BREEDING, GENETICS, AND PHYSIOLOGY

Functional Characterization of OsLti6a Using Yeast Heterologous Expression

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ABSTRACT

OsLti6 genes are related to an evolutionary, conserved abiotic stress-related gene family encoding low-molecular-weight hydrophobic proteins. A high expression level of OsLti6 in a chilling-tolerant rice genotype suggests a role in stress tolerance. Also, stress tolerance and high expression of OsLti6 associated with low electrolyte leakage indicated a possible role of OsLti6 in membrane stability. To study the role of OsLti6 in chilling-stress tolerance, expression effects were tested in a heterologous yeast system. Expression of OsLti6a in yeast allowed higher survival and growth rates when grown under chilling stress compared to the same yeast strain transformed with a control plasmid. Tolerance to other abiotic stresses in the heterologous system, as measured by electrolyte leakage and cell viability, implies a role for OsLti6 in membrane stability during stress.

INTRODUCTION

Sub-optimal environmental conditions cause damage to many plant species including rice. To neutralize the damaging effects of stress conditions, plants have developed complex molecular and biochemical mechanisms (Seki et al., 2003). In Arabidopsis, two genes were isolated from a cDNA library using a subtracted cDNA probe enriched in chilling-induced transcripts (Capel et al., 1997). Functional characterization of the Arabidopsis RCI2A gene in a yeast mutant defective in the RCI2 homologous yeast gene was able to correct the salt-sensitive phenotype of the SNA1 deletion mutant (Nylander et al., 2001). Two homologous rice genes induced by chilling stress, OsLti6a and OsLti6b, were isolated from a subtracted cDNA library (Morsy et al., 2005). Based on hydrophy
plots and subcellular localization in the membrane fraction, OsLti6 proteins are thought to have a role in increasing membrane integrity during stress (Morsy et al., 2005).

Over-expression of stress-responsive genes in a model system may be used to confirm their role in the biology of stress tolerance. The yeast heterologous expression system is a rapid, highly reproducible tool that provides tentative answers concerning the biological function of stress-related genes (Stanasila et al., 1998). Functional characterization of OsLti6 by heterologous expression should provide information for or against the hypothesis of its role in stress tolerance. This paper describes the effects of heterologous expression of OsLti6a in a yeast system.

**PROCEDURES**

**Survival and Growth Rates**

Stress tolerance of the *S. cerevisiae* strain expressing OsLti6 (YRG2-OsLti) was tested under stress conditions and compared to control yeast transformed with the null vector (YRG2-BD). YRG2-OsLti and YRG2-BD yeast strains were grown for 2 to 3 days on SD media lacking tryptophan. One colony of each strain was inoculated into 1 ml YPAD medium, vortexed and transferred into 50 ml YPAD medium. Overnight cultures were collected by centrifugation then resuspended in 1X TE buffer to a final OD\textsubscript{600} of 1.0 (5x10\textsuperscript{7} cell/ml). The yeast suspensions were further diluted by adding 100 µl of each into 50 ml of TE buffer. A 200 µl aliquot of each was spread on YPAD plates (5 plates) to determine the survival rates under various stresses. For chilling treatment, cells were grown for 4 to 5 days at 12°C. For salt and mannitol treatments, YPAD media were prepared with 0.25, 0.5, 0.75, 1, 1.2, or 1.4 M NaCl and 0.5, 0.75, 1, 1.2, or 1.4 M mannitol, and the yeast cultures grown on these media at 30°C for 2 to 3 days. For the growth rate of yeast strains, 100 µl of cell suspension with an OD\textsubscript{600} of 1.0 of each cell strain were added to YPAD broth and grown at 12°C with shaking for 48 hours for chilling treatments. For salt and osmotic stress treatments, liquid medium containing 0.75 M NaCl or 1 M mannitol was used, respectively. Control yeast strains were grown on liquid YPAD medium and kept at 30°C with shaking.

