Plant Omics Journal POJ

POJ 8(6):485-492 (2015) ISSN:1836-3644

# Stress-induced expression in *Arabidopsis* with a Dehydrin LEA protein from *Cleistogenes songorica*, a xerophytic desert grass

**Jiyu Zhang[1], Lingfang Kong[1], Zhipeng Liu[1], Zulfi Jahufer[2], Zhen Duan[1], Yaxin Huo[1], Hongyan Di[1] and Yanrong Wang[1*]**

**[1]State Key Laboratory of Grassland Agro-ecosystems, College of Pastoral Agriculture Science and Technology, Lanzhou University, Lanzhou, 730020, China**
**[2]Grasslands Research Centre, AgResearch Ltd., Palmerston North 4442, New Zealand**

***Corresponding author: yrwang@lzu.edu.cn**

**Abstract**

Late embryogenesis abundant (LEA) proteins were confirmed to be involved in the acquisition of tolerance to drought, cold and high salinity in many different organisms. In this paper, we report on the isolation and characterization of the cDNA clone for a LEA protein (CsLEA) and its putative promoter sequence from *Cleistogenes songorica*, a xerophytic desert grass. CsLEA was predicted as a hydrophilic LEA protein. In addition, semi-quantitative RT-PCR and relative quantitative RT-PCR assays showed that *CsLEA* was only expressed in dehydrated roots samples, while absolute quantitative RT-PCR showed that accumulation of *CsLEA* transcripts was constitutively increased both in leaves and roots during the time-course of drought stress. Transgenic plants of *Arabidopsis* with stress inducible expression of *rd29A*::*CsLEA* were generated and used to study the role of this LEA protein in stress tolerance. Transgenic plants exhibited higher viability than that of WT plants on MS media supplemented with 300mM sorbitol or 50mM NaCl. Significant differences for $CO_2$ assimilation rate ($P<0.05$) and proline concentration ($P<0.01$) were shown between *rd29A*::*CsLEA* transgenics and WT during dehydration and rehydration of the plants. Data presented here suggest that *CsLEA* expression is modulated by drought stress and therefore affects osmotic regulation during water deficit.

**Keywords:** *Cleistogenes songorica*; Drought stress; Gene expression; Late Embryogenesis Abundant protein; Promoter; Transgenic *Arabidopsis*.
**Abbreviations:** ABRE_abscisic acid response element; LEA_Late Embryogenesis Abundant; LTREATLTI78_low temperature responsive element; MYB_myeloblastosis; WT_Wild type.

## Introduction

The Late Embryogenesis Abundant (LEA) proteins were first identified from seeds in the late stages of embryo development (Dure et al., 1981). Most of LEA proteins and their mRNAs accumulate to high concentrations in plant embryo tissues during the last stages of seed development before desiccation and also in vegetative tissues exposed to dehydration, osmotic, and/or low-temperature stress(Ingram and Bartels 1996; Thomashow 1999). LEA homologues have been found in bacteria (Stacy and Aalen 1998) and animals (Hand et al., 2011). *LEA* gene from *Artemia franciscana* was used to improve cells of *Drosophila melanogaster* tolerance to salt and water deficit (Marunde et al., 2013). LEA proteins are intrinsically disordered and show mostly a random coil structure in solution, some members can adopt a degree of conformation during drying or in the presence of a-helix-promoting agents (Shih et al., 2008). LEA proteins are postulated to support the stabilization of other proteins, such as enzymes during periods of water deficit (Tunnacliffe and Wise 2007). LEA proteins are generally grouped based on their similarity to prototypical LEA proteins from *G. hirsutum* (Wise 2003). A genome-wide identification LEA genes in *Prunus mume* revealed that the *PmLEA* genes could be classified into eight groups based on sequence similarity and the presence of particular motifs: LEA_1, LEA_2, LEA_3, LEA_4, LEA_5, PvLEA18, dehydrin and SMP (seed maturation protein) (Du et al., 2013). At the molecular level, the protective effects of LEA proteins from groups 1, 2 and 3 have been proposed to occur through diverse mechanisms. In *Arabidopsis thaliana*, 50 *LEA* genes were represented in nine groups (Bies-Etheve et al., 2008). Based on physicochemical properties, LEA proteins can also be classified as typical and atypical groups. Typical LEA proteins are also called hydrophilins since they are hydrophilic, with a hydrophilicity index greater than 1, and amino acid Gly content is greater than 6% (Battaglia et al., 2008; Wise and Tunnacliffe 2004). Those showing hydrophobic characteristics are known as atypical LEA proteins (Galau et al., 1993; George et al., 2009). LEA proteins have been also postulated to play a protective role under different abiotic stresses (Dalal et al., 2009; George et al., 2009; He et al., 2012; Olvera-Carrillo et al., 2010; Park et al., 2011; Wang et al., 2009). For example, overexpression of *AtLEA3-3* in *Arabidopsis* enhanced tolerance to salt and osmotic stress (Zhao et al., 2011). Ectopic expression of a LEA protein gene *TsLEA1* from *Thellungiella salsuginea* confers salt-tolerance in yeast and *Arabidopsis* (Zhang et al., 2012). Their physiological and molecular functions still remain largely unknown. Moreover, very few studies take transgenic as approach to demonstrate the functional role of *LEA* genes. Available data indicate that the transcripts of LEA protein accumulate during the late stage of seed development in response to stress conditions, such as drought, UV light, salinity, cold, and wounding (Battaglia et al., 2008; Bhardwaj et al., 2013; Costa et al., 2011; He et al., 2012). *Cleistogenes songorica*, a C4 plant, belongs to a group of xerophyte plants growing in the desert grassland environment

