

***RcLEA*, a late embryogenesis abundant protein gene isolated from *Rosa chinensis*, confers tolerance to *Escherichia coli* and *Arabidopsis thaliana* and stabilizes enzyme activity under diverse stresses**

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Abstract The late embryogenesis abundant (LEA) protein family is a large protein family that is closely associated with resistance to abiotic stresses in many organisms, such as plants, bacteria and animals. In this study, we isolated a *LEA* gene, *RcLEA*, which was cytoplasm-localized, from *Rosa chinensis*. *RcLEA* was found to be induced by high temperature through RT-PCR. Overexpression of *RcLEA* in *Escherichia coli* improved its growth performance compared with the control under high temperature, low temperature, NaCl and oxidative stress conditions. *RcLEA* was also overexpressed in *Arabidopsis thaliana*. The transgenic *Arabidopsis* showed better growth after high and low temperature treatment and exhibited less peroxide according to 3, 3-diaminobenzidine staining. However, *RcLEA* did not improve the tolerance to NaCl or osmotic stress in *Arabidopsis*. In vitro analysis showed that *RcLEA* was able to prevent the freeze–thaw-induced inactivation or heat-induced aggregation of various substrates, such as lactate dehydrogenase and citrate synthase. It also protected

the proteome of *E. coli* from denaturation when the proteins were heat-shocked or subjected to acidic conditions. Furthermore, bimolecular fluorescence complementation assays suggested that *RcLEA* proteins function in a complex manner by making the form of homodimers.

Keywords *LEA* · *Rosa chinensis* · Abiotic stress · Resistance · Homodimer

Introduction

Late embryogenesis abundant (LEA) proteins, as their name suggests, accumulate in abundance during the late development stage of seeds (Dure et al. 1989). *LEA* genes can also be induced by abscisic acid, dehydration, osmotic or cold stress in vegetative tissues, implying that they not only function in seed maturation, but also play roles in the abiotic stress tolerance of plants (Godoy et al. 1990; Bostock and Quatrano 1992; Wilhelm and Thomashow 1993; Moons et al. 1997). In the 30 years since the first *LEA* protein was discovered in cotton (Dure and Chlan 1981; Dure and Galau 1981; Dure et al. 1981), hundreds of *LEA* genes from different organisms such as plants, algae, fungi and bacteria have been isolated (Dure 1993; Wise and Tunnacliffe 2004; Battaglia et al. 2008). They are categorized into five or six groups according to similarities in their amino acid sequences and conserved motifs, followed the classical criteria which was established from Dure et al. 1989. However, as more *LEA* proteins were discovered and the development of computational analysis methods, different criteria to classify *LEA* proteins (Tunnacliffe and Wise 2007; Shih et al. 2008) and new group of proteins were demonstrated (Bies-Ethève et al. 2008; Hundertmark and Hinch 2008). *LEA* proteins are highly hydrophilic, and

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rich in certain amino acid residues such as glycine, alanine and glutamate (Dure et al. 1989; Wise 2003; Shih et al. 2008).

Increasing evidence has indicated a positive correlation between the LEA proteins and abiotic stress tolerance, particularly dehydration (Dure et al. 1989; Tunnacliffe and Wise 2007). Overexpression of LEA proteins in *Escherichia coli*, yeast or plants can confer higher tolerance to salt, drought or cold stress (Imai et al. 1996; Xu et al. 1996; Swire-Clark and Marcotte 1999). *E. coli* that was transformed with *PM2* had a higher survival rate under low temperature, high temperature or high salinity conditions (Liu et al. 2010). Overexpression of the sweet potato late embryogenesis abundant 14 (*IbLEA14*) gene increased the osmotic and salt stress tolerance of transgenic calli (Park et al. 2011). However, the precise mechanism how LEA proteins work has not yet been elucidated clearly. They are proposed to protect proteins from irrevocable denaturation or aggregation, stabilize membrane or other cellular components under abiotic stresses by action as a hydration buffer, molecular shields or molecular chaperones (Wolkers et al. 2001; Goyal et al. 2003; Wise and Tunnacliffe 2004; Tolleter et al. 2007; Tunnacliffe and Wise 2007; Shih et al. 2010), although supporting evidence is limited. AavLEA1, from the anhydrobiotic nematode *Aphelenchus avenae*, and Em from wheat protect citrate synthase (CS) from heat-induced aggregation and lactate dehydrogenase (LDH) from cold-induced aggregation, respectively (Goyal et al. 2005). The soybean *PM2* protein prevents LDH from inactivation after freeze–thaw and heat shock treatments (Liu et al. 2010). The K-segment of Maize DHN1 mediates binding to anionic phospholipid vesicles and helps stabilize cellular components, including membranes (Koag et al. 2009). The LEA proteins are rich in Gly and other small residues, which favors a flexible conformation in aqueous solutions (Garay-Arroyo et al. 2000). The conformation of LEA proteins is essential to some of their functions mentioned above, such as binding to macromolecules and protecting enzymes (Hoekstra et al. 2001; Tunnacliffe and Wise 2007; Shih et al. 2008).

Rosa chinensis, a species of *Rosa*, is one of the most important horticultural and ornamental plants in the world. It not only has commercial value, but also has pharmaceutical applications (Gao and Chen 2000). However, the growth of most *R. chinensis* varieties is limited by unfavorable environmental conditions, such as high temperatures and drought. Under stress conditions, these varieties do not blossom, but enter dormancy directly; thus, research to enhance the plant's resistance to these stresses is of interest (Xu et al. 2011).

In this study, we first isolated a *LEA* gene, *RcLEA*, from *R. chinensis* that could be induced by heat shock. It is a D95 type LEA protein and similar to Arabidopsis LEA

group 7 proteins, which are characterized by three conserved motifs, motif 1 (LDK-K-F-A-KL-IP-PE), motif 2 (V-V-NP-IP-S-R-G-IPD-G-L) and motif 3 (D-PVV-TIP-GEIKLP-D) (Bies-Ethève et al. 2008). To characterize the function of *RcLEA*, we transformed it into *E. coli* and Arabidopsis. Compared with the control strain, *E. coli* expressing *RcLEA* showed higher tolerance to abiotic stresses including low and high temperature, high salt and oxidative stress. Overexpression of *RcLEA* in Arabidopsis also improved the resistance of transgenic Arabidopsis to heat and freezing stresses. Moreover, in vitro, purified *RcLEA* protein could protect LDH and CS from inactivation or aggregation and prevent the soluble proteins of *E. coli* from denaturing with various stress treatment. These results suggested that *RcLEA* might play important roles in resistance to abiotic stresses.

Materials and methods

Plant materials and growth conditions

The heat-resistant variety of *R. chinensis*, Sohloss Manneim (SM) was propagated in vitro on Murashige and Skoog (MS) differentiation medium containing 4.4 g L⁻¹ MS salts, 1 mg L⁻¹ 6-benzyl aminopurine (6-BA), 0.2 mg L⁻¹ indol-yl-3-acetic acid (IAA), 3 % sucrose, and 0.7 % agar, pH 6.0, and MS rooting medium containing 2.2 g L⁻¹ MS salts, 0.5 mg L⁻¹ IAA, 3 % sucrose, and 0.7 % agar, pH 6.0. Seedlings of SM were grown in soil in a greenhouse (16 h light/8 h dark, 60 % relative humidity, 22–25 °C).

A. thaliana (ecotype Col-0) was used in this study. Arabidopsis seeds were sown on a potting soil mix (compost soil/perlite/vermiculite, 9/1/3, v/v/v), and grown in a growth chamber under long-day conditions (16 h light/8 h dark) at 22 °C. For growth on agar-solidified medium, surface-sterilized seeds were germinated on MS medium, pH 5.8, solidified with 0.8 % (w/v) agar, in the dark for 2 days at 4 °C, and were then transferred to a growth chamber (22 °C, 16 h light/8 h dark).

Isolation of the *RcLEA* gene

The leaves of SM seedlings which were treated with 38 °C for 3 h were collected and total RNA was extracted from them using the 'Plant RNAout' kit (TianDZ, China). Then the first strand cDNA was synthesized according to the protocol of the PrimeScript™ RT Reagent Kit (TaKaRa, China). To obtain the open reading frame (ORF) of *RcLEA*, PCR was performed with the degenerate primers based on the conserved sequence (GenBank Accession No. BI978651) of *LEA* gene from Old Blush, a *R. chinensis*

cultivar: sense 5'-ATGTCGGAYGAYGARCAYCA-3' and antisense 5'-YTA CTTIGGRCCAATRTCCTTR-3' (R: A and G; Y: C and T). The PCR products were purified and cloned into the pMD18-T vector (TaKaRa, China) for sequencing.

To obtain the genomic DNA sequence of *RcLEA*, genomic DNA was isolated from leaves of SM seedling according to the cetyl trimethyl ammonium bromide (CTAB) method, and then digested with *Sal* I before self-ligated to generate circular molecules. Using the circular molecules as templates and the following primers which were designed from the sequence obtained above:

IN1L: 5'-AGGACAAGAACGACAAGTGG-3';
 IN1R: 5'-GTCATCTCAATCTCAACCT-3';
 IN2L: 5'-CGAGAGAAGCAGCGGCAAGT-3';
 IN2R: 5'-CCTCTTCTTCTTCAGCCCC-3';
 IN3L: 5'-AGGCTGCTATGGAGAATGGA-3'; and
 IN3R: 5'-ACACATGGGCTTCTGGGGTC-3'.

