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Full Length Article

Overexpression of Protein Gene of Stress-Tolerant *Chlorella* Confers Tolerance to NaCl Stress in *Saccharomyces cerevisiae* and *Arabidopsis thaliana*

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Abstract

The late embryogenesis abundant (*LEA*) protein for a novel stress related to gene *ChLEA* was cloned from a stress-tolerant (ST) *Chlorella* cDNA library. The cDNA was 315 bp long and the open reading frame was 627 nucleotides, which encoded a deduced protein of 104 amino acids. Maximum likelihood tree results showed that ST *Chlorella* first clustered with unicellular microalgae and then with plants. *ChLEA* gene expression in the cells of *Chlorella* was strongly induced by NaCl and 4°C according the Northern blot analyses. *ChLEA* overexpression in yeast and *Arabidopsis* plants increased the resistance to NaCl and 4°C. These data raised the possibility of using *ChLEA* to protect the microalga against stress tolerance. © 2017 Friends Science Publishers

Keywords: LEA protein; Stress-tolerant; *Chlorella*

Introduction

The late embryogenesis abundant proteins (LEA) were described for the first time some 35 years ago. The reporter observed that *Gossypium hirsutum* (cotton) embryogenesis accumulated high levels of proteins during the maturation phase (Dure *et al*., 1981).

Deinococcus radiodurans mosses proteins showed sequences similarity to some LEA proteins, which were described in the some other organisms such as bryophyte (Tortula ruralis), higher plants (Deinococcus radiodurans, Megaphorura arctica etc.) and different nematode species (Battista *et al*., 2001; Browne *et al*., 2002; Gal *et al*., 2003, 2004; Oliver *et al*., 2004). LEA proteins have been separated into different groups, because of the appearance on the basis of amino acid composition and different sequence motifs. Genome organization, protein characteristics the evolution and the expression profile of LEA genes has been in Arabidopsis (Bies-Etheve *et al*., 2008; Hundermark and Hincha, 2008). LEA proteins structural and functional characteristics were analyzed, which emphasize characteristics of amino acid composition and conserved motifs. D-7 and D-29 have been categprized as sub-Group 3 or 4 of LEA proteins. (Battaglia *et al*., 2008).

Dehydration was induced by drying, freezing or saline conditions. The LEA proteins can related with the cellular tolerance to dehydration. The LEA proteins and their subclass dehydrins have been reported from the desiccation tolerant bacteria, invertebrates and sugarcane (Galani *et al*., 2013). Overexpression of LEA proteins can improve tolerance to various degrees of hyperosmotic stress (-1 to -6MPa) in vegetative tissues, which induced by freezing, salt or a partial loss of water (Wahid and Close, 2007; Galani *et al*., 2013). The LEA proteins improved tolerance to salt or drought according to the overexpressed in desiccation sensitive system that was Arabidopsis seedlings (Swire and Marcotte, 1999), leaves of rice (Cheng *et al*., 2002) and yeast (Borrell *et al*., 2002).

The late embryogenesis abundant protein gene of stress-tolerant Chlorella is scantily known especial regarding tolerance to NaCl stress in Saccharomyces cerevisiae and Arabidopsis thaliana. In the present study, one gene from stress-tolerant (ST) *Chlorella* cDNA library was prepared in NaCl stress was isolated. The cDNA clone was isolated by a high homology search as the LEA protein hence, we named it *Chlorella* LEA gene (*ChLEA*). *ChLEA* was isolated in NaCl stress from ST *Chlorella* full-length cDNA yeast library. The objective was to determine whether ChLEA could successfully express in yeast and Arabidopsis and could confer high salinity and low temperature tolerance.

