JcLEA, a Novel LEA-Like Protein from *Jatropha curcas*, Confers a High Level of Tolerance to Dehydration and Salinity in *Arabidopsis thaliana*

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Abstract

*Jatropha curcas* L. is a highly drought and salt tolerant plant species that is typically used as a traditional folk medicine and biofuel crop in many countries. Understanding the molecular mechanisms that underlie the response to various abiotic environmental stimuli, especially to drought and salt stresses, in *J. curcas* could be important to crop improvement efforts. In this study, we cloned and characterized the gene for a late embryogenesis abundant (LEA) protein from *J. curcas* that we designated JcLEA. Sequence analyses showed that the JcLEA protein belongs to group 5, a subgroup of the LEA protein family. In young seedlings, expression of JcLEA is significantly induced by abscisic acid (ABA), dehydration, and salt stress. Subcellular localization analysis shows that JcLEA is distributed in both the nucleus and cytoplasm. Moreover, based on growth status and physiological indices, the overexpression of JcLEA in transgenic *Arabidopsis* plants conferred increased resistance to both drought and salt stresses compared to the WT. Our data suggests that the group 5 JcLEA contributes to drought and salt stress tolerance in plants. Thus, JcLEA is a potential candidate gene for plant genetic modification.

Introduction

Water and ion concentrations in soil are important abiotic elements for living organisms. Unlike animals, plants are sessile organisms that are exposed to seasonal and local environmental variations, and their survival and growth are strongly influenced by local stress factors. It has been estimated that 70% of crop yield losses are caused by abiotic stresses, with drought and high salinity being the most serious threats to crop production in many areas of the world [1]. Plants have developed multi-pathway survival strategies, at multiple-levels and on multiple-scales to allow them to grow and reproduce successfully. The late embryogenesis abundant (LEA) proteins are involved in one type of self-protection mechanism. LEA proteins belong to a large protein family that is closely associated with resistances to abiotic stresses, especially to drought, in a range of organisms [2]. The LEA proteins were originally identified in terrestrial plants, including wheat (*Triticum aestivum*) and cotton (*Gossypium hirsutum*), and were subsequently detected in many other species of higher plants [3–8]. LEA proteins mainly accumulate in the late stages of embryogenesis in plant seeds under dehydration stress [5], and have also been found in bacteria [9], slime molds [10], nematodes [11–15], fungi [16], and humans [17].

LEA proteins are localized to various subcellular compartments, including the nucleus, mitochondria, chloroplast, endoplasmic reticulum, vacuole peroxisome, plasma membrane and cytoplasm [18,19]. They function as a protecting factor associated with the desiccation tolerance of seeds, pollen and anhydrobiotic tissues [20,21]. Over the past two decades, remarkable research progress has been achieved in the characterization of LEA proteins, which are considered to be functional effectors in the generation of abiotic tolerance in plants.

The LEA protein super families have a diversity of sequences and functions. Based on the nomenclature introduced by Battaglia, LEA proteins are categorized into seven distinctive groups, which include groups 1 (LEA-5), 2 (dehydrin), 3 (LEA-4), 4 (LEA-1), 5 (A: SMP; B: LEA-5; C: LEA-2), 6 (LEA-6), and 7 (ABA-WDS) [22]. Groups 1, 2, 3, and 4, which share specific sequence motifs within each group, are considered to be typical or genuine hydrophilic LEA proteins, while group 5 LEAs lack significant signature motifs or consensus sequences and are regarded as atypical LEA proteins, containing a significantly higher proportion of hydrophobic residues than typical LEA proteins [22]. It is noteworthy that group 5 proteins are natively folded and are not soluble after boiling, suggesting that they are probably not heat stable [23]. The average molecular weight of the group 5 proteins is 18.1 kDa, and most group 5 LEA proteins are acidic. Based on these features, it is predicted that group 5 proteins play roles in seed maturation, dehydration, and the combining of concentrated ions [8]. At present, a number of group 5 LEA genes have been cloned and characterized from many plant species, including cotton LEA D-
34 [24], maize RAB28 [25], carrot ECP31 [26], Arabidopsis AtECP31 and AtRAB28 [27,28], G. brassicaceae plantagineum plc27-43 [29], tomato ER3 [30], cayenne pepper CaLEA6 [31], Medicago truncatula MrPM23 [32], soybean GmPM22, GmPM24, GmPM25, and GmPM26 [33], and OsLEA5 from rice [34]. Nevertheless, group 5 still attracts fewer investigations compared with the other LEA subcategories mentioned above.

