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RESEARCH PAPER

Verification of the resistance of a *LEA* gene from *Tamarix* expression in *Saccharomyces cerevisiae* to abiotic stresses

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Abstract: The role of late embryogenesis abundant (LEA) proteins in stress tolerance was examined by using a yeast expression system. LEA protein tolerance to the abotic stresses in plants involved in salt, drought and freezing stresses and additional tolerance to heat, $NaHCO_3$ (salt-alkali) and ultraviolet radiation was also investigated. The transgenic yeast harboring the *Tamarix LEA* gene (DQ663481) was generated under the control of inducible GAL promoter (pYES2 vector), yeast cells transformed with pYES2 empty vector were also generated as a control. Stress tolerance tests showed that *LEA* yeast transformants exhibited a higher survival rates than the control transformants under high temperature, $NaHCO_3$, ultraviolet radiation, salt (NaCl), drought and freezing, indicating that the *LEA* gene is tolerant to these abiotic stresses. These results suggest that the *LEA* gene is resistant to a wider repertoire of stresses and may play a common role in plant acclimation to the examined stress conditions.

Keywords: *LEA* gene; abiotic stress; transgenic yeast; stress tolerance

Introduction

Late embryogenesis abundant proteins (LEA) accumulate during the late stage(s) of embryogenesis and they are found in a wide range of plant species. These proteins are highly hydrophilic and are proposed to play a role in desiccation tolerance based on their accumulation and physicochemical properties. Many *LEA* genes were cloned from plants, including *Physcomitrella patens* (Liang et al. 2004), *Pisum sativum* (Grelet et al. 2005), *Glycine max* (Shih et al. 2005), *Capsicum annuum* (Kim et al. 2005), *Oryza sativa* (Moons et al. 1997), *Gossypium hirsutum* (Galau et al. 1993) and *Raphanus sativus* (Raynal et al. 1990). The abiotic stress tolerance of *LEA* proteins has been investigated extensively, and the resistance to stresses of freezing (Shimamura et al. 2006), drought or osmosis (Manfre et al. 2006; Goyal et al. 2005a), and salt (Chourey et al. 2003) has been also confirmed.

The yeast, *Saccharomyces cerevisiae,* is widely used to produce heterologous proteins (Hasegawa et al. 2000), and it is an excellent model organism for studying the mechanisms underlying stress tolerance (Han et al. 1999; Posas et al. 2000; Serrano and Rodriguez-Navarro, 2001; Jeong et al. 2000). As the glyceraldehyde-3-phosphate dehydrogenase gene was transferred into yeast cells, and yeast transformants exhibited significantly higher resistance to cold, salt, heat, and drought stresses than controls. In addition, Mahalakshmi et al. (2006) reported that expression of the serine-rich protein gene from *Porteresia coarctata* conferred increasing NaCl tolerance in yeast. Wang et al. (2005) cloned the novel Ca^{2+}-permeable channel gene, *TaTPC1*, from wheat and expression of *TaTPC1* in a yeast mutant lacking CCH1 recovered its growth under lithium stress. Overexpression of the sugar beet *eIF1A* gene also increased the sodium and lithium salt tolerance of yeast (Rausell et al. 2003). These results illustrate that the yeast expression system is a desirable tool for studying stress tolerance gene. In the present study, we utilized yeast expression system to study the stress tolerance of the *Tamarix LEA* gene.

Materials and methods

Construction of the yeast expression vector and yeast transformation

Yeast strain, *S. cerevisiae* INVSc1 (His-, Leu-, Trp-, Ura-), from Invitrogen, was used for all transformation. The yeast plasmid, pYES2 (Invitrogen), with the inducible GAL1 promoter was used as an expression vector. Primers for amplifying the *LEA* gene were designed according to the sequence of the *LEA* gene (DQ663481) from *Tamarix*: LEA1, 5’CTAGAGGTACCA-

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TGGCTCGCTGCTCTTACTCTAAT3’, (*Kpn*I site underlined) and LEA2, 5’TCTAGCTCGAGTCAGTGAGAGGATCGATT-GAACTTG3’ (*Xho*I site underlined). The *LEA* gene was amplified by PCR method using a cDNA library plasmid (pBluescript; Stratagene) containing the *Tamarix LEA* gene. The amplified product was digested with *Kpn*I plus *Xho*I and ligated to *Kpn*I/*Xho*I -digested pYES2 to construct the expression vector, pYES2-*LEA*. pYES2-*LEA* was transferred into *Escherichia coli* for amplification. To confirm whether the *LEA* gene was correctly inserted into the pYES2 vector, the plasmid was extracted from *E. coli* cells transformed with pYES2-*LEA*, and digested with *Kpn*I and *Xho*I. The pYES2-*LEA* and control plasmid, pYES2 (empty vector), were transformed into *S. cerevisiae* INVSc1 using a lithium acetate method, as described in the manufacturer’s protocol (pYES2, Invitrogen). The selected yeast clones were grown at 30°C in SC-ura medium (containing 2% glucose) without uracil. The transformant of empty pYES2 was used as a control.

