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Full Length Research Paper

LeERF1 improves tolerance to drought stress in tomato (*Lycopersicon esculentum*) and activates downstream stress-responsive genes

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Ethylene responsive factors (ERFs) are important transcriptional regulators involved in plant responses to abiotic and biotic stresses. In this study, the function of the *LeERF1* gene in sense- and antisense-*LeERF1* transgenic tomato plants was analyzed. The results demonstrated that overexpression of *LeERF1* in tomato plants enhanced tolerance to drought stress. The *LeERF1*-overexpressing transgenic plants maintained higher relative water content (RWC), free proline and soluble sugar levels, and showed lower malondialdehyde (MDA) level and electrolyte leakage under drought stress, compared with wild-type and antisense-*LeERF1* tomato plants. Furthermore, overexpression of *LeERF1* in tomato plants activated the expression of stress-related genes, including *P5CS*, *LEA*, *Itpg2* and *tdi-65*. These results suggested that *LeERF1* played a positive role in tolerance to drought stress.

Key words: *LeERF1*, drought tolerance, transcription factor, transgenic tomato.

INTRODUCTION

Ethylene is a gaseous phytohormone that plays an important role in the regulation of growth processes, including seed germination, fruit ripening, and organ senescence and abscission, which are involved in stress and pathogen attack (Johnson and Ecker, 1998; Bleecker and Kende, 2000). Therefore, it is crucial to study the function of ethylene to understand the stress response. The ethylene responsive element binding proteins, first discovered in tobacco as binding proteins, which contain a highly conserved DNA binding domain known as the ethylene responsive factors (ERF) domain (Ohme-Takagi and Shinshi, 1995). The ERF domain is composed of an α-helix and a β-sheet that interacts with the target DNA (Allen et al., 1998). The ERF protein binds to the *cis*-acting element AGCCGCC (GCC box) as the core sequence essential for defense-related genes (Hao et al., 1998).

A large number of ERF proteins were identified from various plants including *Arabidopsis*, tobacco, wheat, rice and hot pepper. For example, overexpression of *AtERF14* in *Arabidopsis* has largely enhanced defense gene expression and regulated the expression of other ERF genes (Onate-Sanchez et al., 2007). *GbERF2* transgenic tobacco plants accumulated higher levels of pathogenesis-related gene transcripts and enhanced resistance to fungal infection (Zuo et al., 2007). Over-expression of *TaERF1* in wheat activated stress-related genes and improved pathogen and abiotic stress tolerance in transgenic plants, suggesting that *TaERF1* might be involved in multiple stress signal transduction pathways (Xu et al., 2007). The expressions of four rice ethylene-responsive transcription factors *OsBIERF1-4* enhanced biotic and abiotic stress (Cao et al., 2006).

In tomato, several ERF proteins have been identified in response to biotic and abiotic stresses. For example, the

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Abbreviations: ERFs, Ethylene responsive factors; **RWC,** relative water content; **MDA,** malondialdehyde; **PR,** pathogenesis-related; **RT-PCR,** reverse transcriptase polymerase chain reaction; **FW,** fresh weight; **TM,** turgid mass; **DW,** dry weight; **MDA,** malondialdehyde.

tomato Pti4-6, specially recognized and bound to a DNA sequence that was present in the promoter region of a large number of genes encoding “pathogenesis-related” (PR) proteins (Zhou et al., 1997). Expression of tomato *JERF3* in tobacco activated the expression of oxidative and osmotic stress-related genes, resulting in decreased accumulation of reactive oxygen species (ROS) and enhanced adaptation to drought, freezing and salt stress (Wu et al., 2008). Tomato transcription factor, JERF1, interacted with multiple *cis*-acting elements and activated the expression of stress responsive genes, ultimately enhancing tobacco tolerance and growth under high salinity and low temperature (Wu et al., 2007). Tomato TERF1 can interact with both GCC-box and DRE to enhance the expression of genes involved in biotic and abiotic stress tolerance (Huang et al., 2004). These results indicated that ERF proteins interacted with downstream partners of various stress-responsive genes, subsequently conferred responses against biotic and abiotic stresses. Previously, an ERF protein LeERF1 was isolated from a cDNA library of tomato fruit, which contained a conserved ERF domain of specific binding to the *cis*-acting element GCC box (Yu et al., 2004). We also obtained sense- and antisense-*LeERF1* transgenic tomato plants, in which *LeERF1* positively modulated ethylene triple response on etiolated seedling, plant development, fruit ripening, and softening in tomato (Li et al., 2007).

