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ORIGINAL ARTICLE

# Ectopic expression of an EAR motif deletion mutant of *SlERF3* enhances tolerance to salt stress and *Ralstonia solanacearum* in tomato

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**Abstract** Ethylene-responsive transcription factors (ERFs) bind specifically to *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, *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 SlERF3ΔRD. We show that SlERF3ΔRD protein retains the character of a transcription factor and becomes a GCC-mediated transcriptional activator. Constitutive expression of full-length *SlERF3* in tomato severely suppressed growth and, as a result, no transgenic plants were obtained. However, no apparent effects on growth and development of *SlERF3ΔRD* transgenic plants were observed. Overexpression of *SlERF3ΔRD* in transgenic tomato induced expression of pathogenesis-related protein genes such as *PR1*, *PR2* and *PR5*, and enhanced tolerance to *Ralstonia solanacearum*. Furthermore, transgenic *Arabidopsis* and tomatoes constitutively expressing *SlERF3ΔRD* exhibited reduced levels of membrane lipid peroxidation and enhanced tolerance to salt stress. In comparison with wild-type plants grown under stress conditions, transgenic *SlERF3ΔRD* 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 |

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## 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 *SlERF3* 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 *SlERF3*, *SlERF3ΔRD*, was generated and characterized for its subcellular localization and transcriptional transactivation activity. Furthermore, transgenic *Arabidopsis* and tomato plants overexpressing *SlERF3ΔRD* 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. CL5915-93$D_4$-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. solanacearum*, 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 (JA) were applied on tomato leafs 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 [$\alpha$-$^{32}$P] 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 *SlERF3* and *SlERF3ΔRD*

Partial *SlERF3* cDNA was identified from subtractive cDNA libraries (Hsieh et al. 2010). Full-length *SlERF3* was isolated by 5′- and 3′-RACE with RNA specimen extracted from leaves of salt-treated wild-type plants following the manufacture's instructions (Clontech, Palo Alto, CA, USA). The re-amplified full-length *SlERF3* was cloned into the pGEM-T easy vector (Promega, USA). To obtain the full-length open reading frame construct, *SlERF3* was amplified using SlERF3 F1 and SlERF3 R1 primers. For *SlERF3ΔRD* construct, *SlERF3ΔRD* was amplified from *SlERF3* full-length cDNA using SlERF3 F1 and SlERF3 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 *SlERF3 or SlERF3ΔRD* as the effector plasmids. The GCC box and sequence from the RD29A gene promoter and mutant GCC box were multimerized 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-transfected into $4 \times 10^4$ 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

*SlERF3* and *SlERF3ΔRD* 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 $T_2$ transgenic *Arabidopsis* and tomato, and untransformed plants. Transgenic *Arabidopsis* was confirmed by RT-PCR with specific primers *SlERF3ΔRD* $F_1$ 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 ($F_v/F_m$ 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) ($A_{600} = 0.6$) by soil-drenching (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 *SlERF3* 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 SlERF3. The SlERF3 protein constitutes 210 amino acids with a predicted molecular mass of 23 kDa. Amino acid sequence alignment showed that SlERF3 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 LeERF3b (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 SlERF3 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 EAR-containing genes from various species were analyzed. SlERF3 shares 40–59% identity with NtEREBP5, CsERF1, AtERF11, and GmEREB4 (Fig. 1a). Phylogenetic tree analysis revealed that SlERF3 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 SlERF3, 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 SlERF3, LeERF3, LeERF3b, and LeERF2 (AY275554) remain unknown.

### *SlERF3* 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 SlERF3 in response to different stimuli, we analyzed the temporal expression patterns of *SlERF3* in tomato leaves under various biotic and abiotic stress conditions using RNA gel blot analysis. As shown in Fig. 2a, the *SlERF3* transcript could barely be detected in leaves in the absence of stress conditions (designated as 0 h). However, under chilling, drought, and salt treatments, *SlERF3* transcripts accumulated substantially within 1 h and peaked at 2, 24, and 12 h, respectively. In addition, *SlERF3* 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 *SlERF3* after exogenous application of ethephon, an ethylene releaser, JA, and SA. As shown in Fig. 2c, the *SlERF3* transcripts were barely affected by ethephon within the first 8 h and increased moderately after 24 h of treatment. By contrast, JA treatment resulted in a rapid accumulation of *SlERF3* transcripts, followed by a fast reduction of expression to a level below (4 and 8 h) and equivalent to (24 h) *SlERF3* expression in the control group. SA application led yet again to a different expression pattern: the expression of *SlERF3* 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.