in North West of China with a mean annual precipitation of 100mm (Zhang et al., 2011). Transgenic *Arabidopsis* plants expressing *ALDH12A1* from *C. songorica* under the abiotic stress inducible *rd29A* promoter showed enhanced tolerance to drought stresses (Zhang et al., 2014). To understand more information associated with drought stress tolerance of this xerophytic grass, we report on the isolation and characterization of the cDNA clone for an LEA protein and its putative promoter sequence from *C. songorica*. This cDNA clone was obtained from a cDNA library created from drought-stressed root tissue of *C. songorica* plants (Zhang et al., 2011). Stress inducible expression of *rd29A*::*CsLEA* in *Arabidopsis* plants was investigated under drought and salt stress.

## Results

### *Cloning and bioinformatics analysis of the CsLEA gene*

The full-length cDNA of the target gene isolated from *C. songorica* was 817 bp, designated as *CsLEA* (GenBank accession no. FJ972827). *CsLEA* consists of a 5' UTR of 95 bp, a 3' UTR of 260 bp, a 462 bp open reading frame, encoding a deduced amino acid sequence of 153 amino acids peptide with a predicted molecular weight of 36.385 kDa and a pI of 5.16. To investigate whether *CsLEA* possessed introns, we isolated the genomic sequences using PCR primers (Table 1) corresponding to the 5′- and 3′-ends of *CsLEA* cDNA. Sequence alignment of the cDNA with its genomic counterpart revealed that *CsLEA* has a single 112 bp intron in the genomic DNA sequence. The conserved domain in the Pfam database revealed that CsLEA has a dehydrin domain, PF00257 and an e-value of 1.2e-36. The NPSA (http://npsa-pbil.ibcp.fr) predicted that the secondary structure of the CsLEA protein was 16.34% α-helices , 77.12% random coil and 6.54% extended strand. Kyte–Doolittle hydropathy plot analysis revealed that the CsLEA protein is highly hydrophilic (Data not presented). CsLEA showed high similarity with the orthologs from *Hordeum vulgare* (69%), *Sorghum bicolor* (67%) and *Zea mays* (65%), respectively (Fig 1A). Alignment of the deduced polypeptide sequence of CsLEA and the other orthologs revealed that they all contain a C-terminal conserved region (Fig 1B). The amino acid segments RSGSSSSSSS-EDDGMGGRRKKG and TGEKKGIMDKIKEKIPGQ were relatively conserved among all the proteins analyzed (Fig 1A). In rice genome, the *OsLEA* genes could be classified into seven groups based on sequence similarity: LEA_1, LEA_2, LEA_3, LEA_4, LEA_5, Dehydrin and SMP (Wang et al., 2007). The phylogenetic relationships between different LEA group proteins was analyzed and an phylogenetic tree was constructed including rice LEA protein sequences available in the GenBank. The results showed that CsLEA is a typical Dehydrin proteins and has close phylogenetic relationship with the Dehydrin homologues of AAD02258, 1488312, 18964 (Fig 1B).

### *Isolation and analysis of the CsLEA promoter*

In order to gain further insight into the mechanism responsible for the transcriptional regulation of *CsLEA*, we isolated a 972 bp promoter region upstream of the *CsLEA* ATG start codon from genomic DNA using a hiTAIL-PCR strategy. Based on the results from hiTAIL-PCR analysis and searches of data bases of Plant Cis-acting Elements, a number of regulatory elements responding to dehydration, low-temperature, and heat shock were recognized, including ABRE (abscisic acid response element), MYB (myeloblastosis), LTREATLTI78 (low temperature responsive element, low-temperature-induced genes in Arabidopsis thaliana), and CCAAT box (Table 2). In addition, a light signal response element and a typical CAAT-box element were identified.

### *Expression analysis of CsLEA genes*

To investigate its temporal and tissue expression patterns, the transcription levels of *CsLEA* in different organs were determined by semi quantitative RT-PCR and real-time quantitative RT-PCR. Semi quantitative RT-PCR and relative quantitative RT-PCR performed using cDNA derived from 10d stressed and non-stressed leaves and roots of *C. songorica* seedlings demonstrated the only inducible of *CsLEA* expression in dehydrated roots samples (Fig 2A, 2B). The absolute quantitative RT-PCR results showed that drought stress stimulated *CsLEA* mRNA expression both in leaves and roots from the 6 d after treatment, then gradually increased to maximum at 10 d after treatment and decreased to zero expression after rehydration (Fig 2C). This indicates that *CsLEA* expression is modulated by drought stress.

### *Phenotypic analysis of transgenic plants under abiotic stress tolerance*

Wild type plants and *rd29A*::*CsLEA* transgenic plants were subjected to salt and osmotic stress tolerance analyses. The stress tolerance of transgenic *Arabidopsis* was evaluated by transferring two week old seedlings to MS agar medium containing 0, 100, 200, 300, 400mM sorbitol, or 0, 10, 20, 50, 100mM NaCl, respectively. No significant differences were observed between transgenic seedlings and WT plants grown on MS agarose only without stress at 0 and 10 days, and MS supplemented with less than 200mM sorbitol or 20 mM NaCl (Fig 3). Transgenic plants exhibited higher growth than that of WT plants both on MS supplemented with 300mM sorbitol (Fig 3A (e)) or 50 mM NaCl (Fig 3 B (e)) at 10 days. Both WT and transgenic plants showed growth restriction under high concentration osmotic stress of 400mM sorbitol (Fig 3A (f)) or 100 mM NaCl (Fig 3 B (f)) at 10 days.