In the present investigation, we found that sense-*LeERF1* transgenic tomato plants showed much more tolerance to drought stress, compared with wild-type and antisense-*LeERF1* transgenic tomato plants. This paper aims to investigate the physiological changes and expression of downstream stress-related genes under drought stress. The results suggest that *SlERF1* might play a key role in tolerance to drought stress.

MATERIALS AND METHODS

Plant materials and growth conditions

Tomato (*Lycopersicon esculentum* cv Zhongshu No.4) plants were grown in the growth chamber at 25°C under 16/8 h light/dark cycle, with a relative humidity of 70%. Four-week-old tomato seedlings (T_3 generation) were used for the drought stress treatment and quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis. Wild-type tomato plants and transgenic tomato plants of sense- and antisense-*LeERF1* were designated as WT, LeERF1-sn and LeERF1-as, respectively.

Analysis of drought tolerance in transgenic tomato plants

The T_3 generation of LeERF1-sn, LeERF1-as and WT lines were treated under drought stress condition. For drought stress treatment, the plants were transplanted to pots filled with soil and vermiculite (1:1). Four-leaf stage tomato seedlings were withheld water for 10 days. Leaves of the tomato plants were harvested, frozen in liquid nitrogen and stored at -80°C until required. About 30 seedlings were used for each transgenic line.

Measurement of the relative water content

The relative water content (RWC) of the wild-type and LeERF1 transgenic tomato plants was performed as described by Yamasaki and Dillenburg (1999). Leaves were obtained from the tomato plants and their fresh weight (FW) was measured. To determine turgid mass (TM), the leaves were floated in deionized water for 7 h. Finally, the leaf samples were dried in an oven at 80°C for 48 h to obtain the dry weight (DW). The RWC was then calculated using the following formula:

$$RWC\ (\%) = [(FW-DW) / (TW-DW)] \times 100$$

Assay of malondialdehyde (MDA) content and electrolyte leakage

MDA content was estimated by thiobarbituric acid (TBA), as described by Peever and Higgins, (1989). The absorbance was determined at 450, 532 and 600 nm, respectively. The content of MDA was calculated as indicated:

$$C\ (\mu mol\ l^{-1})_{MDA} = 6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$$

Electrolyte leakage assay of the leaves of the transgenic and wild-type tomato plants was conducted according to the method of Dionisio-Sese and Tobita (1998). The conductivity of the solution was determined by a conductivity meter (Model DDSJ-308A, Shanghai Precision & Scientific Instrument Co., Ltd., China).

Measurement of the proline and soluble sugar contents

Proline was determined as described by Bates et al., (1973). The absorbance was measured at 520 nm and proline content was calculated using the standard curve. The content of soluble sugar was determined at 620 nm according to the method of Morris (1948).