Jatropha curcas L. is a multi-purpose shrub belonging to the plant family Euphorbiaceae. The species is widespread throughout arid and semi-arid tropical regions of the world, and has been utilized (FC) without drought injury [35]. It has been reported that mechanisms involved in the response of Mediterranean crops [37,38]. However, the molecular mechanisms underlying the drought-tolerant characteristics of J. curcas showed a significant reduction in growth (by 5.82% for every 10 mM increment in NaCl concentration). This finding shows that treated plants (irrigated with NaCl levels of over 30 mM NaCl) showed a significant reduction in growth (by 5.82% for every 10 mM increment in NaCl concentration). This finding shows that grown in a natural environment on farmland in the Renhe (Panzhihua, Sichuan Province, China). No specific permission was required for any of the locations or activities, and the field studies did not involve endangered or protected species. Seeds were germinated in soil in the greenhouse. Roots, leaves and stems were collected and stored at −70°C. Seeds of Arabidopsis thaliana accession Columbia (Col-0) were obtained from the Arabidopsis Biological Resource Center (ABRC: Columbus, OH, USA).

### Materials and Methods

#### Plant Materials and Sampling Locations

Seeds of Jatropha curcas were obtained from the Jatropha curcas Germplasm Resources Preservation Center. The plants were grown in a natural environment on farmland in the Renhe (Panzhihua, Sichuan Province, China). No specific permission was required for any of the locations or activities, and the field studies did not involve endangered or protected species. Seeds were germinated in soil in the greenhouse. Roots, leaves and stems were collected and stored at −70°C. Seeds of Arabidopsis thaliana accession Columbia (Col-0) were obtained from the Arabidopsis Biological Resource Center (ABRC: Columbus, OH, USA).

### Table 1. Oligonucleotide primers and their uses in this study.

| Primers     | Sequence                                                                 | Usage                              |
|-------------|--------------------------------------------------------------------------|------------------------------------|
| LEAF        | 5′-ATHACNATTHGGGWGARGWTRGAR-3′ (H is A, C and T; N is A, C, G and T; W is A and T; R is A and G) | Cloning of JcLEA cDNA             |
| LEAR        | 5′-RSWAGCCACGACCDCCDCWG-3′ (W is A and T; D is A, G and T)                 | Cloning of JcLEA cDNA             |
| JcLEAF1     | 5′-GACTACCTCTCCTGATGTTTCTGAGGGCAG-3′                                   | Cloning of JcLEA cDNA             |
| JcLEAF2     | 5′-GATGCTGAAGGGTTATTGAGGGCAAGAA-3′                                     | Cloning of JcLEA cDNA             |
| JcLEAR1     | 5′-CAGGCATCTCCGGGTTACCTGCTTGGC-3′                                     | Cloning of JcLEA cDNA             |
| JcLEAR2     | 5′-CAGGACCAGAGCAGCACTGACGG-3′                                           | Cloning of JcLEA cDNA             |
| JcLEAfull-F | 5′-ATGAGCCAGGCAACCACGAAGA-3′                                            | Cloning of JcLEA cDNA, Semi-quantitative PCR, Transformsants identification |
| JcLEAfull-R | 5′-TATGGAATCTGATTAAGCTTACGACGTGC-3′                                     | Cloning of JcLEA cDNA, Semi-quantitative PCR, Transformsants identification |
| JcLEAQ1     | 5′-ACGGCGAAACAGAGGTTGGCAGCTG-3′                                        | Cloning of JcLEA cDNA             |
| JcLEA-B     | 5′-GCGgattcATGAGCGAGGGCAACAC-3′                                        | Cloning of JcLEA cDNA             |
| JcLEA-X     | 5′-GCTctagaTTATGGGTTCTCGATTAAGC-3′                                     | Cloning of JcLEA cDNA             |
| LEA-GPF     | 5′-AATGAAAAGAAAAGAAAAAAGCAGC-3′                                        | Construction of JcLEA-GFP          |
| LEA-GFP      | 5′-GGactagtTGGTCTGATTAAGCGTACA-3′                                      | Construction of JcLEA-GFP          |
| 5′-RACE CDS Primer | 5′-AAGCAGCTTGTTATCAACGGCAGAGTACT(3) (1N-3′) | Cloning of JcLEA cDNA             |
| 5′-RACE CDS Primer | 5′-(T)2N-3′                                                             | Cloning of JcLEA cDNA             |
| UPM         | Long: 5′-CTAAATACGACTCAGTATGGAGCAGGGTTATCAACGGCAGAGTACT-3′ | Cloning of JcLEA cDNA             |
| NUP         | 5′-AAGCAGCTTGTTATCAACGGCAGAGTACT-3′                                     | Cloning of JcLEA cDNA             |
| SMARTIA Oligo | 5′-AAGCAGCTTGTTATCAACGGCAGAGTACT-3′                                     | Cloning of JcLEA cDNA             |
| AP          | 5′-GGCAGCGGCTGACTGATCTTTTTTTTTTTTTTTTT-3′                               | Cloning of JcLEA cDNA             |
| JcActin-rF  | 5′-TAACTGGCTCCTTGGATGTC-3′                                              | Quantitative Real-time PCR         |
| JcActin-rR  | 5′-AGAAAAGAAAAAGAAAAAGCAGC-3′                                           | Quantitative Real-time PCR         |
| LEA-R1      | 5′-AGTATGTTTATGAGAAGA-3′                                                | Quantitative Real-time PCR         |
| LEA-R2      | 5′-CCATTGTAATATCCCATACC-3′                                              | Quantitative Real-time PCR         |

