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Expression of Plant Group 2 and Group 3 *lea* Genes in *Saccharomyces cerevisiae* Revealed Functional Divergence among LEA Proteins

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To study functions of late embryogenesis abundant (LEA) proteins, which accumulate in plant cells under water deficit conditions, *in vivo* functional analyses were carried out using a yeast (*Saccharomyces cerevisiae*) heterologous expression system. Two *lea* genes, tomato *le4* (group 2) and barley *HVA1* (group 3), were expressed under the *GAL1* promoter, and the gene products were detected using specific antisera. The growth of the transformants was scored and compared with a control strain to analyze the effect of these proteins on yeast cells under stress conditions. The yeast cells expressing *HVA1* showed shorter lag period when transferred to a medium containing 1.2 M NaCl as compared to a control strain, while the cells expressing *le4* did not show improved growth. Attenuated growth inhibition in a medium containing 1.2 M KCl was observed in the yeast cells expressing *le4* and *HVA1*. No obvious growth improvement was observed in a high sorbitol medium in the cells expressing either *le4* or *HVA1*. Increased freezing tolerance was observed in both *lea*-expressing cells, while no effect on heat tolerance was observed. These results support the hypothesis that different LEA proteins play a distinctive role in the protection against cellular dehydration.

Key words: dehydrin, LEA protein, salt stress, water-deficit.

During periods of water deficit imposed by drought, salinity, and low temperature, plant cells accumulate a set of proteins that are thought to function in protecting cells from dehydration. A number of genes for such proteins have been isolated and characterized (*1*, *2*). A subset of water deficit–induced genes are also expressed in embryos during the late stages of embryogenesis before desiccation, termed *lea* genes (*l*ate *e*mbryogenesis *a*bundant) (*3*). After their first identification in cotton, *lea* or *lea-like* genes were isolated from many plant species (*3*, *4*). LEA proteins are extremely hydrophilic and are boiling-soluble, indicating that the proteins are hydrated and non-globular (*4*). These characteristics have led to the suggestion that LEA proteins are involved in the protection of plant cells from dehydration (*1*, *4*, *5*). Accumulation of LEA proteins coincides with desiccation and freezing tolerance of plants (*6–9*).

LEA proteins are classified into at least seven groups based on the amino acid sequence homology and specific motifs (*1*). Based on the individual amino acid sequences and predicted protein structures, LEA proteins of each group have been suggested to have different functions during water deficit (*1*, *3*, *10*). Group 1 LEA proteins contain a conserved 20-amino-acid sequence with a high glycine content (~20%). Up to 70% of the polypeptide is predicted to form a random coil. Tandem repeats of the conserved 20-amino-acid motif were found one to four times in the middle of the molecules. The hydrophilic and random coil structure indicates a possible function for group 1 LEA proteins as a water-binding protein, which may preserve water in desiccating cells.

Group 2 LEA proteins, also referred to as dehydrins, are characterized by a highly conserved 15-amino-acid lysine-rich sequence, or K-segment, with a consensus of EKKGIMDKIKEKLPG. The putative amphipathic α-helical structure of the K-segment indicates a possible hydrophobic interaction with the surface of macromolecules (*11*). A serine cluster that can be phosphorylated is found in most dehydrins (*12*), although the physiological role of the phosphorylation remains unknown. One of the suggested functions of group 2 LEA protein is to preserve membrane and protein structure under dehydration (*10*, *13*). Localization of specific dehydrins at the plasmamembrane (*14*) and the endomembrane (*10*) is consistent with the former function. The cryoprotective activity of a purified spinach dehydrin supports the latter possibility (*13*).

Group 3 LEA proteins commonly contain a repeat of an 11-mer amino acid motif with the consensus amino acid sequence TAQAAKEKAGE. This is repeated as many as 13 times in Lea76 of *Brassica napus* (*15*). A possible amphiphilic α-helical structure of this motif may allow dimerization of the polypeptides. The arrangement of charged amino acids within the motif suggested a function in sequestering ions that will accumulate under dehydration conditions (*10*).

Group 4 LEA proteins lack repeated motifs, but they contain a well-conserved N-terminal domain. This domain is predicted to form amphiphilic α-helices which may interact with ions or membrane (*1*, *16*). An alternative function of water-binding protein was proposed for the cotton group 4 LEA protein, D113, based on the predicted long random

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Abbreviation: LEA, late embryogenesis abundant.

coil domain at its C-terminal (*4*). Since this domain is not as long in tomato and sunflower proteins, it may not be a common function of group 4 LEA proteins.