Gene expression analysis

Total RNA was extracted from the four-week-old tomato seedlings following the procedure of guanidium thiocyanate (Gregory et al., 1988). RNA treated with DNase was reverse transcribed using M-MLV reverse transcriptase (Promega, USA) with a poly-T primer, according to the instructions of the manufacturer. Quantitative RT-PCR was performed on an ABI PRISM 7000 sequence detection system (Applied Biosystems) by using SYBR Green PCR Master Mix (Toyobo, Japan). The qRT-PCR amplification conditions were set as follows: initial denaturation of 5 min at 95°C, 40 cycles of at 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, with a final extension of 10 min at 72°C. *Actin* was used as a reference gene in each PCR reaction. Each sample was performed with three replicates. The following gene specific primers were used: 5´-TGGGATGATATGGAGAAGATATGG-3´ and 5´-GGCTTCAGT TAGGAGGACAGGA-3´ for *Actin* (U60480); 5´-CGTCCTCTGTT GTCCC-3´and 5´-AGTGAAGGCAATGAAGC-3´ for *tdi-65* (AF 172856); 5´-GTCCCAATCTCCTCCAA-3´ and 5´-CAGGGTAAT CGCATCAG-3´ for *LEA* (Z46654); 5´-TACTGGACC GTTGAGCA-3´ and 5´-GGTGTTGTGGTGGTGTTA-3´ for *ltpg2* (U81996); 5´-CCCACAGCAGCACAA-3´ and 5´-TTCGCAAGGGTATGAAG-3´ for *P5CS* (AY897574).

Statistical analysis

Statistical analyses were carried out via one-way analysis of variance

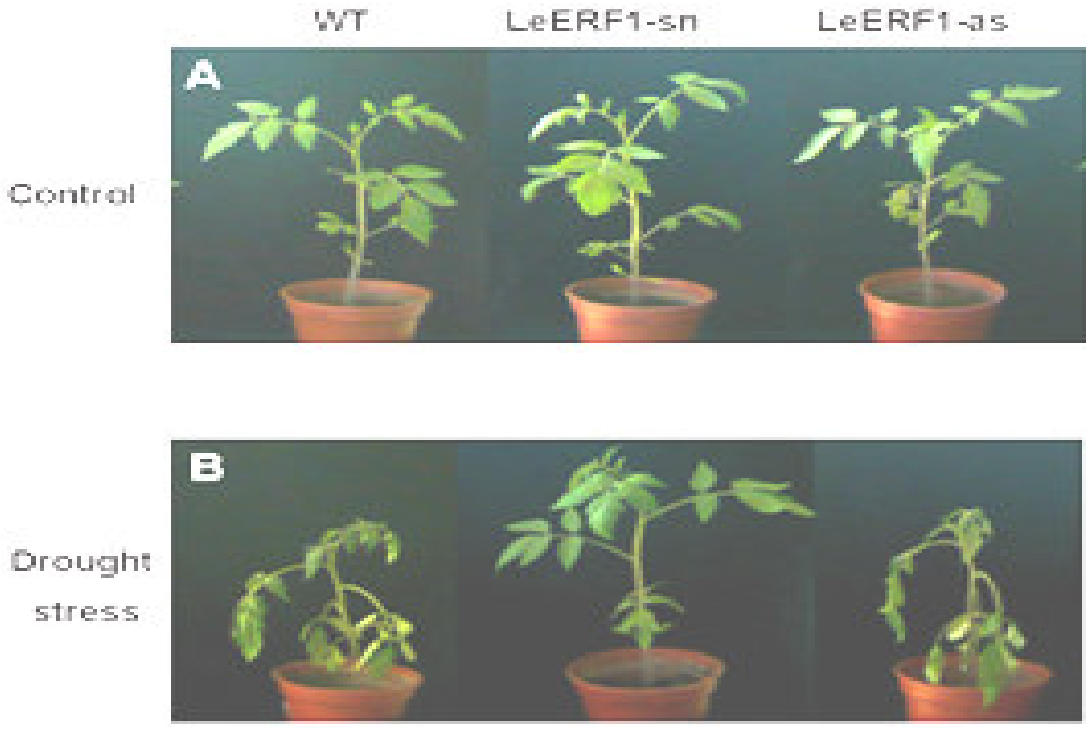

Figure 1. Evaluation of drought tolerance in wild-type (WT), sense-*LeERF1* (LeERF1-sn) and antisense-*LeERF1* (LeERF1-as) tomato plants. Four-week-old tomato seedlings were withheld in water for 10 days. About 30 seedlings were used for each WT and *LeERF1* transgenic tomato plants.

