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EXPRESSION PATTERNS OF AN ABIOTIC STRESS-INDUCIBLE ETHYLENE RESPONSIVE FACTOR-4 GENE, *LeERF4,* IN TOMATO

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ABSTRACT

A full length cDNA encoding *Lycopersicum esculentum* ethylene responsive factor-4 (designated as *LeERF4*) was isolated and investigated the expression pattern of *LeERF4* in tomato plant under different abiotic stresses from the tomato plant. Phylogenetic analysis based on the deduced amino acid sequence of the *LeERF4* cDNA from tomato revealed a high sequence similarity to other ethylene responsive genes. Southern blot analysis showed that *LeERF4* is a duplicate copy gene in the tomato genome. The organ specific expression profiling indicated that *LeERF4* was expressed in all tested organs. The highest expression was found in young leaves and flowers compared to other tested organs. Northern blot analysis revealed that various environmental stresses such as salt, drought, coldness, ethylene and MV induced significant expression of *LeERF4* but not expressed by the treatment of ABA. These results suggested that the *LeERF4* plays an important role in tomato responses to abiotic stress and may be useful in improving plant tolerance to abiotic stress.

Keywords: abiotic stress, ethylene responsive factor-4, expression analysis, tomato, oxidative stress

INTRODUCTION

Ethylene, a gaseous phytohormone, mediates diverse developmental and physiological processes throughout the entire life cycle of plants (Abeles *et al.*, 1992). Ethylene-responsive factors (ERFs) are uniquely present in the plant kingdom and belong to the AP2-type transcription factors, which function as trans-acting factors at the last step of transduction (Ohme-Takagi and Shinshi, 1995). ERF subfamily genes play various roles in plant growth, development, and response to different environmental stress factors (Okamuro *et al.*, 1997).Previous reports have shown that ERF proteins, which contain a conserved AP2 / ERF DNA-binding domain (Riechmann *et al.*, 2000), regulate development and responses to environmental stimuli in plants (Nakano *et al.*, 2006).

ERF subfamily transcription factors have been identified in various plant species, including *Arabidopsis* (Liu *et al.*, 1998), rice (Cao *et al.*, 2006), and cotton (Huang *et al.*, 2007; Jin and Liu, 2008). The proteins of the ERF subfamily were divided into six groups termed B-1 to B-6. The expression and biological functions of genes in the ERF subfamily were summarized by Nakano *et al.* (2006). As an example, transcription of tobacco Tsi1 (for Tobacco stress-induced gene 1) was induced by salt, ethephon (ET), and salicylic acid (SA). Soybean is one of the most economically important crop species in the world and only a few members of the ERF and CBF/DREB subfamily have been characterized in this species (Mazarei *et al.*, 2002; Li *et al.*, 2005), and most of their functions remain to be determined. Ectopic expression of ERF genes such as HARDY, DREB1A from *Arabidopsis*, *HvCBF4* from barley and *TERF1* from tomato in rice confers an increased tolerance to abiotic stresses (Oh *et al.*, 2005). The ERF family is a large gene family of transcription factors and is a part of the AP2/ERF super family, which contains the AP2 and RAV families (Reichmann *et al.,* 2000). The AP2/ERF super family is defined by the AP2/ERF domain, which consists of about 60 to 70 amino acids and is involved in DNA binding. These three families have been defined as follows; the AP2 family proteins contain two repeated AP2/ERF domains, the ERF family proteins contain a single AP2/ERF domain, and the RAV family proteins contain a B3 domain, which is a DNA-binding

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domain conserved in other plant-specific transcription factors, including VP1/ABI3, in addition to the single AP2/ERF domain. The ERF family is sometimes further divided into two major subfamilies, the ERF subfamily and the CBF/ DREB subfamily (Sakuma *et al.*, 2002).

Tomato is one of the most important commercial vegetable crops worldwide and a main component of the traditional human diet. It is one of the vegetables that have excellent nutritive value, higher content of ascorbic acid, required for human nutrition, and there is now strong evidence to link dietary ascorbic acid with protective effects against various oxidative stress-related diseases (Davey *et al.*, 2000). Known for its sensitivity to unfavorable growth conditions, including salinity, drought, cold ABA, H_2O_2 and abscisic acid conditions, it is surprising that the tomato has not been thoroughly investigated with regards to the molecular basis of its abiotic stress tolerance. To the best of our knowledge, no reports have been published on the *LeERF4* transcription factor in tomatoes. Therefore, in this study we isolated the *LeERF4* gene from tomatoes cDNA and analyzed their mRNA expression patterns in response to various abiotic and oxidative stresses.