Level of *LEA* gene expression in *S. cerevisiae* at different inducing time

Northern blot analysis was performed to determine the expression level of *LEA* gene at different inducing time in yeast. Yeast transformants were cultured under different conditions as follows: yeast transformants harboring *LEA* gene were cultured in the induction medium (SC-ura medium supplied with 2% galactose) for 3, 12, 24, 36, 48 and 60 h at 30°C and harvested for RNA isolation. The empty pYES2 transformants were cultured in the induction medium for 24 h at 30°C, then harvested as a control. Total RNA was extracted from each samples using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. RNA (20 μg) was dissolved in denaturing buffer (formamide: formaldehyde [37% solution]: 10 x Mops buffer, 500:162:100 [v/v/v]), heated (65°C, 15 min), fractionated on formaldehyde agarose gels, blotted on Hybond N^+ membranes, and fixed by UV cross-linked (254 nm, 8 min). Probe was labeled with digoxigenin (Roche) using PCR method. After prehybridization at 65°C for 2 h, the membrane was hybridized with probe for 18 h at 65°C. The washing and detection procedures were performed following the manual's instruction (Dig Northern starter kit instruction manual, Roche).

Characterizing the stress resistance of *LEA* gene

Yeast transformants harboring pYES2-*LEA*, or a control vector, were cultured in SC-ura liquid medium containing 2% glucose, and incubated for 24 h at 30°C. The cell densities were measured at OD600, adjusted to OD600 of 0.4 in 5 mL of induction medium (SC-ura medium supplied with 2% galactose), and incubated for 24 h at 30°C to promote further expression of the *LEA* gene. After incubation, cell densities were recalculated at OD600, and the culture samples (cells transformed with the *LEA* gene or the control) were adjusted to an equal cell number (about 1 x 10^7 cells) for the stress experiments.

To measure responses to NaCl stress, the yeast cells were incubated in a 5 mol/L NaCl solution, placed at 4°C for 24 h, and diluted 100- and 1000-fold for spread plates. To measure responses to $NaHCO_3$ stress, yeast cells were added to an 8% or 10% $NaHCO_3$ solution and mixed before being placed at 30°C for 8 h, and diluted 100-fold for spread plates. To calculate responses to drought stress, yeast cells were vacuumed for 8 h, treated with an 8 mol/L sorbitol solution for 24 h, and diluted 1000-fold for spread plates. To quantitate responses to high temperature stress, yeast cells were incubated for 2 or 3 h at 53°C and for measuring freezing stress, yeast cells were placed in an ethanol bath at -20°C for 24 h, and then diluted 100- and 1000-fold for spread plates. Following the above treatments, the stressed cells (100 μl) was spread on SC-Ura solid medium (supplying with 2% glucose) and incubated for 48–52 h at 30°C. To measure ultraviolet radiation stress, yeast cells were spread on SC-Ura solid medium and exposed to ultraviolet radiation at a wave length of 254 nm (100 μJ/ cm^2) for 12 s or 36 s; then the plates were incubated for 48 h at 30°C. The plates were scanned and the survival rates of two samples (*LEA* transformed yeast cells and the control) were compared for the assay of *LEA* gene stress tolerance.

Results

LEA gene reached a high expression level at the induction time of 12 h to 24 h

Yeast transformants harboring the *LEA* gene were induced by galactose at different times (3, 12, 24, 36, 48 and 60 h), and analyzed by northern blot to demonstrate the presence of the RNA transcript during expression of the *LEA* gene. Northern blot results showed that the *LEA* gene was transcribed in yeast cells, with transcription reaching a high level at the induction time of 12 h to 24 h (Fig. 1).

Fig. 1 Analysis of *Tamarix LEA* gene expression in yeast cells by northern blot.

Yeast transformants harboring *Tamarix LEA* gene were induced by galactose for 3, 12, 24, 36, 48 or 60 h; CK, yeast cells transformed with empty pYES2.

