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ORIGINAL PAPER

LchERF, a novel ethylene-responsive transcription factor from *Lycium chinense*, confers salt tolerance in transgenic tobacco

Dianyun Wu · Jing Ji · Gang Wang · Chunfeng Guan · Chao Jin

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Abstract

Key message **An ERF gene, *LchERF*, was cloned from *L. chinense* for the first time. Overexpression of *LchERF* conferred salt stress tolerance in transgenic tobacco lines during seed germination and vegetative growth.**

Abstract Ethylene-responsive transcription factors (ERFs) play important roles in tolerance to biotic and abiotic stresses by regulating the expression of stress-responsive genes. Although the ERF proteins involved in defense responses against biotic stresses have been extensively documented, the mechanisms by which ERF subfamily genes regulate plant responses to abiotic stresses are largely unknown. In this study, a novel ethylene-responsive transcription factor, named *LchERF*, was isolated from *Lycium chinense* (a salinity-resistant plant). Analysis of the LchERF-deduced protein sequence showed that it had a typical AP2/ERF domain and belonged to the B-3 subgroup of the ERF subfamily. The expression of *LchERF* was found to be tissue specific in *L. chinense* under normal conditions. Upon treatment with NaCl, polyethylene glycol (PEG) or ethephon (ET), transcript levels of *LchERF* rapidly increased in *L. chinense*. Overexpression of *LchERF* conferred salt stress tolerance in transgenic tobacco during seed germination and vegetative growth. Compared with control lines, *LchERF*-overexpressing plants showed higher chlorophyll and proline contents, and were associated with lower H_2O_2 content under salt stress. Overall, our results demonstrate that LchERF might play an important role in the regulation of plant responses to abiotic stresses and mediate various physiological pathways that enhance salt stress tolerance in plants.

Keywords Ethylene-responsive factor · *Lycium chinense* · Osmotic pressure · ROS · Salt tolerance

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D. Wu
School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, People's Republic of China

J. Ji (✉) · G. Wang · C. Guan · C. Jin
School of Environmental Science and Engineering, Tianjin University, Tianjin 300072, People's Republic of China
e-mail: jijing@tju.edu.cn

G. Wang
e-mail: wanggangtjdx@126.com

Introduction

Plants are constantly exposed to adverse environmental conditions throughout their life cycle, including biotic and abiotic stresses (Cai et al. 2014; Zhang et al. 2004). However, plants have developed a finely tuned adaptive network to perceive stress signals and regulate the expression of specific stress-related genes during the course of evolution. Transcription factors play key roles in regulating plant stress responses through binding to *cis*-acting elements in the promoters of stress-related genes or interacting with other transcription factors, subsequently activating or repressing the expression of related genes (Chen and Zhu 2004; Schenk et al. 2000; Yamaguchi-Shinozaki and Shinozaki 2006). For example, MYB, NAC, bZIP (zinc finger proteins), ABA responsive element (ABRE)-binding proteins and AP2/ERF proteins are known to play important roles in regulating the expression of specific stress-related genes.

AP2/ERF transcription factors form one of the largest plant transcription factor families. The AP2/ERF

superfamily is defined by the AP2/ERF domain, which consists of about 60–70 amino acids and is involved in DNA binding (Nakano et al. 2006). Based on differences in the binding domain, the AP2/ERF superfamily can be divided into five main subfamilies: AP2 (APETALA2), CBF/DREB, ERF (ethylene-responsive factor), RAV and one very specific gene, At4g13040 (Nakano et al. 2006; Sakuma et al. 2002). The AP2 subfamily proteins, which contain two AP2/ERF domains, mainly participate in the regulation of developmental processes. RAV subfamily proteins contain a B3 domain and one AP2/ERF domain, and are involved in ethylene, brassinosteroid, and biotic and abiotic stress responses. Although both the ERF and CBF/DREB subfamily proteins only have one AP2/ERF domain each, the highly conserved 14th and 19th amino acids distinguish the ERF proteins (alanine and aspartic acid, respectively) from CBF/DREB (valine and glutamic acid, respectively) proteins. The members of the CBF/DREB subfamily play vital roles in plant abiotic stress resistance by recognizing the dehydration responsive or cold-repeat element (DRE/CRT) with a core motif of A/GCCGAC (Hu et al. 2012; Kishor et al. 1995; Mishra et al. 2013). Ethylene-responsive transcription factors (ERFs) were first identified from tobacco as GCC box-binding proteins, and can either induce or repress the expression of genes containing the GCC box and related elements in their promoters. Sequence analysis showed that ERFs contained a highly conserved, plant-specific DNA-binding domain consisting of 58–59 amino acids (Ohme-Takagi and Shinshi 1995). The tertiary structure of the ERF domain in AtERF1 has been analyzed. It consists of a three-stranded anti-parallel β-sheet and an α-helix packed approximately parallel to the β-sheet (Allen et al. 1998). Based on the sequence identities of their DNA-binding domains, the ERF subfamily members can be divided into six small subgroups (B-1 through B-6) (Sakuma et al. 2002). The ERF subfamily members are mainly involved in responses to biotic stresses by recognizing the *cis*-acting element AGCCGCC, known as the GCC box (Hao et al. 1998). Certain ERF subfamily members also can bind DRE/CRT elements (Lee et al. 2004; Zhang et al. 2009).

Ethylene-responsive transcription factors (ERFs) have been identified in many plant species, such as *Arabidopsis thaliana* (Oñate-Sánchez and Singh 2002), *Nicotiana tabacum* (Fischer and Dröge-Laser 2004), and *Lycopersicon esculentum* (Tournier et al. 2003). The ERF proteins involved in defense responses against biotic stresses have been extensively documented (Gutterson and Reuber 2004; Park et al. 2001). For example, overexpression of ERF genes in transgenic *Arabidopsis* or tobacco plants can induce the expression of several PR (pathogenesis-related) genes, resulting in enhanced resistance of transgenic plants to bacterial, fungal or viral pathogens (Berrocal-Lobo et al. 2002; Fischer and Dröge-Laser 2004; Yi et al. 2004; Zuo et al. 2007). The expression of the wheat ERF gene *TaPIE1* was induced following *Rhizoctonia cerealis* infection. *TaPIE1*-overexpressing transgenic wheat exhibited significantly enhanced resistance to *R. cerealis* (Zhu et al. 2014). Recent studies have found that certain ERF proteins participate in abiotic stress responses in plants (Schmidt et al. 2013). For example, transcripts of *GhERF4* from *Gossypium hirsutum* accumulated rapidly to high levels when plants were treated with salt stress (Jin and Liu 2008), and overexpression of *JcERF* gene isolated from *Jatropha curcas* seedlings in transgenic *Arabidopsis* enhanced salt tolerance (Tang et al. 2007). Seedlings of *TaERF3*-overexpressing transgenic wheat exhibited significantly enhanced tolerance to both drought and salt stresses compared with untransformed lines. Conversely, *TaERF3*-silencing wheat plants showed more sensitivity to drought and salt stresses compared with the control plants (Rong et al. 2014). However, abiotic stresses are complex stimuli that can induce many different yet related physiological changes (e.g. ionic imbalance and osmotic stress) (Zhu 2001). Thus, the mechanisms by which ERF subfamily genes regulate plant responses to abiotic stresses need to be further examined.