Functional analyses of LEA proteins are being carried out both *in vitro* and *in vivo*. A group 3 LEA protein from *Craterostigma plantagineum*, pcC3-06, was successfully accumulated in transgenic tobacco to a level comparable to that in *C. plantagineum*. However, the transgenic plants did not show improved drought tolerance (*17*). Transgenic rice plants producing HVA1, a barley group 3 LEA protein, were also constructed. Growth rates of the transgenic lines after osmotic and water deficit stresses were higher than that of non-transformed plants. The levels of HVA1 accumulation correlated with increased tolerance to the stresses. These data suggested an *in vivo* function of HVA1 in the stressed cells (*18*). Only a few reports on biochemical analyses using purified LEA proteins are available (*13*, *19*). In an earlier study, we tested the effect of overproduction of LE25, a group 4 LEA protein from tomato, in *Saccharomyces cerevisiae*. The yeast cells producing LE25 protein displayed shorter lag times for growth than control cells when challenged by high NaCl concentration. It was proposed that LE25 protein acts as an ion-scavenger (*16*). Recently, another group reported a similar effect of yeast cells expressing *Em*, a group 1 LEA (*20*).

To better understand the function of group 2 and group 3 LEA proteins during stresses, we have produced *S. cerevisiae* cells expressing *le4* from tomato (group 2) and *HVA1* from barley (group 3) and compared the growth of the yeast strains under ionic, osmotic, freezing, and heat-stress conditions. We found substantial difference in the stress tolerance between group 2 and group 3 *lea*-expressing cells. These results support the hypothesis that different LEA proteins play a distinctive role in the protection against cellular dehydration.

MATERIALS AND METHODS

Strain, Media and Plasmids—*S. cerevisiae* EH13-15 (*MATα, trp1*) (*21*) cells were used as the host strain. The plasmid pYPR3831X (*16*) was used to express *le4* (*22*) *and HVA1* (*23*) cDNAs under the control of the *GAL1* promoter. The recombined plasmids pYPR38LE4 and pYPR38HVA1 were constructed as described (*16*). These plasmids were transformed into *S. cerevisiae* EH13-15 using a lithium acetate method (*24*). Cells were cultured in YNBD medium (0.67% yeast nitrogen base without amino acid, and 2% glucose) or in YNBG medium (0.67% yeast nitrogen base without amino acid, and 2% galactose).

Total RNA Isolation and Northern Blot Hybridization—The transformants were cultured in YNBD medium until an OD_{660} of 0.7 was reached, when part of the culture was switched to YNBG medium to activate the *GAL1* promoter. After 6 h of induction, total RNA was isolated by a hot phenol method (*25*). Total RNA was separated on a denatured 1% agarose gel and blotted onto Hybond N^+ membrane (Amersham). The cDNA fragments from pLE4 (*26*), pHVA1 (*23*), and a *HSP12* genomic fragment (mentioned below) were ^{32}P-labeled with Random Primer DNA Labeling kit (Takara, Kyoto) and used as probes. Overnight hybridization and washing was carried out as described (*16*). A 745-bp *HSP12* fragment was amplified by PCR from the *S. cerevisiae* genomic DNA with sense (5′-ACGGTGAATTGCGTTCTACTTC-3′) and anti-sense (5′-TTTAAGCTGGAGTGAAATAG-3′) primers.

Immunodetection of LEA Proteins in S. cerevisiae—The yeast cells harboring pYPR38LE4 and pYPR38HVA1 were precultured in YNBD medium until an OD_{660} of 0.7 was reached, when the cells were transferred to YNBG medium. Total proteins were extracted from the YNBG culture after specific time periods (*27*), separated by 0.1% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, then blotted onto Hybond C nitrocellulose membrane (Amersham). The ECL Western blotting system (Amersham) was used for detection according to the suppliers instructions. Anti-dehydrin and anti-HVA1 antibodies were used to detect the target proteins. Anti-dehydrin antisera and anti-HVA1 antisera were obtained from Dr. T.J. Close, University of California, Riverside and Dr. David Ho, Washington University, respectively. Anti-dehydrin antisera recognize the K-segment, which is conserved among group 2 LEA proteins including LE4 (*12*).

Growth under Ionic and Osmotic Stress—*S. cerevisiae* cells harboring pYPR38LE4, pYPR38HVA1, and pYPR-3831X were grown in YNBG medium for 2 days. Two milliliters of the culture was inoculated into YNBG (200 ml) supplemented with either 1.2 M NaCl, 1.2 M KCl, or 2.0 M sorbitol. At each time point, 1 ml of culture was removed, and OD_{660} was measured with a spectrophotometer. Growth was measured three times, and essentially same growth curve was obtained each time.