### *SlERF3ΔRD* acts as a GCC-mediated transcriptional activator

Sequence analysis showed that SlERF3 contains an EAR motif. To understand the function of the EAR domain within SlERF3, we generated full-length *SlERF3* cDNA and EAR motif-deleted cDNA (*SlERF3ΔRD*). 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 SlERF3

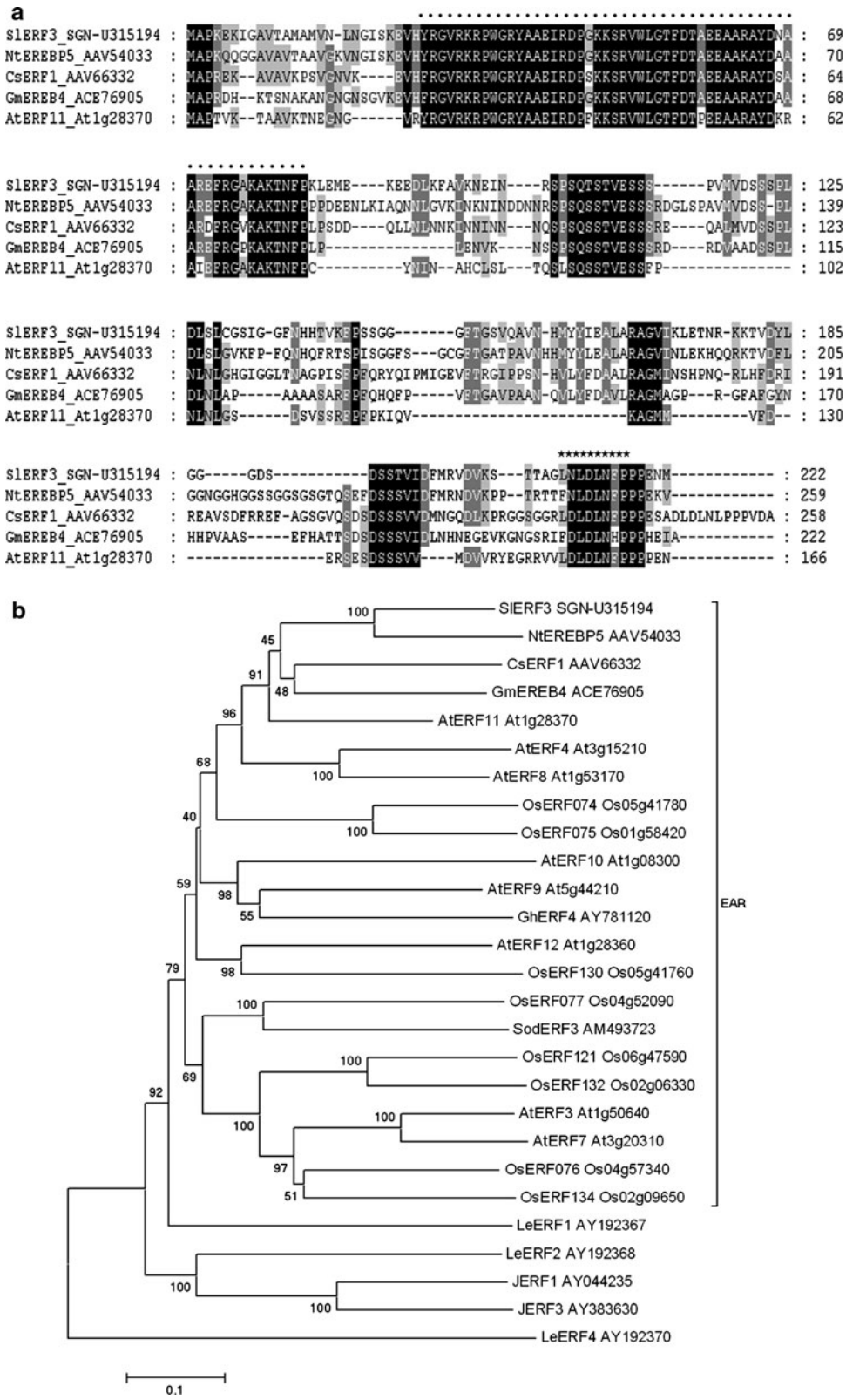


**Fig. 1** Amino acid alignment and phylogenetic tree of SlERF3 and ERF proteins from various species. **a** Comparison of the derived amino acid sequence of selected EAR motif-containing genes that have highly sequence similarity with tomato SlERF3. *Dots* indicate the conserved AP2/ERF DNA-binding domain and *asterisks* mark the EAR motif. Amino acids identical in all proteins are shown in *black*; those conserved in at least three sequences are *shaded*. **b** Phylogenic comparison of SlERF3 protein and some ERF-related proteins. The ERF proteins used for construction of phylogenetic trees are identified from tomato (SlERF3, LeERFs, and JERFs), Arabidopsis (AtERFs), rice (OsERFs), *Cucumis sativus* (CsERF1), *Glycine max* (GmEREBP4), *Gossypium hirsutum* (GhERF4), *Nicotiana tabacum* (NtEREBP5), and *Saccharum officinarum* (SodERF3). Proteins containing EAR motifs are labeled as EAR

should not affect the nuclear localization of SlERF3ΔRD. Indeed, no difference in localization between SlERF3 and SlERF3ΔRD 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,