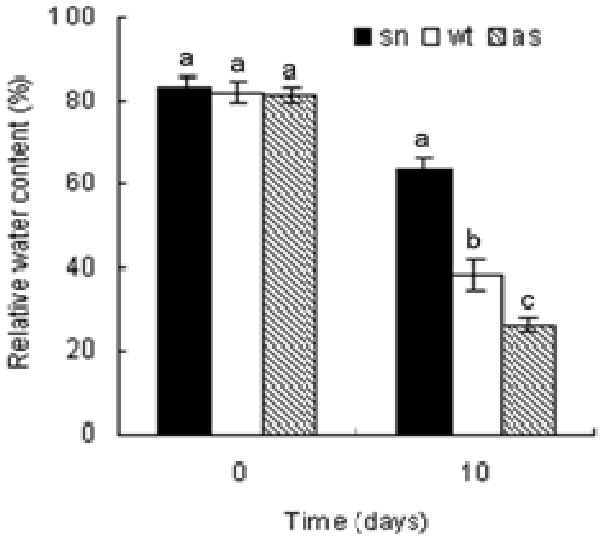

Figure 2. The relative water content of wild-type (WT), sense-*LeERF1* (sn) and antisense-*LeERF1* (as) transgenic tomato plants grown under drought stress for 10 d. The bars represent the mean ± SD of three individual measurements. Columns with different letters indicate significant difference at *P*<0.05 (Duncan test).

and Duncan's multiple range test. The statistical significant was evaluated at the P<0.05 level.

RESULTS

Overexpression of *LeERF1* in tomato plants enhanced tolerance to drought stress

To investigate the response of *LeERF1* transgenic plants to drought stress, the four-week-old seedlings were grown under water-deficit stress for 10 d. After drought-stress, the leaves of the WT and LeERF1-as transgenic lines wilted, rolled and exhibited chlorosis, while only a few of the leaves of LeERF1-sn transgenic lines rolled (Figure 1). To further study the capacity of osmotic adjustment in transgenic tomato plants, relative water content was measured under drought stress condition. The results showed that no significant differences exist between WT and transgenic plants under normal condition. However, the RWC of LeERF1-sn plants was reduced to 20%, whereas WT and LeERF1-as plants declined to 43.7 and 54.9% under drought stress, respectively (Figure 2). These results suggested that LeERF1-sn transgenic tomato plants were more tolerant to drought stress, compared with WT and LeERF1-as plants.

Overexpression of *LeERF1* in tomato plants maintained the stability of cell membrane