MATERIALS AND METHODS

Plant materials, growth conditions, and stress treatments

Seeds of tomato (*Lycopersicum esculentum* L.) were surface-sterilized for 5 min in 1% (w/v) sodium hypochloride and finally washed with distilled water. Then seeds were cultured in Murashige and Skoog (MS) medium (pH 5.8) including 3% sucrose and 0.8% agar. The germinated plants were transferred to pots and kept in culture room at 25°C for 4 weeks. Drought was induced by removing plants from the pots and placing them on filter paper at 25°C under dim light. Tomato leaves were collected after 0 (untreated, control), 3, 6, 12, 24, and 48 hr after removing from pots as a drought treatment. For cold treatment, the leaves were placed in distilled water and kept in a 4°C cold chamber under dim light and photoperiodic conditions and sampling leaves as described previously. For oxidative stresses such as salinity, abscisic acid (ABA), ethylene, and methyl viologen (MV) treatments were applied by submerging the whole seedlings continuously in a water solution of 250 mM NaCl (salt), 100 μM ABA, 100 μl of ethylene and 50 μM MV, respectively. Treated samples were collected after indicated time periods. Sterile water was used as a control for all treatments. All stress treated plant materials were immediately frozen in liquid nitrogen and stored at −80°C until further use.

RNA isolation and amplificaiton of the full length *LeERF4* cDNA

Total RNA was isolated from young tomato leaves using TRI-reagent® according to the manufacturer's instructions (MRC, USA). From the DNase-treated total RNA (1 μg), first-strand cDNA was synthesized using the AccuPower® PCR PreMix (Bioneer, South Korea), containing oligo (dT) primers and Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Invitrogen, USA). Sequences were obtained from *Lycopersicon esculentum* ethylene responsive factor-4 gene in tomato (Accession number: AY192370). Isolation of the *LeERF4* gene was carried out by amplifying the target region using forward (5′- ATGACGAAACAAGATGAAGGA -3′) and reverse (5′- CTACACCAACTCCATCTTGTT-3′) primers, with the cDNA as template. The PCR reaction was carried out as follows: an initial 5 min of denaturation at 94°C; 25 cycles at 94°C for 1 min, 55°C for 45 sec, and 72°C for 1.5 min; and a final 5 min incubation at 72°C. The reaction products were separated on 1% agarose gels and visualized after staining with ethidium bromide. To confirm the nucleotide sequence similarity, PCR product was extracted from gel and purified, then cloned into pGEM-T Easy Vector (Takara) and sequenced.

Phylogenetic analysis

BLASTP search was conducted against deduced amino acid sequences of characterized homologous dehydrin responsive element protein. Multiple alignments and construction of a phylogenetic tree were generated according to the program http://www.ebi.ac.uk/tools/t-coffee, from the proteomics server of the European Bioinformatics Institute (EBI). The sequences were pepper *CaEREB-3* (AAX20036); Cotton

GhEREB (AAO59439), Carrot *DcERF1* (BAF75651), *Arabidopsis AtERF5* (BAA97157), and Cucumber *CmERF1* (BAD01555).

Northern blot analysis

Total RNA was isolated from stress treated and control tomato plants using TRI-reagent according to the manufacturer's instructions (MRC, USA). To ensure approximately equal loading of RNA, 20 µg of total RNA was loaded onto 1.2% (w/v) denaturing formaldehyde agarose gels (Sambrook et al., 1989) and transferred to Hybond-N^+ membranes (Amersham Pharmacia Biotech, UK) with a transfer solution of 20 × SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0). It was cross-linked to the membranes by baking for 2 hr at 80°C. RNA blots were pre-hybridized overnight at 65°C in a hybridization buffer with 50% formamide, 5 × SSPE, 5 × Denhardt's, and 0.1% SDS. PCR products corresponding to *LeERF4* cDNAs were labeled with [α-^{32}P] dCTP by random priming (Promega, USA).