Lycium chinense Mill, belonging to the family Solanaceae, is a drought-resistant shrub or small tree that is extensively distributed in the northwestern areas of China and other warm and subtropical countries such as Japan, Korea and some European countries. The fruit of *L. chinense* has not only nutrient but also medicinal value. It can reduce blood glucose and serum lipids, and is therefore considered as an anti-aging, immunomodulation, antitumor and male-fertility-facilitating agent (Gan et al. 2004). Moreover, *L. chinense* has great capability for environmental adaptation, so it is a good choice for studying plant environmental resistance. Previous studies on this plant have mainly focused on the extraction of active components, pharmacology and medical functions, cultivation and so on (Chang and So 2008; Gan et al. 2004; Li et al. 2007). However, our understanding of the molecular mechanisms of *L. chinense* responses to abiotic stresses such as salt and drought stress is limited. Additionally, although there has been substantial progress in the identification and functional analysis of ERF genes in model plants, the ERF genes in *L. chinense* have not been characterized. Therefore, it is important to clone and characterize ERF genes from *L. chinense*.

In this study, a novel ERF gene was cloned from *L. chinense* and designated *LchERF*. The expression patterns of *LchERF* in response to abiotic stresses and its function in transgenic tobacco were investigated.

Materials and methods

Plant material, growth conditions and treatments

Lycium chinense seedlings were grown at 25 °C with a 16-h light/8-h dark cycle and relative humidity of 60–75 %. Experiments were carried out using 10-week-old plants with 26–30 leaves.

Seeds from transgenic plants were surface sterilized and sown on Murashige and Skoog (MS) medium for germination under a 16/8-h light/dark cycle at 22/25 °C. Two-week-old *Nicotiana tabacum* seedlings were transplanted into soil and further grown in the greenhouse under a 16/8-h light/dark cycle at 25 °C and 60–75 % relative humidity. All seeds used in this experiment were harvested at the same stage and stored similarly and for the same duration, thus guaranteeing same seed viability.

Before stress treatment, *L. chinense* plants were carefully uprooted and transferred to Hoagland's solution for 7 days under same conditions as described above. For salt and drought treatments, the *L. chinense* plants were placed in different culture bottles wrapped with aluminum foil containing 300 mM NaCl or 20 % PEG 6000 solution; for the ethylene treatment, the *L. chinense* plants were sprayed with 100 μM ethephon (ET) solution. All plants were cultured at 25 °C with a 16-h light/8-h dark cycle and relative humidity of 60–75 %. Stress treatments were given for different time durations (0, 1, 3, 6, 12, 24 h). Sample treated at 0 h was used as control. The fourth to fifth leaves from the top of the first lateral branch were harvested at the indicated times and immediately frozen in liquid nitrogen before further analysis. For each treatment, ten independent plants at the same age were treated simultaneously. Each treatment was repeated at least three times independently.

Cloning procedure and sequence analysis of LchERF gene

Total RNA was extracted from the leaves of *L. chinense* using the RNeasy plant mini kit (QIAGEN) according to the manufacturer's instructions. The isolated total RNA was used as the template for first-strand cDNA synthesis using a TaKaRa RNA PCR Kit (AMV) Ver. 3.0. A gene-specific upstream primer was designed based on the Unigene of the EST of *L. chinense* sequenced by BGI (Beijing Genomics Institute). The M13 Primer M4 in the TaKaRa RNA PCR Kit (AMV) Ver. 3.0 was used as the downstream primer. All primer sequences are presented in Table 1. Amplification was performed with *L. chinense* cDNA templates and polymerase chain reaction (PCR) products were purified directly using a MiniBEST DNA Fragment Purification Kit Ver.4.0 (TaKaRa). After being cloned into the pMD18-T vector using a pMD™ 18-T Vector Cloning Kit (TaKaRa), the *LchERF* gene sequences were confirmed by sequencing three clones one time from three independent PCRs. The nucleotide and predicted amino acid sequences were analyzed using DNAMAN. Multiple alignments of the deduced amino acid sequence were performed using the Clustal W and DNAMAN programs, and a phylogenetic tree was constructed with the neighbor-joining (NJ) method using the MEGA 4.1 software with the following parameters: Poisson correction, complete deletion, and bootstrap (500 replicates) (Zhuang et al. 2013).

Semi-quantitative RT-PCR analyses

To investigate the differential expression patterns of *LchERF* in different tissues (root, stem and leaf) of *L. chinense* plants under normal conditions, the total RNA of roots, stems and leaves was extracted using the RNeasy plant mini kit (QIAGEN) from the same *L. chinense* plant, which was not treated with any stress. To detect *LchERF* differential expression patterns between the control and stress-treated plants, total RNA of treated *L. chinense* plants was extracted. For semi-quantitative reverse-transcriptase RT-PCR analysis, total RNA was used as the template for first-strand cDNA synthesis using the TransScriptII one-step gDNA removal and cDNA synthesis SuperMix with random primers (TransGen Biotech). All protocols were performed according to the manufacturer's instructions.

PCR amplification was carried out for 4 min at 94 °C, followed by 28 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. A final extension was performed at 72 °C for 8 min. All primer sequences are presented in Table 1. The constitutively expressed *β-actin* (GenBank accession number: KM011338) gene was used as an internal control. The RT-PCR product of the *LchERF* gene was sequenced to verify the specificity of PCR amplification. The experiment was repeated at least three times using independently isolated total RNAs. The cycle numbers of the PCR were adjusted to obtain barely visible bands in agarose gels. The PCR products were loaded on 0.75 % agarose gels and stained with ethidium bromide.