Measurements of Freezing- and Heat-Stress Tolerance—When the yeast culture grown in YNBG reached an OD_{660} of 0.7, cells in 1 ml of culture were collected by centrifugation and resuspended in the same volume of sterile water. Four aliquots of 0.1 ml of suspension were spread on YNBD agar plates after appropriate dilution. Another four aliquots were frozen in a metal block cooled at −20°C. After 24 h, the frozen aliquots were thawed in a water bath at 30°C for 5 min and plated on YNBD after appropriate dilution. Colonies that appeared on the plates were counted, and their numbers were averaged. For heat treatment, similarly prepared aliquots were subjected to heat shock at 50°C for 0, 2, 4, 6, or 10 min, then incubated at 30°C for 5 min. Aliquots were spread on YNBD agar plates and survivors were counted.

RESULTS

Production of LE4 and HVA1 Proteins in S. cerevisiae—To achieve high level expression of *lea* genes in *S. cerevisiae*, cDNAs from pLE4 and pHVA1 were introduced into pYPR3831X to obtain pYPR38LE4 and pYPR38HVA1, respectively (Fig. 1). These recombinant plasmids were transferred into the *S. cerevisiae* strain EH13-15. Expression of the introduced genes were detected by RNA gel blot analyses. The mRNAs of 0.9 and 1 kb for *le4* and *HVA1*, respectively, were detected when induced in galactose medium (Fig. 2, A and B). To determine if LE4 and HVA1 proteins were accumulated, total protein was extracted from the transformants and analyzed by SDS–polyacrylamide gel electrophoresis. In contrast to the case with LE25 (*16*), bands corresponding to LE4 or HVA1 could not be detected by staining with Coomassie Brilliant Blue (data not shown). Using antisera against dehydrin and HVA1, the production of the proteins was detected after 4 h and 16 h

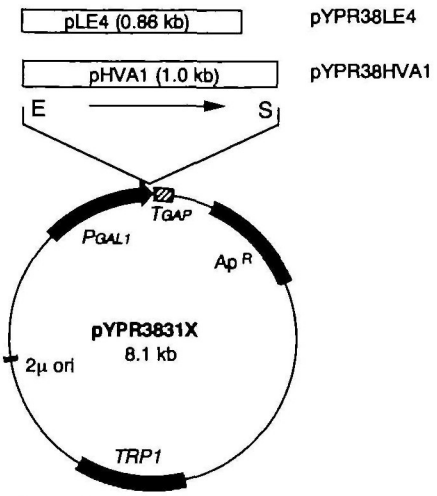

Fig. 1. **The plasmid vector used to express *le4* and *HVA1* in *S. cerevisiae*.** The cDNA clones, pLE4 and pHVA1, were placed between the promoter of the *GAL1* gene (P_{GAL1}) and the terminator of the glyceraldehyde 3-phosphate dehydrogenase gene (*TDH3*) (T_{GAP}) of the multicopy plasmid pYPR3831X, resulting in pYPR38LE4 and pYPR38HVA1.

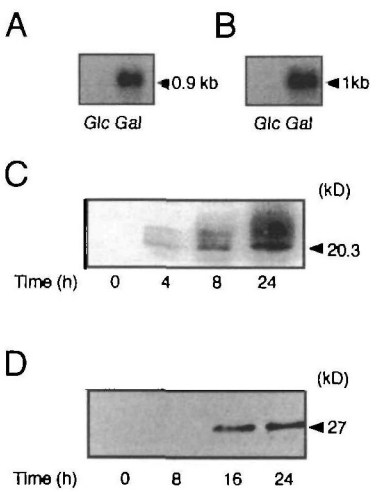

Fig. 2. **Galactose-induced expression of *le4* and *HVA1* in *S. cerevisiae*.** (A) RNA gel blot analysis of *S. cerevisiae* cells expressing *le4*. EH13-15/LE4 cells were grown in either YNBD to OD_{660} 0.7 (Glu) or further grown in YNBG for 6 h (Gal). Ten micrograms of the total RNA was loaded in each lane and probed with pLE4. (B) RNA gel blot analysis of *S. cerevisiae* cells expressing *HVA1*. EH13-15/HVA1 cells were grown in either YNBD to OD_{660} 0.7 (Glu) or further grown in YNBG for 6 h (Gal). Ten micrograms of the total RNA was loaded in each lane and probed with pHVA1. (C) Western blot analysis of total protein isolated from EH13-15/LE4 cells at 0, 4, 8, or 24 h after transfer to galactose medium. Ten micrograms of total protein was separated on SDS-PAGE and transferred. The membrane was hybridized with anti-dehydrin antibodies. Apparent molecular mass in kDa is indicated with an arrowhead. (D) Western blot analysis of total protein isolated from EH13-15/HVA1 cells at 0, 8, 16, or 24 h after transfer to galactose medium. Ten micrograms of total protein was separated on SDS-PAGE and transferred. The membrane was hybridized with anti-HVA1 antibody. Apparent molecular mass in kDa is indicated with an arrowhead.

of induction, respectively (Fig. 2, C and D). The nucleotide sequences of pLE4 and pHVA1 indicated that they have an open reading frame encoding 13.9-kDa (*26*) and 22-kDa (*28*) polypeptides, respectively. Like some other LEA proteins, they migrated slower on SDS-PAGE due to their biased amino acid compositions (*28*, *29*) or modification of the proteins.