Drought stress tolerance analysis was also performed on *rd29A*::*CsLEA* transgenic plants under soil conditions. A preliminary experiment of drought stress treatment was carried out to decide the tolerance of WT plants. In this experiment, drought stress was executed by controlling watering up to 14 days, and then following a 3 days recovery. Transgenic *Arabidopsis* plants overexpressing *CsLEA* showed enhanced drought tolerance and recovery. Severe wilting was observed in the majority of WT plants, while two transgenic *Arabidopsis* lines, rd29-LEA-1 and rd29-LEA-3, maintained growth and development both at the end of drought stress and after recovery (Fig 4A). The $CO_2$ assimilation rate (*A*) and leaf proline content of WT and *rd29A*::*CsLEA* transgenic plants, during the 17 days of stress treatment, are presented in Fig 4B and Fig 4C. Although no significant differences in $CO_2$ assimilation rates was observed in 29A-LEA-1, 29A-LEA-3 and WT during the first 9 days of the dehydration stress treatment, significant differences ($P<0.05$) existed after 14 days dehydration and 3 days rehydration (Fig 4B). The value of *A* decreased in all the experimental lines with the 14 days dehydration stress treatment. The photosynthetic ability of transgenic lines resumed after the 3 day recovery period. The WT line did not show any change in photosynthetic activity post recovery. These results associate LEA protein with a role of protecting the plant photosynthetic mechanisms from dehydration

**Table 1**. Primers used to clone and analyze *CsLEA*.

| Primer name | (5'-3') Nucleotide sequence | Purpose |
|---|---|---|
| CsLEA_F | ATGGAGCACCAGGGACGGTAC | cDNA Cloning of *CsLEA* |
| CsLEA_R | GGGCTGGCCGGGGAGCTTCT | |
| CsLEA_$F_{gDNA}$ | AATTGGCCCAATGGCCCAA | gDNA Cloning of *CsLEA* |
| CsLEA_$R_{gDNA}$ | CATACAGACTCTTCACACTA | |
| 0a | CATTACATACAGACTCTTCACACTA | Promotor Cloning of *CsLEA* |
| 1a | ACGATGGACTCCAGTCCGGCCCAAATAAACAAGTAGCAGTATG | |
| 2a | CTTACTGGATTGCTGCAACTTTTGG | |
| CsLEA_$F_{rt}$ | GATACACGGCACGGGTACAG | RT-PCR of *CsLEA* |
| CsLEA_$R_{rt}$ | TGTGACGCGCTTAAGAACAC | |
| CsGAPDH_$F_{rt}$ | CTCTGCCCCTAGCAAAGATG | RT-PCR of *CsGAPDH* |
| CsGAPDH_$R_{rt}$ | GAGCTTGCCCTCAAAAACAG | |
| CsLEA_$F_{Qrt}$ | TACGTGCGAACAGTCTGTGA | Q-RT-PCR of *CsLEA* |
| CsLEA_$R_{Qrt}$ | TGTGACGCGCTTAAGAACAC | |
| CsGAPDH_$F_{Qrt}$ | GTCAGCCAAGGACTGGAGAG | Q-RT-PCR of *CsGAPDH* |
| CsGAPDH_$R_{Qrt}$ | ACACATCGACTGTTGGGACA | |
| CsEIF5_1_F | CAACACCAAGCCCACTCC | Q-RT-PCR of *CsEIF5_1* |
| CsEIF5_1_R | AGGGTTGAGGAGGACAGCAC | |
| CsEIF5_2_F | GAGCGTGCTGTCCTCCTC | Q-RT-PCR of *CsEIF5_2* |
| CsEIF5_2_R | GAATCCCTTGCCCACACC | |
| rd29A_F | AGAATCTCAAACACGGAG | Cloning of *rd29A* |
| rd29A_R | ACTAAGTTTATAGAGAGACTG | |
| CsLEA_$F_{BamHI}$ | GCGGGATCCATGGAGCACCAGGGACGGTAC[a] | Sub-cloning *CsLEA* into vector |
| CsLEA_$R_{SacI}$ | GCGGAGCTCTTAGGGCTGGCCGGGGAG[b] | |

[a,b] Underlined sequences, restriction site of BamHI and SacI

**Table 2.** Analysis of putative cis-acting elements in the *CsLEA* promoter.

| Transcription element | Motif | Function |
|---|---|---|
| CACTFTPPCA1 | YACT | Cis-acting elements |
| GTGANTG10 | GTGA | Cis-acting elements |
| ARR1AT | NGATT | Cis-acting elements |
| ACGTATERD1 | ACGT | Cis-acting elements |
| GT1CONSENSUS | GRWAAW | Transcription regulation |
| CAAT-box | CAAT | Transcription occurs |
| POLLEN1LELAT52 | AGAAA | Transcription regulation |
| ROOTMOTIFTAPOX1 | ATATT | Transcription occurs |
| DOFCOREZM | AAAG | Transcription occurs |
| GATABOX | GATA | Transcription occurs |
| SITEIIATCYTC | TGGGCY | Transcription factor |
| TAAAGSTKST1 | TAAAG | Transcription factor |
| CCAATBOX1 | CCAAT | Heat shock response element |
| CURECORECR | GTAC | Signal response element |
| -10PEHVPSBD | TATTCT | Light signal response element |
| SORLIP2AT | GGGCC | Phy control element |
| ABRELATERD1 | ACGTG | Dehydration response |
| MYBCORE | CNGTTR | Dehydration response |
| LTREATLTI78 | ACCGACA | Stress(low temperature) response |

