-containing ERFs can play an important role in the regulation of defense- and stress-related genes in plants. For instance, SodERF3 can be induced by ABA, salt, and wounding. Constitutive expression of sugarcane SodERF3 increased tolerance to drought and osmotic stress in transgenic tobacco (Trujillo et al. 2008). The transcripts of cotton GhERF4 gene are rapidly increased after salt, ethylene, cold, drought, and ABA treatment (Jin and Liu 2008). The expression of rice OsBIERF4 genes is induced by salicylic acid (SA) and by Magnaporthe grisea infection (Cao et al. 2006). Additionally, RNA expression of LeERF3b is regulated by fruit ripening and environmental stresses (Chen et al. 2008). The function of EAR-containing genes in response to biotic and abiotic stresses remains to be individually clarified. Recently, repressors have been considered to function as safety controllers that prevent damage from activation of programmed cell death caused by runaway response pathways in plants grown under biotic or abiotic stresses (Thiel et al. 2004; Kazan 2006). Whether the ERF-mediated altered responses of transgenic plants to biotic and/or abiotic stresses are mediated by the EAR motif is unclear.

The aim of the present study is to gain further insight into the function of class II ERFs and the role of the EAR domain in plant response to environmental stresses and pathogen infection. Therefore, the tomato SIERF3 identified by our previous microarray analysis was isolated and analyzed for its expression under biotic and abiotic stresses. In addition, an EAR-deleted version of SIERF3, SIERF3ARD, was generated and characterized for its subcellular localization and transcriptional transactivation activity. Furthermore, transgenic Arabidopsis and tomato plants overexpressing SIERF3ARD were generated and assessed for their response to salinity and bacterium infection. The data obtained in this study demonstrate that EAR-containing proteins may find use in crop improvement for broad-spectrum of stress tolerance through the manipulation of the EAR repressor domain.

Materials and methods

Amino acid alignment and phylogenetic tree

ERF protein sequences were obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), the Arabidopsis Information Resource (http://www.Arabidopsis.org/), the Sol genomic network (http://sgn.cornell.edu/index.pl), and the Rice Genome Annotation (http://rice.plantbiology.msu.edu/). Alignment of amino acid sequence was performed using the Clustal X program (Thompson et al. 1997) and further adjusted by GeneDoc software. The phylogenetic tree analysis was conducted using MEGA3.1 (Kumar et al. 2004). The phylogenetic tree was generated using the neighbor-joining method created with 1,000 bootstrap trials by use of the neighbor-joining algorithm. Percentages of bootstrap values are indicated on the tree.
Plant materials and experimental treatment

Seeds of tomato [Solanum lycopersicon (L.) Miller cv. CL5915-93D4-1-0-3] were kindly provided by the AVRDC-The World Vegetable Center (Tainan, Taiwan). Four-week-old wild-type tomato plants were raised from seeds in controlled environment chambers under a 16-h light/8-h dark cycle at 24°C (about 120 μmol m⁻² s⁻¹), with 50% relative humidity. For chilling, salt, R. solana-cearum, and hormone treatments, plants were grown in soil. Ethephon, an ethylene releaser, was used as ethylene replacement (Zhang and Wen 2009). Ethephon, SA, and jasmonic acid (IA) were applied on tomato leaves by spraying. For drought treatment, plants were air-dried in the growth chamber after removal from Hoagland’s nutrient solution. The gene expression analyses were made using the leaf samples collected after each treatment.

RNA isolation and gene expression analysis

Total RNA isolation and northern blot analysis were performed as described previously (Hsieh et al. 2002a, b). For northern blot analysis, total RNA was separated on a 1% agarose gel and then transferred to a nylon membrane. Probes were labeled with [γ-³²P] dCTP by a random labeling method (Feinberg and Vogelstein 1983). For real-time PCR analysis, quantitative PCR was performed in triplicates with SYBR green on the ABI 7500 Real-Time PCR System (Applied Biosystems, USA) following the ABI standard protocol.