To study the stability of membrane in transgenic and WT

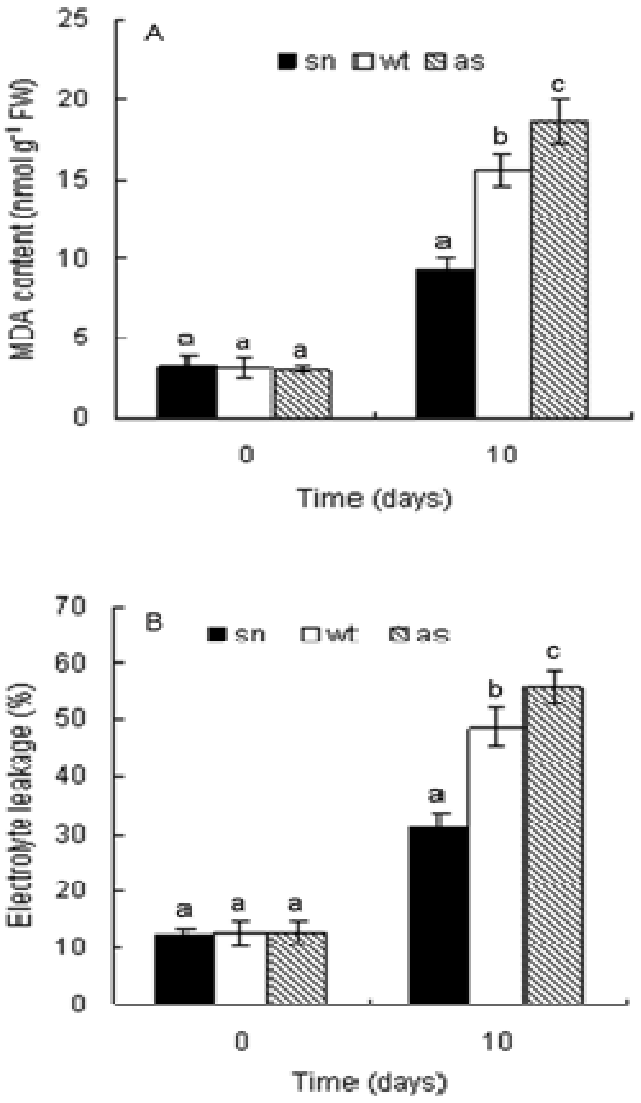

Figure 3. MDA content (A) and electrolyte leakage (B) of wild-type (WT), sense-*LeERF1* (sn) and antisense-*LeERF1* (as) tomato plants under drought stress for 10 d. The results are the mean ± SD of three individual measurements. Columns with different letters indicate significant difference at $P<0.05$ (Duncan test).

plants under drought stress, MDA content and electrolyte leakage as an indication of membrane damage were determined. The results indicated that MDA content and electrolyte leakage increased in tomato plants under drought stress. However MDA content and electrolyte leakage in WT and LeERF1-as plants increased more than those in LeERF1-sn transgenic lines. The amount of MDA in LeERF1-sn transgenic plants increased only 2.7-3.1 folds, compared to non-treated plants under drought stress (Figure 3a). Electrolyte leakage increased 31.3% in LeERF1-sn transgenic plants, thus in WT and LeERF1-as lines increased 48.7 and 55.8% under drought stress, respectively (Figure 3b). The results indicated that the LeERF1-sn transgenic tomato plants were subjected to less damage to the cell membrane under drought stress.

Accumulation of free proline and soluble sugar in sense-*LeERF1* transgenic tomato plants

Proline and soluble sugar are osmolyte in plants and accumulate in response to stress treatment. After being withheld in water for 10 days, the proline content in both *LeERF1* transgenic lines and WT lines increased constantly (Figure 4a), whereas the level of proline in the LeERF1-sn lines was significantly higher than that in WT and LeERF1-as lines. In the LeERF1-sn transgenic tomato plants, the proline content increased 4.6 - 4.7 folds under drought stress, compared with the non-treated lines. However, in the WT and LeERF1-as tomato plants, the proline content was only increased from 3.0 - 3.5 folds and 2.4 - 2.7 folds compared with the non-treated lines, respectively. The levels of soluble sugar in the LeERF1-sn, WT and LeERF1-as lines under drought stress showed, respectively, 6.3 - 7.4 folds, 2.6 - 3.6 folds and 2.8 - 3.7 folds higher than those of the non-treated lines (Figure 4b). There was no significant difference in the levels of free proline and soluble sugar between WT and LeERF1 transgenic lines under normal condition.

Activation of stress-related genes in *LeERF1*-overexpressing transgenic tomato plants

Overexpression of *TERF1* in rice activated the expression of stress-responsive genes and enhanced tolerance to abiotic stress (Gao et al., 2008). To further elucidate the role of *LeERF1* gene involved in drought stress, we analyzed the expression of several stress-related genes, such as *P5CS*, *LEA*, *ltpg2* and *tdi-65* in WT and *LeERF1* transgenic lines. As shown in Figure 5, the expression levels of these genes in LeERF1-sn transgenic lines were higher than those in WT plants under normal condition. Especially, the expression level of *ltpg2* in LeERF1-sn plants was nearly 13 folds compared to WT lines under normal condition. In contrast to WT and LeERF1-as plants, the expression levels of these four genes were significantly activated in LeERF1-sn transgenic tomato plants under drought stress.