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Plant Growth Conditions

For gene expression analysis, 30-day-old *J. curcas* plants were treated with 300 mM NaCl for 1 day, 100 μM ABA (Abscisic acid) for 3 hours and 30% PEG-4000 for 3 days, respectively. For root length determination, surface-sterilized *Arabidopsis* seeds were germinated on MS (Murashige and Skoog) medium (pH 5.8) containing 2% phytagel with 50 mM, 100 mM, and 150 mM NaCl or 10% and 15% PEG-4000. The seedlings were grown under a 16 h light/8 h dark photoperiod at a temperature of 22 °C with a light intensity of 150 μmol m⁻² s⁻¹ in a plant growth cabinet. Plants were exposed to drought and salt stress for 14 days and then photographed, and root length was measured using ImageJ software.

Two transgenic *Arabidopsis* lines overexpressing *JcLEA* were used for physiological tests of tolerance to high salinity and dehydration. Seedlings growing in the greenhouse under a 16 h light/8 h dark cycle at 22 °C for 4 weeks were watered with 50 mM, 100 mM, 150 mM, or 200 mM NaCl for 25 days and photographed and analyzed for survival rates at the 25th day of salt treatment. For drought treatment, water was withheld from 4-week-old plants for 14 days, after which they were rewatered for 10 days. The survival rate determinations and physiological measurements were carried out at the 14th day under the drought treatment and the 10th day after rewatering. Physiological measurements including the relative water content, electrolyte leakage, glucose content, sodium content, and potassium content were measured at the 14th day of the dehydration treatment, the 10th day after rewatering, and the 25th day of salt treatment. Whole plants were used for investigation of sodium content and potassium content, and leaves were used for the other physiological measurements.

RNA Extraction

Total RNA of *J. curcas* was extracted as previously reported [39]. Total RNA was extracted from *A. thaliana* leaves using the

![Figure 1. Nucleotide and deduced protein sequences of the cloned JcLEA gene. The start and stop codons are indicated in bold.](https://doi.org/10.1371/journal.pone.0083056.g001)
Figure 2. Alignment of JcLEA with other plant group 5 LEA proteins sequences. The alignment was performed using the published sequences of LEAs including RcLEA-D34 from *Ricinus communis* (XP_002520716), AhLEA-5 from *Arachis hypogaea* (ADQ91843), MtLEA-D34 from *Medicago truncatula* (XP_003590628), CsLEA-D34 from *Cucumis sativus* (XP_004146999), GmLEA-D34 from *Glycine max* (XP_003536910), VvLEA-D34.
Plant RNA Mini Kit (Aidlab Biotecnologies Co., Ltd., China). RNA quality and concentration were analyzed by agarose gel electrophoresis (EC250-90, E-C Apparatus Corporation) and spectrophotometry (WFZUV-2100, Unico™ Instruments Inc.). The RNA samples were stored at −70°C prior to use.