Stress tolerance analysis of the *LEA* gene in transgenic yeast cells

The stress tolerance of *LEA* gene, or control transformant yeast cells was tested by treating them with a variety of stress inducers. The cells were then spread on plates, incubated for 48 h at 30°C, and the survival rates of *LEA* transformant and control cells were compared. The results showed that there was no difference in survival rate between the transgenic and nontransgenic yeast under non-stress conditions (Figs. 2–7), indicating that the transgenic and nontransgenic yeasts have an identical grow rate. While the survival rate of the transgenic and nontransgenic

yeasts varied significantly when treated with different stress.

Resistance to NaCl stress

There was no difference in survival rate between the transgenic and nontransgenic yeast under non-stress conditions. Following exposure to 5 mol/L NaCl at 4°C for 24 h, the survival rate of yeast cells transformed with the *LEA* gene was about 6-fold higher than that of the control transformants (Fig. 2), indicating that the expression of *LEA* gene enhances the salt tolerance of yeast cells.

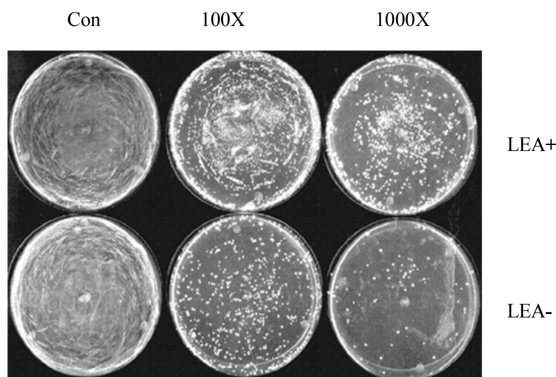

Fig. 2 NaCl tolerance of yeast cells

Yeast cells were incubated in a 5 mol/L NaCl solution, placed for 24 h at 4˚C, diluted 100- and 1000-fold, and plated on SC-Ura medium. LEA+, *LEA* transformed yeast cells; LEA-, yeast transformed with the empty pYES2; Con, untreated yeast cells; 100 x, 1000 x, cells were diluted 100-, 1000-fold and plated.

Resistance to $NaHCO_3$ stress

Yeast cells were treated with an 8% or 10% $NaHCO_3$ solution and incubated at 30°C for 8 h. No difference in survival rate between the transgenic and nontransgenic yeast were found under non-stress conditions. The survival rate of *LEA* yeast transformants was significantly higher than the control transformants, especially after exposure to 10% $NaHCO_3$ solution (Fig. 3).

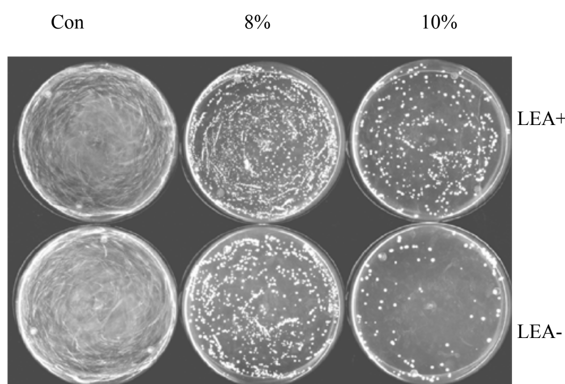

Fig. 3 $NaHCO_3$ tolerance of yeast cells

Yeast cells were added to an 8% or 10% $NaHCO_3$ solution, incubated at 30˚C for 8 h, and plated on SC-Ura medium. LEA+, *LEA* transformed yeast cells; LEA-, yeast transformed with the empty pYES2; Con, untreated yeast cells.

At 8% $NaHCO_3$ stress the survival rate of *LEA* yeast transformants was more than 2-fold higher than that of control cells. At 10% $NaHCO_3$ stress, the survival rate of the *LEA* gene transformants was about 5-fold that of the control cells. The results show that the *LEA* gene has significant salt-alkali stress tolerance.

Resistance to Sorbitol stress

The vacuum-treated yeast cells were incubated in 8 mol/L sorbitol solution for 24 h at 4°C. The transgenic and nontransgenic yeast were shown as Fig. 4. The cells were then diluted 1000-fold and plated on medium. The survival rate of the *LEA* transformed yeast cells was about 2.4-fold that of control cells, showing that expression of *LEA* gene enhances the drought tolerance of yeast cells.