DISCUSSION

Majority of studies demonstrated that ERF proteins played an important role in plant abiotic and biotic stress responses and developmental processes (Chakravarthy et al., 2003; Alonso and Stepanova, 2004). Our cloned *LeERF1* gene encoded the conserved ERF domain of specific binding to the *cis*-acting element GCC box (Yu et al., 2004). We have described previously that the ERF transcription activator *LeERF1* positively mediated ethy-

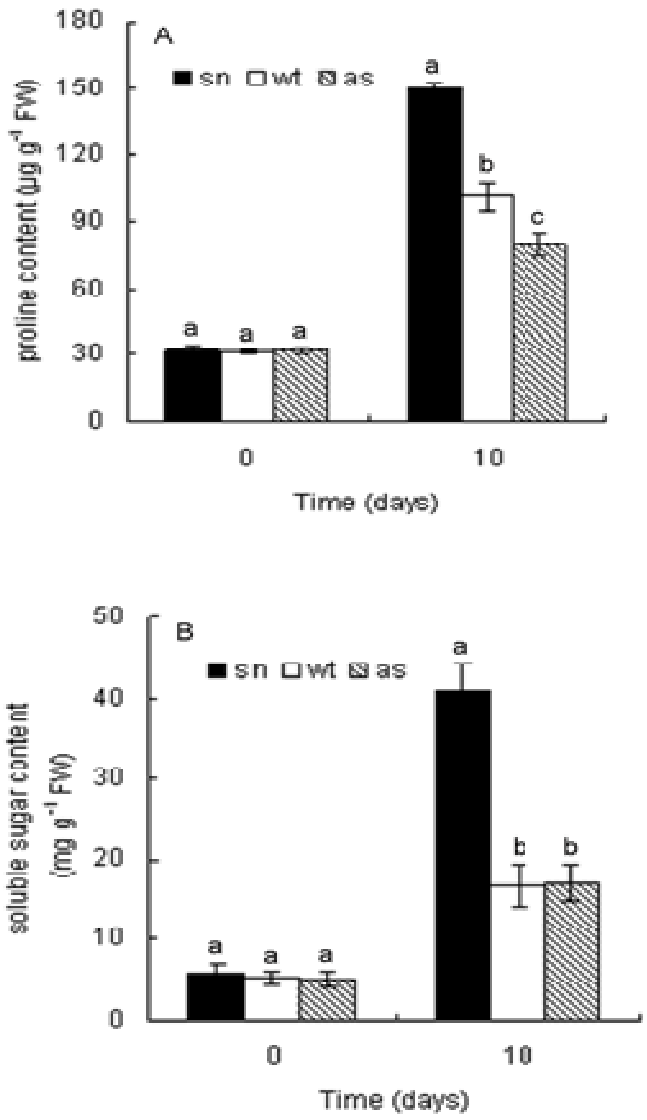

Figure 4. Levels of proline (A) and soluble sugars (B) in wild-type (WT), sense-*LeERF1* (sn) and antisense-*LeERF1* (as) tomato plants under drought stress for 10 d. The results are the mean ± SD of three individual measurements. Columns with different letters indicate significant difference at $P < 0.05$ (Duncan test).

lene signals and was a downstream component in the ethylene signaling pathway in tomatoes (Li et al., 2007). In this study, overexpression of *LeERF1* in tomato plants enhanced tolerance to drought stress and up-regulated downstream stress-related genes.