Isolation and generation of SIERF3 and SIERF3ARD

Partial SIERF3 cDNA was identified from subtractive cDNA libraries (Hsieh et al. 2010). Full-length SIERF3 was isolated by 5′- and 3′-RACE with RNA specimen extracted from leaves of salt-treated wild-type plants following the manufacturer’s instructions (Clontech, Palo Alto, CA, USA). The re-amplified full-length SIERF3 was cloned into the pGEM-T easy vector (Promega, USA). To obtain the full-length open reading frame construct, SIERF3 was amplified using SIERF3 F1 and SIERF3 R1 primers. For SIERF3ARD construct, SIERF3ARD was amplified from SIERF3 full-length cDNA using SIERF3 F1 and SIERF3 R2 primers and cloned into the pGEM-T easy vector. All primer sequences are listed in Supplemental Table 1.

Transactivation assays

For transactivation assay, the Luc gene in pJD301 (Luehrsen et al. 1992) was replaced by SIERF3 or SIERF3ARD as the effector plasmids. The GCC box and sequence from the RD29A gene promoter and mutant GCC box were multi-merized four times and placed upstream of the minimal –42 to +8 TATA box from the cauliflower mosaic virus (CaMV) 35S promoter. This construct was substituted for the CaMV 35S promoter in pJD301, and fused to the firefly luciferase (LUC) gene as the reporter plasmid. The pBI221 plasmid containing the β-glucuronidase (GUS) gene driven by the CaMV 35S promoter was used as an internal control (Hsieh et al. 2010). Transactivation assay was performed by the polyethylene glycol-mediated transformation method (Abel and Theologis 1994). Ten micrograms of reporter plasmid and 5 μg of effector plasmid or control plasmid (pUC18) were co-transformed into 4 × 10⁴ protoplasts with 10 μg internal control plasmid pBI221. The transfected cells were incubated at 22°C in light for 18–20 h, harvested by centrifugation at 100 g for 2 min, and lysed in lysis buffer (Promega). Luciferase activity was measured using the Promega luciferase assay kit (E1500) on Luminometer (Berthold, Germany) according to the manufacturer’s instructions, and GUS activity was determined as described (Lu et al. 1998).

Generation and molecular characterization of transgenic plants

SIERF3 and SIERF3ARD were cloned into pCAMBIA1390 driven by the CaMV35S promoter (Hsiao et al. 2007), and transgenic Arabidopsis and tomato were generated by Agrobacterium-mediated transformation as described (Hsieh et al. 2002a, b). Total RNA was isolated from leaves of T₂ transgenic Arabidopsis and tomato, and untransformed plants. Transgenic Arabidopsis was confirmed by RT-PCR with specific primers SIERF3ARD F₁ and Nos-3. Primers for actin (Act) and hygromycin resistance gene (Hpt) are listed in Supplemental Table 1. The probes used for hybridization were tomato β-tubulin, hygromycin resistance gene (Hpt), tomato pathogenesis-related protein 1 (PR1; accession number: AJ011520), PR2 (β-1,3-glucanase, accession number: CK664757), and PR5-like (accession number: AY257487) (Schaller et al. 2000).

Stress response assays and measurement of growth characteristics of transgenic plants

Seeds of transgenic Arabidopsis were surface-sterilized as described (Brini et al. 2007) and grown under a 16-h light/8-h dark cycle at 24°C. For germination assays, seeds were plated for 7 days on MS medium (Murashige and Skoog 1962) containing 150 mM NaCl. For other analyses, 10-day-old Arabidopsis was treated with 150 mM NaCl agar medium for 7 days. The chlorophyll content, fluorescence (F₁/F₀ ratio), and relative malondialdehyde (MDA) level were measured as described (Sanjaya et al. 2008). Transgenic and wild-type tomato were directly sown in soil for 2 weeks and soaked with 250 mM NaCl solution for a few seconds at 2-day intervals for 14 days, and then chlorophyll content and fluorescence were measured.
For bacterial wilt test, 3-week-old transgenic tomato plants whose roots were severed were inoculated with *R. solanacearum* strain Pss4 (race 1, biovar 3) 

(Chan et al. 2005). Wilted symptoms were observed from days 7 to 35 post-inoculation. The growth characteristics were measured for 3-month-old plants (the time includes stress treatment).