All membranes were hybridized at 65°C with hybridization buffer containing labeled DNA probe. Hybridization was performed for 3-4 days at 65°C in 5% dextran sulfate, 0.25 M disodium phosphate (pH 7.2), 7% (w/v) SDS, and 1 mM EDTA. After hybridization, the filter was washed twice with 2 × SSC and 0.1% SDS for 10 min each at room temperature, and twice with 0.1 × SSC and 0.1% SDS for 5 min each at 65°C. The dried blots were placed on X-ray film for a week at −80°C and developed.

Southern blot analysis

Genomic DNA was isolated from mature tomato leaves (Dellaporta *et al.*, 1983). Genomic DNA samples (12 µg) were completely digested with *Eco*RI, *Hind*III and *Xba*I. Digested genomic DNA was separated by electrophoresis on 1% agarose gel, denatured, and blotted onto a nylon membrane (Amersham Pharmacia, Uppsala). Membranes were then hybridized with the full-length of *LeERF4* cDNA probe labeled with [α-^{32}P] dCTP. Hybridization was performed for 3-4 days at 65°C in 5% dextran sulfate, 0.25 M disodium phosphate (pH 7.2), 7% (w/v) SDS, and 1 mM EDTA. After hybridization, the blot was washed twice with 2 × SSC and 0.1% SDS for 10 min each at room temperature and twice with 0.1 × SSC and 0.1% SDS for 5 min each at 65°C. The blots were then dried and developed on X-ray film incubated for 1 week at –80°C.

RESULTS AND DISCUSSION

Isolation and phylogenetic analysis of *LeERF4*

LeERF4 (GenBank accession no. AY192370) was obtained by RT-PCR amplification of total RNA prepared from tomato leaf cDNA. The complete sequence of *LeERF4* was comprised of 606 bp encoding 202 putative amino acids. Sequence analysis revealed that the deduced amino acid sequences of the cDNA clone contain an Ap2/EREBP domain that is a potential transcription activation domain (Figure 1).

To determine whether *LeERF4* is an ethylene responsive factor, its predicted amino acid sequence was compared to that of previously characterized homologous dehydration responsive element-binding proteins. A homology search revealed that *LeERF4* was similar to many plant ethylene responsive factor proteins, especially in the ERF domains (Figure 2). The amino acid sequence of *LeERF4* was then compared with the other ethylene responsive factor. The putative protein encoded by *LeERF4* shares 60% identity with pepper *CaEREB-3* (AAX20036), 54% identity with Cotton *GhEREB* (AAO59439), 60% similarity with *Arabidopsis AtERF5* (BAA97157), 52% identity with Carrot *DcERF1* (BAF75651) and 59% homology with Cucumber *CmERF1* (BAD01555).

To investigate the evolutionary relationship among plant ethylene responsive factor proteins involved in stress response, a phylogenetic tree was constructed using the neighbor- joining method and full-length amino acid sequences (Figure 3). Results revealed that *LeERF4* was clustered with *Capsicum annum* ethylene responsive element binding factor-3 whereas other stress and ethylene responsive factor proteins were categorized into another branch. This suggests that the *LeERF4* gene codes an ethylene responsive factor protein capable of responding to abiotic stresses.

Fig. 1. Sequence analysis of the *LeERF4* full-length cDNA. The ERF domains are underlined. The protein-coding regions are in upper case letters and the 5'- and 3'-flanking regions are in lower case letters. Putative open reading frame is shown below the nucleo-tide sequence in one-letter symbol of amino acids. The stop codon is marked by an asterisk (*). Nucleotide and amino acid numbers are on the left side.