Growth Performance of Transformed S. cerevisiae Strains under Ionic and Osmotic Stress—The result described above demonstrated that the regulated expression of *le4* and *HVA1* driven by the *GAL1* promoter led to the accumulation of LE4 and HVA1 proteins in *S. cerevisiae*. We tested

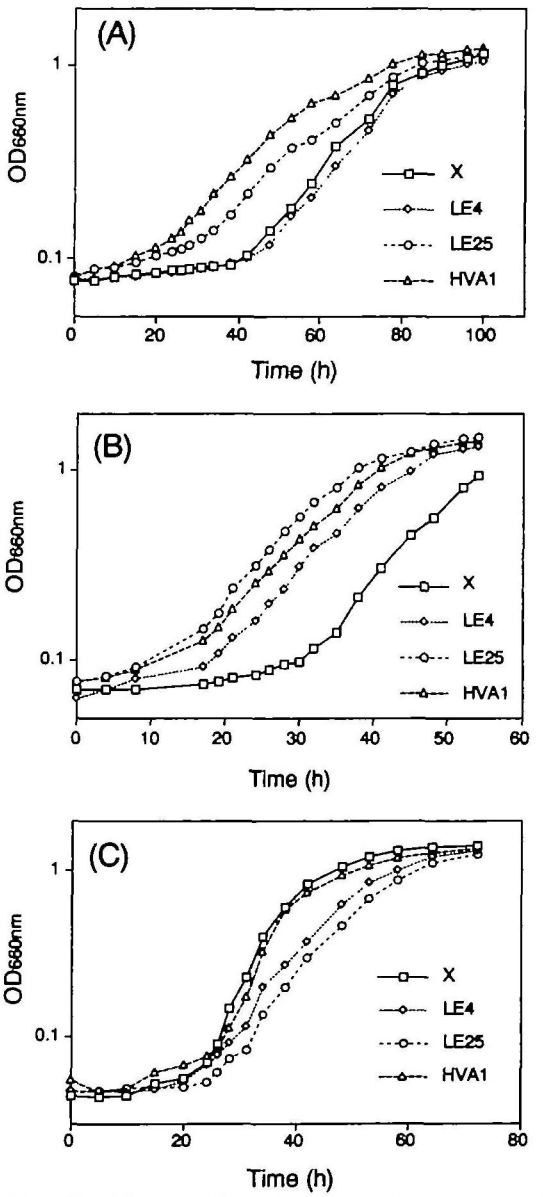

Fig. 3. **Growth of *S. cerevisiae* cells expressing *le4*, *le25*, *HVA1*, and the control cell in the medium containing 1.2 M NaCl (A), 1.2 M KCl (B), and 2.0 M sorbitol (C).** Yeast cells were grown in YNBG medium for 2 days to reach full growth. Two milliliters of the culture was inoculated into YNBG (200 ml) supplemented with either 1.2 M NaCl, 1.2 M KCl, or 2 M sorbitol. At each time point, 1 ml of culture was removed and OD_{660} was measured with a spectrophotometer.

the growth performance of the yeast cells producing LE4 (EH13-15/LE4) and HVA1 (EH13-15/HVA1) proteins under ionic and osmotic stress conditions. Evaluation of growth performance was compared to the strain transformed with pYPR3831X (EH13-15/X). Growth of the same strain producing LE25 (EH13-15/LE25) (*16*) was also measured for comparison.