**Statistical analysis**

Data were analyzed by a Student’s pair wise *t* test. Statistically significant difference between treatments is indicated as follows: *P* < 0.05 and **P** < 0.01.

**Results**

Isolation of SIERF3 containing the EAR domain

Recently, using microarray data from a subtractive library, it has been shown that the expression of a tomato *ERF* mRNA was highly induced by salt and drought stress (Hsieh et al. 2010). Further characterization reveals that this gene (Unigene number SGN-U315194) encodes a protein called SIERF3. The SIERF3 protein constitutes 210 amino acids with a predicted molecular mass of 23 kDa. Amino acid sequence alignment showed that SIERF3 shares high similarity with LeERF3 (96.4%, GenBank accession number: AY192369, isolated from *S. lycopersicon* cv. Microtom), LeERF2 (94.6%, GenBank accession number: AY275554, isolated from *S. lycopersicon* cv. Lichun), and LeERF2b (94.6%, GenBank accession number: AY559314, isolated from *S. lycopersicon* Mill cv. Alisa Craig) (Tournier et al. 2003; Zhang et al. 2005; Chen et al. 2008) (Supplemental Fig. 1). The variance in the four genes might result from different tomato cultivars or sequencing errors; alternatively, it might represent different genes with similar transcripts in tomato.

To determine the relationship between SIERF3 and other ERFs, alignment and phylogenetic analyses were carried out. Tomato ERFs identified from recent studies (Tournier et al. 2003; Wang et al. 2004; Zhang et al. 2004, 2005), class II ERFs of *Arabidopsis* (Fujimoto et al. 2000), rice subgroup VIIIa ERFs (Nakano et al. 2006), and ERF-containing genes from various species were analyzed. SIERF3 shares 40–59% identity with NtEREBP5, CsERF1, AtERF11, and GmEREB4 (Fig. 1a). Phylogenetic tree analysis revealed that SIERF3 is most similar to tobacco NtEREBP5 (Fig. 1b). We should note that there are two entries of LeERF2 present in GenBank: one (GenBank accession number: AY275554) (Zhang et al. 2005) contains an EAR motif which is highly similar to SIERF3, the other (GenBank accession number: AY192368) (Tournier et al. 2003; Zhang et al. 2009; Zhang and Huang 2010) contains no EAR motif (Supplemental Fig. 1). Although the LeERF2 (AY192368) has been evidenced to modulate ethylene biosynthesis to enhance freezing tolerance (Pirrello et al. 2006; Zhang et al. 2009; Zhang and Huang 2010), the functions of SIERF3, LeERF3, LeERF3b, and LeERF2 (AY275554) remain unknown.