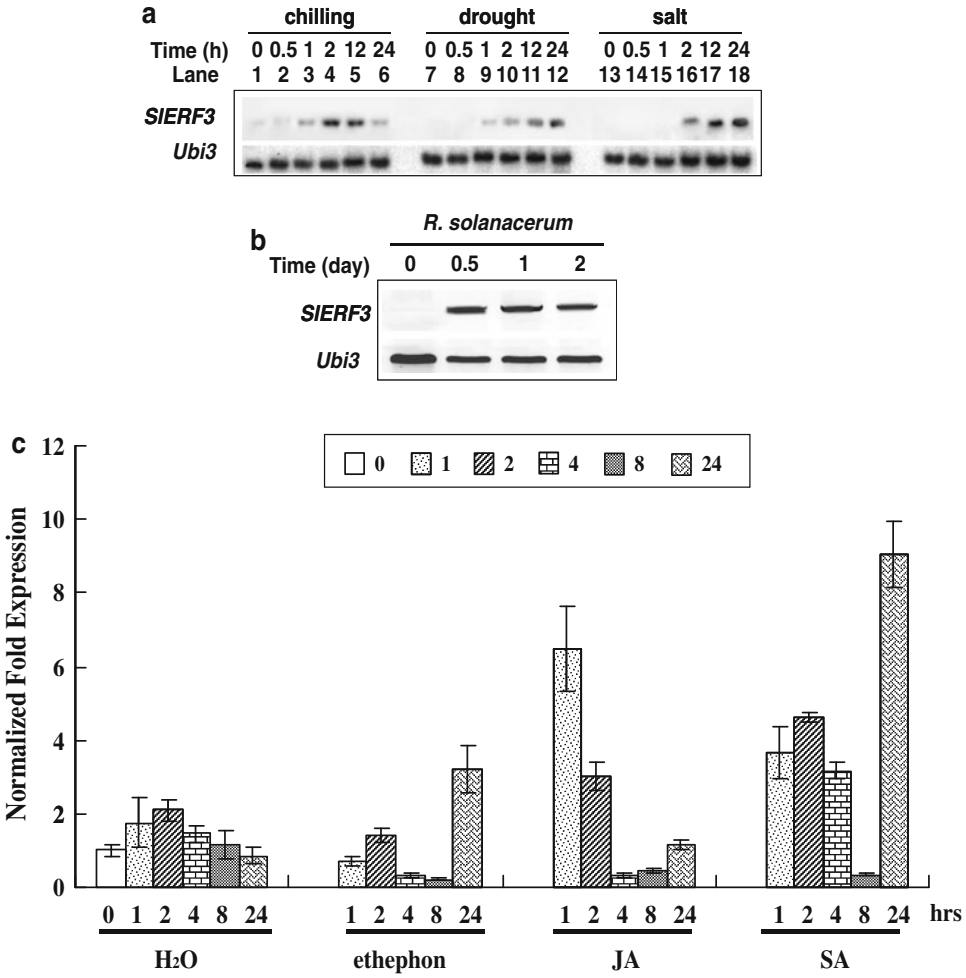


**Fig. 2** Expression patterns of *SlERF3* under biotic and abiotic stresses, and hormone treatments, as assessed by northern blot analysis or real-time RT-PCR. **a** Induction of *SlERF3* after chilling (4°C), drought (air-drying), or salt treatments (200 mM NaCl). **b** Induction of *SlERF3* after root-invading inoculation with the pathogen *R. solanacearum*. Ubi3 was used as an internal control in both panels. **c** Relative expression levels of *SlERF3* after treatment with 1 mM ethephone, 0.1 mM methyl jasmonic acid (JA), or 1 mM salicylic acid (SA). Relative transcripts levels were measured by real-time PCR and normalized by that of the reference gene

effector plasmids with *SlERF3* or *SlERF3ΔRD* (Fig. 3a) were used to performed transactivation assay in *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 SlERF3ΔRD 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 35Sm and mGCC35 m 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.

## *SlERF3ΔRD* transgenic tomato exhibits increased pathogenesis-related (*PR*) gene expression and enhanced resistance to *R. solanacearum*

In order to understand how the EAR domain of SlERF3 contributes to plant stress response, we generated transgenic tomato plants with constitutive expression of *SlERF3* or *SlERF3ΔRD*. However, *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 *SlERF3* transgenic tomato under stresses were prohibited. On the other hand, no apparent effects on growth and development of *SlERF3ΔRD* transgenic plants were observed. After antibiotic selection and genomic PCR of several *SlERF3ΔRD*-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 pathogenesis-related (PR) genes containing GCC boxes, including *PR1*, *PR2*, *PR3*, and *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 SlERF3ΔRD protein can enhance downstream PR genes expression and pathogen resistance, homozygous progenies of *SlERF3ΔRD* transgenic plants were subjected to further analyses of PR gene expression. As shown in Fig. 4a, expression of *PR1*, *PR2*, and *PR5* genes, which