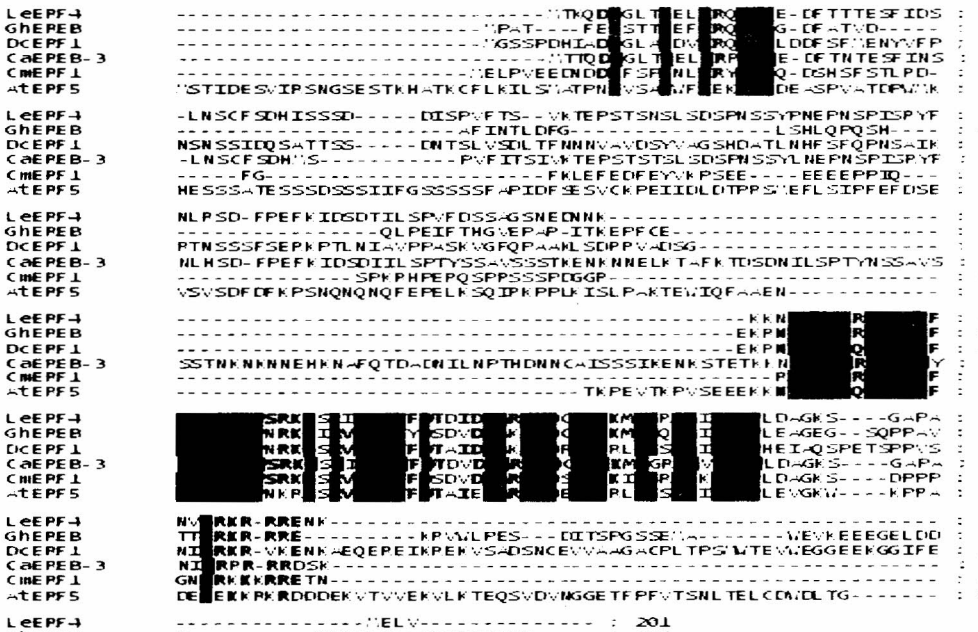

Fig. 2. Deduced amino acid sequences of *LeERF4* and its alignment with Pepper *CaEREB-3* (AAX20036); Cotton *GhEREB* (AAO59439), Carrot *DcERF1* (BAF75651), *Arabidopsis AtERF5* (BAA97157), and Cucumber *CmERF1* (BAD01555). Bootstrap values are indicated for each branch divergence. Number of the amino acid residues is shown to the right of each sequence. Fully conserved residues among the different sequences are shown with a black background. Chemically similar residues are denoted with a gray background. Dashes represent gaps introduced to maximize similarities of the ERF family genes.

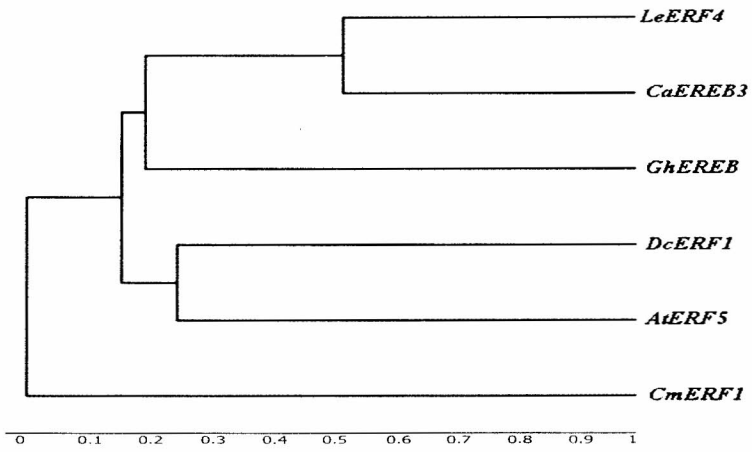

Fig. 3. Phylogenetic relation among ethylene responsive factor-4 . Dendrogram was based on the amino acid sequence alignment of the following proteins: The sequences were Pepper *CaEREB-3* (AAX20036); Cotton *GhEREB* (AAO59439), Carrot *DcERF1* (BAF75651), *Arabidopsis AtERF5* (BAA97157), and Cucumber *CmERF1* (BAD01555). Bootstrap values are indicated for each branch divergence. Tree was obtained from amino acid sequences and constructed using T-coffee. Scale indicates the branch length.

Genomic organization and organ-specific expression of *LeERF4*

To estimate the copy number of *LeERF4* in the tomato genome, DNA gel blot analysis was performed with tomato genomic DNA (Figure 4A). The probe used was a 606 bp PCR fragment corresponding to the C-terminal coding region of *LeERF4* cDNA. A double-hybridized band was observed, indicating that duplicate copies of *LeERF4* may exist in the tomato genome. The expression of *LeERF4* mRNA was examined in various tomato tissues by RNA gel blot analysis using a 606 bp cDNA fragment as a probe (Figure 4B). *LeERF4* was expressed in all tissues tested, with the highest expression in flower and lowest expression in the mature leaves of tomato plant. These tissue-specific *LeERF4* expression patterns are probably associated with differential functions. Hence, diversity in the expression of tomato *LeERF4* proteins reflects their function in specific cells and tissues.