**SIERF3** expression is induced by biotic, abiotic stresses and hormones

ERFs have been shown to play a direct regulatory role in response to multiple signal stimulation. To clarify the potential function of SIERF3 in response to different stimuli, we analyzed the temporal expression patterns of SIERF3 in tomato leaves under various biotic and abiotic stress conditions using RNA gel blot analysis. As shown in Fig. 2a, the SIERF3 transcript could barely be detected in leaves in the absence of stress conditions (designated as 0 h). However, under chilling, drought, and salt treatments, SIERF3 transcripts accumulated substantially within 1 h and peaked at 2, 24, and 12 h, respectively. In addition, SIERF3 expression was induced within 12 h after challenge with the bacterial pathogen *R. solanacearum* and this induction was maintained at about the same level for at least 2 days (Fig. 2b). The inductions of ethylene, JA, and SA have been shown to correlate with the onset of plant defense responses (Koornneef and Pieterse 2008). Therefore, we used quantitative RT-PCR to test the expression patterns of SIERF3 after exogenous application of ethphon, an ethylene releaser, JA, and SA. As shown in Fig. 2c, the SIERF3 transcripts were barely affected by ethphon within the first 8 h and increased moderately after 24 h of treatment. By contrast, JA treatment resulted in a rapid accumulation of SIERF3 transcripts, followed by a fast reduction of expression to a level below (4 and 8 h) and equivalent to (24 h) SIERF3 expression in the control group. SA application led yet again to a different expression pattern: the expression of SIERF3 increased moderately and rapidly, remained constant for several hours, fell below control levels at 8 h of treatment, and showed a strong increase after 24 h.

**SIERF3**ΔRD acts as a GCC-mediated transcriptional activator

Sequence analysis showed that SIERF3 contains an EAR motif. To understand the function of the EAR domain within SIERF3, we generated full-length SIERF3 cDNA and EAR motif-deleted cDNA (SIERF3ΔRD). As the nuclear localization sequence of ERF family proteins is likely located within the AP2/ERF domain (Matsuo and Banno 2008), deletion of the EAR motif from SIERF3
should not affect the nuclear localization of SIERF3ΔRD.
Indeed, no difference in localization between SIERF3 and SIERF3ΔRD could be detected (Supplemental Fig. 2).

The EAR domain was suggested to be responsible for the GCC-mediated transcriptional repression of AP2/ERF proteins (Ohta et al. 2001; Song et al. 2005). Therefore,
effector plasmids with \( \text{SlERF3} \) or \( \text{SlERF3}^{\text{RD}} \) (Fig. 3a) were used to perform transactivation assay in \textit{Arabidopsis} protoplasts. A reporter gene with four tandem copies of the GCC box or a mutated GCC box (mGCC) was also used (Fig. 3b). Similar to other class II ERFs that act as transcriptional repressors, SlERF3 appeared to repress reporter gene expression since in its presence luciferase expression was reduced to 30% of the control level, whereas \( \text{SlERF3}^{\text{RD}} \) led to a 3.8-fold higher transactivation activity as compared with the control (Fig. 3c). By contrast, luciferase expression remained unchanged in reporter constructs \( 35\Sigma \text{m} \) and \( \text{mGCC}35\Sigma \) in the absence or presence of effector. These data indicate that the EAR motif is also responsible for transcriptional activation/repression of tomato AP2/ERF genes.

\( \text{SlERF3}^{\text{RD}} \) transgenic tomato exhibits increased pathogenesis-related (\( \text{PR} \)) gene expression and enhanced resistance to \( \text{R. solanacerum} \)