The control strain, EH13-15/X, was grown in galactose (YNBG) medium to an early stationary phase, then transferred to YNBG containing 1.2 M NaCl. The growth of the control strain was initially arrested; exponential growth resumed after a lag phase of 40 h (Fig. 3A). The EH13-15/HVA1 cells displayed improved growth as compared to the control strain. After transfer to 1.2 M NaCl, the lag phase of EH13-15/HVA1 was about 20 h (Fig. 3A), which was shorter than that observed with the control strain. It is interesting to note that the lag phase of EH13-15/HVA1 was shorter than that of EH13-15/LE25, which may indicate that production of HVA1 protein was more effective in protecting yeast cells under high NaCl stress than LE25. In contrast, there was no difference in the length of the lag phase between EH13-15/LE4 and EH13-15/X (Fig. 3A). Similar experiments were carried out to investigate the effect of high KCl concentration on growth characteristics. In galactose medium containing 1.2 M KCl, both EH13-15/LE4 and EH13-15/HVA1 displayed shorter lag periods (10–20 h) than the control strain (30 h). Growth of EH13-15/LE25 strain in the high KCl medium was similarly improved (Fig. 3B). In the hyperosmotic medium containing 2 M sorbitol, EH13-15/HVA1 exhibited almost the same lag period and growth rate in the exponential phase as the control strain (Fig. 3C). In contrast, EH13-15/LE4 and EH13-15/LE25 had similar lag phase periods as the control strain but growth rate at the exponential phase was slower than the control strain (Fig. 3C). When EH13-15/HVA1 was grown in YNBG without sorbitol, it exhibited slightly lower growth rate than the control strain. The doublng time of EH13-15/HVA1 at mid log phase was 178 min, while that of EH13-15/X was 152 min, suggesting that the production of HVA1 only has a positive effect on growth under osmotic stress. The yeast strains producing LEA proteins showed no difference in their growth on solid YNBG containing NaCl and sorbitol ranging from 0.2 and 2.0 M (data not shown). This may reflect the difference in the total stress environment in the liquid and the solid cultures. An alternative explanation is that production of LE4 and HVA1 proteins in *S. cerevisiae* only provides an advantage during the lag phase before the native yeast mechanism for stress adaptation is in place.

The production of heterologous stress proteins may trigger intrinsic stress responses such as the production of glycerol and heat shock response, which can contribute to ionic and osmotic stress tolerance. To check this possibility, we analyzed expression of the *HSP12* gene, which is activated by both the high osmolarity glycerol (HOG) and the heat shock pathways (*30*). In addition, we have found that overexpression of *HSP12* in yeast confers salt tolerance (Imai *et al.*, unpublished observations). Figure 4 shows that the expression of *lea* genes is greatly induced in YNBG, while the level of *HSP12* expression remains unchanged in YNBG. This suggested that overexpression of LEA proteins in yeast does not activate the HOG or heat shock pathways.

Temperature-Stress Tolerance of S. cerevisiae Strains Expressing the lea Genes—Many reports have shown that LEA proteins accumulate during cold acclimation. Thus, a function in freezing tolerance was suggested (*31*). It is interesting to determine if the expression of LEA-encoding cDNAs alters freezing stress tolerance in yeast. Yeast cells grown in galactose medium to an exponential phase were frozen rapidly and stored at –20°C for 24 h. The thawed cells were diluted and spread on plates to calculate percent

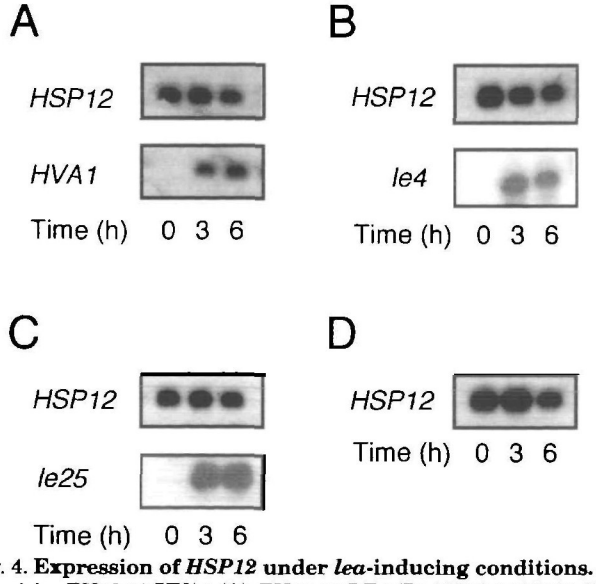

Fig. 4. **Expression of *HSP12* under *lea*-inducing conditions.** *S. cerevisiae* EH13-15/HVA1 (A), EH13-15/LE4 (B), EH13-15/LE25 (C), EH13-15/X (D) were grown in YNBD medium until OD_{660} 0.7, then each culture was divided into three parts: one was unchanged (0 h), and the others were switched to YNBG medium and incubated for 3 and 6 h. Total RNA samples (10 μg) from the respective cultures were fractionated on 1% agarose-formaldehyde gels, blotted, and hybridized with respective probes as indicated.