Materials and Methods

Chlorella LEA Gene Isolation

The ST *Chlorella* was isolated from extreme saline-alkali soil (pH>10) in Songnen Plain (46°27′N and 125°22′E Heilongjiang Province, China), which is rich in NaCl

(Wang *et al*., 2011; Qiao *et al*., 2015). ST *Chlorella* was screened and fostered in liquid Bold's basal medium (Bold and Wynne, 1978). All the cultures were maintained at 22±1°C under the 8:16 h light/dark photoperiod by fluorescent white light of 80 μmol photons $m^{-2}s^{-1}$. A full-length cDNA library of ST *Chlorella* was constructed. One gene was isolated from ST *Chlorella* cDNA library under the NaCl stress condition (Qiao *et al*., 2015), which closely matched the sequences of other species LEA protein genes. The gene was named *ChLEA*.

Sequence Analysis

The analysis of complete nucleotide sequence of *ChLEA* was performed using BlastX in non-redundant amino acid sequence database of the NCBI website (http://www.ncbi.nlm.nih.gov). The deduced amino acid sequence and ORF (open reading frame) were analyzed by the DNASTAR Lasergene v7.1 software (http://dnastar.com/t-products-lasergene.aspx) to examine the alignments of multiple sequences. The maximum likelihood (ML) phylogenetic tree was constructed by Mega 5.

RNA Isolation and Northern Blot Analyses

To investigate the *ChLEA* expression level under abiotic stresses (low temperature and high salinity stresses) the microalgal samples were treated in the medium containing with 200 mM NaCl and 4°C, respectively. Each treatment sample was collected at 0, 6, 12, 24 and 48 h. The *ChLEA* gene-specific primers used were forward (5′-ATGGCCGGCAACAAGCCCATC-3′) and reverse (5′-TTAGCGGGTCGCGTTCGGTCG-3′). Hybridization was performed as specified by the Roche manufacturer and detected with the CDP Star by Luminescent Image Analyzer LAS-4000 (Fujifilm, Japan).

Localization of ChLEA Protein in Cell

For the construction of the expression plasmid pYES2-*ChLEA*-GFP and pBI121-*ChLEA*-GFP the *ChLEA* full-length sequence was amplified from plasmid pYES2-*ChLEA* by PCR using the *ChLEA*-specific primers GFP-F (5′-GGATCCATGGGCCTCAAGGAAGACTTTG-3′) and GFP-R (5′-GGTACCGGAGCGTACTTCGCCTTCAGCG-3′; NotI site underlined). The PCR product was ligated into the pEGFP plasmid (Clontech) at the BamH1 and NotI sites. The *ChLEA*–pEGFP fusion fragment was digested by NotI and BamHI and transferred into pYES2 subsequently (Invitrogen).

Plasmid Construction, Yeast Transformation and Stress-tolerance Assays

ChLEA was linked to the vector of pYES2 (Invitrogen). The plasmid pYES2-*ChLEA was constructed by* EcoRI and BamHI for yeast transformation. The aim to analysis the effects of the yeast cells, transgenic yeast containing pYES2 and pYES2-*ChLEA* vector, which incubated in liquid SD-Uracil (yeast nitrogen base without amino acid, 0.77 g/L; -Ura Do supplement and 0.077 g/L; pH 5.8) medium at 30°C overnight. The overnight cultures were adjusted to OD_{600} at 0.5 and diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} with sterile H_2O. Afterward, about 5 μL of each dilution series was spotted onto solid yeast extract/peptone/galactose media (YPG) supplemented with 1 M NaCl and 10°C as indicated. The pYES2 empty vector yeast was used as control. Growth was monitored for 7–30 days at 30°C.

Generation of ChLEA-overexpressing Transgenic Arabidopsis

For the construction of expression plasmid pBI121-*ChLEA* a *ChLEA* full-length cDNA was amplified from plasmid pYES2-*ChLEA* by PCR using *ChLEA*-specific primers pBI-F (5′-GGATCCATGGCCGGCAACAAGCCCATC-3′; BamHI site underlined) and pBI-R (5′-GTCGACTTAGCGGGTCGCGTTCGGTCG-3′;). The PCR product was ligated into the pBI121 plasmid (Clontech) at the BamHI and SalI sites.