stress. Proline concentration increased during the dehydration stress treatment and reached a peak at 14 days of severe moisture stress (Fig 4C). There was a decrease in proline expression after the 3 day recovery period. Similarly to the $CO_2$ assimilation rate, significant ($P$<0.01) differences in Proline concentration was shown between the two transgenic lines and WT after 14 days of dehydration and 3 days of rehydration. These differences were not observed during the initial 9 days of dehydration (Fig 4C). The Proline content of the two transgenic *Arabidopsis* lines on the 14th day of dehydration stress was on average 4 fold higher than in WT. It can be concluded that osmotic regulation was induced by water deficit.

**Discussion**

LEA proteins have been classified into at least five groups based on amino acid sequence homology and specific structural features (Dure et al., 1989). Analyzing the *Arabidopsis thaliana* genome sequence to detect LEA protein genes, 50 genes were classified into nine groups according to their similarities and the presence of conserved domains (Bies-Etheve et al., 2008). In our paper, we described the identification of a dehydration inducible gene encoding a LEA protein from the xerophytic grass *C. songorica*, and showed increased osmotic stress tolerance in transgenic *Arabidopsis* plants. The calculated molecular weight of *CsLEA* is 36.385 kDa. LEA proteins can also be referred to according to their molecular weights, such as D-7, D-11, D-19, D-29, D-34, D-73, D-95, and D-113 (Cuming 1999). Most LEA proteins are highly hydrophilic and remain soluble after boiling (Dure 1993). All LEA proteins with a higher content of hydrophobic residues than typical LEA proteins are included in atypical LEA proteins. Atypical LEA proteins have been implicated in cellular functions different from those of the hydrophilic LEA proteins (Galau et al., 1993; Zegzouti et al., 1997). Dehydrin LEA are also found in rice (Wang et al., 2007) and algae and share a highly conserved 15-amino-acid lysine-rich sequence, or called K-segment

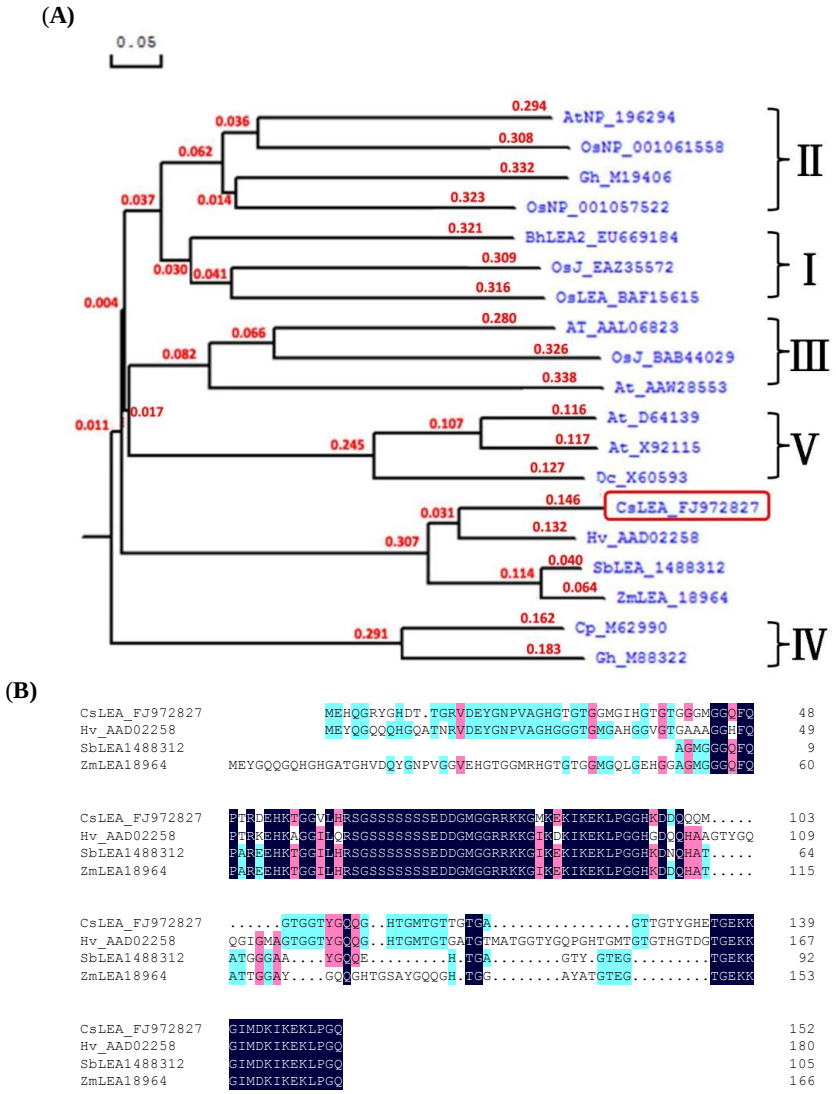


**Fig 1.** Phylogenetic analysis and amino acid sequence alignment and of CsLEA and homologous proteins. (A) Phylogenetic analyses of selected LEA family in *Oryza sativa*. Ⅰ~Ⅴ represent 5 LEA groups in rice (Wang et al., 2007). GenBank accession nos. 196294, 001061558, M19406, 001057522, EU669184, EAZ35572, BAF156615, AAL06823, BAB44029, AAW28553, D64139, X92115, X60593, FJ972827, AAD02258, 1488312, 18964, M62990, M88322. (B) Alignment of the deduced amino acid sequences of LEA in different plants. Identical amino acids showed conserved motif are shaded in black, and similar amino acids are shaded in purple and blue. Dashes show gaps in the amino acid sequences introduced to optimize alignment.