Plants respond to abiotic and biotic stress in their natural environment, affecting various physiological and biochemical changes (Fujita et al., 2006; Knight and Knight, 2001). Our results showed that WT and LeERF1-as tomato plants displayed growth inhibition, chlorosis and rolled under drought stress, whereas the LeERF1-sn transgenic lines grew normally (Figure 1). RWC as a general measure of plant water status was determined.

The results indicated that LeERF1-sn transgenic tomato plants maintained a significantly higher RWC in their leaf tissue, as compared with the WT and LeERF1-as lines (Figure 2). Furthermore, abiotic stresses can cause oxidative damage to cell membrane (Chinnusamy et al., 2005). It has been shown that constitutive expression of *OsDREB1B* in tobacco plants showed significantly lower MDA content than the wild type plants under osmotic stress (Linga and Arjula, 2008). In this study, the levels of MDA and electrolyte leakage in WT and LeERF1-as tomato plants were higher than those in LeERF1-sn transgenic plants (Figure 3). It implied that over-expression of *LeERF1* in tomato plants maintained the stability of cell membrane under drought stress condition.

Under a variety of abiotic stress, plants accumulated osmolytes including proline and soluble sugar (Bonhert and Jensen, 1996). These osmolytes function as osmo-protectants to improve tolerance to stress responses by their own protective mechanisms (Hasegawa et al., 2000; Bhatnagar-Mathur et al., 2008; DaCosta and Huang, 2006; Mahajan and Tuteja, 2005). It is known that the higher levels of proline and soluble sugar were detected in transgenic plants under stress conditions (Hseih et al., 2002; Vannini et al., 2007; Xu et al., 2008). Similarly, our results demonstrated that LeERF1-sn transgenic tomato plants accumulated higher levels of proline and soluble sugar, compared with WT and LeERF1-as tomato plants under drought stress (Figure 4). The increased levels of proline and soluble sugar stabilized membranes and enhanced tolerance to drought stress in LeERF1-sn transgenic tomato plants.

Increasing studies indicated that many ERF transcription factors activated the expression of stress-responsive genes (Huang et al., 2004; Hu et al., 2008). We analyzed the expression of several stress-related genes in WT and *LeERF1* transgenic lines. In contrast to WT and LeERF1-as transgenic lines, LeERF1-sn transgenic plants showed significant activation of several stress-related genes, such as *P5CS*, *LEA*, *ltpg2* and *tdi-65*. For example, as a key gene of proline synthetase, the expression level of P5CS in LeERF1-sn transgenic plants was higher than that in WT and LeERF1-as transgenic lines under drought stress (Kavi et al., 1995). The results indicated that *LeERF1* might activate proline synthetase to enhance tolerance to drought stress in tomato plants. Furthermore, the expression levels of *LEA* encoding a late embryo-genesis protein, *ltpg2* encoding a lipid transfer protein, and *tdi-65* encoding a drought-induced cysteine protease in LeERF1-sn transgenic plants were higher than those in WT and LeERF1-as transgenic lines under drought stress (Wang et al., 2006; Yubero-Serrano et al., 2003; Harrak et al., 2001). These results indicated that *LeERF1* might up-regulate the expression of stress-related genes and resulted in the tolerance to drought stress in LeERF1-sn transgenic tomato plants.

In conclusion, the *LeERF1* gene is an important element for tolerance to drought stress. These results demon-strated that overexpression of *LeERF1* in tomato plants