PCR was performed. The program was: 4 min at 94 °C; 10 s at 98 °C; 15 min at 65 °C for 30 cycles; and 10 min at 72 °C. First round of PCR was performed with primers IN1L/IN1R and the products were diluted 100-fold for the second round of amplification with primers IN2L/IN2R, and the products were diluted to 100-fold as template for the third round amplification with IN3L/IN3R primers. The final PCR products were purified and cloned into pMD18-T vector for sequencing.

To obtain the 3' end of *RcLEA*, 3'-RACE was performed using a 3'-Full RACE Core Set (TaKaRa, China). 3'-RACE nested PCR was performed using three nested primers mentioned above IN1F, IN2F, IN3F combined with AUAP (5'-GGCCACGCGTCGACTAGTAC-3') with the following programs: 4 min at 94 °C; 30 cycles of 30 s at 94 °C; 40 s at 55 °C; 1 min at 72 °C; and 10 min at 72 °C. The final PCR products were purified and cloned into pMD18-T vector for sequencing.

The complete ORF of *RcLEA* was obtained by RT-PCR using two primers, 5'-ATGGCATCCTATGATGACT-3' and 5'-AAATCCAGTGTAGAGATTA-3', under the following amplification conditions: 4 min at 94 °C; 30 cycles of 30 s at 94 °C; 40 s at 55 °C; 1 min at 72 °C; and 10 min at 72 °C. The PCR products were purified and cloned into pMD18-T vector for sequencing.

Sequence alignment, phylogenetic and hydropathy analysis

The deduced amino acid sequence of *RcLEA*, along with sequences of other reported plant LEA proteins, was aligned using ClustalX. A phylogenetic tree was constructed using the Neighbor-Joining method provided by the MEGA5.1 program. The grand average of hydropathy

(GRAVY) analysis of deduced amino acid sequence was performed by using the ExPasy (<http://www.expasy.org/tools>), PSORT (<http://psort.ims.u-tokyo.ac.jp>), and SoftBerry (<http://www.softberry.com>) programs.

RNA isolation and expression analysis

Total RNA was extracted from the leaves of the three *R. chinensis* varieties, SM, Las Vegas (LV) and Kodes Perfecta (KP) under the control and heat-shock conditions respectively, by using the 'Plant RNAout' kit (TianDZ, China). The first strand cDNA was synthesized as the method described above. Then PCR was performed using the cDNA as template with *RcLEA* gene-specific primers 5'-ATGTCGCTTATCCCAAATT-3' and 5'-AAATCCAGTGTAGAGAT-3'. The primers 5'-TCTGGCATCATACCTTCTACA-3' and 5'-GGATGGCTGGAAGAGGAC-3' were used for control amplification of *RcACTIN*.

Constitutive expression of *RcLEA* cDNA in *E. coli*

*Eco*R I and *Sal* I restriction sites were added to the 5' and 3' ends of the *RcLEA* ORF by PCR. The PCR product was inserted into pET32a, a prokaryotic expression vector. The recombinant construct pET32a-*RcLEA* and pET32a were both transformed into *E. coli* strain BL21.

For thermotolerance assays, *E. coli* transformed with pET32a-*RcLEA* and pET32a were cultured in liquid Luria-Bertani (LB) medium supplemented with ampicillin (Amp, 100 mg L⁻¹) at 37 °C respectively. Isopropyl-beta-D-thiogalactopyranoside (IPTG, 1 mM) was added to the culture when OD₆₀₀ = 1.0. Two hours later, cultures were incubated at 50 °C. One milliliter of the culture was taken at 0, 0.5, 1, 2, 3, 4 or 5 h respectively and centrifuged at 4500 r/min for 5 min. The precipitate was resuspended with equivalent sterilized water and diluted until there were about 1 × 10³ cells in 1.0 ml solution. Spread 100 μl of the final dilution onto a LB solid medium with 100 mg L⁻¹ Amp. Incubate plates upside down at 37 °C overnight. Counter the colony of triplicate plates.

For cold treatments, aliquots from IPTG-induced cultures were plated and kept at 4 °C for 0, 2, 4, 6, 8, 12 days and cell viability was measured as described above.

For other stress treatments, aliquots from IPTG-induced cultures were streaked on LB medium with different stress substances, 550 mM NaCl or 400 μM H₂O₂ and photos were taken after 12 h of cultivating at 37 °C.

Construction of expression vectors and plant transformation

To construct the expression vectors, the pMD18-T vector containing the ORF of *RcLEA* was used as a template and

a *Hind* III restriction site was added to the 5' end of the sense primers, a *Bam*H I site was added to the 5' end of the antisense primer. The PCR product was digested with *Hind* III/*Bam*H I and was subcloned into pHB vector, pre-digested with *Hind* III/*Bam*H I.

The plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101. Arabidopsis plants were transformed by means of floral dip (Clough and Bent 1998). Transgenic plants were selected using 25 mg L⁻¹ hygromycin.

Stress treatments of the transgenic Arabidopsis plants

For heat and chilling treatments, 4-week-old WT and homozygous transgenic Arabidopsis plants grew under normal conditions (22 °C) were shifted to 45 °C for 24 h or -20 °C for 4 h, and then transferred to normal conditions for recovery. For salt and osmotic stress treatments, seeds of WT and transgenic Arabidopsis were germinated on MS medium containing 0 (control), 200, 300 mM mannitol or 100, 150 mM NaCl for 9 days. Soil-cultivated 4-week-old Arabidopsis plants were irrigated with 200 mM NaCl or 400 mM mannitol for 14 or 5 days respectively, and then leaves were sampled.

Catalase activity assay (CAT)

0.1 g of Arabidopsis leaves were homogenized with 1 ml ice cold 150 mM sodium phosphate buffer (pH 7.0). The homogenate was centrifuged at 1,000 rpm for 20 min at 4 °C and the supernatant was crude enzyme extract used for determination of CAT activity. Add 50 µl crude enzyme extract to the reaction buffer which contains 150 mM sodium phosphate buffer (pH 7.0), 100 mM 30 % H₂O₂. CAT activity was determined by recording the decline in A₂₄₀ according to Nakno and Asada (1981) and Ai et al. (2008).

LDH activity

The LDH (Roche, USA) was diluted to 50 mg L⁻¹ with 100 mM sodium phosphate buffer (pH 7.0). RcLEA proteins were added to equal volumes of LDH at mass ratios of 0:1, 1:5, 1:1, 5:1 or 10:1 (RcLEA:LDH). The freeze-thaw, thermo-denaturation and high-salinity assay, and the measurement of LDH activity were according to Liu et al. (2010).

Thermal aggregation of CS

The CS was diluted to 0.043 mg mL⁻¹ with 50 mM Tris, 100 mM NaCl, pH 7.5 and then incubate in 45 °C without additions or in the presence of 0.043 or 0.43 mg mL⁻¹ RcLEA for 30 min. A400 was measured for every 5 min.

Stability of soluble proteome of *E. coli*

The soluble proteins were extracted from *E. coli* cells and the amount of soluble proteins was quantified by the Bradford protein assay and diluted to 0.372 g L⁻¹. RcLEA proteins were added to equal volumes of proteome at mass ratios of 0:1, 1:3, 3:5, 1:1 (RcLEA: proteome). The protein mixture was kept in 60, 80 or 100 °C for 2 min, or blended with 3 M HCl, 2 M NaOH or 30 mM AgNO₃. Then after centrifuged at 10,000 rpm for 20 min at 4 °C, the Bradford protein assay was used to quantify the content of soluble proteins.

Subcellular localization and bimolecular fluorescence complementation (BiFC) assays

The coding region of *RcLEA* was in-frame fused downstream to the yellow fluorescent protein (YFP) sequence in the 1300-super-nYFP vector. The resulting constructs containing *YFP-RcLEA* were transformed into *Nicotianabenthiana* leaf epidermal cells, essentially as described by Sheen and Tang (Sheen 2001; Tang et al. 2010). Protoplasts were prepared 3 days after infiltration by cutting leaf discs into small pieces and incubating for 3 h in the enzyme solution (0.4 M mannitol, 20 mM MES-K, 10 mM CaCl₂, 5 mM β-mercaptoethanol, 0.1 % BSA, 1 % cellulase R10, 0.3 % macerozyme R10, pH5.7). Fluorescence of YFP in the leaf epidermal cells and the isolated protoplasts was imaged by a confocal laser scanning microscope (Zeiss LSM510; Carl Zeiss Micro-Imaging GmbH, Jena, Germany).

For generation of the BiFC vectors, the stop-codonless coding region of *RcLEA* was subcloned via *Bam*HI-*Sal*I into p131YNE-35S and p131YCE-35S, resulting in *RcLEA-YFP^N* and *RcLEA-YFP^C* fusions. The ORF of *RcHSP17.8* whose protein is cytoplasm-localized was fused to *YFP^N* as a negative control. Infiltration of *N. thamniana* leaves was performed as previously described by Tang et al. (2010). Fluorescence of YFP in the leaf epidermal cells was imaged as before.