Cloning of JcLEA Gene from J. curcas

Primers LEAF and LEAR were used in a reverse transcription polymerase chain reaction (RT-PCR) to obtain a partial fragment of the JcLEA cDNA. This pair of primers corresponded was designed from highly conserved amino acid sequences of LEA proteins from Ricinus communis (XP_002520716), Glycine max (XP_003536910), Medicago truncatula (XP_003590628) and Gossypium hirsutum (P09444). Amplifications were performed at 94°C for 4 min, followed by 50 cycles of amplification (94°C for 40 s, 55°C for 30 s, and 72°C for 40 s). Following this, 5'- and 3'-rapid amplifications of cDNA ends were conducted using SMART technology (SMART™ RACE cDNA Amplification Kit) to produce a full-length JcLEA cDNA. The JcLEA cDNA was sequenced using the DYEnamic Direct dGTP Sequencing Kit (Amersham Pharmacia, UK) and a 373A DNA sequencing instrument. The sequences were then compared with known sequences in the NCBI database using blastp (Standard Protein-DBLAST) on NCBI (www.ncbi.nlm.nih.gov). The conserved domains were searched with RPS-BLAST (Search the Conserved Domain Database) in the NCBI database. The phylogenetic tree of LEA proteins sequences was constructed based on their conserved properties and homology. The representative protein sequences of several previously reported groups of LEA proteins were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/). MEGA 5.0 was used as a computational phylogenetics tool to construct the maximum likelihood tree [40]. The classification of LEA groups was performed as previously described [22]. The sequences of all primers used in this paper are listed in Table 1.

Plant Transformation Constructs

The open reading frame (ORF) fragment of JcLEA was amplified using the gene-specific primers JcLEA-B and JcLEA-X and Pfu DNA polymerase (Promega). The resulting fragment was digested with BamHI/Xhol and ligated into the corresponding restriction sites of the pCAMBIA1304 vector under control of the CaMV35S promoter. For production of the JcLEA-GFP fusion protein, the JcLEA coding region was amplified using primers LEA-GFPF and LEA-GFPR. The PCR product was digested with NdeI/SphI and inserted into the pCAMBIA1302 vector. Expression of the JcLEA-GFP protein fusion was driven by the CaMV35S promoter. These constructs were sequenced to confirm the amplification fidelity and the exact insertion point of JcLEA in the pCAMBIA vectors.

Plant Transformation and Confirmation

For stable transformation of Arabidopsis, Arabidopsis Col-0 was transformed using the Agrobacterium-mediated floral dip method. Agrobacterium tumefaciens strain LBA4404 carrying the plasmid 35S::JcLEA was cultured overnight to an OD600 of 1.2 to 1.6. The Agrobacterium cells were collected by centrifugation for 10 min at room temperature at 3000 rpm and then resuspended to an OD600 of approximately 0.8 in floral dip inoculation medium (2.16 g 1−1 MS powder, 0.5 g 1−1 2-(N-Morpholino) ethanesulfonic acid, 50 g 1−1 sucrose, 10 μg 1−1 6- benzylaminopurine and 0.02% v/v silwet L-77, pH 5.7). The T1 plants were selected on hygromycin (30 mg l−1) and further confirmed by PCR using primers JcLEA-full-F and JcLEA-full-R. T2 lines showing 3:1 segregation were carried forward to the T3 generation. PCR positive homozygous T3 and T3 lines were used for functional analyses of JcLEA.

For transient gene expression in Nicotiana tabacum callus, leaves of 4-week-old sterilized tobacco plants were infiltrated with A. tumefaciens GV3101 carrying the JcLEA-GFP fusion vector. The leaves were placed on MS solid medium in the dark at 25°C for two days before the imaging analysis.

Fluorescence and Luminescence Imaging

Fluorescence signals were imaged using a confocal laser scanning microscope (Zeiss 710, Germany) at a magnification of 20x and analyzed with Zen software. Before the detection of the fluorescence signals, the tobacco leaf pieces were stained with DAPI for 40 min to facilitate the observation of leaf cell nuclei.

Western Blot Analysis

Plant nuclear and cytosolic proteins were extracted from N. tabacum leaf disks using a Nuclear and Cytoplasmic Protein Extraction Kit (Shanghai Sangon Biotech, China). A 50 ng sample of the extracted protein was boiled in SDS gel loading buffer and electrophoresed on a 15% polyacrylamide gel for three hours. Proteins were then electroblotted onto PVDF membrane using the Mini Trans-Blot™ Electrophoretic Transfer Cell System (Bio-Rad). The membranes were then blocked in Tris- HCl-buffered saline (TBS: 10 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 1% bovine serum albumin (BSA) for ≥2 h. The anti-GFP antibody (#G1112 Santa Cruz Biotechnology Inc., CA, USA) was incubated with the membranes at a 1:200 dilution in TBS containing 3% BSA overnight at 4°C. After washing, a 1:5000 dilution of anti-rabbit IgG HRP secondary antibody (#E1012 Santa Cruz Biotechnology) was incubated for 1 hour at room temperature in TBST containing 3% BSA. The filter was then washed and incubated with a WestDura Femto ECL kit (Fisher Scientific, Leicestershire, UK), and bands were visualized using a ChemiScope 3400 Mini Imaging System (Clinx Science Instruments Co., Ltd., China).