Transgenic Plants Stress Tolerance Assays

For the measurement of root length and growth half-strength MS medium was used as a control. The T_3 generation transgenic *Arabidopsis* seeds and WT were germinated on half-strength MS solid agar plates supplemented with 125 mM NaCl and 14°C at 7–30 days, respectively and maintained at 22±1°C under a 8:16 h photoperiod of light/dark. The plates were positioned vertically on shelves for root growth comparison.

Results

Ch LEA Full-length cDNA Sequence with the Deduced Amino Acids

The sequence was 627 bp in length with an ORF of 315 nucleotides, which encode 104 amino acids protein. The protein had a predicted molecular weight of 10.86 kDa and its isoelectric point was 6.349 (Fig. 1). The cDNA sequence contained a 76 and 236 bp 5′- and 3′-UTRs, respectively. *ChLEA* comprises 14 basic, 16 acidic, 33 hydrophobic and 23 polar amino acids. On the basis of multiple sequence alignment, *ChLEA* amino acid sequence shared close identities with the previously published *ChLEA* sequences: 85% with *Chlorella variabilis* and 48% with *Auxenochlorella protothecoides*. ML tree results showed that ST *Chlorella* first clustered with unicellular microalgae and subsequently with plants (Fig. 2).

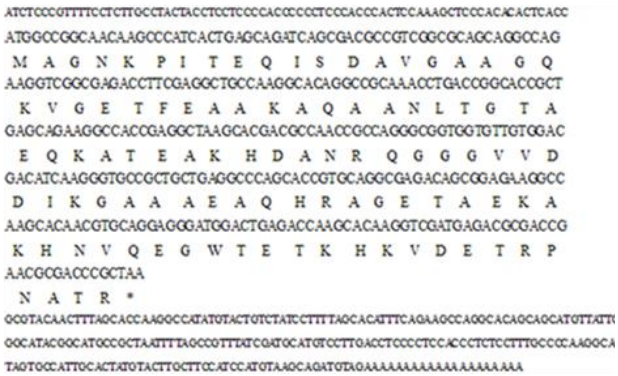

Fig. 1: Nucleotides and deduced amino acid sequences of the *Chlorella* LEA protein gene. The asterisk marked stop codon. The accession No. KT934396 was assigned by GenBank database

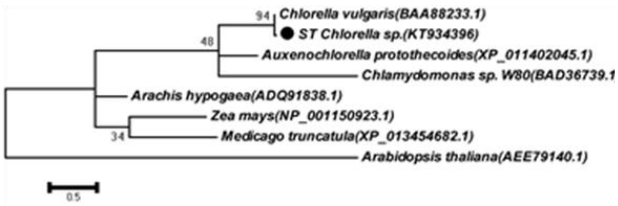

Fig. 2: Maximum likelihood phylogenetic relationship of LEA protein gene in ST *Chlorella* with various LEAs from other species. Bootstrap values are calculated 1,000 times

Expression of ChLEA Inducible by NaCl and Low Temperature Stresses

The ST *Chlorella* cells exposed to NaCl and 4°C for different time (0, 6, 12, 24 and 48 h) indicated that *ChLEA* mRNA expression obviously increased upon the treatment of 200 mM NaCl from 6 h to 48 h compared with the control (0 h). *ChLEA* mRNA expression was higher than that of the control at 12 h and 24 h occurred at the highest level. The expression declined at 48 h but still remained higher than that of the control (Fig. 3). Subsequently, the expression increased gradually at 6, 12, 24 and 48 h under 4°C, but it reached the highest level at 48 h. These data suggested that *ChLEA* expression was induced by NaCl and 4°C (Fig. 3).

Localization of ChLEA in the Cytosol

According to the PSORT Web server (http://psort.nibb.ac.jp) ChLEA was predicted to be localized to the cytosol. To verify this observation, pYES2-*ChLEA*-GFP and pBI121-*ChLEA*-GFP construct was generated by fusing the *ChLEA* coding region to the auto fluorescent protein tag pEGFP (Clontech, USA). Transgenic *Arabidopsis* plants expressing pBI121-*ChLEA*-GFP were generated by *Agrobacterium tumefaciens*-mediated transformation.