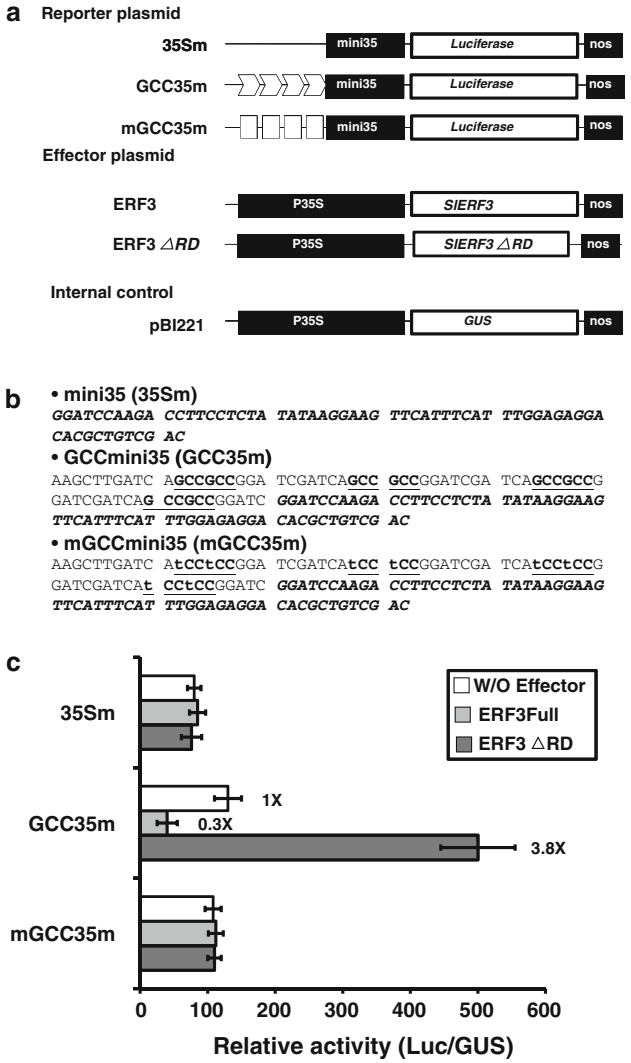


**Fig. 3** Transactivation of GCC-mediated transcription by *SlERF3* or *SlERF3ΔRD* in *Arabidopsis* protoplasts. **a** Schematic diagram of reporter, effector and internal control plasmid constructs. Reporter plasmids GCC35m and mGCC35m contain four repeats of the wild-type or mutant GCC box sequence. 35Sm and mini35 denote unmodified and minimal CaMV35S promoter, respectively. GUS, beta-glucuronidase. **b** Partial sequences of reporter constructs. Sequences denoting the CaMV35S minimal promoter are shown in *italics* and in *boldface*, while GCC boxes are *underlined* and in *boldface*. **c** Relative luciferase activities in transactivation assays. The effector plasmid encoding *SlERF3* or *SlERF3ΔRD*, and the reporter plasmid were co-transfected into protoplasts with internal control plasmid by polyethylene glycol-mediated transformation. Transcriptional activation is expressed as ratio of Luciferase (Luc) to GUS activity

contain a GCC box in their promoters, was greatly increased in *SlERF3ΔRD* transgenic tomato but barely detected in wild-type plants grown under regular conditions. These data indicate that SlERF3ΔRD could act as a transcriptional activator to increase expression of defense genes.

To examine whether expression of *SlERF3ΔRD* 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 *SlERF3ΔRD* 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 *SlERF3ΔRD* 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 SlERF3ΔRD protein in tomato plants.

## Constitutive expression of *SlERF3ΔRD* 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 *SlERF3ΔRD* changes the response of a heterologous plant to salt stress, three *SlERF3ΔRD* transgenic *Arabidopsis* lines (AER1, AER2, and AER3) were selected and analyzed. The constitutive overexpression of *SlERF3ΔRD* under normal growth conditions was confirmed by RT-PCR (Fig. 5a). Under salt stress, the *SlERF3ΔRD* 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 *SlERF3ΔRD*-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 *SlERF3ΔRD* under normal conditions was detected. Our data clearly demonstrate that overexpression of *SlERF3ΔRD* enhanced salt tolerance in transgenic *Arabidopsis* plants.

## Ectopic expression of *SlERF3ΔRD* enhances salt tolerance in transgenic tomato

*SlERF3ΔRD* transgenic tomatoes were further subjected to analysis for their response to salinity. While wild-type