(EKKGIMDKIKEKLPG) present in one or several copies. CsLEA also contain K-segment and an S-segment (polyserine stretch) that can undergo extensive phosphorylation. Many dehydrins also contain a Y-domain (DEYGNP) similar to the nucleotide-binding site of plant and bacterial chaperones. In perennial ryegrass, LEA protein has potential to enhance cellular water retention benefiting drought tolerance by forming non-polar residues (G, A) to polar residues (S, T) (Yu et al., 2013). CsLEA classified as a hydrophilic protein, is induced by drought stress in *C. songorica*. LEA proteins have been associated with abiotic stress tolerance in many studies (Dalal et al., 2009; He et al., 2012; Park et al., 2011; Wang et al., 2009). A genome-wide identification and analysis of 30 *LEA* genes in *Prunus mume* revealed that most *PmLEA* genes were highly expressed in flowers (22/30) and its expression up-regulated by ABA treatment (19/30) (Du et al., 2013). However, *LEA* genes in *Arabidopsis* are expressed in vegetative tissues in the absence of any abiotic stress. *LEA* genes from the same group do not present identical expression profiles and regulation of these genes with apparently similar expression patterns does not systematically involve the same regulatory pathway (Bies-Etheve et al., 2008). Dries et al., (2011) comparatively analyzed *LEA-like 11-24* gene expression and regulation in three resurrection plants *Craterostigma plantagineum*, *Lindernia brevidens* and *L. subracemosa* and showed that the *LEA-like 11-24* gene was differentially regulated in these plants. We analyzed the expression patterns of *CsLEA* in response to drought stress in *C. songorica*. The results of absolute quantification RT-PCR indicated that *CsLEA* was drought-induced with high expression only in the leaf and root tissue of severely stressed seedlings. The broad stress adaptive capacity of the *C. songorica* may due to a combination of *CsLEA* with other stress related genes. The LEA group 4B genes show responses to ABA, dehydration, and high salinity in vegetative tissues, suggesting a key role under salt and drought stress (Battaglia et al., 2008; Dalal et al., 2009).

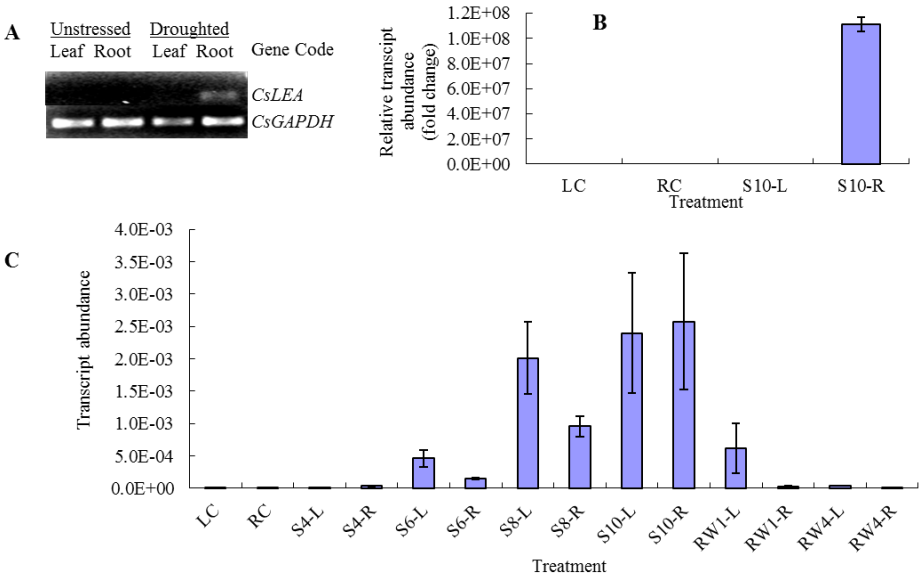


**Fig 2.** Expression patterns of *CsLEA* in *C. songorica* during the drought stress and recovery stages by semi-quantitative RT-PCR and Q- RT-PCR. Error bars represent standard error of the mean (n=6). (A) semi-quantitative RT-PCR of *CsLEA*, *CsGAPDH* as a housekeeping gene. (B) relative Q-RT-PCR of *CsLEA*, (C) absolute Q- RT-PCR of *CsLEA*. LC and RC, leaf and root of non-stressed seedlings; S4-L and S4-R, leaf and root of 4d stressed seedlings; S6-L and S6-R, leaf and root of 6d stressed seedlings; S8-L and S8-R, leaf and root of 8d stressed seedlings; S10-L and S10-R, leaf and root of 10d stressed seedlings; RW1-L and RW2-R, leaf and root of 1d rewatered seedlings; RW4-L and RW4-R, leaf and root of 4d rewatered seedlings.