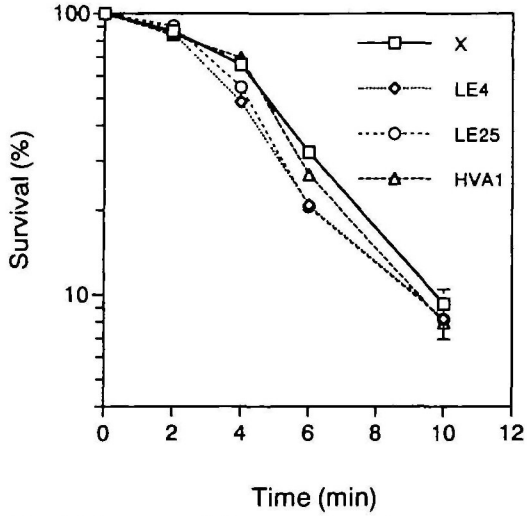

Fig. 5. **Heat tolerance of *S. cerevisiae* cells expressing *le4*, *le25* *HVA1*, and the control cells EH13-15 (pYPR3831X).** Yeast cells were subjected to heat shock at 50°C for 0, 2, 4, 6, or 10 min, followed by treatment at 30°C for 5 min. Colonies that appeared on YNBG agar plates were counted. Data from four independent experiments were averaged. Bars represent standard errors of means.

survival. The EH13-15/LE4 and EH13-15/HVA cells exhibited 5.2 ± 1.4% (mean ± standard error) and 5.8 ± 1.4% survival, which are about two times higher than that of the control cells (2.4 ± 0.4%). Thus, expression of *le4* and *HVA1* conferred freezing-stress tolerance in yeast cells. However, the survival rate was lower than that of EH13-15/LE25 (8.4 ± 1.7%). This may indicate LE25 has a different function than the others, which may be closely associated with amelioration of the effect of freezing, although the difference in the levels of accumulation has to be considered.

Induction of heat shock proteins (HSPs) in *S. cerevisiae* cells confers improved tolerance to freezing, suggesting that a class of HSPs is commonly involved in both heat and freezing tolerance (*32*). Thus, we have tested if the yeast cells producing LEA proteins are heat tolerant. Surviving cells after 50°C treatment were counted on plates after appropriate dilution. Figure 5 shows that no heat tolerance was acquired in the *lea*-expressing cells, suggesting that the protective function of the LEA proteins is specific to dehydration-related stresses.

DISCUSSION

In plant cells, a number of proteins are induced during stresses involving dehydration such as drought, salt and cold. LEA proteins are a subset of such stress-induced proteins, which can be divided into several different groups based on amino acid sequence. LEA proteins have attracted interest because they are extremely hydrophilic and are predicted to protect cell functions under stress conditions. However, clear experimental evidence supporting the function of LEA proteins is still lacking. To initiate the functional analysis of LEA proteins, *S. cerevisiae* cells have been used as a model organism. Yeast cells have been considered a good model system for eukaryotic cells and used to address the function of some heterologous genes (*33*). In the present study, we expressed two plant *lea* genes, *le4* and *HVA1*, in *S. cerevisiae* and examined their effects on growth performance under ionic, osmotic and temperature stress.

LE4 and HVA1 proteins were produced in *S. cerevisiae* by the *GAL1* promoter, but not to the level of LE25 (*16*). We cannot yet explain the difference in the protein levels, while similar levels of mRNA accumulation were observed. However, in the sequence preceding the translation start codon of *le25*, there is a polyA cluster, which is frequently found in highly expressed yeast genes this cluster was not found in *le4* or *HVA1*. Thus, it is possible that the efficiency of translation initiation is different.

Growth measurements in ionic and osmotic stress media revealed that LE4 and HVA1 may have different protective functions in *S. cerevisiae*. When *S. cerevisiae* cells are exposed to high osmoticum, an adaptation process is initiated. This includes activation of the HOG pathway, which induces accumulation of glycerol, a compatible solute, within the cell (*34*). The shortened lag periods in the stress media may be interpreted as a result of mitigation of detrimental effects of the stress or enhancement of yeast intrinsic adaptation mechanisms. Although it seemed possible that overexpression of *le4* and *HVA1* enhances activation of the HOG pathway or heat shock response, Northern blot analysis showed that there is no significant difference in the levels of the HOG- and heat shock-regulated *HSP12* expression between the *lea*-expressing and the control cells (Fig. 4). Thus, it is less likely that the observed effects of *lea*-overexpression in yeast cells were due to the activation of HOG and heat shock pathways.