Results

Isolation and sequence analysis of *RcLEA*

A cDNA containing a 981 bp ORF was isolated from *R. chinensis* (SM variety) that was treated at 38 °C for 3 h. It was subcloned into pMD18-T and sequenced. The cDNA encoded a new LEA protein of 326 amino acids with a predicted molecular mass of 36.0 kDa and a pI of 4.52. It shared the most similarity with At2g44060 among the LEA proteins in Arabidopsis according to a BLAST

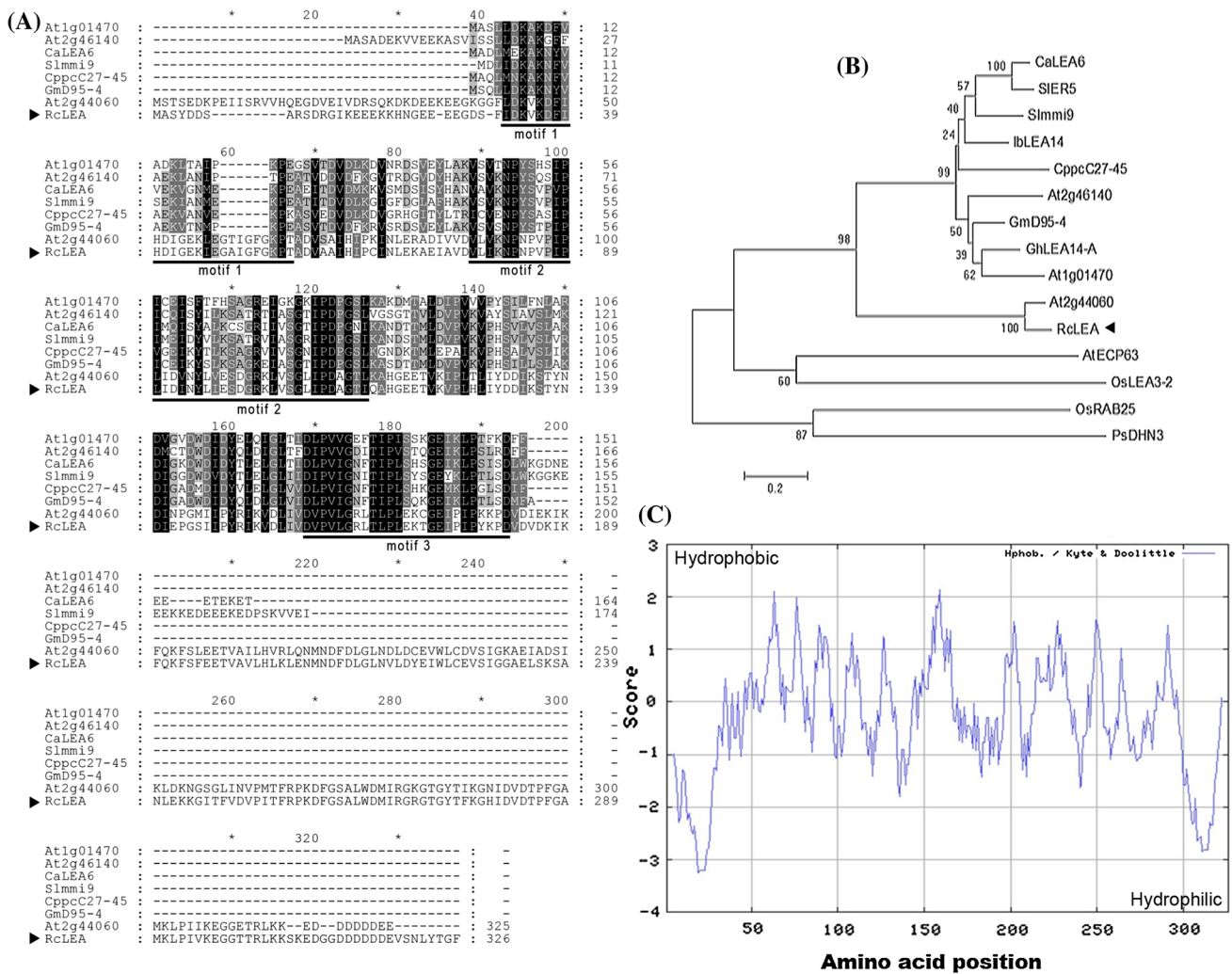


Fig. 1 Alignment, phylogenetic analysis and hydropathy analysis of the deduced amino acid sequences of RcLEA and other plant LEAs. **a** Alignment of RcLEA with other plant LEAs. **b** Phylogenetic tree of RcLEA and other plant LEAs. **c** Hydropathy analysis of RcLEA protein. The GenBank accession numbers are as follows: Slmml9 (*Lycopersicon esculentum*, Z46654), D95-4 (*Glycine max*, U08108), CaLEA6 (*Capsicum annuum*, AF168168), LEA14-A (*Gossypium hirsutum*, M88322), ER5 (*Lycopersicon esculentum*, U77719), pcC27-45 (*Craterostigma plantagineum*, M62990), AtECP63 (*Arabidopsis thaliana*, NM_129219.2), At1g01470 (*Arabidopsis thaliana*, BT015111), At2g46140 (*Arabidopsis thaliana*, NM_130176), At2g44060 (*Arabidopsis thaliana*, BT024723), IbLEA14 (*Ipomoea batatas*, GU369820), OsLEA3 (*Oryza sativa*, AAD02421), OsRAB25 (*Oryza sativa*, X57327), DHN3 (*Pisum sativum*, X63063)

search. At2g44060 belongs to the Group 7 which is related to cotton D95 (Bies-Ethève et al. 2008). Amino acid sequence alignment of RcLEA and some reported LEA proteins similar to D95 showed three conserved motifs (Fig. 1a). A phylogenetic tree was constructed (Fig. 1b), which showed that RcLEA was more closely related to At2g44060 than other LEA proteins (Fig. 1b). LEA proteins are hydrophilic, glycine-rich proteins. RcLEA showed a preponderance of Asp, Ile, Gly, Lys, Glu, Leu and Val, which constituted 10.7, 10.4, 8.9, 8.9, 8.3, 8.0, and 7.1 % of the total amino acids, respectively, and lacked Trp. The GRAVY of RcLEA was -0.283 , suggesting that RcLEA was a neutral protein, not a typical hydrophilic LEA protein (Fig. 1c).

Subcellular localization of RcLEA

The ORF of RcLEA was fused in-frame to the 3' end of YFP sequence. The YFP-RcLEA fusion protein was expressed in *N. benthamiana* leaf epidermal cells by infiltration with *A. tumefaciens* strain GV3101 harboring the recombinant construct. Additionally, we also delivered the chimeric construct into *N. benthamiana* mesophyll protoplasts by the polyethylene glycol transformation method (Tang et al. 2010) to express the fusion protein. The YFP fluorescence was examined by confocal laser-scanning microscopy. In the positive control YFP alone, a diffuse green fluorescence, typical cytosolic location was observed with a tendency to accumulate in the nucleus and near the plasma