Several putative cis-acting DNA elements were identified in the upstream sequence and 5' UTR of *CsLEA*, including ABRE, MYB, LTREATLTI78, and ACGTATERD1. The ACGT element is involved in early response to drought of *erd1*, an *Arabidopsis* gene (Simpson et al., 2003) and might contribute to the up-regulation of *PjLEA3* at PEG stress in *P. juliflora* leaves (George et al., 2009). ABRE elements were found functionally conserved between *C. plantagineum* and *L. brevidens* (Dries et al., 2011), and wheat (Min et al., 2012). This supports the hypothesis that functionally important cis-acting regulatory elements are often evolutionarily conserved as a result of functional constraints (Ludwig 2002).
Overexpression of plant LEA proteins in yeast and bacteria has been described as conferring increased tolerance to osmotic or salt stresses (Liu and Zheng 2005; Swire-Clark and Marcotte Jr 1999; Zhang et al., 2012; Zhao et al., 2011). Over expression of certain *LEA* genes in transgenic plants resulted in enhanced tolerance to abiotic stresses (Borrell et al., 2002; Liu et al., 2009; Puhakainen et al., 2004; Qu et al., 2012; Xu et al., 1996) , while *Arabidopsis* plants deficient in LEA 4 group genes were more susceptible than wild-type plants under water stress (Olvera-Carrillo et al., 2010). Heterologous expression in *E. coli* for *OsLEA5*, the hydrophobic LEA protein from *Oryza sativa* L. exhibited improved resistance against diverse abiotic stresses: high salinity, osmotic, freezing, heat, and UV radiation (He et al., 2012). In this report, we overexpressed *CsLEA*, an hydrophilic protein gene in *C. songorica*, increased the tolerance to drought and salt stress in transgenic *Arabidopsis*. Furthermore, compared with the wild-type, transgenic plants (*rd29A*::*CsLEA)* were able to protect their photosynthetic machianisms and osmotic regulation during dehydration stress.

## Materials and methods

### *Plant material and drought stress treatment*

Seedlings of *C. songorica* were grown in a sand vermiculite mixture (1:1 by volume) and maintained according to protocols by (Zhang et al., 2011). Drought stress was applied to 55 d old plants by withholding watering for up to 10 d, and then rewatering once on day 10. At 0, 4, 6, 8, and 10 d of drought stress, and 1, and 4 d after rewatering, leaves and roots were sampled, freeze dried, and stored at −80 ℃. Wild-type *Arabidopsis thaliana* (genotype Col-0) seeds were used for genetic transformation using the floral dip method.

### *Cloning of the CsLEA cDNA, gDNA, promotor sequence and sequence analysis*

Total RNA was isolated using a RNeasy plant mini kit (Qiagen, Germany). The quality and quantity of the RNA was verified using agarose gel electrophoresis and spectrophotometric analysis followed by treatment with RNase-free DNase I. About 1 μg of total RNAs was used for first strand cDNA synthesis. Genomic DNA was extracted from the fresh leaf samples using the Plant Genomic DNA kit (Tiangen, China) according to the manufacturer's recommendations. An EST similar to LEA protein from a drought-induced cDNA library (Zhang et al., 2011) of *C. songorica* seedlings was used as a template for rapid amplification of full length cDNA sequence using specific primers (Table 2). Genomic DNA sequence was also isolated using genomic DNA as template and the gene specific primers shown in Table 1. To obtain the upstream fragment of *CsLEA*, hiTAIL-PCR was applied according to Liu and Chen (Liu and Chen 2007) using genomic DNA. The arbitrary degenerate primers and specific primers shown in Table 1 were used in hiTAIL-PCR. Putative cis-acting elements in the promoter region were searched using databases of Plant Cis-acting Elements, PLACE (https://sogo.dna.affrc.go.jp/cgi-bin/sogo.cgi?sid=&lang=en&pj=640&action=page&page=newplace). The amplified PCR products were cloned into a pGEMT-Easy vector (Promega Corp., Madison, WI) and sequenced at Shanghai Shenggong Biotechnological Ltd. (Shanghai, China). Sequence similarities were examined using the GenBank database via the BLAST program