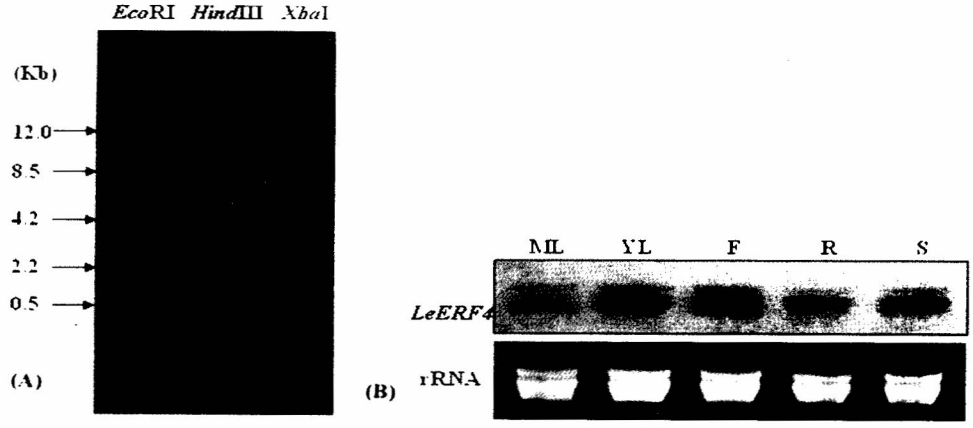

Fig. 4. Gel blotting analysis of *LeERF4* (A) Southern blotting analysis: Genomic DNA was digested with *Eco*RI, *Hind*III and *Xba*I loaded on an agarose gel and hybridized with the ^{32}P-labeled probe corresponding to *LeERF4* cDNA. (B) Tissue specific RNA expression of the *LeERF4* gene in tomato: Twenty micrograms RNA were monitored in different plant oregans (YL, young leaves; ML., mature leaves; R, roots; S, stems; and F, flowers. rRNA bands are shown in lower part of each panel indicating equal loading of RNA.

Expression of *LeERF4* mRNA in response to various abiotic and oxidative stresses

The expression of *LeERF4* in response to various abiotic stresses was analyzed in leaves by RT-PCR analyses (Figures 5A-F). The expression of *LeERF4* gradually increased until 12 h, thereafter slightly declined until 48 h following salt stress (Figure 5A). Salt stress of 12 h generated higher expression followed by a decline. In addition, other ERFs displayed improved tolerance to salt stress as well as to pathogen infection in transgenic tobacco plants, such as *Tsi1* (Park *et al.* 2001) and *CaERFLP1* (Lee *et al.* 2004). Transcript levels of *LeERF4* were markedly increased by 3 h of drought and were maintained until 24 h and then declined trends (Figure 5B). This indicated that the mRNA transcript level was highest after 6 h and 24 h of drought. Mechanical wounding has been shown to trigger the activation of a large array of genes (Titarenko *et al.*, 1997). In response to cold treatment, *LeERF4* mRNA levels were gradually increased until 24 h, but then dramatically decreased at 48 h, reaching their highest level at 12 h and 24 h (Figure 5C). ERE binding factor (ERF) is a major subfamily in the ERF/AP2 family. Genome projects have provided sufficient information about ERF proteins which played key roles in resistance to various environmental stimuli in most plant species, although the expression of class III ERF5 in *Arabidopsis* did not induce by exogenous ABA, NaCl and drought (Fujimoto *et al.* 2000). Expression levels during ABA treatment were similar until 48 h (Figure 5D). Therefore, there was no significant induction of *LeERF4* gene expression in response to ABA. *OsAP25* could be induced by ABA, which showed that it was involved in ABA-dependent signal transduction pathway in rice. So the expression pattern of *OsAP25* was different from the *AtERF5* in *Arabidopsis*.