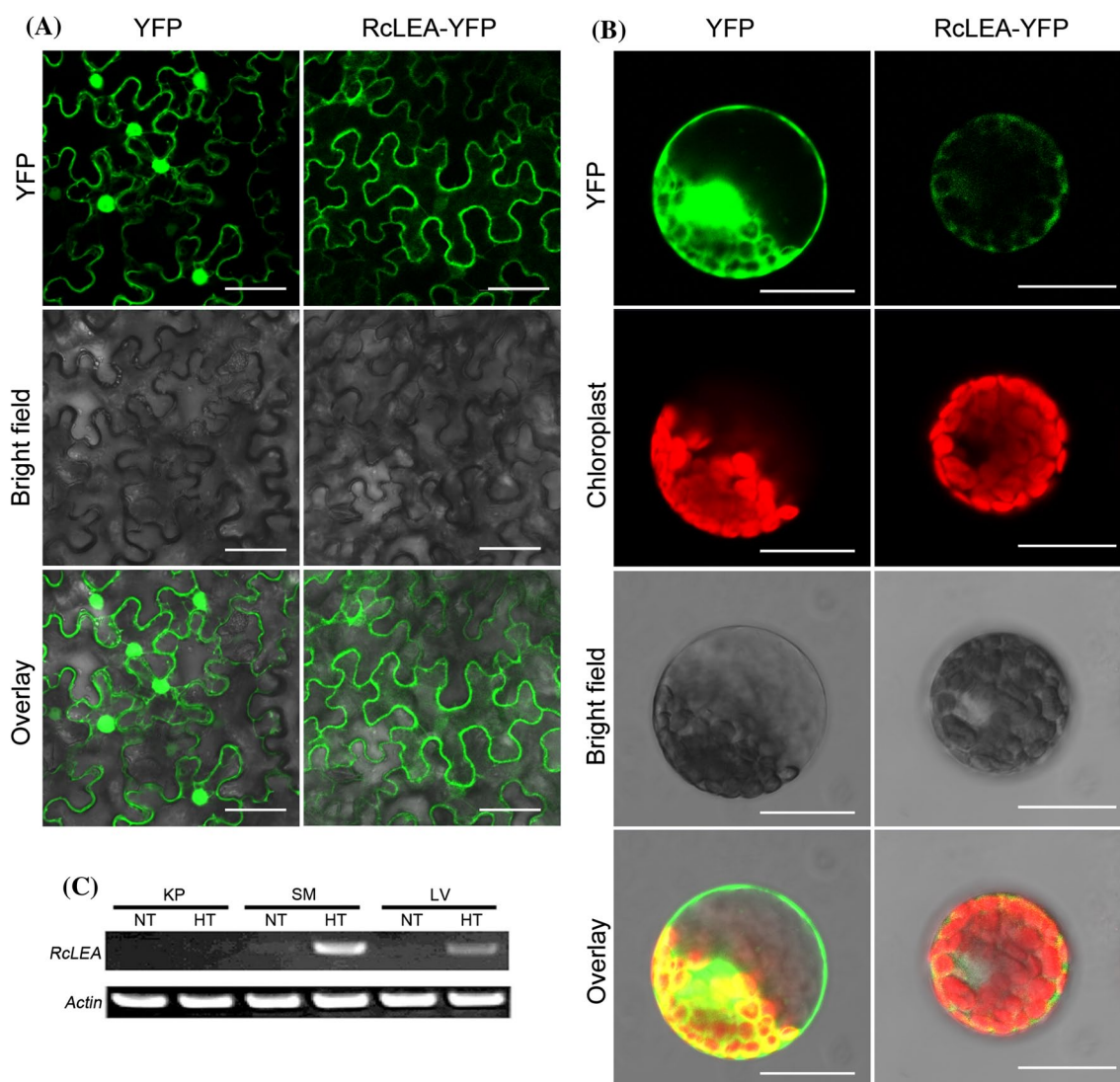


Fig. 2 Subcellular location and heat induced expression of *RcLEA*. **a** Subcellular localization of RcLEA-YFP fusion protein in tobacco (*N. benthamiana*) leaf epidermal cells. Bar 50 μm . **b** Subcellular localization of RcLEA-YFP fusion protein in tobacco (*N. benthamiana*)

leaf protoplasts. Bar 25 μm . **c** RT-PCR analysis of the expression of *RcLEA* under control and high temperature in different *R. chinensis* varieties. mRNA was extracted from plantlets of SM, LV and KP grown in soil with or without 38 $^{\circ}\text{C}$ treatment for 3 h

membrane as expected (Fig. 2a left panels; Fig. 2b left panels). When fused to RcLEA, the YFP fluorescence was detected only in cytoplasm (Fig. 2a right panels; Fig. 2b right panels). These results demonstrated that RcLEA was a cytoplasm-localized protein.

Up-regulation of *RcLEA* in heat-resistant *R. chinensis* under high temperature conditions

The expression of *RcLEA* was strongly induced after 38 $^{\circ}\text{C}$ treatment for 3 h in the SM variety and weakly induced in LV, but in KP, it could not be detected with or without treatment (Fig. 2c). In heat-treated SM, RcLEA protein accumulated to higher level than that in KP based

on the results of two-dimensional electrophoresis gel (Fig. S1). In addition, SM showed higher heat resistance than LV or KP, indicating that RcLEA may act in the heat shock response and help *R. chinensis* survive under such conditions.

Overexpression of *RcLEA* enhanced the viability of *E. coli* cells under thermal, salt and oxidative stress conditions

To characterize the function of *RcLEA* and find out whether it plays roles in responses to abiotic stresses, the ORF of *RcLEA* was cloned into pET32a and transformed into *E. coli* strain BL21. After heat (50 $^{\circ}\text{C}$) or cold (4 $^{\circ}\text{C}$) treatment for different periods of time, the survival rate

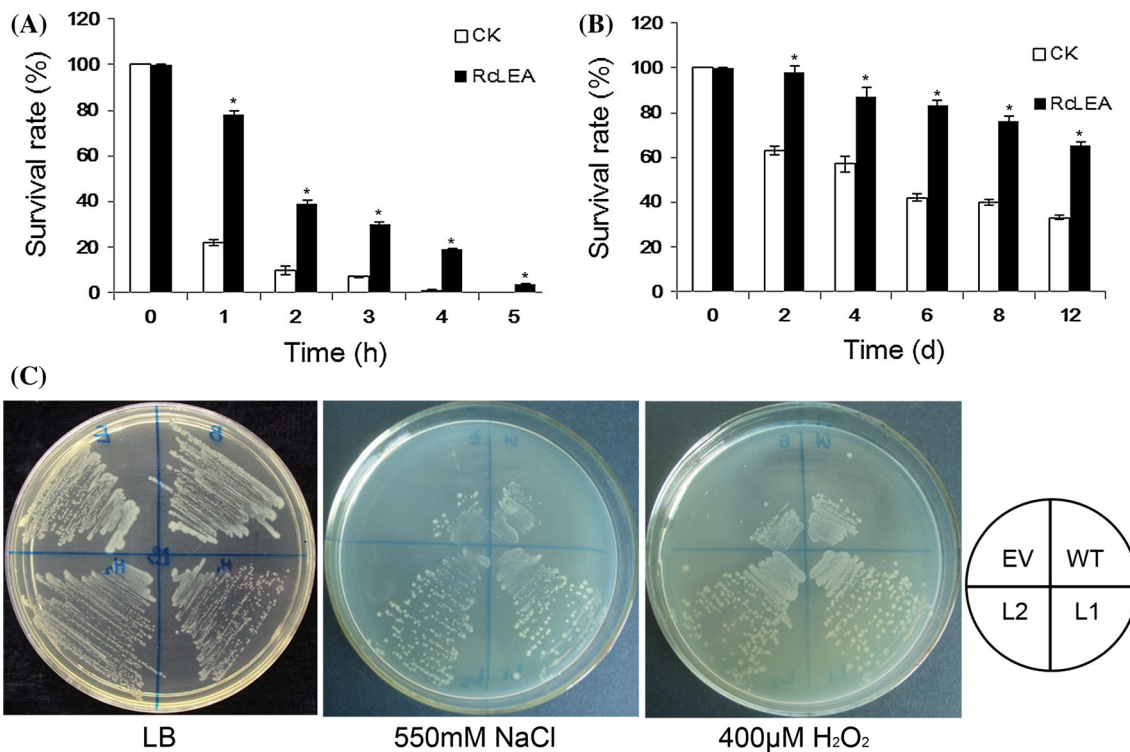


Fig. 3 Heterogeneous expression of *RcLEA* in *E. coli* strain BL21 enhanced the host's stress tolerance. **a** Survival rate of *E. coli* BL21 transferred with empty vector (CK) or *RcLEA* treated by high temperature (50 °C) for different times (*x* axis). **b** Survival rate of *E. coli* BL21 transferred with empty vector (CK) or *RcLEA* treated by low

temperature (4 °C) for different times (*x* axis). Results were averages of three biological replicates. Bars show mean \pm SD. * indicated a significant difference ($P < 0.05$). **c** Growth of *E. coli* strain BL21 on LB medium with 0, 550 mM NaCl or 400 μ M H₂O₂

of *E. coli* was measured by counting the colony-forming units. *E. coli* in which *RcLEA* was overexpressed showed a higher survival rate than the control *E. coli* that harbored the empty vector after heat shock for 1, 2, 3, 4 or 5 h, but the difference in survival rate narrowed as the treatment time increased (Fig. 3a). Similarly, the survival rate of *E. coli* harboring pET32a-*RcLEA* decreased at a clearly slower rate compared with cells harboring the pET32a empty vector under cold conditions (Fig. 3b). Even after 12 days of treatment, the colony number of *E. coli* expressing *RcLEA* was still twice that of the control. These results indicated that *RcLEA* could enhance the viability of *E. coli* transformants under heat and cold stresses.

To examine whether *RcLEA* could confer resistance to other abiotic stresses, cultures of wild type (WT) *E. coli* and *E. coli* carrying the empty vector or the pET32a-*RcLEA* construct were streaked on plates of LB medium alone or LB supplemented with 550 mM NaCl or 400 μ M H₂O₂ (Fig. 3c). More cells of the two *E. coli* lines (L1 and L2) expressing *RcLEA* survived than those of the wild type or empty vector when treated with NaCl or H₂O₂. These results revealed that *RcLEA* could increase the tolerance of *E. coli* to salt and oxidative stresses.

RcLEA overexpression in *A. thaliana* increased resistance to high temperature

To analyze the function of *RcLEA* in plants, *RcLEA* was overexpressed in Arabidopsis under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter. The expression level of *RcLEA* in transgenic Arabidopsis was examined by semi-quantitative RT-PCR (Fig. S2). Three lines designated L1, L2 and L3, with high, moderate and low expression levels, were selected for the subsequent experiments. To demonstrate whether *RcLEA* is involved in tolerance to abiotic stresses, transgenic Arabidopsis in which *RcLEA* was expressed and the WT were treated with high and low temperatures, NaCl and mannitol.