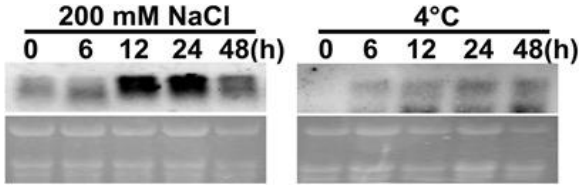

Fig. 3: Northern blot analyses, the expression of the *LEA* gene in ST *Chlorella* cells using a digoxigenin-labeled *ChLEA* cDNA probe exposed to NaCl and 4°C stresses. Extracted the total RNA (5 µg) from *Chlorella* cells treated with 4°C and 200 mM NaCl and at indicated time point

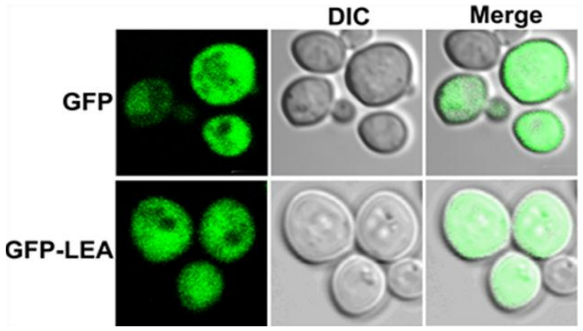

Fig. 4: Subcellular localization of ChLEA in yeast and *Arabidopsis* protoplast. Confocal microscopy of yeast pYES2-*ChLEA*-GFP cloned with the GFP gene in the pYES2 vector and expressed in the yeast strain INVSc1. Bars=5 µm

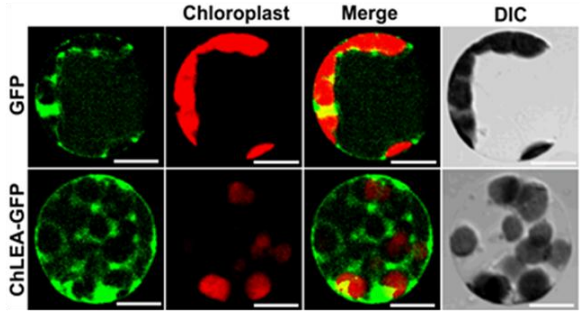

Fig. 5: ChLEA–GFP localization was in the cytosol using confocal microscopy for pBI121-*ChLEA*-GFP. At the top of the image, it was showed GFP vector-transformed *Arabidopsis.* Bars=20 µm. Left was fluorescent; second was merged and right was bright-field (DIC) images

The GFP signal was examined by confocal laser scanning microscopy and fluorescence was detected primarily in the cytosol. The GFP control showed expression in cytosol (Fig. 4 and 5).

Induction of NaCl and Low Temperature Tolerance by Overexpression of ChLEA Gene

Five serial dilutions of the cells were plated and each panel was the five columns corresponding columns. The growth

of *ChLEA* transformants yeast cells was similar to the empty vector at the normal conditions (upper left panel, Fig. 6) The *ChLEA* transformants grew better than the controls in the presence of 1 M NaCl and 10°C (Fig. 6).

Full-length cDNA of *ChLEA* was expressed from the binary vector pBI121 of CaMV 35S promoter for the transformation of *Arabidopsis*. To evaluate the tolerance after stress treatments half-strength MS medium was used as control. There was no difference between T_3 generation transgenic lines (T1, T2 and T3) overexpressing *ChLEA* and WT of seedlings (Fig. 7). The root length of *ChLEA* overexpression in *Arabidopsis* was remarkably better than that in WT at 125 mM NaCl and 12°C treatment (Fig. 7). The results suggested that *ChLEA* overexpression may enhance the stress tolerance of plants.