Previous studies indicated that accumulation of group 3 LEA proteins coincides with salt and desiccation tolerance (*7*, *35*). The group 3 LEA proteins have an 11-mer amino acid motif with the consensus sequence TAQAAKEKAGE (*10*). In HVA1, this motif is repeated as many as 13 times. Dure (*10*) pointed out that the 11-mer repeated sequence of group 3 LEA proteins could form an alpha helical dimer, which is suitable for the accommodation of positively and negatively charged ions. Thus, he proposed that a putative function of group 3 LEA proteins is sequestration of ions (*10*). Growth of *HVA1*-expressing cells displayed shortened lag periods after being transferred to both high NaCl and high KCl media. This supported the putative function of HVA1 in ion-sequestration. Xu *et al.* (*18*) have shown that expression of barley HVA1 in transgenic rice plants confers water deficit and salt stress tolerance. Our results with yeast were consistent with those observed with transgenic plants.

Group 2 LEA proteins or dehydrins have been identified in many plant species. Based on the primary structure, a function was proposed for group 2 LEA to stabilize cellular structures including proteins and membranes under dehydration (*11*). The expression of *le4* in yeast cells confers tolerance to high concentration of KCl but not to NaCl or sorbitol. It is possible that LE4 may interact directly with K^+, but the structural feature of group 2 LEA proteins is not ideal for the ion-binding protein (*11*). Rather, it may interact with proteins or membranes, which may be structurally altered by a high concentration of KCl.

The problem in using transgenic plant to address the functions of LEA protein is that LEA proteins are highly accumulated in the stressed cells, and this makes it difficult to see the effect of overexpression of single *lea* gene. The multigene family of *lea* hampers knocking out of the specific group of LEAs. To determine the physiological role of LEA proteins under stress conditions, the heterologous expression system described here will be useful.

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REFERENCES

1. Bray, E.A. (1993) Molecular responses to water deficit. *Plant Physiol.* **103**, 1035–1040
2. Ingram, J. and Bartels, D. (1996) The molecular basis of dehydration tolerance in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**, 377–403
3. Dure, L. III., Crouch, M., Harada, J., Ho, T.H.D., Mundy, J., Quatrano, R., Thomas, T., and Sung, Z.R. (1989) Common amino acid sequence domains among the LEA proteins of higher plants. *Plant Mol. Biol.* **12**, 475–486
4. Dure, L. III. (1993) Structural motifs in Lea protein in *Plant Responses to Cellular Dehydration During Environmental Stress* (Close, T.J. and Bray, E.A., eds.) pp. 91–103, American Society of Plant Physiologists, Rockville, MD
5. Baker, J., Steele, C., and Dure, L. III (1988) Sequence and characterization of Lea proteins and their genes from cotton. *Plant Mol. Biol.* **11**, 277–291