For heat stress treatment, four-week-old transgenic and WT Arabidopsis seedlings grown in soil were heat-shocked at 45 °C for 24 h. Under normal conditions, no phenotypic differences were observed between the transgenic and WT plants, but after the treatment, the leaves of the WT wilted more severely (Fig. 4a). The electrolyte leakage of transgenic Arabidopsis leaves, especially L2, was much lower than wild-type leaves after plants were exposed to high temperature, while there were no differences among them without treatment (Fig. 4c). These results indicated that the leaves of

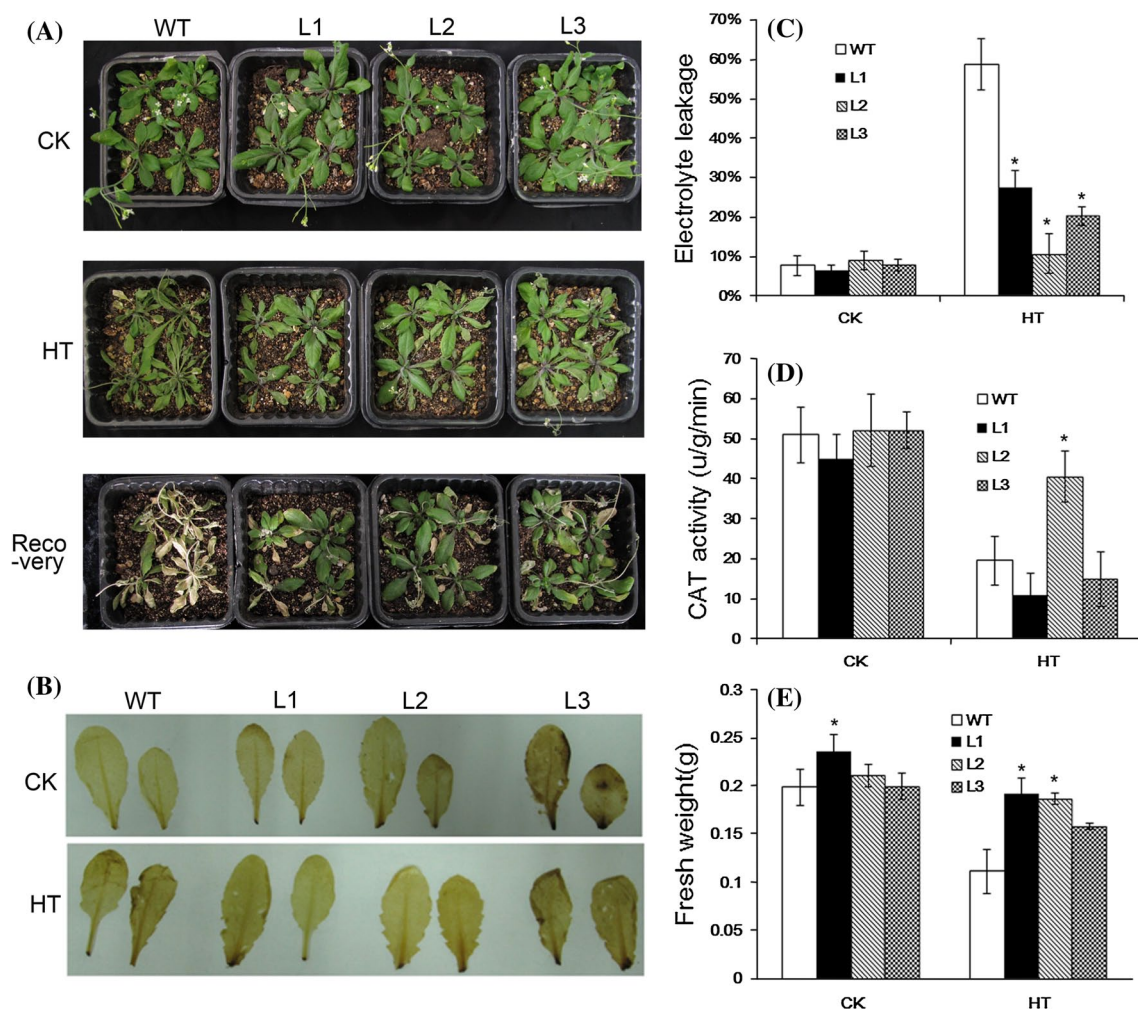


Fig. 4 Ectopic expression of *RcLEA* in *Arabidopsis* affected the plants' thermotolerance. Four-week-old plants grown under at 22 °C were shifted to a non-permissive temperature (45 °C) for 24 h, then transferred to 22 °C for recovery. **a** The growth performance of WT and transgenics with *RcLEA* grown under normal conditions, 24 h with heat-shock and 3 days after recovery. **b** DAB staining assay on the leaves of WT and the transgenic lines treated or not. **c** Electro-

lyte leakage of rosette leaves of the plants under normal and high temperature conditions. **d** CAT activity of the plants with or without heat treatment. **e** Fresh weight of rosette leaves of heat treated and control plants after recovery for 10 days. WT wild-type, L1, L2, L3 overexpression lines of transgenic *Arabidopsis*, CK control, HT high temperature. Results were averages of five biological replicates. Bars show mean \pm SD. * indicated a significant difference ($P < 0.05$)

the WT were impaired more significantly compared with the transgenic plants. CAT is an important antioxidative enzyme that helps to mitigate the ROS-mediated oxidative damage caused by abiotic stresses. CAT activity was reduced notably in the stressed plants, but was higher in L2 plants than in the WT and the other transgenic lines (Fig. 4d), implying that the expression of *RcLEA* protected the enzyme activity of CAT. DAB staining was used to detect peroxide, and showed that the accumulation of peroxide in the *Arabidopsis* plants was opposite to the CAT activity (Fig. 4b). Under standard conditions, the WT and transgenic *Arabidopsis* leaves exhibited weak DAB staining, whereas in L3 the staining was a little darker. After heat stress, compared to the *RcLEA* overexpression lines, more intense DAB staining was observed on WT leaves. All of the treated plants were transferred to

normal conditions for recovery. Three days later, photos were taken. As shown in Fig. 4a, the transgenic lines grew better than the WT. The fresh weight of L2, measured after 10 days recovery, was significantly greater than that of the WT, while the weight difference between the WT, L1 and L3 was not that marked (Fig. 4e). In summary, overexpression of *RcLEA* in *Arabidopsis* enhanced the heat stress resistance of transgenic plants.

RcLEA overexpression in *A. thaliana* increased resistance to low temperature

We next analyzed the role of *RcLEA* in the freezing stress response. Four-week-old transgenic and WT *Arabidopsis* seedlings grown in soil were treated at -20 °C for 4 h

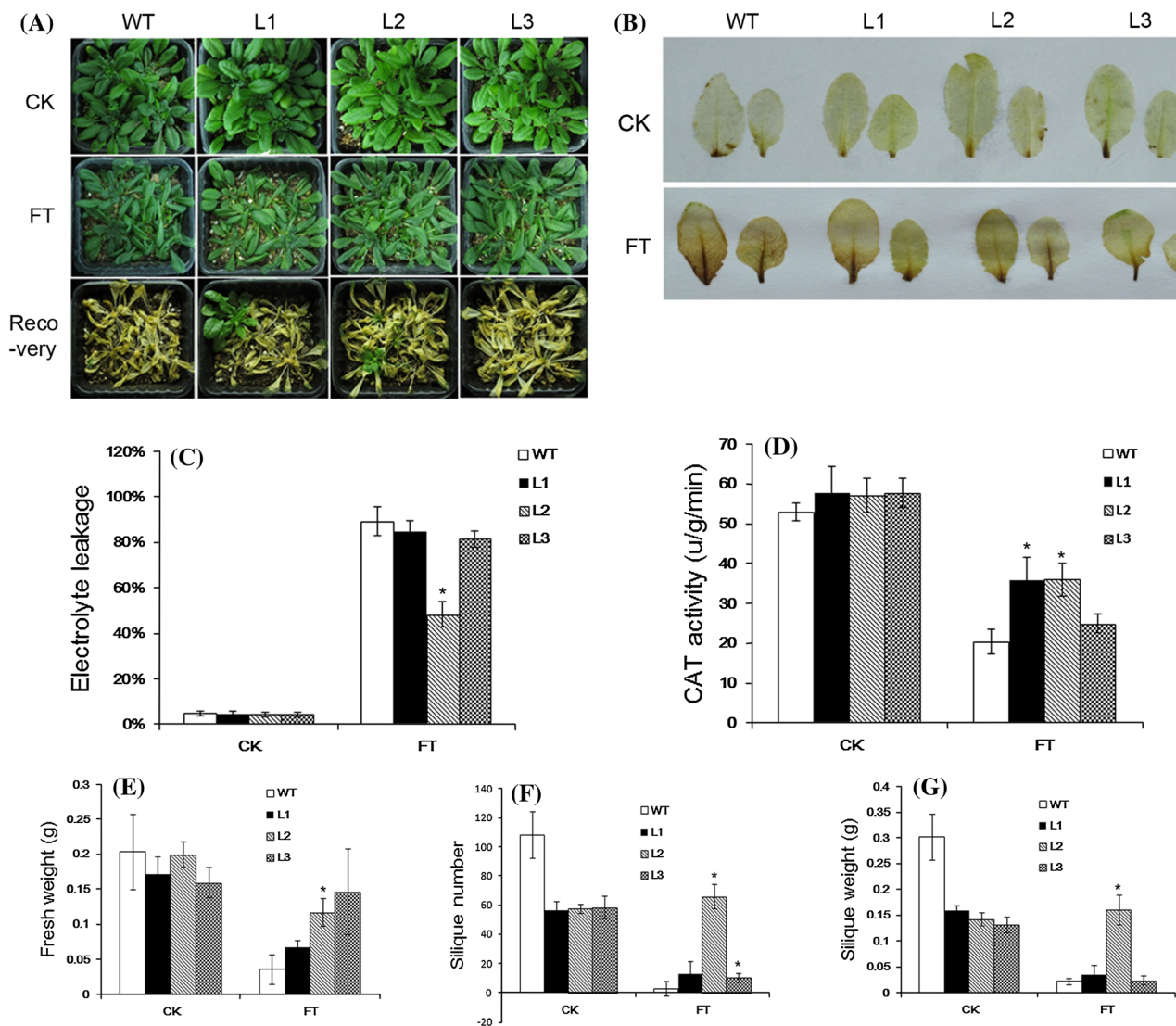


Fig. 5 Overexpression of *RcLEA* in Arabidopsis affected the plants' tolerance to freezing stress. Four-week-old plants grown under at 22 °C were treated at −20 °C for 4 h, and transferred to normal temperature (22 °C) for recovery. **a** The growth performance of WT and transgenics with *RcLEA* grown under normal conditions, 4 h with freezing treatment and 3 days after recovery. **b** DAB staining assay on the leaves of WT, the transgenic lines treated or not. **c** Electrolyte leakage of rosette leaves of the plants under normal and freezing con-