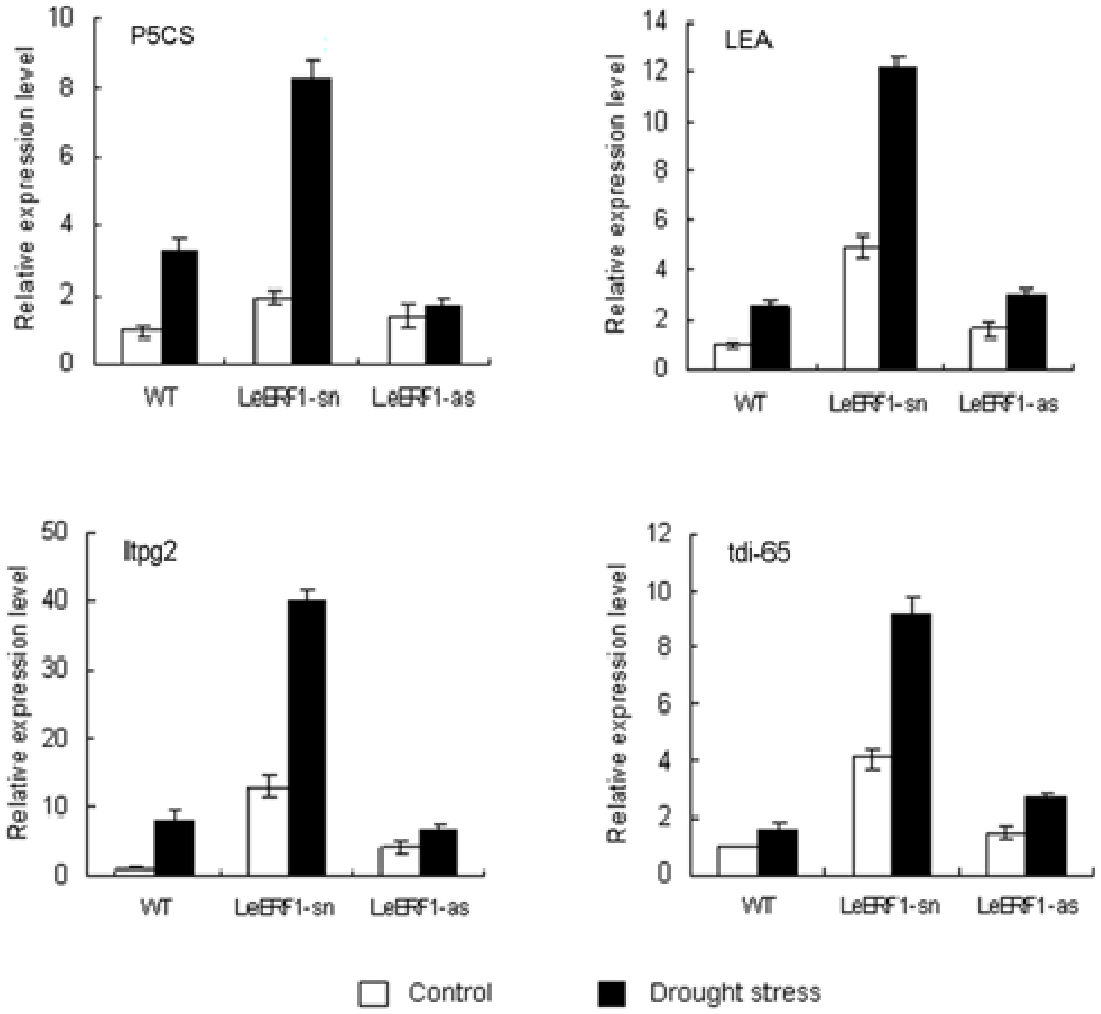

Figure 5. The expression levels of stress-related genes in wild-type (WT), sense-*LeERF1* (LeERF1-sn) and antisense-*LeERF1* (LeERF1-as) tomato plants under control and drought stress conditions. The expression levels of *LEA*, *P5CS*, *ltpg2* and tdi-65 transcripts were assessed by qRT-PCR. The results are the mean ± SD of three individual measurements.

led to the accumulation of osmolytes including free proline and soluble sugar, prevented increased of level MDA and electrolyte leakage, and up-regulated several stress-related genes in tomato plants under drought stress. To further understand the mechanism of the *LeERF1*-mediated signaling pathway in response to stresses, it will be essential to identify the stress-related genes directly activated by *LeERF1*.

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