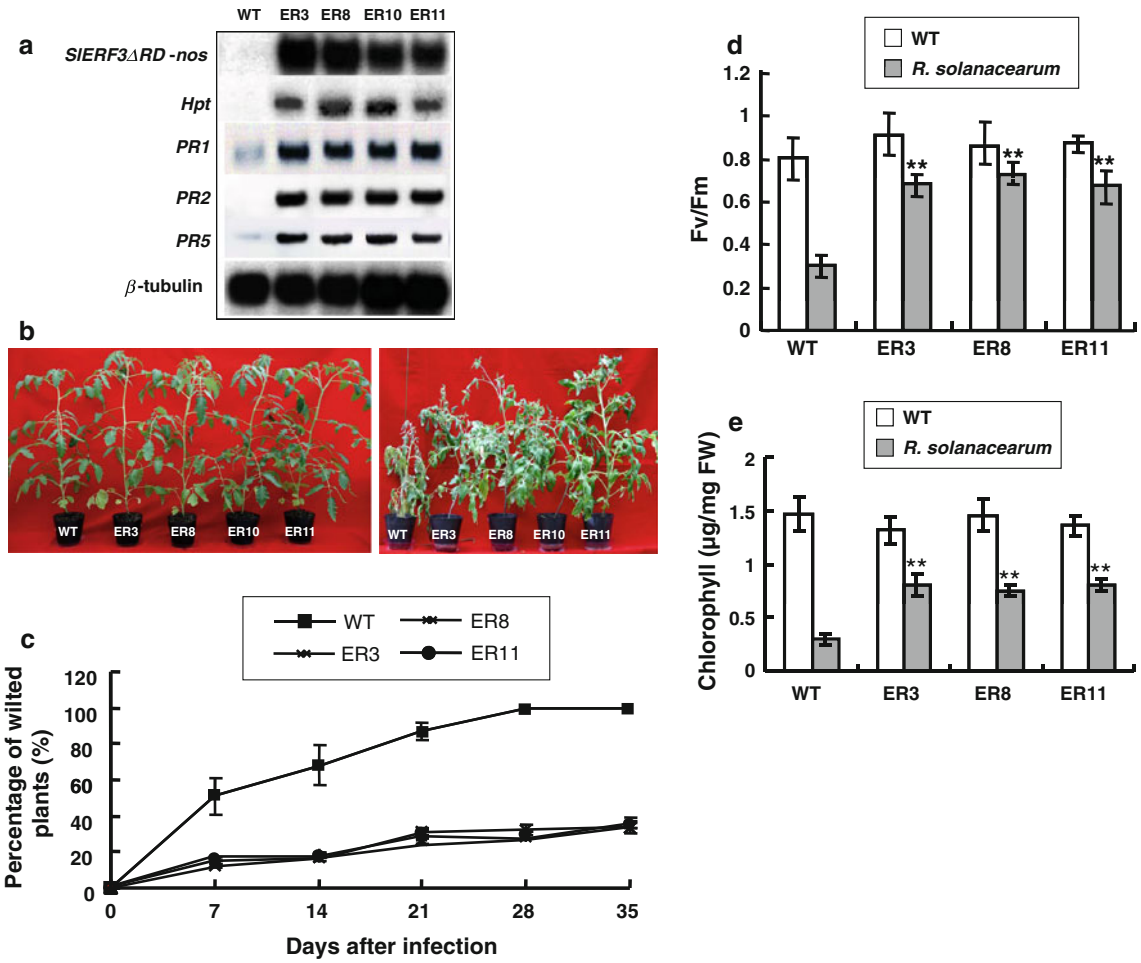


**Fig. 4** Overexpression of *SlERF3ΔRD* enhanced tolerance to bacterial wilt in transgenic tomato. **a** Northern blot analysis of *SlERF3ΔRD*, *Hpt, PR1*, *PR2*, and *PR5* expression in wild-type plants and in *SlERF3ΔRD* transgenic lines. *β*-tubulin was used as a loading control. **b** Phenotype of 3-week-old transgenic tomato and wild-type plants treated with $H_2O$ (*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 ($F_v/F_m$ ratio) and **e** Chlorophyll content of *SlERF3ΔRD* transgenic lines and wild type were measured at day 7 post-inoculation ($^{**}P < 0.01$)

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 *SlERF3ΔRD*-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 *SlERF3ΔRD*. These results, combined with the results described above, clearly show that the expression of *SlERF3ΔRD* improves salt tolerance in transgenic tomato.

## Growth characteristics of *SlERF3ΔRD* transgenic tomato

The enhancement of tolerance to salt and *R. solanacearum* implied that *SlERF3ΔRD* may be a good candidate for tomato improvement. To further examine how *SlERF3ΔRD* affects tomato quality, several growth characteristics of wild-type and *SlERF3ΔRD* transgenic tomato under different treatments were measured. Under normal growth conditions, *SlERF3ΔRD* overexpression lines showed no significant difference with wild type in fruit number, seed number, and fresh weight. However, after