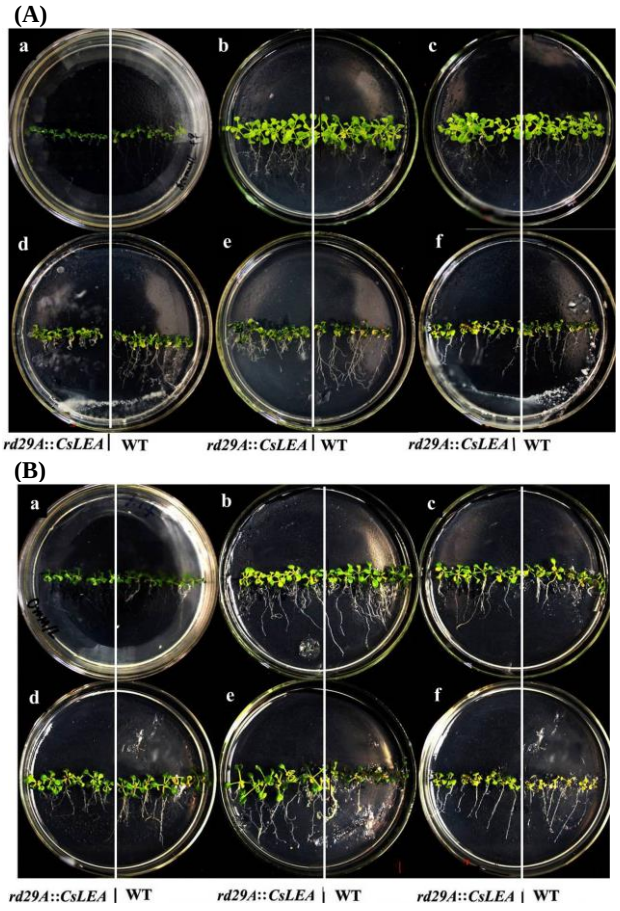


**Fig 3.** Osmotic and salt stress tolerance of transgenic *rd29A*::*CsLEA Arabidopsis.* Two-week-old seedling of wild type (WT) and *Arabidopsis* transgenics were transplanted on MS + sorbitol media. (a) Seedling growth on MS only (control) at 0 days after-sowing (DAS), (b) MS only at 10 DAS, (c) MS+ 100mM sorbitol at 10 DAS, (d) MS+ 200mM sorbitol at 10 DAS, (e) MS+ 300mM sorbitol at 10 DAS, and (f) MS+ 400mM sorbitol at 10 DAS. (B)Two-week-old seedling of wild type (WT) and *Arabidopsis* transgenics were transplanted on MS + NaCl media. (a) Seedling growth on MS only (control) at 0 days after-sowing (DAS), (b) MS only at 10 DAS, (c) MS+ 10mM NaCl at 10 DAS, (d) MS+ 20mM NaCl at 10 DAS, (e) MS+ 50mM NaCl at 10 DAS, and (f) MS+ 100mM NaCl at 10 DAS.

(http:/ /blast.ncbi.Nlm.nih.gov/Blast.cgi). For nucleic acid and amino acid sequence alignment, DNAMAN 5.2.2 software (Lynnon Biosoft) was used. A hydropathy plot was generated using the online PROTSCALE program (www.expasy.ch/ tools/protscale.html). Phylogenetic relationship was analyzed by multiple alignments of plant *LEA* cDNAs via the Vector NTI advance 10 Suit (Invitrogen, Carlsbad, CA).

***Transcription analysis of CsLEA in C. songorica seedlings***

Semi-quantitative and real time RT-PCR were used to test the expression profile of *CsLEA*. Total RNA was extracted from the control and stress treated leaf and root tissues using the method described above. The semi-quantitative PCR was carried out in a total volume of 30 µL including 0.005 µmol $L^{-1}$ dNTP, 3 µL 10×Dynazyme buffer, 1 U *Taq* polymerase, 0.5 µmol $L^{-1}$ of each primer, and 0.5 µg cDNA. A putative GAPDH encoding sequence (*CsGAPDH*, FJ972819) from *C. songorica* (Zhang et al., 2009) was used as a constitutively expressed reference gene. The relative real-time PCR were assayed in a 10 µL qRT-PCR reaction containing 5 µL 2×SYBR Green mix (Applied Biosystems) and 1 µL cDNA (1:10 dilution). To test the absolute quantification of *CsLEA* expression, serial timepoint leaf and root samples were collected at 0, 4, 6, 8 and 10 d of drought stress, and 1 and 4 d after rewatering. Three 'housekeeping' genes, *CsGAPDH*, *CsEIF5_1* and *CsEIF5_2* were used to normalize target gene quantities according to (Zhang et al., 2009). The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative changes in gene expression from quantitative real-time PCR experiments (Livak and Schmittgen 2001; Zhang et al., 2009). *CsLEA* and reference gene specific primers mentioned above are listed in Table 1. All experiments were conducted with three biological and two technical replicates.

***Induced expression of CsLEA12A in Arabidopsis***

*CsLEA* was PCR-amplified from the pGEMT- *CsLEA* vector with the CsLEA_$F_{BamHI}$ and CsLEA_$R_{sacI}$ primers as mentioned in Table 1, restricted with BamHI and SacI, and cloned into modified binary vectors pBI121-*rd29A*::*GUS*::*NOS*, by replacing *GUS* with *CsLEA*. Thus, *CsLEA* was cloned using the stress-inducible *Arabidopsis rd29A* promoter in pBI121-*rd29A*::*GUS*::*NOS*. The binary vectors were transformed into the *Agrobacterium* strain *GV3101* and used for transformation into *Arabidopsis thaliana* ecotype Columbia 0 using the floral dip transformation method (Clough and Bent 1998). The T1 plants were selected on Kanamycin (50 $\mu gml^{-1}$) and confirmed by PCR. The T2 lines showing a 3:1 segregation for Kanamycin resistance were carried forward to the T3 generation. Presence of the transgene in transgenic *Arabidopsis* was confirmed by PCR using promoter specific forward primers and the *CsLEA*-specific reverse primer. PCR-confirmed homozygous T3 were used for functional validation of *CsLEA*.

***Stress treatment of transgenic plants***

To evaluate the stress tolerance of transgenic *Arabidopsis* plants containing the stress response gene *CsLEA*, two-week-old seedlings (grown on MS media) of wild type (WT) and T3 transgenics were transplanted on to MS with 0, 10, 20, 50, 100mM NaCl or with 0, 100, 200, 300, 400mM sorbitol media, and subjected to stress for 10 days. Photographs were taken to record the differences in growth after 10 days.