Quantitative Real-time PCR and Semi-quantitative RT-PCR

Quantitative real-time PCR (qPCR) was used to analyze gene expression levels as described previously [41]. cDNA was synthesized using PrimeScript™ RT Master Mix (TaKaRa, China) in a 20 μl-volume according to the manufacturer’s instructions. The qPCR was carried out using primers JcLEA-F and JcLEA-R in SYBR® Premix Ex Taq™ II (Perfect Real-Time; TaKaRa, China) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) with three replicates. The PCR procedure was 95°C for 30 s, 40 cycles of 95°C for 5 s, and 60°C for 34 s, followed by 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. The actin gene from J. curcas was used as the internal control.

The relative expression of the JcLEA gene was analyzed in four transformed lines of Arabidopsis by semi-quantitative RT-PCR.
Figure 3. Phylogenetic tree of LEA protein sequences from different plant species. MEGA 5.0 was used to create an alignment of the protein sequences downloaded from the NCBI database (http://www.ncbi.nlm.nih.gov/). The JcLEA protein cloned from J. curcas shown boxed in group 5.

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using the primers JcLEA-full-F and JcLEA-full-R with the following PCR protocol: 94°C for 5 min, followed by 20 cycles of 94°C for 30 s, and 55°C for 30 s. The tubulin gene from *Arabidopsis* was used as the internal control.

**Electrolyte Leakage Measurement**

Ion leakage was determined by the method of Zhou et al. [42]. Leaves from four plants of each line were sampled for each treatment and rinsed with distilled deionized water to remove surface ions before being incubated in 8 ml deionized water at room temperature for 12 h. The sample conductivities (C1) were measured with a DDS-11A conductivity meter (Shanghai SUOSHEN Electrical Equipment Co., Ltd., China). The samples were then incubated for 1 h at 100°C and conductivities (C2) were measured when they had cooled to room temperature. The relative electrolyte leakage was calculated using the following formula: (C1/C2) ×100%. The whole assay was repeated three times and the data analyzed using the Student’s *t*-test.

**Relative Water Content Measurement**

Leaves from four plants of each Arabidopsis line were collected for each treatment and the fresh weights (FW) were determined. The samples were then incubated in 8 ml deionized water at room temperature for 12 h at room temperature. The turgid weights (TW) of the leaf samples were measured. All samples were oven-dried at 65°C for 30 h, and the dry weights were measured. The relative water content (%) was calculated as = 100% × (FW-DW)/ (TW-DW) [43]. Three replicates were performed, and the data was analyzed by Student’s *t*-test.

**Glucose Content Assay**

Glucose content was assayed with a Glucose (HK) Assay kit (Sigma-Aldrich, Inc.). Leaves from four different plants of each Arabidopsis line for each treatment were collected and incubated in 1.5 ml distilled deionized water and the absorbance of NADH was measured when they had cooled to room temperature. The turgid weights (TW) of the leaf samples were measured. The absorbance of NADH was measured with a BioPhotometer Plus (Eppendorf, Germany) [43]. Three replicates were performed, and the data was analyzed by Student’s *t*-test.

**Potassium and Sodium Ion Contents**

A minimum of 30 plants from each transgenic line were treated with 50 mM, 100 mM, and 150 mM NaCl. Whole plants were washed three times with distilled deionized water to remove any possible surface ions before drying, and 0.5 g to 1 g tissues samples were then used for measurements. Samples were digested in 30 ml mixed acid digestion solution (HNO3:HClO4) and the mixed solutions were heated on the electro-thermal board to clarification. The potassium or sodium standard concentration liquid was then used for measurements. Samples were digested in 30 ml mixed acid digestion solution (HNO3:HClO4) and the mixed solutions were heated on the electro-thermal board to clarification. The potassium production was determined at 766 nm using a flame photometer (FP6410, China). The potassium and sodium contents of the samples were calculated against standard curves for potassium and sodium.

**Results**

**Molecular Cloning of the Full-length cDNA of JcLEA**

*J. curcas* belongs to the botanical family Euphorbiaceae and grows widely in tropical and sub-tropical areas of Central and South America, Africa, India, and Southeast Asia. *J. curcas* has been considered to be a type of crop that is highly tolerant to both dehydration [44] and high levels of salinity [37]. To understand the role of the LEA protein from *J. curcas*, we first cloned and sequenced the full-length JcLEA cDNA based on the highly conserved amino acid sequences of known plant LEA proteins in the NCBI database using the SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). The full-length JcLEA cDNA comprised a 763 bp open reading frame (ORF), a 50 bp 5’-untranslated region (UTR), and a 247 bp 3’-UTR with a poly (A) tail (Fig. 1). The ORF encoded a polypeptide of 254 amino acids, for which the calculated isoelectric point and molecular mass were predicted to be 4.64 and 26.5 kDa, respectively (pl/Mw Tool at www.expasy.org). The predicted JcLEA protein contained 33.46% hydrophobic residues, including alanine, isoleucine, leucine, phenylalanine, and valine residues, and no cysteine, histidine or tryptophan residues.