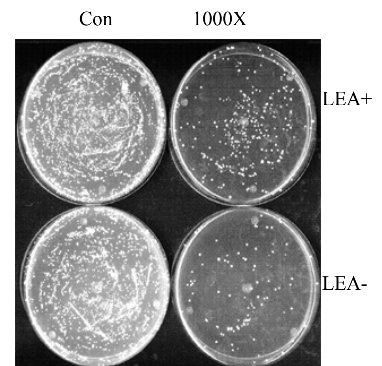

Fig. 4 Drought resistance of yeast cells

Yeast cells were treated with 8 mol/L sorbitol solution for 24 h and plated on SC-Ura medium. LEA+, *LEA* transformed yeast cells; LEA-, yeast transformed with the empty pYES2; Con: untreated yeast cells; 1000X: cells were diluted 1000 fold and plated.

Ultraviolet radiation stress resistance

The yeast cells were treated with ultraviolet radiation (100 μJ/cm$^{2)}$ for 12 s or 36 s. Although *LEA* transformed and control yeast cells all suffered serious injury, the survival rate of *LEA* transgenic cells was about 2-fold greater than that of the control cells when exposed to ultraviolet radiation for 12 s. After exposed to ultraviolet radiation 36 s, there were 9 colonies of *LEA* transformants in plate, while only 1 colony of control was found (Fig. 5). These results suggest that the *LEA* gene also has ultraviolet radiation stress resistance.

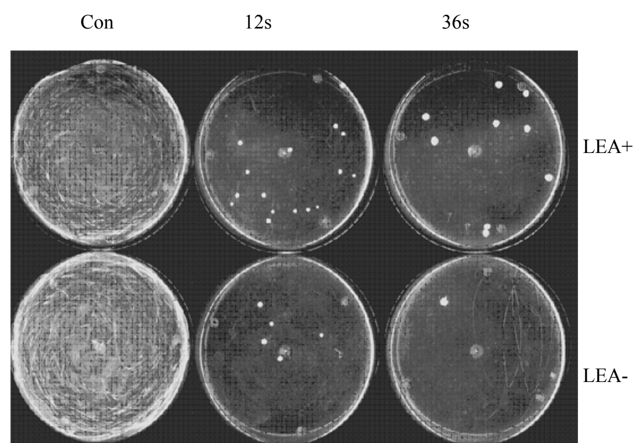

Fig. 5 Ultraviolet radiation resistance of yeast cells.

Yeast cells were spread on SC-Ura solid medium and exposed to ultraviolet radiation (254 nm, 100 μJ/ cm^2) for 12 s or 36 s, incubated for 48 h at 30˚C. LEA+, *LEA* transformed yeast cells; LEA-, yeast transformed with the empty pYES2; Con, untreated yeast cells.

High temperature (53 ℃) stress resistance

Yeast cells were incubated at a 53°C water batch for 2 h and 3 h, and plated on culture medium. No differences between *LEA* transformed and control cells were found under non-stress condition. However, after 2 h and 3 h, the *LEA* transformed cells exhibited significantly higher survival rates than the control cells (Fig. 6). After the 3-h incubation, the survival rate of *LEA* transgenic cells was more than 10-fold greater than that of the control cells. The results clearly indicate that the *LEA* gene induces high tolerance to heat.

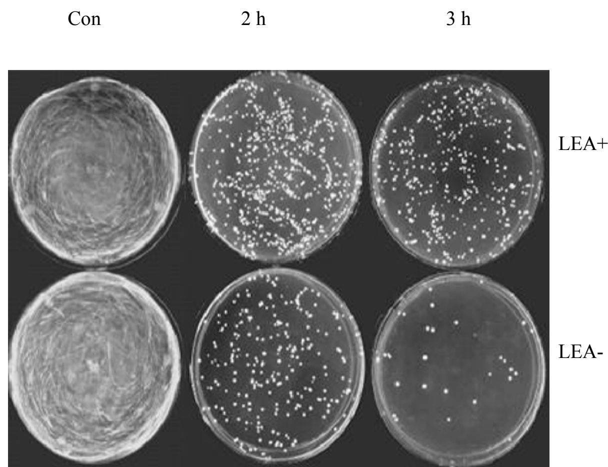

Fig. 6 High temperature resistance of yeast cells

Yeast cells were incubated at 53˚C for 2 or 3 h and plated on medium. LEA+, *LEA* transformed yeast cells; LEA-, yeast transformed with the empty pYES2; Con, untreated yeast cells

Freezing stress resistance

After incubation in a -20°C alcohol bath for 24 h, the yeast cells were diluted 100- and 1000-fold and plated on SC-Ura culture medium. The survival rate of *LEA* transgenic cells was about 3-fold greater than that of the control cells, indicating that the *LEA* gene enhances the freezing tolerance of yeast cells (Fig. 7).