Construction binary vector and transformation of tobacco

The open reading frame (ORF) of *LchERF* was amplified by PCR using a specific primer pair (LchERFf2 and LchERFr2) modified to include *Bam*HI and *Sal*I restriction sites. The fragment was inserted into the binary vector pCAMBIA2300 under the control of the *Cauliflower mosaic virus* (CaMV35S) promoter. Then, the recombinant plasmid pCAMBIA2300-*LchERF* was introduced into

Table 1 Oligonucleotide primers used in this study

Primer	Primer sequences 5′ 3′
LchERFf	ATGTATCAACCCATTTCTACTGAGTTAGC
M13Primer m4	GTTTTCCCAGTCACGAC
LchERFf1	TTGGAACTTACGAGACGGCT
LchERFr1	CAGCTGATCGTCGCTTAACC
ActinF	GGAAACATAGTGCTCAGTGGTG
ActinR	GCTGAGGGAAGCCAAGATAG
LchERFf2	CGCGGATCCATGTATCAACCCATTTCTACTGAGTTAGC
LchERFr2	ACGCGTCGACTTAACTGACCAATAGCTGCTCGCCA
LchERFf3	TCTACAAGACGCTTTAAATAT
LchERFr3	GCTTAACCGTCACTCGAACG
Vf	ATACCGTAAAGCACGAGGAAG
Vr	CTGAAGCGGGAAGGGACT

LchERFf gene-specific upstream primer, *LchERFf1* semi-quantitative RT-PCR forward primer of *LchERF*, *LchERFr1* semi-quantitative RT-PCR reverse primer of *LchERF*, *ActinF* semi-quantitative RT-PCR forward primer of *β-actin*, *ActinR* semi-quantitative RT-PCR reverse primer of *β-actin*, *LchERFf2* the forward primer for binary vector construction (*Bam*HI site underlined), *LchERFr2* the reverse primer for binary vector construction (*Sa1*I site underlined), *LchERFf3* the forward primer for transgenic plant detection, *LchERFr3* the reverse primer for transgenic plant detection, *Vf* the forward primer for Vec plant detection, *Vr* the reverse primer for Vec plant detection

Agrobacterium tumefaciens (strain C58). As a control (Vec), the empty vector pCAMBIA2300 was introduced into *A. tumefaciens* (strain C58) simultaneously. Agrobacterium-mediated transformation tobacco was performed by the leaf disc method (Horsch et al. 1985). Putative transgenic seedlings were screened on 100 mg/L kanamycin MS agar medium. The T_0 kanamycin-resistant seedlings were confirmed by PCR analysis using the specific primers. The seeds of all transgenic lines were harvested for subsequent analysis. The seeds of T_1 progeny transgenic lines were used in the present study. All of the primers used in this study are presented in Table 1.

Salt stress assays

Seeds from T_1 progeny transgenic lines were surface sterilized and sown on MS agar medium containing different concentrations of NaCl (0, 50, 150, and 200 mM). The germination rate was determined after 15 days.

Seedlings of transgenic lines grown on normal MS medium for 7 days were transferred onto MS agar medium supplemented with different concentrations of NaCl (0, 50, 150, and 200 mM) for 14 days. At the end of the salt treatment, the length of the roots was measured, and then all leaves were excised from the treated seedlings and immersed in 95 % ethanol for 24 h to extract chlorophyll. The total chlorophyll in the leaves was determined as described below.

Eight-week-old transgenic plants planted in soil were irrigated with 200 mM NaCl solution for 30 days at 3-day intervals. After NaCl treatment for 16 days (a total of four irrigations), the third to fourth leaves from the top of the transgenic plants were harvested for physiological analysis. The contents of proline and H_2O_2 in the leaves were examined as described below. Following irrigation with 200 mM NaCl solution for an additional 14 days (a total of eight irrigations), the survival percentage (the number of surviving plants relative to the total number of treated plants) was counted.

Measurement of proline, chlorophyll and H_2O_2 concentrations

Proline concentration was determined according to Bates et al. (1973). The content of H_2O_2 was estimated by monitoring the absorbance at 415 nm of the titanium peroxide complex as described by Jiang and Zhang (2001). The total chlorophyll was measured according to Wintermans and De Mots (1965). At least ten independent plants were evaluated in each test, and all tests were repeated three times.

Results

Isolation and sequence analysis of *LchERF*

The *LchERF* gene (GenBank accession number KJ584446) was isolated from *L. chinense* for the first time. *LchERF* had an open reading frame (ORF) of 678 bp encoding a protein of 225 amino acids with a predicted molecular mass of 24.71 kDa, and a pI of 8.62. The predicted LchERF protein had a typical conserved DNA-binding domain (AP2/ERF domain) of 59 amino acids. Moreover, LchERF