**Homology Analysis of Amino Acid Sequences**

A database search using Blastx (http://www.ncbi.nlm.nih.gov/) showed that there was a relatively high similarity between the predicted JcLEA protein and other plant LEA proteins. Notably, the LEAs identified in the blast analysis all belonged to the group 5 subfamily, and included the following nine plant proteins: ReLEA-D34 from *R. communis* (XP_002520716), ArLEA-5 from *Anchis hypogena* (ADQ919814), MtLEA-D34 from *M. truncatula* (XP_003590628), CsLEA-D34 from *Cuscutis sativus* (XP_004146999), GmLEA-D34 from *Glycine max* (XP_0003536910), VvLEA-D34 from *Vitis vinifera* (XP_0003632934), LEA34_GOSHI from *G. hirsutum* (p00444), AtLEA-5 from *A. thaliana* (BAB01464) and DeECP31 from *Daucus carota* (BAD86645). A number of gaps and insertions were needed to optimize the alignment. The amino acid identities between JcLEA and ReLEA-D34, AtLEA-5, MtLEA-D34, CsLEA-D34, GmLEA-D34, VvLEA-D34, LEA34_GOSHI, AtLEA-5, and DeECP31 were found to be 76%, 64%, 64%, 63%, 62%, 61%, 57%, and 57%, respectively. Moreover, three SMP-like motifs that are generally present in LEA proteins were also detected (Fig. 2).

**Phylogenetic Analysis**

As there was a relatively high degree of similarity between JcLEA and the other group 5 LEA proteins, we constructed a phylogenetic tree to determine the specific group to which JcLEA belongs. The representative protein sequences of seven groups of LEA protein were used to perform Blast analyses so that LEA proteins from various plant species in each group could be identified. The maximum likelihood tree constructed with MEGA 5.0 showed that the JcLEA protein is related to group 5 LEA proteins and clustered together with the representative proteins of this group. In addition, we observed from the phylogenetic tree...
that the proteins within one group shared high levels of similarity, but that the similarities between groups were relatively low (Fig. 3).

Expression Pattern of JcLEA under Different Abiotic Stress Treatments

Previous reports have shown that the expression of LEA-like genes could be induced by ABA, cold, drought, and high salt treatments [45,46]. As J. curcas can tolerate dehydration and high levels of salinity, we have tested the possibility that JcLEA is involved in the plant response to drought and high salt. The gene expression patterns of JcLEA in response to 30% PEG for 3 days [47], 100 µM ABA for 3 hours [42], and 300 mM NaCl for 1 day [48] were investigated in young leaves of J. curcas seedlings. The relative expression of JcLEA was significantly increased in response to drought, ABA, and NaCl treatments, suggesting that JcLEA might play a role in dehydration and high salt tolerance (Fig. 4).

Subcellular Localization of JcLEA Protein

Information on the subcellular localization of proteins can be important to elucidate the functional roles proteins play in plant cells [43]. In order to determine the localization of the JcLEA protein, the JcLEA-GFP translational fusion was constructed and transiently expressed under the control of the 35S promoter in tobacco leaves. Confocal laser fluorescence microscopy showed that the JcLEA-GFP fusion protein was localized to the cytosol and the nucleus (Fig. 5). In order to localize the JcLEA-GFP fusion protein more precisely, we tested 35S::JcLEA-GFP and 35S-GFP protein expression in transiently transformed tobacco, by extracting proteins and performing western blot analysis using a GFP polyclonal antibody (Fig. 6). These data showed that JcLEA-GFP was localized to both the cytosol and the nucleus, similar to the GFP control.

Preparation of JcLEA overexpressing Arabidopsis Lines

To study the contributions of JcLEA to drought and salt tolerance, we produced JcLEA-overexpressing lines of Arabidopsis Col-0 that were verified by genomic DNA PCR and semi-quantitative RT-PCR (Fig. 7a–c). The transgenic lines had different JcLEA expression levels. Among these, lines 25–29 and 25–33 showed higher expression levels of JcLEA, and JcLEA transcripts were not detected in the WT (Wild type) plants. Homozygous seeds of lines 25–29 and lines 25–33 were collected for further investigation. One set was germinated on soil for physiological measurements, and another set was germinated on MS medium for root length observation under conditions of drought and salt stress.