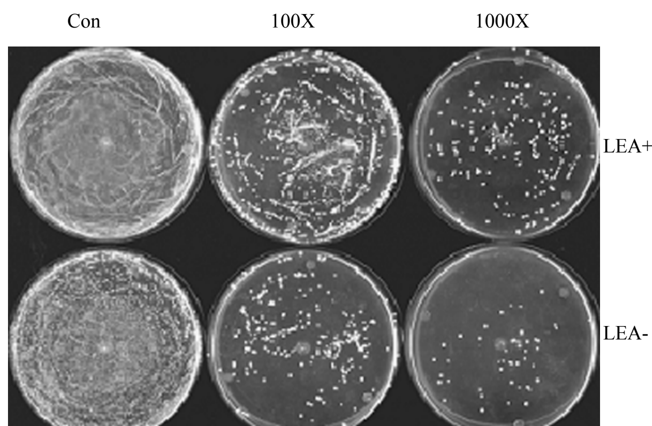

Fig. 7 Freezing tolerance of yeast cells

Yeast cells were placed in an ethanol bath for 24 h at -20˚C and diluted 100- or 1000-fold for spread plates. LEA+, *LEA* transformed yeast; LEA-, yeast transformed with the empty pYES2; Con, untreated yeast cells; 100 X, 1000 X, cells were diluted 100-, 1000 fold and plated.

Discussion

The precise role of LEA proteins is not fully defined (Goyal et al. 2005a), although their various functions have been proposed. It is thought that LEA proteins confer stress resistance by stabilizing membranes and protein structures (Serrano and Montesinos 2003; Danyluk et al. 1998; Grelet et al. 2005; Babu et al. 2004); preferentially hydrating at moderate desiccation and replacing water at extreme desiccation (Serrano and Montesinos 2003). LEA proteins can also adopt certain structures by interacting with macromolecules from other plants, thus promoting protection from stress-induced damage (Soulages et al. 2002). Goyal et al. (2005b) found that LEA proteins can act as molecular chaperones or shields that might prevent irreversible protein aggregation. Goyal et al. (2005a) further illustrated that LEA proteins can prevent aggregation by binding to non-native proteins, and maintaining them in a folding competent state. In addition, LEA proteins are shown to sequester ions, protecting enzymes from desiccation, and replacing water (Grelet et al. 2005) when the plant is under stress-inducing conditions. These studies primarily focus on LEA protein tolerance to the abotic stresses of salt, drought and freezing in plants. We confirm that the *Tamarix LEA* gene is tolerant to these stresses and illustrate that it has additional tolerance to heat, $NaHCO_3$ (salt-alkali) and ultraviolet radiation.

Our study shows that expression of the *Tamarix LEA* gene confers heat tolerance and enhances the survival of yeast cells. *Tamarix* can endure a high temperature in desert, implying that they have developed an efficient heat resistance system. Through this study, we suggest that the *LEA* gene may contribute to heat stress resistance in *Tamarix*. High temperature stress affects plant growth and development and is a common environmental stress to plants in summer season. The finding and study of heat tolerance gene will be helpful for us to enhance the heat stress tolerance of plants by genetic methods.

Our study also shows that the *LEA* protein has $NaHCO_3$ tolerance. Expression of the *LEA* gene in yeast cells significantly enhanced their survival rate in response to $NaHCO_3$ stress. Thus, it appears to be an attractive candidate gene in improving plant salt-alkali tolerance by genetic engineering. The ability to make transgenic plants with tolerance to salt-alkali stress has important implications. According to incomplete statistics of UNESCO and FAO, 950 million ha (6.4%) of the world's land area has saline-alkali soil (Zhang et al. 2006). In saline-alkali soil, plants endure concomitant saline and alkali stress; consequently it is difficult to grow in the saline-alkali soil. The *Tamarix LEA* gene has high saline-alkali resistance, and may be used to genetically engineer plants that will survive these soil conditions.

The studies confirmed that the *LEA* gene was tolerant to many kinds of environmental stress, suggesting that the *LEA* gene may contribute to the ability of adapting to stressful environments of plants. This study improves our understanding of how the *LEA* gene functions during stress, and has implications for the genetic engineering of plants with enhanced stress tolerance.

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