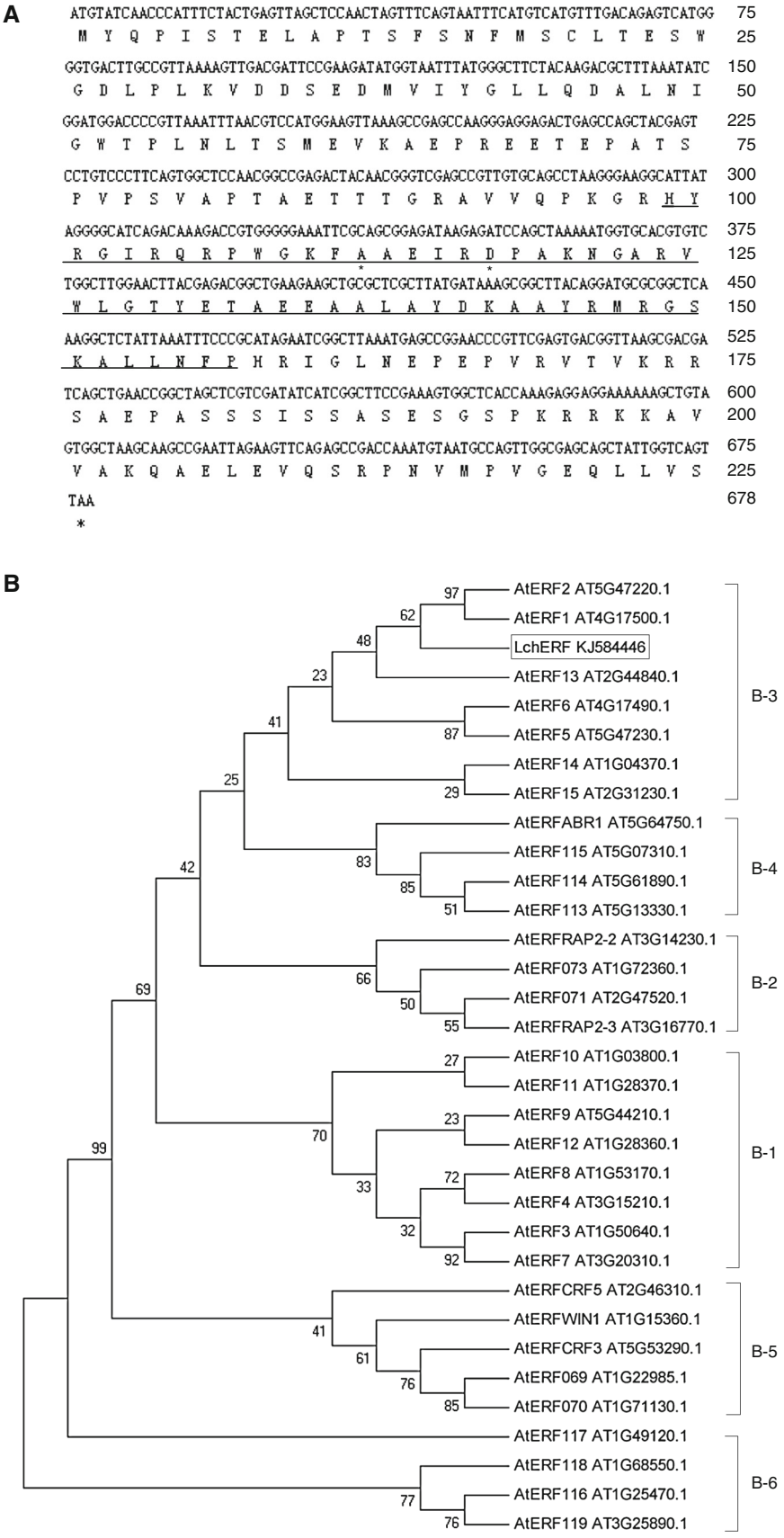
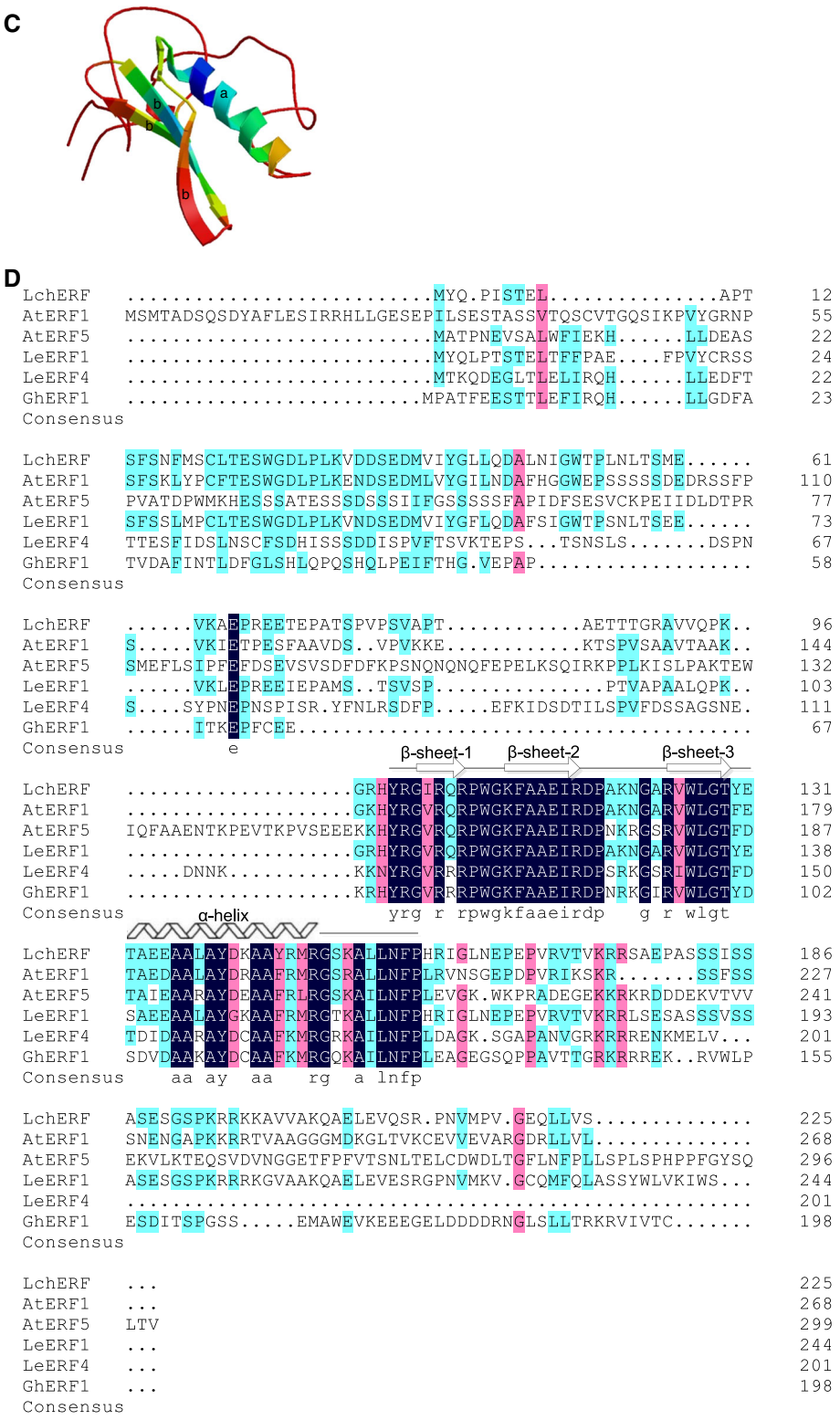