Overexpression of JcLEA Enhanced Drought Tolerance in Arabidopsis

When the transgenic Arabidopsis seedlings were grown on MS medium containing 10% PEG and 15% PEG to be exposed to drought conditions, they exhibited much better growth status than did WT Arabidopsis plants, especially for root length, which is sensitive to dehydration (Fig. 8a & b). After water was withheld from the Arabidopsis plants for 14 days, the transgenic lines suffered less from dehydration compared with the WT plants, in which

Figure 5. Subcellular localization of the JcLEA protein in tobacco leaf cells. GFP fluorescence of JcLEA: GFP and the GFP control are shown. DAPI staining was used to visualize the nucleus. doi:10.1371/journal.pone.0083056.g005

Figure 6. Detection of LEA-GFP fusions proteins extracted from tobacco leaves. Western blot analysis of proteins extracted from LEA-GFP or GFP transiently expressed in tobacco leaf cells, respectively. doi:10.1371/journal.pone.0083056.g006

Figure 7. Incorporation and expression of JcLEA in transgenic Arabidopsis. (a) Schematic diagram of the 35S::JcLEA DNA vector construct. (b) Verification of transfectants from T1-generation regenerated Arabidopsis plants by PCR amplification from genomic DNA. Lane 1 contains a fragment size marker. (c) Expression levels of JcLEA in four transgenic lines as determined by semi-quantitative RT-PCR. doi:10.1371/journal.pone.0083056.g007
many of the leaves were bleached and withered (Fig. 8c). After watering again for 10 days, several of the withered leaves on the transgenic plants recovered and turned green, and the survival rates of the 35S::JcLEA seedlings were higher than those of WT (Fig. 8c & 8d). For further physiological investigation, the relative water content, electrolyte leakage, and glucose content were analyzed as markers of drought adaptation. The relative water content of transgenic *Arabidopsis* was significantly higher than that of WT drought treatment and drought plus rewatering, indicating that JcLEA could help to prevent water loss in plants (Fig. 8e). Electrolyte leakage, which is an indicator of the capacity to protect the plasma membrane integrity under stress, was also significantly lower in transgenic plants, demonstrating that JcLEA reduced damage to cell membranes in transgenic plants (Fig. 8f). Moreover,
Figure 9. Phenotypes and physiological indices of transgenic Arabidopsis plants bioassayed for high salt tolerance. (a and b) Phenotypes and root lengths of WT and transgenic plants treated with 0, 50, 100, 150, and 200 mM NaCl on MS medium (pH 5.8) at 22°C for two weeks. Error bars represent the standard deviation (SD, n = 6). (c and d) Phenotypes and survival rates of plants treated with different concentrations of NaCl (SD, n = 3). (e and f) Measurements of relative water content (e), electrolyte leakage (f) and glucose content (g) in plants treated with different concentrations of NaCl (SD, n = 3).
in response to dehydration stress, the transgenic plants accumulated more glucose, which contributed to the stability of the internal milieu of the plant cells (Fig. 8g). These physiological measurements indicated that the overexpression of JcLEA significantly improved the drought tolerance in Arabidopsis.

Overexpression of JcLEA Enhanced Salt Tolerance in Arabidopsis

Similar to the investigation of drought resistance, phenotypes and physiological indices in 35S::JcLEA plants treated with NaCl were evaluated to test whether the JcLEA protein functions in salt tolerance. When the treated with NaCl solutions, the transgenic lines showed significantly higher levels of tolerance and higher survival rates than did the WT plants (Fig. 9a–d). WT plants displayed severe damage at ≈100 mM NaCl, while nearly 50% of the 35S::JcLEA plants survived even when treated with 200 mM NaCl. The physiological indices including relative water content, electrolyte leakage, and glucose content also indicated that JcLEA contributed to cellular protection during salt stress (Fig. 9e–g). When exposed to high levels of NaCl, plant cells attempt to maintain high concentrations of K$^+$ and low concentrations of Na$^+$ in the cytosol in order to establish intracellular K$^+$ and Na$^+$ homeostasis, which is crucial for the protection of cytosolic enzymes and osmotic pressure stability [49]. We evaluated the Na/K ratio in transgenic and control seedlings based on the Na$^+$ and K$^+$ contents. Our results showed that presence of JcLEA reduced the Na/K ratio below 150 mM NaCl, suggesting a protective function for this protein through exclusion of extra Na$^+$. However, JcLEA failed to exclude Na$^+$ in the 200 mM NaCl treatment, which might be an upper limit for the capacity of ionic adjustment (Fig. 9h).