In order to understand how the EAR domain of SlERF3 contributes to plant stress response, we generated transgenic tomato plants with constitutive expression of \( \text{SlERF3} \) or \( \text{SlERF3}^{\text{RD}} \). However, \( \text{SlERF3} \) transgenic tomato was difficult to shoot and no transgenic plant was obtained under both selection medium and normal growth condition (Supplemental Fig. 3). Therefore, further experiments of \( \text{SlERF3} \) transgenic tomato under stresses were prohibited. On the other hand, no apparent effects on growth and development of \( \text{SlERF3}^{\text{RD}} \) transgenic plants were observed. After antibiotic selection and genomic PCR of several \( \text{SlERF3}^{\text{RD}} \)-overexpressing tomatoes, four lines (ER3, ER8, ER10, and ER11) were selected for northern blot analysis (Fig. 4a). It has been suggested that ERF proteins may play a role in the regulation of pathogenesis-related (PR) genes containing GCC boxes, including \( \text{PR1}, \text{PR2}, \text{PR3}, \) and \( \text{PR5} \), and thus may increase plant resistance to pathogen attack (Ohme-Takagi and Shinshi 1995; Gu et al. 2000; Park et al. 2001). To test whether the \( \text{SlERF3}^{\text{RD}} \) protein can enhance downstream PR genes expression and pathogen resistance, homozygous progenies of \( \text{SlERF3}^{\text{RD}} \) transgenic plants were subjected to further analyses of PR gene expression. As shown in Fig. 4a, expression of \( \text{PR1}, \text{PR2}, \) and \( \text{PR5} \) genes, which
To examine whether expression of $SIERF3AR$ in tomato plants can enhance pathogen resistance, we performed a pathogen inoculation assay. To this end, transgenic and wild-type tomato plants were inoculated with a virulent strain of $R. solanacearum$, Pss4, by soil-drenching. As shown in Fig. 4b, 70% of the wild-type plants displayed typical wilting symptoms 14 days post-inoculation, while only 20% of the homozygous $SIERF3AR$ transgenic plants showed a wilting phenotype. The enhancement of disease-tolerant phenotype was further confirmed by disease incidence assay. All of the wild-type plants wilted 28 days after inoculation, whereas only 30% of the transgenic plants showed symptoms 35 days post-inoculation (Fig. 4c). Consistent with these results, the photosynthetic efficiency ($F_v/F_m$ ratio) and chlorophyll content of $SIERF3AR$ transgenic plants after bacterium infection were higher compared to wild-type plants (Fig. 4d, e), indicating that the level of cellular damage due to pathogen infection was much lower in the transgenic lines as compared to wild-type plants. Taken together, the results demonstrate an enhanced disease tolerance conferred by the overexpression of $SIERF3AR$ protein in tomato plants.

Constitutive expression of $SIERF3AR$ enhances salt tolerance in transgenic Arabidopsis

It has been reported that the expression of several GCC box-containing PR genes (e.g., osmotin) is normally regulated upstream in response to not only pathogen but also osmotic stress (Jia and Martin 1999). To test whether expression of $SIERF3AR$ changes the response of a heterologous plant to salt stress, three $SIERF3AR$ transgenic Arabidopsis lines (AER1, AER2, and AER3) were selected and analyzed. The constitutive overexpression of $SIERF3AR$ under normal growth conditions was confirmed by RT-PCR (Fig. 5a). Under salt stress, the $SIERF3AR$ transgenic Arabidopsis showed normal germination and growth, while the germination and growth of the wild-type plants and the vector-only transgenic line (1301) were inhibited (Fig. 5b). In addition, the photosynthetic efficiency and chlorophyll content of $SIERF3AR$-overexpressed lines were significantly higher than that of the control plants under high salt conditions (Fig. 5c, d). In addition, no photosynthetic defect associated with overexpression of $SIERF3AR$ under normal conditions was detected. Our data clearly demonstrate that overexpression of $SIERF3AR$ enhanced salt tolerance in transgenic Arabidopsis plants.

Ectopic expression of $SIERF3AR$ enhances salt tolerance in transgenic tomato

$SIERF3AR$ transgenic tomatoes were further subjected to analysis for their response to salinity. While wild-type
plants wilted and showed necrotic and bleached leaves 14 days after treatment with 250 mM NaCl, all of the transgenic plants (ER3, ER8, and ER11) remained healthy, with no signs of phenotypic damages (Fig. 6a). The photosynthetic efficiency (Fig. 6b) and chlorophyll content (Fig. 6c) of transgenic tomato were also higher than the corresponding values of wild-type plants under salinity treatment.

To further characterize these salt-tolerant transgenic plants, the level of malondialdehyde (MDA), an indicator of lipid peroxidative damage in plant tissues, was measured. We found that transgenic SIERF3ARD-expressing seedlings had significant lower MDA levels compared to wild-type seedlings under salt stress (Fig. 6d). The reduction in MDA levels indicates a decrease in lipid peroxidation in transgenic plants overexpressing SIERF3ARD.

These results, combined with the results described above, clearly show that the expression of SIERF3ARD improves salt tolerance in transgenic tomato.