ditions. **d** CAT activity of the plants with or without freezing treatment. **e** Fresh weight of rosette leaves of freezing treated and control plants after recovery for 2 weeks. **f** Silique number per plant two weeks after recovery. **g** Silique weight per plant. WT wild-type, L1, L2, L3 overexpression lines of transgenic, CK control, FT freezing treatment. Results were averages of five biological replicates. Bars show mean ± SD. * indicated a significant difference ($P < 0.05$)

(Fig. 5a). Leaves were collected immediately after the treatment for DAB staining (Fig. 5b), electrolyte leakage measurements (Fig. 5c), and CAT activity assay (Fig. 5d). The electrolyte leakage of freezing-stressed plants was much higher than that of untreated plants, but the leakage of L2 plants was lower than that of WT, L1 and L3. CAT activity in L1 and L2 plants was impaired less than in the WT and L3 by freezing stress. Correspondingly, more peroxide was accumulated in the WT, for DAB staining was more apparent in WT leaves and was much weaker in the transgenic Arabidopsis leaves under freezing

stress. After 3 d for recovery, the transgenic line L1 and L2 showed better growth performance than the WT and L3 (Fig. 5a). As a consequence, the three transgenic lines produced more biomass (Fig. 5e), as suggested by the measurement of fresh weight after the plants were grown in normal conditions for two weeks. Moreover, the L2 plants produced more siliques and seeds (Fig. 6f, g). The silique number of L2 was also higher than that of the WT, L1 and L3. Taking these results together, overexpression of *RcLEA* in Arabidopsis conferred higher tolerance to freezing stress.

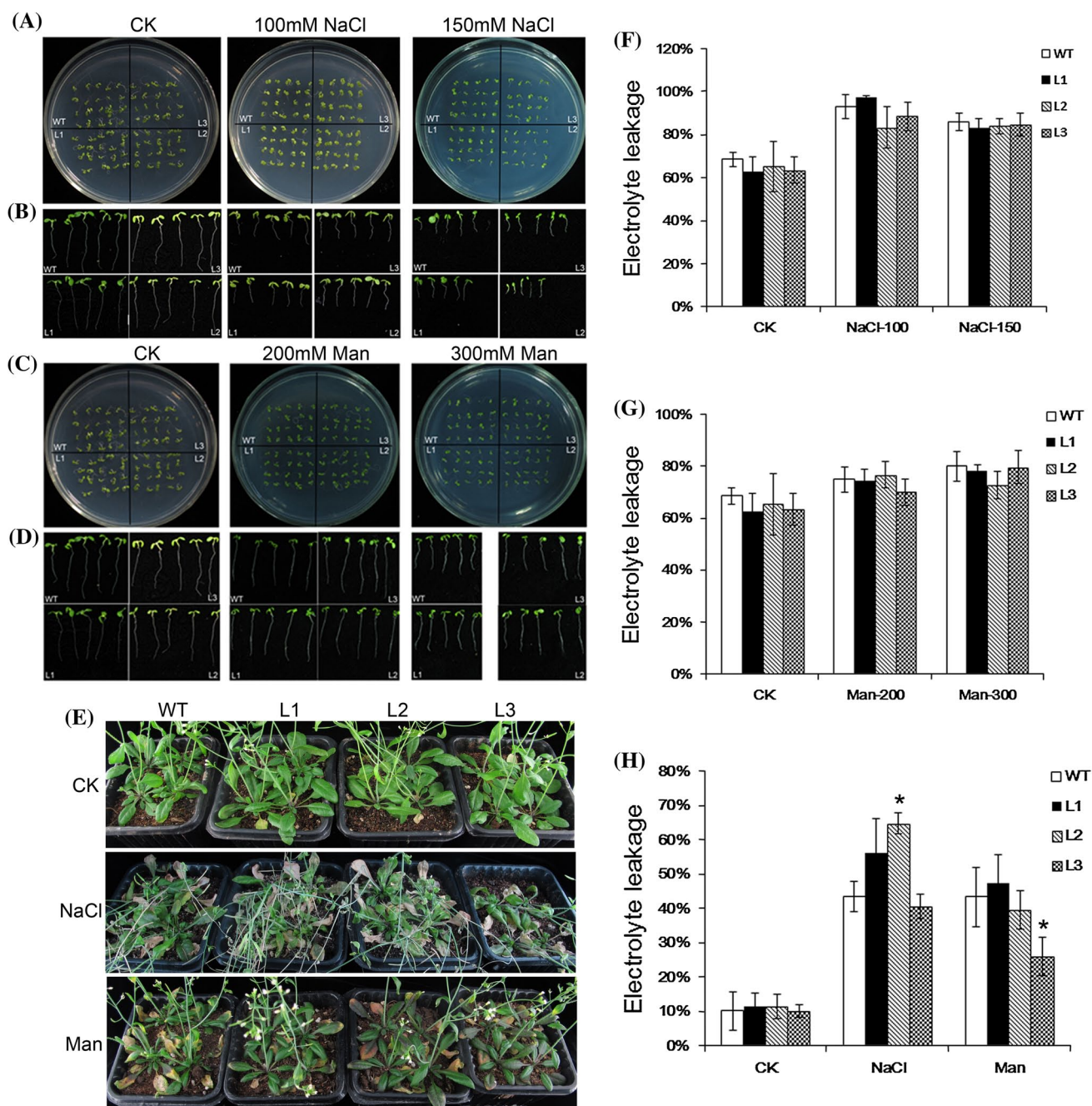


Fig. 6 NaCl or mannitol sensitivity of wild type and transgenic seedlings. **a–d** The growth performance of the 9-days-old seedlings. Seeds of WT and transgenic *Arabidopsis* were germinated on MS medium containing 0 (control), 200, 300 mM mannitol or 100, 150 mM NaCl for 9 days. **e** The growth performance of 4-week-old WT and transformants with *RcLEA* grown under normal conditions, irrigated with 200 mM NaCl or 400 mM mannitol for 14 or 5 days.

f Electrolyte leakage of the 9-days-old seedlings treated with NaCl or not. **g** Electrolyte leakage of the 9-d-old seedlings treated with mannitol or not. **h** Electrolyte leakage of the 4-week-old seedlings treated with NaCl or mannitol or not. WT wild-type, L1, L2, L3 over-expression lines of transgenic *Arabidopsis*, CK control, Man mannitol. Results were averages of five biological replicates. Bars show mean \pm SD. *indicated a significant difference ($P < 0.05$)

RcLEA overexpression in *A. thaliana* had no effect on resistance to NaCl or osmotic stress

To reveal the role *RcLEA* plays in resistance to NaCl and osmotic stress, seeds of the WT and the three transgenic

lines were germinated on MS medium with 0 (control), 200 or 300 mM mannitol, or with 100 or 150 mM NaCl. Nine days later, there were no phenotypic differences between the WT and the *RcLEA* overexpression lines with or without stresses (Fig. 6a–d). The measurement of electrolyte

leakage also demonstrated that the WT and the transgenic lines were impaired to the same degree (Fig. 6f, g). Soil-cultivated 4-week-old plants were irrigated with 200 mM NaCl or 400 mM mannitol for 14 or 5 days respectively. The leaves of WT and transgenic *Arabidopsis* both wilted and exhibited chlorosis (Fig. 6e). The electrolyte leakage of L1, L2 was even higher than WT and L3 under NaCl stress conditions, while under osmotic stress conditions, there was no significant difference of electrolyte leakage between WT and the transgenic L1, L2, in which *RcLEA* was highly expressed (Fig. 6g). These results showed that *RcLEA* overexpression in *Arabidopsis* had no effect on resistance to NaCl or osmotic stress.