Discussion

The LEA proteins are a family of hydrophilic proteins that are presumed to play a protective role during exposure to different abiotic stresses. This large protein family can be classified into seven groups, designated groups one to seven [22]. Among these, the roles of groups 1, 2, and 3 have been extensively studied in relation to the abiotic stress response. HVA1 from Hordeum vulgare, belonging to group 3, confers increased drought tolerance in wheat and rice [50,51], and enhanced drought, salinity, and cold tolerance when expressed in mulberry [52]. In the group 2 LEAs, PMA80 from wheat increases dehydration tolerance of transgenic rice [53], and DHN24 from Solanum solanum improves the cold tolerance of transgenic cucumber [54]. Similarly, the group 1 protein PMA1959 from wheat enhances tolerance to dehydration and salinity in transgenic rice [53]. On the other hand, some of the LEA proteins appear to contribute little to plant stress tolerance. The overexpression of two cold-induced LEA proteins CAP160 and CAP85 from Spinacia oleracea and three desiccation-induced pC proteins from C. plantagineum failed to change the freezing or drought tolerance in transgenic tobacco plants [55,56]. Thus, LEA family members may have different functions in plant stress responses. In comparison, the role of group 5 LEA proteins in abiotic stress tolerance in plants is not well known. Here, we show that a novel JcLEA protein isolated from J. curcas possesses high level of amino acid sequence identity and has similarity to other group 5 LEA proteins from plants. The ABA-, drought-, and salt-induced expression patterns of JcLEA are also typical of other LEA-like genes. Treatments with ABA, drought, salt, and extreme temperatures caused the accumulation of many type of LEA proteins [19,34,57,58]. ABA is a key signaling phytohormone involved in the drought and salt responses in plants [8]. Previous reports have shown that the promoter regions of the group 5 LEA genes generally contain the ABA responsive element (ABRE), which causes the induction of these genes in response to various abiotic stress conditions [59]. The group 5 JcLEA genes are strongly induced by ABA, drought, and salinity, indicating a potential role for JcLEA in the response to drought and salt stress in J. curcas, which is consistent with the functions of characterized LEAs from other plant species [25].

In Arabidopsis, the protective function of JcLEA was confirmed, based on the plant growth status and physiological indices. JcLEA effectively prevents the loss of water and cytoactive components to maintain the integrity of plasma membranes and the homeostasis of plant cells under dehydration as well as high salt stress. The contribution of JcLEA to drought tolerance is consistent with the strong dehydration resistance observed in J. curcas. During salt stress, the accumulated excess Na$^+$, which is toxic to enzymes, is extruded or compartmentalized in the vacuole to prevent growth cessation or cell death [60]. The measurements of potassium and sodium contents show that JcLEA may regulate Na$^+$ transport and maintain the intracellular K$^+$ concentration to stabilize the ionic balance of plant cells. The function of JcLEA is associated with its subcellular localization. It has been reported that LEA proteins, which are non-trans membrane proteins, are widely localized in multiple subcellular compartments in various plant species [19]. The PsLEAm protein from pea is localized in the mitochondrial matrix of seeds, and is the first mitochondrial precursor in a plant LEA protein [61]. Group 1 (AfrLEA1-1) and group 3 (AfrLEA3-4) LEA proteins from Artemia franciscana are also localized in mitochondria [62], while many kinds of group 2 LEA proteins are localized in the nucleus [22]. The respective subcellular locations of LEA proteins may be correlated with the different aspects of cellular protection during various abiotic stresses. JcLEA appears to be localized mainly to the nucleus and cytoplasm, which suggests that it has comprehensive protective functions in plant cells. This finding is also consistent with the water and ionic homeostasis in transgenic Arabidopsis plants that express JcLEA.

In summary, JcLEA is a novel LEA protein belonging to group 5. The constitutive expression of JcLEA does not cause growth repression in host plants. From the results of this study, it can be concluded that JcLEA has a huge potential for transgenic breeding based on (1) the fact that the gene is induced by exposure to drought and salt, (2) the subcellular localization of JcLEA and (3) the improved tolerance to dehydration and high salt levels observed in transgenic Arabidopsis plants.

Author Contributions

Conceived and designed the experiments: J. Lin J. Liang MZ. Performed the experiments: J. Liang MZ XZ YJ MX. Analyzed the data: J. Liang XZ. Contributed reagents/materials/analysis tools: MZ XZ. Wrote the paper: J. Lin J. Liang MZ.

concentrations of NaCl (SD, n = 3). (h) The Na/K ratio of plants treated with 150 mM and 200 mM NaCl. * P<0.05; ** P<0.01.
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