Growth characteristics of SIERF3ARD transgenic tomato

The enhancement of tolerance to salt and R. solanacearum implied that SIERF3ARD may be a good candidate for tomato improvement. To further examine how SIERF3ARD affects tomato quality, several growth characteristics of wild-type and SIERF3ARD transgenic tomato under different treatments were measured. Under normal growth conditions, SIERF3ARD overexpression lines showed no significant difference with wild type in fruit number, seed number, and fresh weight. However, after

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Fig. 4 Overexpression of SIERF3ARD enhanced tolerance to bacterial wilt in transgenic tomato. a Northern blot analysis of SIERF3ARD, Hpt, PR1, PR2, and PR5 expression in wild-type plants and in SIERF3ARD transgenic lines. β-tubulin was used as a loading control. b Phenotype of 3-week-old transgenic tomato and wild-type plants treated with H2O (left panel) or a virulent strain of R. solanacearum by root invasion for 14 days (right panel). c Percentage of wilted plants at different time points of infection. Wilted plants were defined as plants that showed more than 50% of leafs with wilted symptoms. Data were collected from at least 20 plants for each line. Three independent experiments were performed. d PSII photochemical efficiency (Fv/Fm ratio) and e Chlorophyll content of SIERF3ARD transgenic lines and wild type were measured at day 7 post-inoculation (**P < 0.01)
**Discussion**

Cross talk between induced ethylene, SA, and JA defense-signaling pathways is thought to contribute to induction of a powerful defense response in plants (Koornneef and Pieterse 2008). ERF genes have been proven to play key roles as regulators in three defense-signaling pathways. Two ERFs, ERF1 and ORA59, individually integrate defense signals from ethylene (ET) and jasmonate pathways and induce downstream defense-related genes including plant defensin1.2 (PDF1.2) (Lorenzo et al. 2003; Pre et al. 2008). Members of the ERF family can control defense genes positively or negatively. For example, the expression of PDF1.2, the marker gene of the ET and JA defense pathways, is induced by constitutive overexpression of ERF2 but repressed by overexpression of ERF4 in transgenic plants (Brown et al. 2003; McGrath et al. 2005). Usually, EAR-containing ERFs are involved in the repression mechanism (Ohta et al. 2001; McGrath et al. 2005).
One example is AtERF7 which has been suggested to recruit a co-repressor and a histone deacetylase to block transcriptional activation (Thiel et al. 2004; Song et al. 2005). In our study, tomato SlERF3 contains an EAR motif. Our results show that overexpression of SlERF3 leads to repression of GCC-mediated transcription, suggesting that SlERF3 might act as an active repressor. Here, we showed that expression of PR1, PR2, and PR5 genes were significantly induced in SlERF3 

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Data in each column show, from top to bottom, fruit number (FN) per plant, seed number (SN) per fruit, and fresh weight (FW, g) per plant. Each value represents the mean ± standard deviation (n = 30 individual plants). Measured plants were 3 months old, which includes the time of stress treatment.

The genetic modification of higher plants through gene engineering has become a valuable tool for the development of pathogen-resistant or stress-tolerant plants. Sweet pepper ferredoxin-like protein ( pfpl ) gene increased the tolerance of orchid ( Oncidium ) to Erwinia carotovora, a plant pathogen with a wide host range ( Liao et al. 2003; You et al. 2003 ). Overexpression of Arabidopsis transgenes was shown to confer resistance to calcium stress ( Sanjaya et al. 2008 ). In this paper, we demonstrated that a tomato gene reversed its role from a transcriptional repressor to an activator after repressor-domain deletion. Overexpression of SlERF3ARD enhanced tolerance to salinity and to pathogen infection in transgenic tomatoes, while agronomical traits were largely maintained. Thus, EAR motif-containing genes could be new candidates for crop improvement or plant breeding programs aimed at developing plants with superior, broad-range stress tolerance traits.

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