Fig. 1 **a** Nucleotide sequence and deduced amino acid of the *LchERF* gene from *L. chinense*. The AP2/ERF conserved domain is underlined, the conserved 14th alanine and 19th aspartic acid are marked with *asterisks*. **b** The phylogenetic relationships between LchERF and other ERF subfamily TFs from *Arabidopsis*. The phylogenetic tree was created with the neighbor-joining method using the MEGA 4.1 software. The *numbers above or below the branches* indicate the bootstrap values from 500 replicates. LchERF is *boxed*. **c** The 3D structure of LchERF, *a* α-helix, *b* β-stranded sheet. **d** Alignment of the amino acid sequence of LchERF with other ERFs belonged to the B-3 group. Amino acid residues that are conserved in at least three of the six sequences are colored, whereas amino acids identical in all six proteins are shown with a *dark background*. The putative three β-stranded sheets are shown with *arrows above* the sequences; the α-helix is marked with a helical *curve above*. The GenBank accession numbers of all proteins used here are LchERF (KJ584446), AtERF1 (AB008103), AtERF5 (AB008107), LeERF1 (AY192367), LeERF4 (AY192370) and GhERF1 (AY181251). The database of *A. thaliana* ERF subfamily protein sequences was downloaded from the DATF (the database of Arabidopsis transcription factors) website

Fig. 1 continued



had conserved alanine (A) and aspartic acid (D) residues at the 14th and 19th positions in the AP2/ERF domain, which are believed to contribute to functional GCC box and CRT/DRE motif-binding activity in many ERF subfamily members (Fig. 1a).

Based on the similarity of the amino acid sequences of their DNA-binding domains, ERF subfamily proteins in *Arabidopsis* are divided into six subgroups (B-1 through B-6) (Sakuma et al. 2002). A phylogenetic tree was created from the deduced amino acid sequences of LchERF and

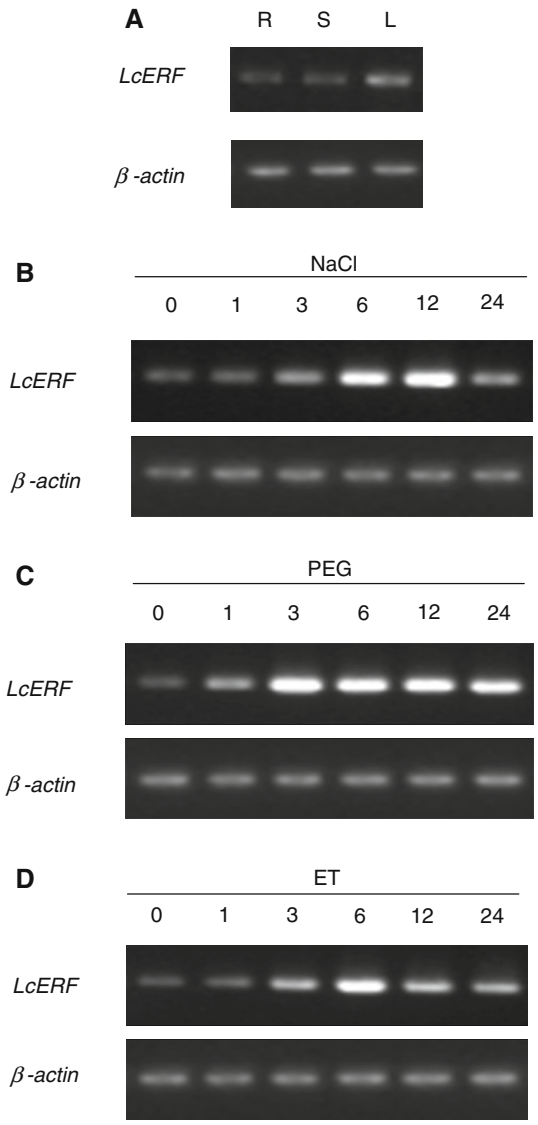


Fig. 2 Expression profiles of *LchERF* in different tissues and under abiotic stresses. **a** The tissue-specific expression of *LchERF* under normal conditions. Total RNA was extracted from the roots, stems and leaves of 11-week-old *L. chinense* plants, and was subjected to RT-PCR analysis. Analysis of *LchERF* transcription in leaves of *L. chinense* plants under various stress conditions. For the stress treatments, 11-week-old *L. chinense* plants were subjected to treatment with 300 mM NaCl (**b**), 20 % PEG 6000 (**c**), and 100 μM ethylene (ET), released from ethephon (**d**). Total RNA was isolated from the fourth to fifth leaves from the top of the first lateral branch at the indicated times following the initiation of treatment and was subjected to RT-PCR analysis

other ERF proteins from *Arabidopsis*. The result revealed that LchERF belonged to the B-3 group of the ERF subfamily (Fig. 1b).

The *A. thaliana* ethylene-responsive transcription factor AtERF1 is a unique AP2 superfamily protein, and its crystallographic structure has been analyzed. The DNA-binding domain (DBD) of AtERF1 consists of a three-stranded anti-parallel β-sheet and an α-helix packed approximately parallel to the β-sheet (Allen et al. 1998). Using Swiss model, we constructed a tertiary structure model of LchERF and found that the DBDs of LchERF and AtERF1 shared 82.61 % identity. The DNA-binding domain of LchERF contained a three-stranded anti-parallel β-sheet and an α-helix that lay parallel to the β-sheet, like in AtERF1 (Fig. 1c). However, the Val at position 149 in AtERF1 was replaced by Ile 103 in LchERF (Fig. 1d). In addition, some other amino acids in the DBD domain of LchERF were not identical to those in AtERF1; these residues may be responsible for binding specificity (Wang et al. 2010).

Expression patterns of *LchERF* in *L. chinense*

To investigate the expression patterns of *LchERF* in *L. chinense*, the level of *LchERF* mRNA in different organs was monitored by semi-quantitative RT-PCR. The results of these experiments are shown in Fig. 2a. *LchERF* mRNA was expressed in the root, stem and leaf. *LchERF* transcript levels in the stem and root were nearly the same but the transcript level in the leaf was higher than in the root and stem. These results suggested that the expression of *LchERF* in *L. chinense* was tissue specific.

Changes in *LchERF* transcript levels in response to abiotic stress were investigated. As shown in Fig. 2b, after salt treatment, *LchERF* mRNA accumulation increased with time and reached a maximum at 12 h. The transcript level then decreased, but remained higher than that in the control (0 h). Upon treatment with 20 % PEG 6000, the transcript level of *LchERF* was induced obviously within 1 h and reached a peak at 3 h, then reduced slightly at 6 h and remained no changed until 24 h. After *L. chinense* plants were sprayed with 100 mM ethephon solution, the transcript level of *LchERF* was also induced, with an induction peak at 6 h, and then declined with time until 24 h. These results indicated that the *LchERF* gene was involved in responses to abiotic stresses.