For assaying drought stress tolerance, two-week-old seedlings of WT and transgenic *Arabidopsis* plants containing the stress response gene *CsLEA*, were transplanted into soil medium (soil vermiculite, 3:1) and allowed to grow for two more weeks under non stress conditions in a plant growth chamber at 22/20 ℃ day/night, 16/8 h day/night and light density of $80\mu mol \cdot m^{-2} \cdot s^{-1}$. Water-deficit stress was imposed by withholding irrigation for 14 days. To evaluate the recovering ability of these plants, irrigation was resumed and photographs were taken 3d after irrigation. During the 0d, 9d, 14d, and 17d of stress treatment, the rates of $CO_2$ assimilation (*A*) were measured using a closed infrared gas analysis system (LI-COR 6400XL; LI-COR, Lincoln, NE, USA) set up with a whole-leaf clamp-on chamber. The measurements were taken on a young, fully expanded rosette leaf (5 plants/line) at an ambient $CO_2$ concentration of 360 µmol $mol^{-1}$ and a chamber temperature ranging from 22°C to 23°C. Proline content was estimated spectrophotometrically by the ninhydrin reaction under conditions described by (Maiale et al., 2004).

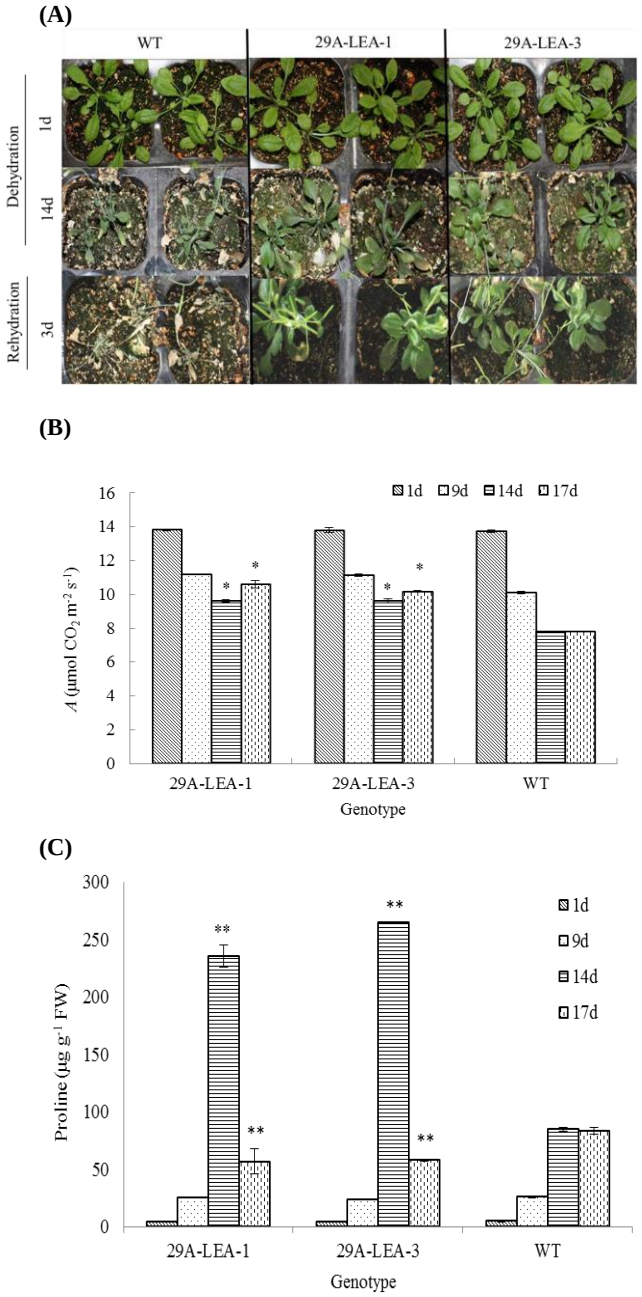


**Fig 4.** Stress induced expression of CsLEA improves drought tolerance in transgenic Arabidopsis. (A) Phynotype of wild type and transgenic Arabidopsis plants under dehydrated at 1d, 9d, 14d and rewatered at 3d (17d). (B) $CO_2$ assimilation rate (A, μmol $CO_2$ $m^{-2}$ $s^{-1}$)as measured by gas exchange using the LI-COR 6400XL system. (C) Leaf proline content (μg $g^{-1}$ FW). WT, wild type Arabidopsis; 29A-LEA-1 and 29A-LEA-3, independent transgenic Arabidopsis overexpressing CsLEA under stress inducible rd29A promoter. Error bars indicate mean ± SD (n = 5). Asterisk denotes significant differences (*P < 0.05, **P < 0.01) between transgenics and WT plants according to ANOVA results.

***Data analysis***

The data were analyzed by one way analysis of variance (ANOVA) followed by LSD calculation at $P< 0.05$ (or 0.01) level to compare the means, using SPSS 13.0 for Windows.

**Conclusion**

In conclusion, this study isolated *CsLEA* cDNA and its promoter sequence. Absolute quantitative RT-PCR showed that accumulation of *CsLEA* transcripts was constitutively increased both in leaves and roots during the time-course of drought stress. Stress inducible expression of *rd29A*::*CsLEA* in *Arabidopsis* plants enhanced drought and salt tolerance by decreasing $CO_2$ assimilation rate and increasing proline concentration to affect osmotic regulation. Further studies are also required to understand the function of different motif in promoter sequence under drought stress treatment, to examine stress tolerance of *CsLEA* transgenic crop plants.

**Acknowledgments**

This work was supported by grants from National Basic Research Program (973) of China (2014CB138704), the National Natural Science Foundation of China (31101759), Program for Changjiang Scholars and Innovative Research Team in University (IRT13019), the Department of Agriculture and Animal Husbandry, Gansu province (GNSW-2011-16), and Fundamental Research Funds for the Central Universities (lzujbky-2013-87).

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