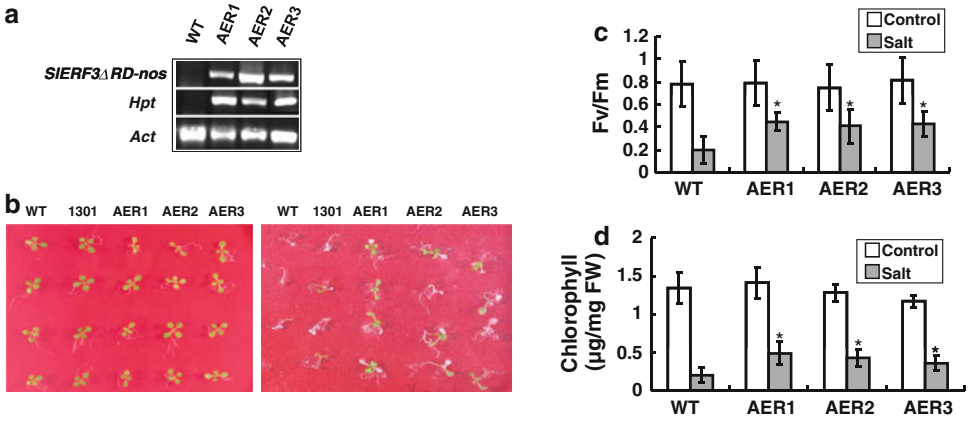


**Fig. 5** *SlERF3ΔRD* transgenic *Arabidopsis* enhanced tolerance to salt stress. **a** RT-PCR analysis of *SlERF3ΔRD* and hygromycin resistance gene (*Hpt*) expression in wild-type and in transgenic lines. **b** Phenotype of *SlERF3ΔRD* transgenic, wild type, and 1301 (vector-only transgenic line) *Arabidopsis* seeds germinated for 7 days on MS medium in the absence (*left panel*) or presence of 150 mM NaCl (*right panel*). **c** PSII photochemical efficiency of *SlERF3ΔRD* transgenic lines and wild type. **d** Chlorophyll content of *SlERF3ΔRD* transgenic lines and wild type (*$P < 0.05$)

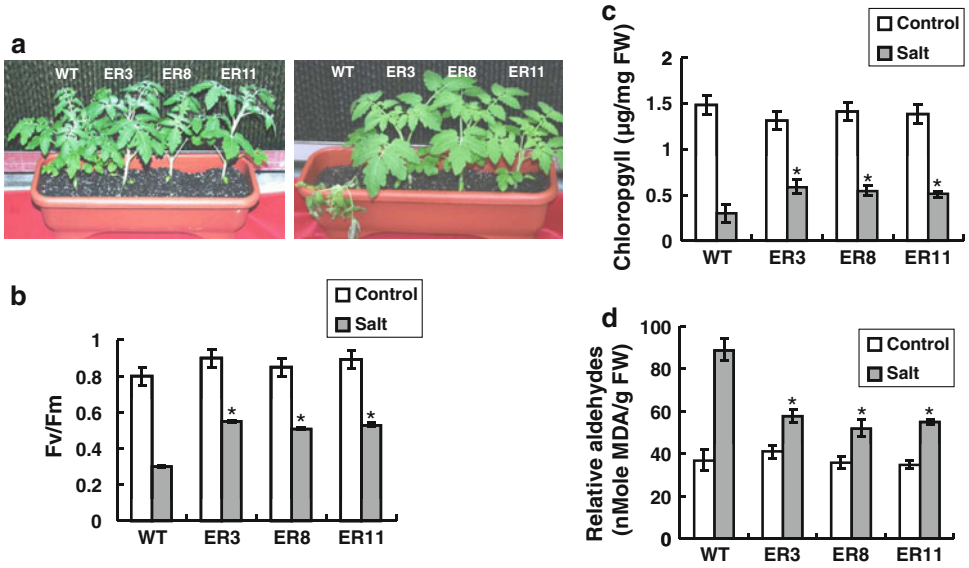


**Fig. 6** Overexpression of *SlERF3ΔRD* enhanced salt tolerance in transgenic tomato. **a** Phenotype of 2-week-old *SlERF3ΔRD* transgenic and wild-type tomato plants treated with $H_2O$ (*left panel*) or 250 mM NaCl (*right panel*) for 14 days. **b** PSII photochemical efficiency of *SlERF3ΔRD* transgenic lines and wild type. **c** Chlorophyll content of *SlERF3ΔRD* transgenic lines and wild type. **d** Lipid peroxidation expressed as malonaldehyde (MDA) content in *SlERF3ΔRD* transgenic lines and wild type (*$P < 0.05$)

*R. solanacearum* infection, wild-type tomato produced no fruit or seeds, while the three growth characteristics of *SlERF3ΔRD* transgenic lines were maintained to 60–70%. Under salt stress, wild-type tomato grew few fruits and seeds. *SlERF3ΔRD* transgenic lines produced much more tomato fruits and seeds compared with wild-type (Table 1). These data imply that ectopic expression of *SlERF3ΔRD* not only enhances salt and *R. solanacearum* tolerance but also maintains important agronomic traits of tomato.