RcLEA protein prevents LDH from inactivation under freezing stress in vitro

To examine whether RcLEA could protect proteins from aggregation, *RcLEA* was expressed in *E. coli* and purified (Fig. 7a, b). The activity of LDH is impaired when it is frozen in liquid N₂ followed by thawing, or heat shocked at high temperature (Goyal et al. 2005); hence, it was used as the substrate for the RcLEA protein. The RcLEA protein was mixed with LDH at different mass ratios, and then thermo-denaturation (Fig. 7c), freeze–thaw (Fig. 7d), and high-salinity (Fig. 7e) assays were performed. The RcLEA protein could not prevent LDH from denaturation by heat-shock, because the activity of LDH reduced to the same level after treatment at 62 °C for 5 min whether with or without RcLEA, even if the amount of RcLEA was ten times that of LDH. However, RcLEA had a protective effect on LDH activity during 10 cycles of freeze–thaw treatment. LDH activity was impaired after the treatment in the absence of RcLEA, but it remained at the same level as it under control conditions when RcLEA was added. The 1 M NaCl treatment had no effect on the activity of LDH.

Thermal aggregation of CS

CS is sensitive to high temperature, which makes it suitable for analyzing the protective effect of LEA proteins. CS was incubated at 45 °C and the increase of A₄₀₀ was measured to indicate the aggregation of CS. RcLEA at same concentration as CS showed significant protective activity, slowing the rate of aggregation, compared to its control. This effect was higher when the concentration of RcLEA increased (Fig. 7f).

The protective effect of the RcLEA protein on the *E. coli* soluble proteome

Soluble proteins will denature and aggregate under high temperature, acid, alkaline or heavy metal conditions. The

soluble proteins extracted from *E. coli* were treated by high temperature, 60, 80, or 100 °C for 2 min (Fig. 7g). The soluble protein level decreased notably after the treatment compared with the control according to a Bradford protein assay, and the higher the treatment temperature was, the lower the soluble protein content remaining. However, more soluble protein remained in the presence of the RcLEA protein, and the amount of remaining soluble protein increased as the percentage of RcLEA protein was raised, indicating that RcLEA could enhance the thermostability of the *E. coli* soluble proteins. In addition, the RcLEA protein could protect the soluble protein of *E. coli* when 3 M HCl was added, but there were no differences in soluble protein content whether RcLEA was mixed or not when 30 mM AgNO₃ supplemented and 3 M NaOH did not effect the content of the *E. coli* soluble proteins (Fig. 7h).

RcLEA protein functions in a complex manner by making form of homodimers

Late embryogenesis abundant proteins generally form homodimers to perform its roles in plant cell (Olvera-Carrillo et al. 2011). In order to determine whether RcLEA can form homodimers, BiFC assays were performed. RcLEA protein fused with YFP N-terminal or YFP C-terminal were co-expressed in *N. benthamiana* leaf epidermal cells via different combinations. Cytoplasm-localized protein, RCHSP17.8 was used as a negative control. When RcLEA-YFP^N and RCHSP17.8-YFP^C were co-expressed, no fluorescence was detected. The YFP fluorescence could be observed only when both RcLEA-YFP^N and RcLEA-YFP^C fusion proteins were present in cytoplasm of transfected cells (Fig. 8). This result not only showed that RcLEA interacted with itself and formed homodimers, but also reinforced the evidence that RcLEA located in cytoplasm.

Discussion

Rosa chinensis is an important flowering plant with horticultural, ornamental and commercial value. However, abiotic stresses such as high and low temperature or osmotic stress can affect the growth or bloom of *R. chinensis*. Thus, it is desirable to enhance the tolerance of these roses to abiotic stresses using genetic engineering tools.

The *LEA* gene was first discovered during the desiccation phases of seed development (Dure and Chlan 1981; Dure and Galau 1981; Dure et al. 1981), implying that it plays a protective role when water is limited. Later, more and more *LEA* genes were isolated from different organisms. According to numerous reports, the expression of many *LEA* genes is induced by abiotic stresses, suggesting that they might function in abiotic stress responses

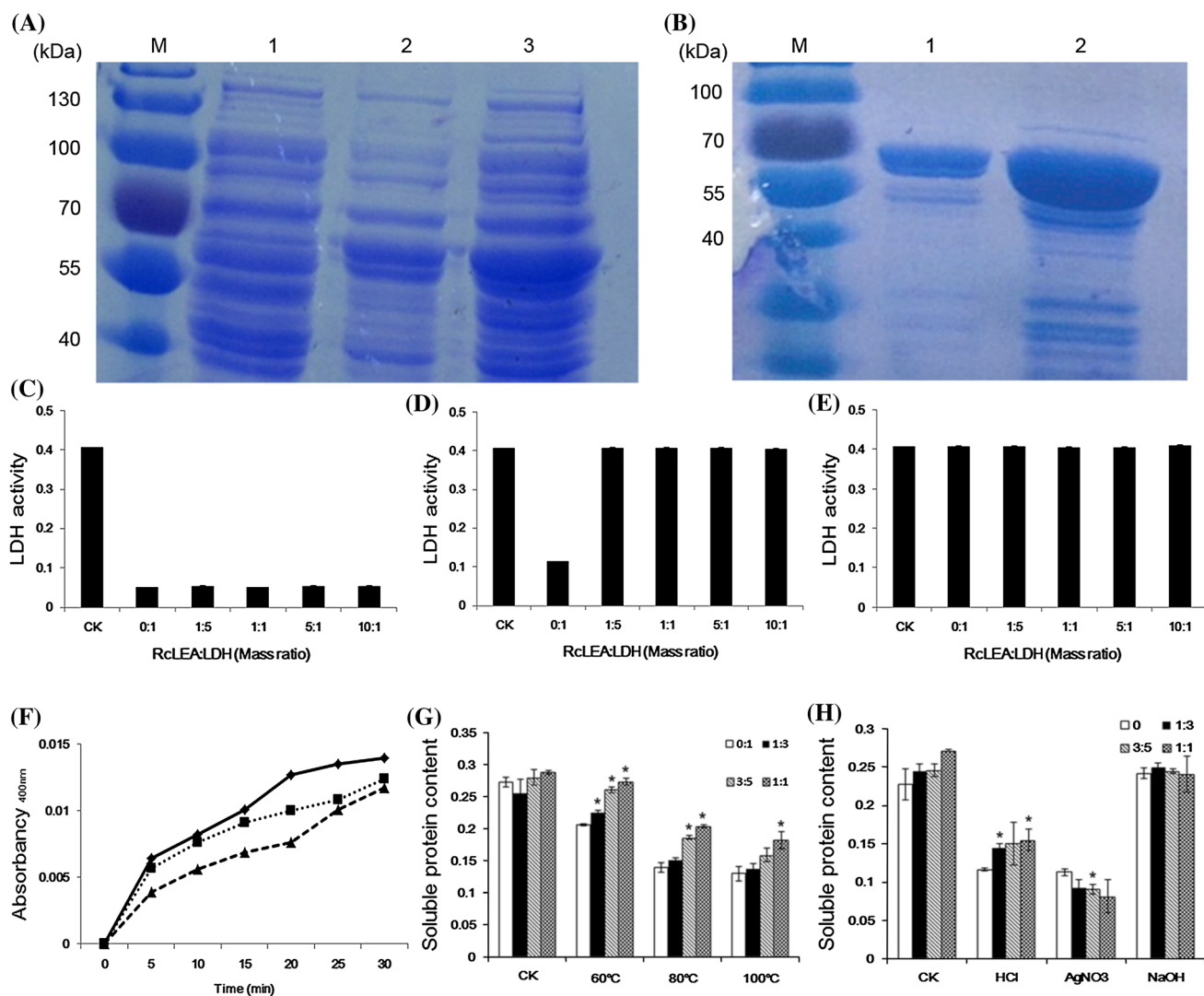


Fig. 7 RcLEA protected LDH, CS from denaturation and stabilize the *E. coli* soluble proteome in vitro. **a** SDS-PAGE analysis of RcLEA protein expression. M, protein molecular weight marker; 1, soluble supernatant from the non-induced *E. coli* BL21 culture without IPTG; 2, 3, soluble supernatant from the induced *E. coli* BL21 culture with IPTG for 6 or 12 h respectively. **b** SDS-PAGE analysis of RcLEA protein purification. 1, purified RcLEA protein; 2, purified and concentrated RcLEA protein. **c** RcLEA protein had no protective effect on LDH activity under high temperature. **d** The protective effect of the RcLEA protein on LDH activity after 10 freeze–thaw cycles. **e** The LDH activity was not affected by 1 mM NaCl. **f** Effects