Identification of transgenic plants

To investigate the function of the *LchERF* gene, we overexpressed its ORF under the control of the CaMV 35S promoter in tobacco. A total of 30 independent transgenic lines (T_0) were selected by kanamycin-resistance screening, and then these transgenic lines were confirmed by PCR detection with the genomic DNA of *N. tabacum* as a template (data not shown). Ten T_1 progeny plants from *LchERF*-overexpressing lines were randomly selected to detect the *LchERF* expression levels in leaves by semi-quantitative RT-PCR. As shown in Fig. 3, six lines showed high *LchERF* expression; no LchERF expression was

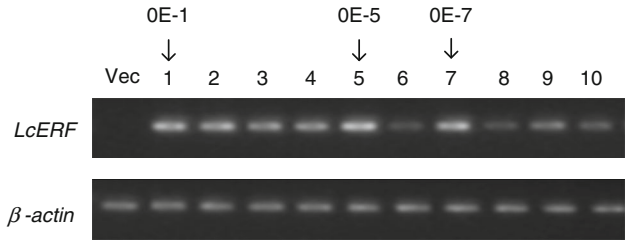


Fig. 3 Analysis of the *LchERF* expression level in transgenic tobacco plants by semi-quantitative RT-PCR. Total RNA was extracted from leaves of T_1 progeny transgenic tobacco plants grown under normal conditions

detected in empty vector plants. The seeds from three representative *LchERF*-overexpressing lines (OE-1, OE-5, OE-7) were selected for further functional analysis.

Overexpression of *LchERF* enhanced the salt tolerance of transgenic plants

Because the expression of *LchERF* in *L. chinense* was significantly induced by salt treatment, we investigated whether *LchERF* could increase salt tolerance in transgenic tobacco plants.

Seeds from three *LchERF*-overexpressing lines (OE-1, OE-5, OE-7) and control lines were planted on MS plates containing different concentrations of NaCl. No significant difference was observed between the control and *LchERF*-overexpressing lines on MS plates with 0 or 50 mM NaCl during germination. However, on the MS plates containing 150 and 200 mM NaCl, the *LchERF*-overexpressing lines showed a significantly higher germination rate compared with the control lines. The germination rate of all *LchERF*-overexpressing lines was more than 70 % on the MS plates with 150 mM NaCl, but was only 50.7 % for the control. When the concentration of NaCl was increased to 200 mM, the germination rate of all *LchERF*-overexpressing lines was still more than 40 %, but only 13.1 % of the empty vector seeds germinated (Fig. 4a, b).

The sensitivity of seedling growth to salt stress was also assayed. The seeds planted on normal MS agar medium for 7 days were transferred onto MS agar medium supplemented with different concentrations of NaCl for 14 days. There was no significant difference in root length among all tested plants under 0 or 50 mM NaCl stress conditions. Although 150 and 200 mM NaCl stress suppressed root growth in all tested plants, the *LchERF*-overexpressing lines had longer roots than the control lines, and the control seedlings had smaller cotyledons than the *LchERF*-overexpressing lines (Fig. 4c, d). In addition, after salt treatment, the total chlorophyll content was also measured. There was no significantly difference

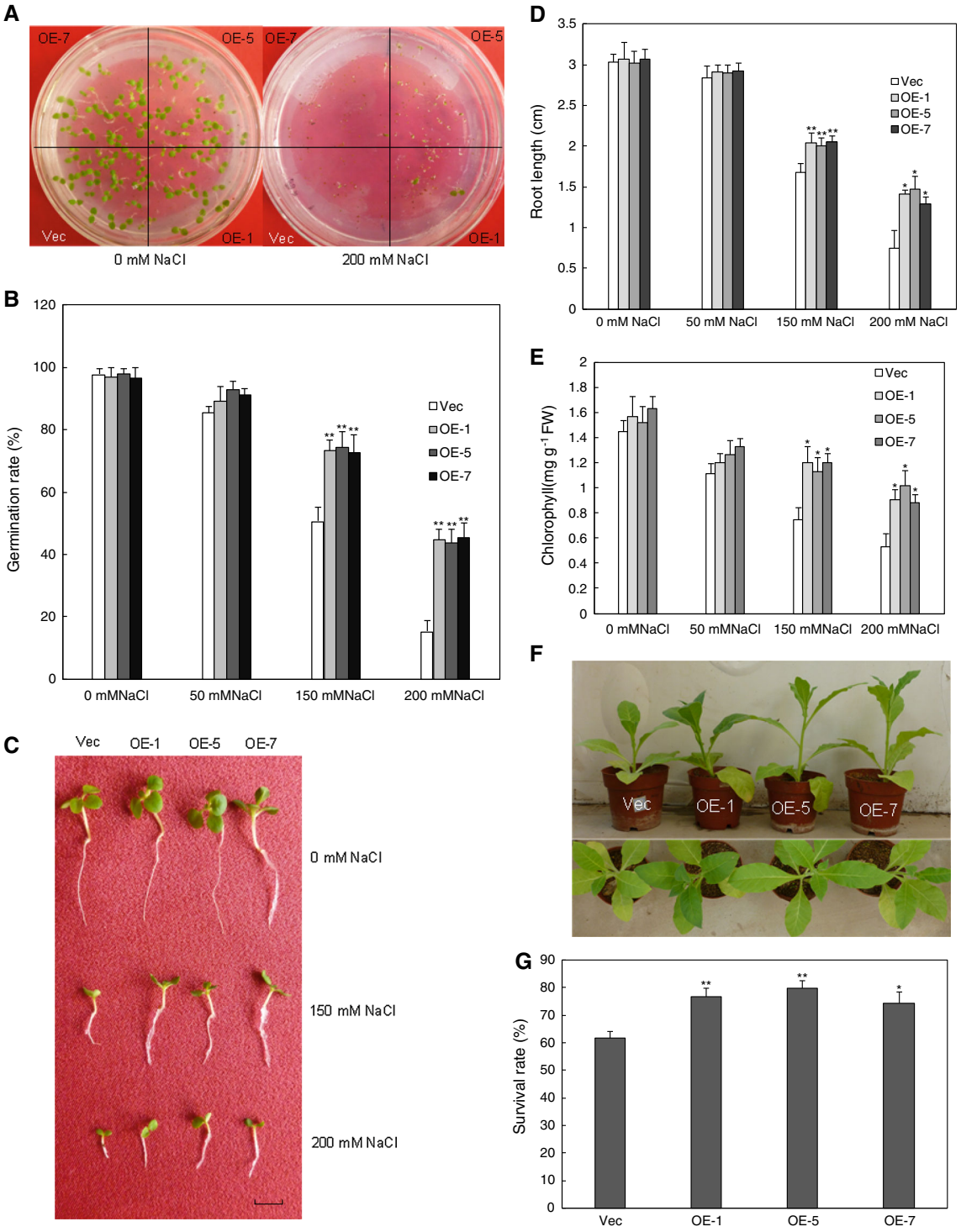
Fig. 4 Salt tolerance tests comparing Vec and *LchERF*-overexpressing *N. tabacum* plants. **a** Phenotypic analysis of Vec and *LchERF*-overexpressing seeds on MS medium supplemented with 0 mM and 200 mM NaCl. The seeds were allowed to grow for 15 days before the photographs were taken. **b** Germination rates of the Vec and *LchERF*-overexpressing lines under normal conditions and different NaCl concentration treatments. The germination rate was counted 15 days after sowing. **c** The root morphology of the Vec and *LchERF*-overexpressing lines on MS medium containing different concentrations of NaCl. Seeds planted on MS medium for 7 days were transferred to MS medium supplemented with different concentrations of NaCl, and the photograph was taken 14 days after transfer. *Scale bar* 1 cm. **d** Primary root lengths of the seedlings 14 days after transfer to MS medium containing different NaCl concentrations. **e** Total chlorophyll content of the Vec and *LchERF*-overexpressing lines after salt treatment for 14 days. **f** Photograph of representative 8-week-old Vec and *LchERF*-overexpressing plants irrigated with 200 mM NaCl solution for 16 days (four times of irrigations). **g** Survival rates of 8-week-old transgenic plants irrigated with 200 mM NaCl solution for 30 days (eight times of irrigations). Each column represents the mean of three independent experiments, and *bars* indicate SDs. *Asterisks* above the column indicate significant differences in comparison with the control ($*P < 0.05$, $**P < 0.01$)