6. Bartels, D., Singh, M., and Salamini, F. (1988) Onset of desiccation tolerance during development of the barley embryo. *Planta* **175**, 485–492
7. Ried, J.L. and Walker-Simmoms, M.K. (1993) Group 3 late embryogenesis abundant proteins in desiccation-tolerant seedlings of wheat (*Triticum aestivum* L.). *Plant Physiol.* **102**, 125–131
8. Houde, M., Daniel, C., Lachapella, M., Allard, F., Lalibertee, J., and Sarhan, F. (1995) Immunolocalization of freezing-tolerance associated proteins in the cytoplasm and nucleoplasm of wheat crown tissue. *Plant J.* **8**, 583–593
9. van Zee, K., Chen, F.Q., Hayes, P.M., Close, T.J., and Chen, T.H.H. (1995) Cold-specific induction of a dehydrin gene family member in barley. *Plant Physiol.* **108**, 1233–1239
10. Dure, L. III (1993) A repeating 11-mer amino acid motif and plant desiccation. *Plant J.* **3**, 363–369
11. Campbell, S.A. and Close, T.J. (1997) Dehydrins: genes, proteins, and associations with phenotypic trait. *New Phytol.* **137**, 61–74
12. Close, T.J., Fenton, R.D., Yang, A., Asghar, R., DeMeson, D.A., Crone, D.E., Meyer, N.C., and Moonan, F. (1993) Dehydrin: the protein in *Plant Responses to Cellular Dehydration During Environmental Stress* (Close, T.J. and Bray, E.A., eds.) pp. 104–118, American Society of Plant Physiologists, Rockville, MD
13. Kazuoka, T. and Oeda, K. (1994) Purification and characterization of COR85-oligomeric complex from cold-acclimated spinach. *Plant Cell Physiol.* **35**, 601–611
14. Danyluk, J., Perron, A., Houde, M., Limin, A., Fowler, B., Benhamou, N., and Sarhan, F. (1998) Accumulation of an acidic dehydrin in the vicinity of the plasma membrane during cold acclimation of wheat. *Plant Cell* **10**, 623–638
15. Harada, J.J., Delisle, A.J., Baden, C.S., and Crouch, M.L. (1989) Unusual sequence of an abscisic acid-inducible mRNA which accumulates late in *Brassica napus* seed development. *Plant Mol. Biol.* **12**, 395–401
16. Imai, R., Chang, L., Ohta, A., Bray, E.A., and Takagi, M. (1995) A *lea*-class gene of tomato confers salt and freezing tolerance when expressed in *Saccharomyces cerevisiae*. *Gene* **170**, 243–248
17. Iturriaga, G. and Bartels, D. (1992) Expression of desiccation-related proteins from the resurrection plant *Craterostigma plantagineum* in transgenic tobacco. *Plant Mol. Biol.* **20**, 555–558
18. Xu, D., Duan, X., Wang, B., Hong, B., Ho, T.H.D., and Wu, R. (1996) Expression of a late embryogenesis abundant protein gene, HVA1, from barley confers tolerance to water deficit and salt stress in transgenic rice. *Plant Physiol.* **110**, 249–257
19. Lisse, T., Bartels, D., Kalbitzer, H.R., and Jaenicke, R. (1996) The recombinant dehydrin-like desiccation stress protein from the resurrection plant *Craterostigma plantagineum* displays no defined three-dimensional structure in its native state. *Biol. Chem.* **377**, 555–561
20. Swire-Clark, G.A. and Marcotte, Jr.W.R. (1999) The wheat LEA protein Em functions as an osmoprotective molecule in *Saccharomyces cerevisiae*. *Plant Mol. Biol.* **39**, 117–128
21. Fukuda, R., Horiuchi, H., Ohta, A., and Takagi, M. (1994) The prosequence of *Rhizopus niveus* aspartic proteinase-I supports correct folding and secretion of its mature part in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **269**, 9556–9561
22. Cohen, A. and Bray, E.A. (1990) Characterization of three mRNAs that accumulate in wilted tomato leaves in response to elevated levels of endogenous abscisic acid. *Planta* **182**, 27–33
23. Hong, B., Uknes, S.J., and Ho, T.H.D. (1988) Cloning and characterization of a cDNA encoding a mRNA rapidly-induced by ABA in barley aleurone layers. *Plant Mol. Biol.* **11**, 495–506
24. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153**, 163–168
25. Schmitt, M.E., Brown, T.A., and Trumpower, B.L. (1990) A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **18**, 3091–3092
26. Cohen, A., Plant, A.L., Moses, M.S., and Bray, E.A. (1991) Organ-specific and environmentally regulated expression of two abscisic acid-induced genes of tomato. *Plant Physiol.* **97**, 1367–1374
27. LaMantia, M., Miura, T., Tachikawa, H., Kaplan, H.A., Lennarz, W.J., and Mizunaga, T. (1991) Glycosylation site binding protein and protein disulfide isomerase are identical and essential for cell viability in yeast. *Proc. Natl. Acad. Sci. USA* **88**, 4453–4457
28. Hong, B., Barg, R., and Ho, T.H.D. (1992) Developmental and organ-specific expression of an ABA- and stress-induced protein in barley. *Plant Mol. Biol.* **18**, 663–674
29. Mundy, J. and Chua, N.-H. (1988) Abscisic acid and water-stress induce the expression of a novel rice gene. *EMBO J.* **7**, 2279–2286
30. Verela, J.C., Praekelt, U.M., Meacock, P.A., Planta, R.J., and Mager, W.H. (1995) The *Saccharomyces cerevisiae HSP12* gene is activated by the high-osmolarity glycerol pathway and negatively regulated by protein kinase A. *Mol. Cell Biol.* **15**, 6232–6245
31. Thomashow, M.F. (1998) Role of cold-responsive genes in plant freezing tolerance. *Plant Physiol.* **118**, 1–8
32. Komatsu, Y., Kaul, S.C., Iwahashi, H., and Obuchi, K. (1990) Do heat shock proteins provide protection against freezing? *FEMS Microbiol. Lett.* **72**, 159–162
33. Gellissen, G., Melber, K., Janowicz, Z.A., Dahlems, U.M., Weydemann, U., Diontek, M., Strasser, A.W.M., and Hollenberg, C.P. (1992) Heterologous protein production in yeast. *Anton. Van Leeuwenhock* **62**, 79–93
34. Albertyn, J., Hohmann, S., Thevelein, J.M., and Prior, B.A. (1994) *GPD1*, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. *Mol. Cell Biol.* **14**, 4135–4144
35. Moons, A., Bauw, G., Prinsen, E., Van Montagum, M., and Straeten, D.V.D. (1995) Molecular and physiological responses to abscisic acid and salts in roots of salt-sensitive and salt-tolerant indica rice varieties. *Plant Physiol.* **107**, 177–186