Discussion

LEA protein has a wide range of sequence diversity, intracellular localizations and expression patterns. The high fraction of retained duplicate genes and inferred functional diversification indicate that they confer an evolutionary advantage for an organism under varying stressful environmental conditions (Michaela and Dirk, 2008). Our results showed that *ChLEA* comprises 14 basic, 16 acidic, 33 hydrophobic and 23 polar amino acids (Fig. 1). From Fig. 2 the ML tree results showed that ST *Chlorella* first clustered with unicellular microalgae and subsequently with plants. The cold response modes vary widely in Chlorella and the adaptation of Chlorella vulgaris to Antarctica may serve as a model system for the evolution of antifreeze mechanisms in a single species of photosynthetic microorganism (Hu *et al*., 2008).

In severely dehydrated wheat seedlings the accumulation of high levels of group three LEA proteins is correlated with tissue dehydration tolerance (Jeffrey and Walker, 1993). C. vulgaris C-27 was studied for the development of freezing tolerance in plant. The result suggested that a sufficiently repeated 11-mer motif was required for the cryoprotective activities of Chlorella LEA proteins (Honjoh *et al.*, 2000). Our results showed that *ChLEA* expression was induced by NaCl and 4°C (Fig. 3). The results suggested that *ChLEA* overexpression may enhance the stress tolerance of plants (Fig. 6 and 7). Several types of proteins that are modulated by abscisic acid accumulate in the developing embryos of maize (Esteis *et al.*, 1992). In this study, transgenic *Arabidopsis* plants expressing pBI121-*ChLEA*-GFP were generated by *A. tumefaciens*-mediated transformation. As shown from Fig. 4 and Fig. 5 the GFP control showed expression in cytosol.

Conclusion

The cDNA of ChLEA was 315 bp long and ORF comprised 627 nucleotides. *ChLEA* overexpression in yeast and *Arabidopsis* plants increased the resistance to NaCl and 4°C. These data raised the possibility of using *ChLEA* to protect microalga and possibly higher plants from stress damage.

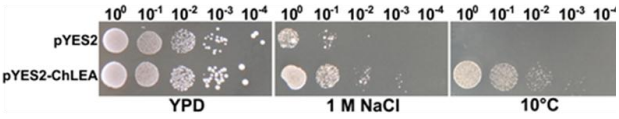

Fig. 6: *ChLEA*-overexpressing yeast cells to NaCl and 10°C stresses. Serial dilutions of the cells were spotted onto yeast extract/peptone/glucose media YPG agar plates with NaCl and 10°C at indicated concentrations. Solid YPG was used as control

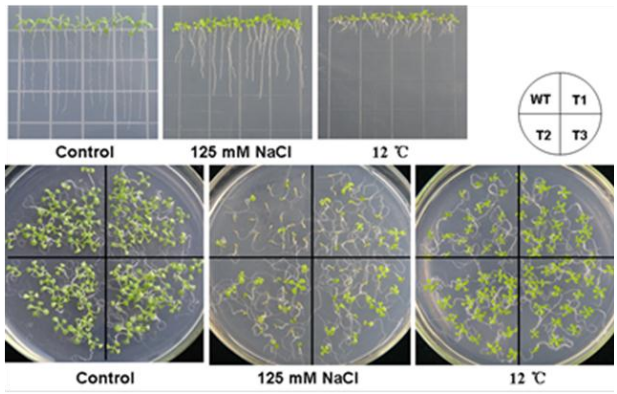

Fig. 7: Effects of abiotic stresses on root lengths and growth of WT and T_3 generation transgenic plants overexpressing *ChLEA*. WT and transgenic seeds were grown on half-strength MS solid a gar plates supplemented with 125 mM NaCl and 10°C, respectively. Seedlings were grown for 7–30 days. The root lengths and growth of WT and *ChLEA*-transgenic plants after treatments are indicated

Acknowledgments

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