in total chlorophyll content between *LchERF*-overexpressing and the control plants under 0 and 50 mM NaCl stress conditions. Although the total chlorophyll content was reduced under 150 and 200 mM stress condition in all tested plants, the extent of this decline in *LchERF*-overexpressing lines was much less than that in the control plants (Fig. 4e).

To further verify that *LchERF* enhanced the salt tolerance of the overexpression lines, 8-week-old transgenic plants planted in soil were irrigated with 200 mM NaCl solution for 30 days at 3-day intervals. After NaCl treatment for 16 days (a total of four irrigations), the control lines showed obvious growth retardation and the leaves were chlorotic, whereas the majority of the *LchERF*-overexpressing lines grew normally (Fig. 4f). Following irrigation with 200 mM NaCl solution for an additional 14 days (a total of eight irrigations), the survival rate of the Vec plants was only 61.8 %, but more than 80 % of the *LchERF*-overexpressing plants survived (Fig. 4g). These results showed that overexpression of *LchERF* could enhance the salt tolerance of transgenic plants during both seed germination and vegetative growth.

Physiological changes in transgenic plants under salt stress

Free proline content and the redox homeostasis of cells in plant leaves are important physiological indices of drought and salinity tolerance (Chang and So 2008; Mao et al. 2011; Szabados and Savouré 2010). To explore the physiological mechanism of the salt stress tolerance conferred by *LchERF* overexpression, the proline and H_2O_2 contents



in transgenic plants were measured before and after 200 mM NaCl treatment. No significant difference in proline and H_2O_2 contents between *LchERF*-overexpressing and Vec plants was observed before salt stress. The proline content increased after salt stress in both control and *LchERF*-overexpressing plants, but *LchERF*-overexpressing lines accumulated higher levels of proline compared with control lines (Fig. 5a). These results indicated that overexpression of *LchERF* could enhance proline accumulation in the leaves of transgenic plants under

salt stress. Although the H_2O_2 content increased after salt stress in all tested lines, the extent of this enhancement in all *LchERF*-overexpressing plants was much less than that in the Vec plants. The H_2O_2 content in control plants doubled after 200 mM NaCl treatment, whereas the *LchERF*-overexpressing plants only showed 56.2, 35.4 and 49.1 % increases in H_2O_2 content under the same salt stress (OE-1, OE-5 and OE-7, respectively; Fig. 5b). These results suggested that overexpression of the *LchERF* gene could suppress H_2O_2 accumulation in transgenic plants under salt stress.

Discussion

The ERF subfamily is a large family of plant transcription factors that belongs to the AP2/ERF superfamily (Riechmann and Meyerowitz 1998). Early studies indicated that the ERF subfamily members primarily participated in biotic stress responses by recognizing the *cis*-acting element AGCCGCC, known as the GCC box (Hao et al. 1998). Recent investigations have shown that certain ERF subfamily proteins are involved in both biotic and abiotic stress responses by regulating specific stress-related genes (Li et al. 2007; Rong et al. 2014; Yamamoto et al. 1999). However, our understanding of how ERF subfamily genes regulate plant responses to abiotic stresses is still limited. *L. chinense* has great capability for environmental adaptation. Therefore, investigation into the mechanisms by which *L. chinense* ERFs regulate stress responses is vital for understanding plants adaptation to environmental stresses. However, the ERF genes of *L. chinense* and the physiological processes they regulate under abiotic stresses have not been reported.

In this study, a novel *LchERF* gene was isolated from *L. chinense*. Sequence alignment showed that the predicted LchERF protein had a single conserved DNA-binding domain (AP2/ERF domain) of 59 amino acids, which contained two key conserved amino acid residues (position 14-alanine and position 19-aspartic acid). In addition, phylogenetic tree analysis showed that *LchERF* was a member of the ERF B-3 subgroup. These data indicate that *LchERF* is a novel gene of the ERF subfamily belonging to AP2/ERF superfamily. This is the first time that a *L. chinense* ERF gene has been identified.