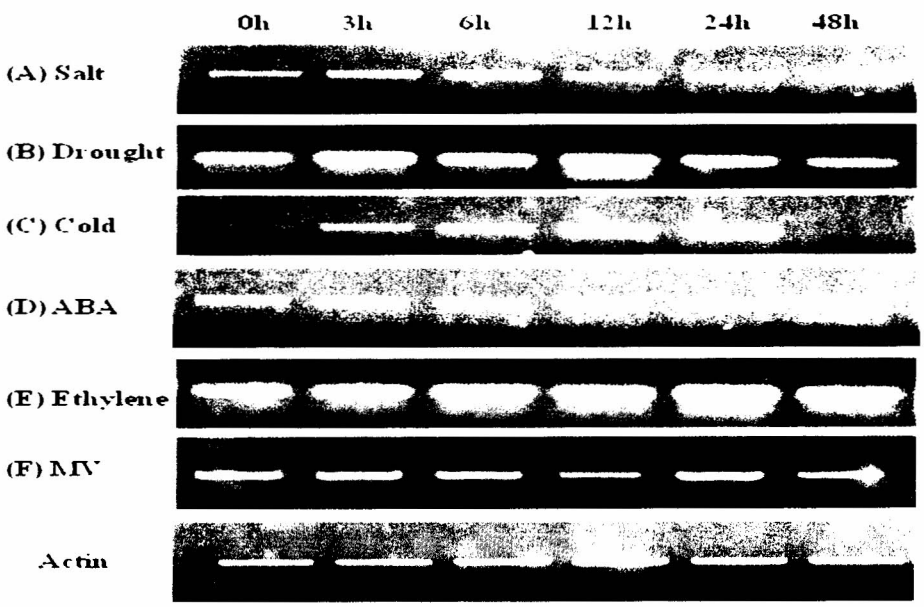

Fig. 5. Expression of *LeERF4* in tomato plants are treated with various abiotics stresses related to oxidative and osmotic stresses. Total RNA was extracted from leaves exposed to (A) 200 mM NaCl, (B) Drought, (C) low temperature, (D) 100 μM ABA (E) 100 uM Ethylene and (F) 50 μM MV at the indicated times after treatment. Twenty micrograms of total RNA was loaded in each lane. The blot was hybridized with probe of tomato *LeERF4* gene. Ethidium bromide-stained rRNA is shown as a control for loading.

Exposure of plants to osmotic stress elevated ABA biosynthesis and the increased ABA levels then induced a number of genes (Bray, 1993; Cohen and Bray, 1990). The fact that *SodERF3* is induced by ABA and wounding could indicate the possibility that this ERF played an integral role in both biotic and abiotic signaling pathways and might be responsible for regulating the possible antagonisms between them

(Anderson *et al.* 2004). ABA is the major plant hormone related to water stress signaling and regulates plant water balance and osmotic stress tolerance (Denekamp and Smeekens, 2003). However, the mRNA expression level was gradually increased by ethylene treatment, reaching its highest expression at 24 h and then slightly declined (Figure 5E). ERF protein has been demonstrated to be induced by ethylene and JA (Ohme-Takagi *et al.* 1995; Lorenzo *et al.* 2003). There was no detectable change in mRNA transcript levels following treatment with methyl viologen (MV), suggesting that MV does not influence the mRNA expression of *LeERF4* (Figure 5F). All of the LeERFs showed specific transcript accumulation patterns, while most ripening-associated genes in climacteric fruit displayed ethylene responsiveness (Giovannoni, 2001). Typically, ERFs influence ethylene-regulated responses to both biotic and abiotic stresses (Riechmann and Meyerowitz, 1980, Singh *et al.*, 2002). In this study, *LeERF4* is up-regulated by ethylene and drought. Therefore, these results showed different expression profiles under different stress conditions upon induction of the *LeERF4* gene. These findings suggest that *LeERF4* is responsive to oxidative and osmotic stresses likely salt, ethylene and MV respectively. *LeERF4* transcripts were induced by abiotic stresses, although certain treatments resulted in a gradual decrease in expression following the initial induction of transcription. Additionally, increased production of ethylene increased the activities of enzymes involved in the biosynthesis of salicylic acid (Summermatter *et al.*, 1995). The intention was to test whether overexpression of *LeERF4* in transgenic tomato plants enhances their resistance to abiotic stresses. In addition, an *in vivo* analysis of different gene-silencing phenotypes will help clarify the role of *LeERF4* in its responses to abiotic stresses.

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