## 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).

**Table 1** Effects of various treatments on the growth characteristics in transgenic and wild-type tomato plants

| Treatment | WT | E3 | E8 | E11 |
|---|---|---|---|---|
| Control | 20.3 ± 2.0 | 24.8 ± 3.6 | 22.4 ± 3.2 | 17.4 ± 5.8 |
| | 23.5 ± 9.2 | 21.2 ± 3.0 | 20.6 ± 2.2 | 20.0 ± 1.3 |
| | 168.4 ± 5.1 | 163.4 ± 10.8 | 168.7 ± 9.2 | 167.0 ± 10.5 |
| *Ralstonia* | 0 ± 0 | 16.3 ± 3.1 | 15.3 ± 3.1 | 13.1 ± 2.5 |
| | 0 ± 0 | 20.6 ± 3.2 | 15.8 ± 6.2 | 12.3 ± 4.2 |
| | 59.6 ± 5.3 | 111.4 ± 3.0 | 125.2 ± 5.3 | 112.3 ± 4.2 |
| Salt | 5.0 ± 1.0 | 16.3 ± 3.1 | 15.3 ± 3.1 | 13.1 ± 2.5 |
| | 8.0 ± 0.5 | 23.6 ± 3.5 | 22.8 ± 4.2 | 22.3 ± 2.2 |
| | 89.6 ± 4.2 | 132.4 ± 4.0 | 133.2 ± 2.1 | 141.5 ± 3.2 |

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

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 *SlERF3ΔRD* transgenic tomato. Taken together, these results suggest that resistance to *R. solanacearum* in tomato might be achieved, at least partially, by triggering the SA-defense-signaling pathway.

In several recent studies, transgenic plants that constitutively express EAR-containing genes were generated. However, overexpression of a full-length EAR-containing gene seems to influence plant growth. Overexpression of two AP2/ERF family genes, *AtERF7* and *DEAR1*, reduced the plant size of transgenic Arabidopsis (Song et al. 2005; Tsutsui et al. 2009). Transgenic Arabidopsis constitutively expressing Zat7 or Zat10, both of which are Cys2/His2 zinc finger proteins containing an EAR motif, also showed growth suppression (Mittler et al. 2006; Ciftci-Yilmaz et al. 2007). In our study, full-length *SlERF3* transgenic tomato exhibited very slow growth performance and did not develop roots; as a result, plants could not be successfully obtained. These results indicate that a precise control of the expression of EAR-containing genes is essential for the development of normal plants.

While this paper was being written, another group reported that virus-induced gene silencing of the *SlERF3* gene in *R. solanacearum*-resistant tomato cultivar decreased the resistance of tomato (Chen et al. 2009). The result implied that both SlERF3 and SlERF3ΔRD might contribute to *R. solanacearum* tolerance. Taking together, deletion of EAR motif of SlERF3 led to promoting growth of transgenic tomato without affecting *R. solanacearum* tolerance. A similar result has previously been reported with the zinc finger protein AtZat7 which also contains an EAR motif. Constitutive expression of AtZat7 resulted in growth suppression and enhanced salinity tolerance in transgenic *Arabidopsis*, while the deletion of the EAR motif of AtZat7 led to salt susceptibility without affecting growth suppression (Ciftci-Yilmaz et al. 2007). Collectively, these results show that EAR-containing genes may play multiple roles in response to biotic and abiotic stresses. Only partial response has been achieved by constitutively expressing genes in which the EAR motif has been deleted. Therefore, EAR motif-containing genes might act through pathways that are dependent or independent of the EAR motif.

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 (*pflp*) gene increased the tolerance of orchid (*Oncidium*) to *Erwinia carotovora*, a plant pathogen with a wide host range (Liau et al. 2003; You et al. 2003). Overexpression of Arabidopsis tryptophan synthase beta 1 (*AtTSB1*) in tomato confers tolerance to cadmium 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 *SlERF3ΔRD* 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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