Plant adaptation to external stimuli is regulated via a network of various signaling pathways, in which different pathways can converge on ERF proteins through complex interactions (Zhang et al. 2004). For example, TSRF1 is a molecular node of signal integration for ethylene signaling pathways (Schmidt et al. 2013). Previous studies have revealed that many ERF genes can be induced by exogenous hormones and abiotic stresses. For example, the

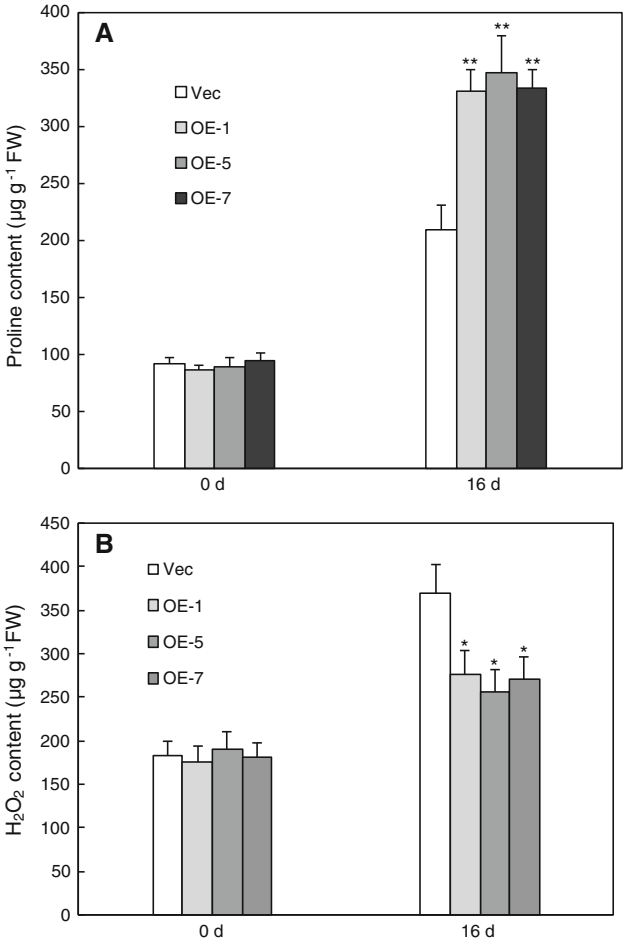


Fig. 5 Proline and H_2O_2 contents in the leaves of Vec and *LchERF*-overexpressing tobacco plants after 0 and 16 days of 200 mM NaCl stress treatment. **a** Proline content, **b** H_2O_2 content. Each column represents the average of three replicates, and *bars* indicate SDs. ** and * indicate significant differences in comparison with the control at $P < 0.01$ and $P < 0.05$, respectively

transcripts levels of *AtERF1*, *AtERF2* and *AtERF5* increased two to threefold after 12 h of ethylene treatment (Fujimoto et al. 2000), and expression of *SlERF5* was induced by abiotic stresses, such as high salt, drought and cold (Pan et al. 2012). In the present study, semi-quantitative RT-PCR analysis showed that the transcript levels of *LchERF* were clearly induced by treatment with PEG, NaCl or ethylene. The expression patterns of *LchERF* were largely similar to those of *GhERF1*/*GhERF6,* which was shown to be induced by salt, drought or ethylene (Jin et al. 2010; Qiao et al. 2008). Our data suggest that *LchERF* might play an important role in protecting *L. chinense* against abiotic stresses, and the LchERF protein might link different pathways to positively regulate plant responses to abiotic stresses. Although soil salinity existed long before humans and agriculture, the problem has been aggravated by agricultural practices such as irrigation. Today, ~20 %

of the world's cultivated land and nearly half of all irrigated lands are affected by salinity (Krasensky and Jonak 2012; Zhu 2001). These findings compelled us to do in-depth work on elucidation of the potential role of the *LchERF* gene in enhancing salt tolerance. Therefore, we generated *LchERF*-overexpressing tobacco lines via transformation. As shown in Fig. 4, the results indicated that overexpression of *LchERF* conferred salt stress tolerance of transgenic plants during the seed germination and vegetative growth periods.

Proline accumulation is an important parameter in determining salt tolerance in plants, and proline may act as a radical scavenger or cellular osmotic regulator (Shan et al. 2007; Yamamoto et al. 1999). The overproduction of proline can increase tolerance against abiotic stresses in transgenic tobacco plants (Kishor et al. 1995). Previous studies have indicated that overexpression of ERF subfamily genes can elevate proline content; the level of salt tolerance and the accumulation of proline parallel each other (Jin et al. 2013; Rong et al. 2014). In this study, proline contents in the leaves of *LchERF*-overexpressing lines were significantly higher than those in control plants under salt stress conditions (Fig. 5a), suggesting that overexpression of *LchERF* enhances the proline accumulation in plants under salt stress. Therefore, overexpression of the *LchERF* gene could increase the salt tolerance of transgenic tobacco by controlling the osmotic pressure of cells.

Previous studies have shown that abiotic stresses can result in damage to plants via oxidative stress involving the generation of ROS (Mao et al. 2011; Rong et al. 2014). The accumulation of H_2O_2 is often used as an indicator of ROS production. The accumulation of H_2O_2 due to salt stress has been reported in many plants. For example, overexpression of the *TaERF3* gene could reduce the accumulation of H_2O_2 in transgenic wheat under salt stress (Hu et al. 2012; Mishra et al. 2013; Rong et al. 2014). In the present study, the control plants accumulated more H_2O_2 than *LchERF*-overexpressing lines under salt stress conditions. This observation implies that overexpression of *LchERF* either inhibits ROS generation or effectively scavenges excess ROS.

Salinity interferes with plant growth as it leads to ion toxicity and osmotic stress. Both ion toxicity and osmotic stress can cause oxidative damage (Huang et al. 2012; Zhu 2001). Overexpression of *LchERF* could enhance salt tolerance in tobacco via ROS scavenging. Additionally, it was reported that hybrid poplar overexpressing *JERFs* can avoid salt damage by accumulating Na^+ in their vacuoles (Li et al. 2009); however, whether the *LchERF* gene functions in coping with the ionic toxicity from salt stress need to be further studied.

Conclusion

In summary, a novel ERF subfamily gene, *LchERF*, was identified and characterized. The results of this study demonstrated that *LchERF* was responsive to abiotic stresses. Overexpression of *LchERF* conferred salt stress tolerance to transgenic plants during seed germination and vegetative growth. Moreover, *LchERF*-overexpressing plants showed higher chlorophyll and proline contents, which were associated with lower H_2O_2 content under salt stress. This demonstrates that LchERF can mediate various physiological pathways to enhance the salt tolerance of transgenic plants. These results broaden the function of ethylene-responsive factors in plants. Furthermore, this study provides a basis for fundamental studies on *L. chinense* regulatory responses to environmental stresses and a prospective gene for efforts to improve salt tolerance in other plants.

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Conflict of interest The authors declare that they have no conflict of interest.

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