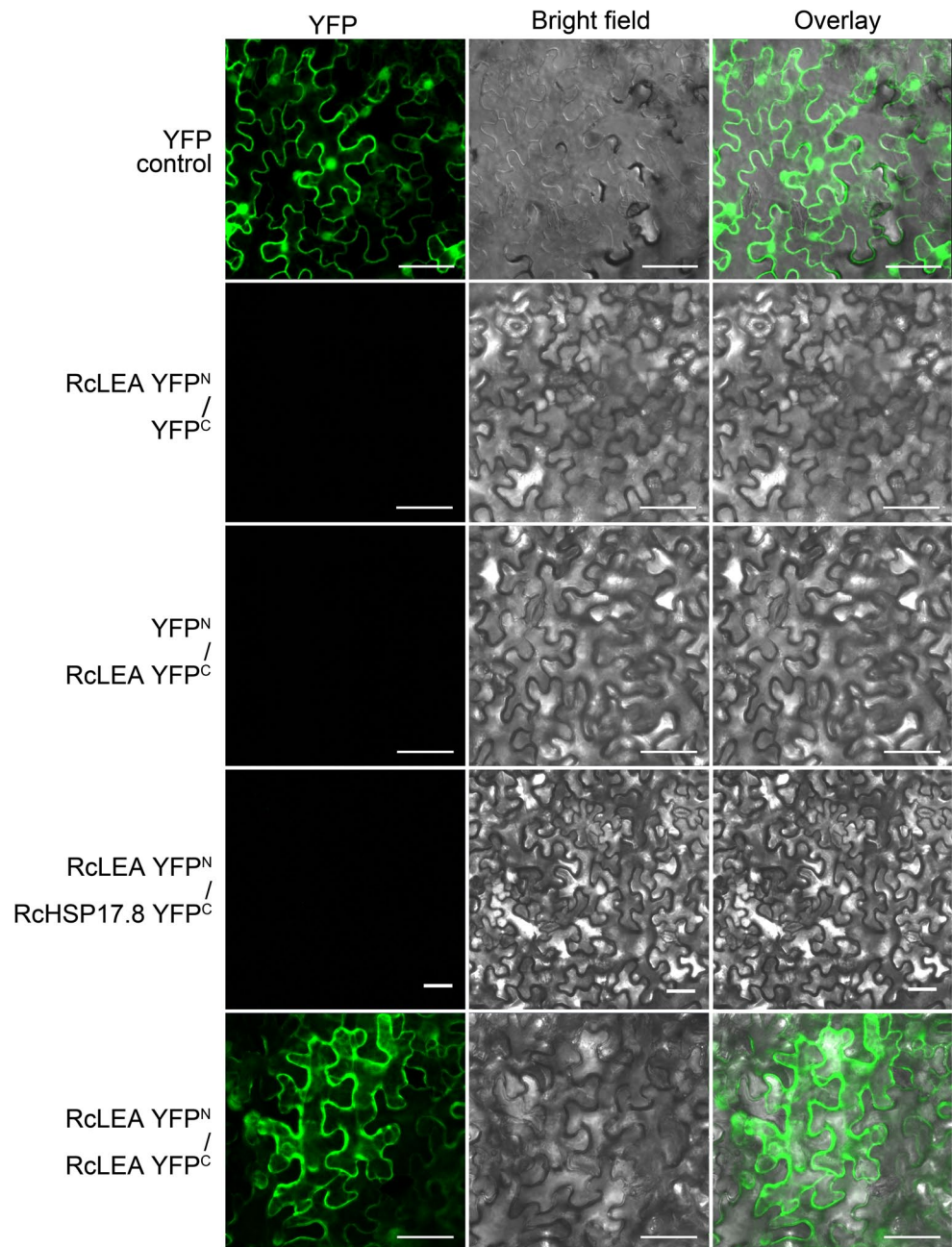
of RcLEA on the heat-induced aggregation of CS. 0.043 mg mL^{-1} CS was incubated at 45°C alone (filled diamond) or in the presence of 0.043 mg mL^{-1} (filled square), or 0.43 mg mL^{-1} RcLEA (filled triangle). **g** The protective effect of the RcLEA protein on the soluble proteome of *E. coli* exposed to high temperature. **h** The RcLEA protein showed protective effect on acid treated *E. coli* proteome, but not alkaline or heavy metal treated proteome. 0:1, 1:3, 3:5, 1:1 indicated the mass ratio of RcLEA: *E. coli* proteins. Results were averages of three biological replicates. Bars show mean \pm SD. *indicated a significant difference ($P < 0.05$)

(Godoy et al. 1990; Bostock and Quatrano 1992; Wilhelm and Thomashow 1993; Moons et al. 1997). In this study, we cloned a *LEA* gene, *RcLEA*, from a heat-resistant *R. chinensis* variety, SM. When SM was cultured at normal temperatures, the expression of the *RcLEA* gene was low, but its mRNA was accumulated to high levels after heat shock. Moreover, the induction level was dependent on the *R. chinensis* variety. In LV, *RcLEA* was induced to a lesser extent than in SM, while it could not be detected

in KP even when heat shock was applied (Fig. 2c). RcLEA protein was also in abundance in SM after heat shock (Fig. S1). KP is the most sensitive and SM the most tolerant to high temperature among the three varieties, which suggests a close relationship between RcLEA and heat resistance.

The function of LEA proteins in abiotic stresses has been reported. Overexpression of a member of the group 4 LEA proteins in *Arabidopsis* enhanced tolerance to severe drought (Olvera-Carrillo et al. 2010). Deficiency of LEA

Fig. 8 BiFC assays of RcLEA. Empty YFP construct (as a positive control) or different combinations of the N-YFP and C-YFP fusion constructs as indicated were co-transfected into the tobacco (*N. benthamiana*) leaves. Bar 50 μm



proteins in this group led to susceptible phenotypes upon water limitation and exhibited a reduced number of floral and axillary buds compared with wild-type plants after recovery from drought stress. Overexpression of wheat dehydrin DHN-5 confers oxidative stress tolerance in transgenic Arabidopsis (Brini et al. 2011). Yeast, Arabidopsis and rice transformed with OsLEA3-2 showed increased tolerance to salt or osmotic stress (Duan and Cai 2012). In particular, the survival rate of the transgenic rice was significantly higher than the WT under drought treatment and the transgenic lines produced more grains after recovery. To determine whether *RcLEA* functioned similarly to other

LEA genes, we overexpressed it in *E. coli* and Arabidopsis. The *E. coli* cells with *RcLEA* survived better than those with an empty vector under heat, cold, salt and oxidative stresses (Fig. 3). According to the electrolyte leakage, the transgenic Arabidopsis overexpressing *RcLEA* suffered less impairment after heat and cold treatment. Hence, they produced greater biomass and more siliques than the WT after recovery for a couple of days (Figs. 4, 5). However, the three transgenic lines exhibited the same growth performance as the WT under salinity or osmotic stress (Fig. 6). These results demonstrated that *RcLEA* played important roles in tolerance to high and low temperature in plants

but not to salinity or osmotic stress, which was in accordance with its expression pattern (it is strongly induced by heat shock in the heat resistant variety SM). The function of LEA proteins in the heat-shock response has been rarely studied. Our study discovered new roles for LEA in heat stress that differs from previous reports.

In general, biomacromolecules, like proteins, nucleic acids or lipids, are damaged through denaturation or dysfunction when cells are subjected to diverse abiotic stresses (Goyal et al. 2005; Liu et al. 2009). The mechanism by which LEA proteins work to enhance the resistance of organisms to abiotic stresses might be that LEA proteins can function in stabilizing proteins, nucleic acids or cell membranes and maintaining redox balance (Tolter et al. 2007; Tunncliffe and Wise 2007; Shih et al. 2010). A mitochondrial late embryogenesis abundant protein, PsLEAM, from *Pisum sativum* stabilizes model membranes in a dry state (Tolter et al. 2010). The Arabidopsis dehydrins ERD10 and ERD14 show chaperone activity with ion-binding properties (Kovacs et al. 2008). The protective function of LEA proteins is related to their structure. Typical LEA proteins are highly hydrophilic and intrinsically unstructured in aqueous solution, but when the environment changes they form homodimer structures that assist them to interact with and protect their substrates (Olvera-Carrillo et al. 2011). To determine how RcLEA protects target proteins, as a homodimer or oligomer, BiFC assay was performed and indicated that RcLEA can form a homodimer to play its protective function (Fig. 8). More detailed analyses are necessary to clarify the function of RcLEA proteins, including the target-binding specificity of homodimer.

Catalase is an important antioxidant enzyme that scavenges the increased amounts of toxic reactive oxygen species (ROS) produced under stress conditions. Transgenic Arabidopsis overexpressing *RcLEA* exhibited higher CAT activity and lower peroxide levels than the WT after heat or freezing treatment, which demonstrated that the RcLEA protein stabilized CAT in vivo (Figs. 4d, 5d). The RcLEA protein could also prevent LDH aggregation during in vitro experiments in which freezing temperatures were imposed; LDH in the presence of the RcLEA protein retained higher activity compared to that in the absence of RcLEA (Fig. 7d). RcLEA also prevented the heat-induced aggregation of CS (Fig. 7f). Furthermore, the RcLEA protein had a protective effect on the *E. coli* soluble proteome under heat and acidic conditions in vitro (Fig. 7g, h), which may be the reason why the cells of *E. coli* overexpressing *RcLEA* survived better than those harboring the empty vector. All of these results suggested that the RcLEA protein was able to maintain the conformation of other proteins, preventing them from denaturation or aggregation upon abiotic stresses.

In conclusion, RcLEA functioned as a protein stabilizer to maintain LDH activity, CS conformation and to protect the soluble proteome of *E. coli*, which helped *E. coli* cells survive under abiotic stresses. Moreover, RcLEA was involved in the stress response pathways of plants and contributed actively to heat and freezing stress tolerance, assisting the plants to survive, recover and produce seeds after abiotic stresses, in which way many LEA genes work in Arabidopsis (Olvera-Carrillo et al. 2010), rice (Duan and Cai 2012), wheat (Brini et al. 2011) and sweet potato (Park et al. 2011). Hence, there is a great chance that RcLEA could function in improving the adaptability of crops or flowers to the environment through genetic engineering. However, further experiments to explore the molecular mechanisms by which RcLEA acts in abiotic stresses, such as examining its ion binding properties and phosphorylation status, need to be performed.

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