**Yeast Viability Test**

One colony each of YRG2-OsLti and YRG2-BD yeast were grown separately to late log phase in YPAD broth. Subsequently, 50 µl of culture were added to 1 ml of sterile water containing 2% D (+) glucose and 10mM Na-HEPES (pH 7.2) in a microfuge tube, centrifuged for 5 minutes at 1000xg and then resuspended in 1 ml of the same mix. Yeast suspensions were combined with 20 µM of FUN-1 cell viability stain (Molecular Probe, Eugene, Ore.), mixed, and incubated at 30°C in the dark for 30 minutes. Number of cells in 5 µl of stained yeast were determined by a hemocytometer slide under an Axioskop 2 Plus fluorescent microscope (Carl Zeiss, Thornwood, N.Y.)
equipped with a fluorescein filter set with excitation at 480 nm and emission at 530 nm. Five squares of the hemocytometer were counted, and an average number of cells in 1 ml was estimated to be 22 X10^6 for each yeast strain per treatment. The stained cells were analyzed with Auto Montage Pro software and scored for dead and living cells and percentage of dead cells was calculated.

**Electrolyte Leakage**

A similar colony of each yeast strain was grown on YPAD liquid media for 24 hours. Cells were collected and the OD_{600} of each was adjusted to 1.0 with TE buffer as described previously. One hundred µl of each strain were inoculated into 25 ml liquid YPAD medium and grown for another 24 hours at 30°C. For the chilling treatment, cultures were moved to 12°C with shaking for 12 hours. For salt and osmotic stress, cells were collected, supernatant was discarded, and new 25 ml medium with salt (0.75 M NaCl) or mannitol (1M mannitol) was added. The cells were gently resuspended and grown for 12 hours at 30°C. Control yeast strains were grown at 30°C in standard YPAD medium. The electrical conductivity (EC) of the cell suspensions was measured at the start and at the end of the 12 hours’ incubation period. Total electrolyte from each treatment was measured after boiling for 30 minutes. The control values from medium containing no yeast were subtracted from the initial and total conductivity before calculation of the percentage of electrolyte leakage. Percent electrolyte leakage (EL) due to stress was expressed as EC before boiling divided by EC after boiling × 100. Ten cultures were measured for each treatment, and the whole experiment was conducted twice.

**Statistical Analysis**

Environmental stress treatments are reported as the average of five separate replications for each strain-treatment. ANOVA and Student-t tests were used to determine differences among treatments and yeast strains.

**RESULTS AND DISCUSSION**

OsLti6 belongs to the UPF007 protein family that is present in many organisms, including animals, fungi, and plants, and may share common function. We previously proposed that OsLti6 proteins contribute to the biochemical processes involved in preserving the structural and functional integrity of the plasma membrane during chilling stress in rice seedlings (Morsy et al., 2005). In this paper, we test the hypothesis by inducing over-expression of OsLti6a in yeasts and subjecting these to abiotic stress.

Survival of yeast strains YRG2-OsLti and YRG2-BD was similar (measured as number of colonies) at 30°C (Fig. 1a). Exposure of these strains to low-temperature stress (12°C) decreased survival significantly compared to growth at 30°C. However, after exposure to chilling temperatures, survival of control yeast with the null plasmid
was only about half that of YRG2-OsLti (Fig. 1a). YRG2-OsLti yeast grown on medium containing mannitol up to 1 M or 0.25 M NaCl was able to maintain survival at the control level (Fig 1b, 1c). These results are supported by the report of Navarre and Goffeau (2000) that the OsLti6 homolog RCI2 from Arabidopsis could functionally complement a yeast mutation in Pmp3p, which is sensitive to salt stress. The YRG2-BD yeast experienced a reduction in survival when grown on medium containing as low as 0.5 M mannitol. Increases in mannitol concentration beyond 1 M or NaCl over 0.25 M led to reductions in survival of both strains, although survival of YRG2-OsLti was higher (Fig. 1b, 1c). At 1.4 M NaCl, the highest concentration used, some YRG2-OsLti cells survived whereas the control yeast had zero survival at this concentration (Fig. 1c). Similar results were reported by Imai et al. (2006) where they found that the OsLti6 homolog from wheat can partially complement a NaCl-sensitive mutant.

A reduction in growth (measured by OD\textsubscript{600}) occurred under chilling stress in both yeast strains, but after 48 hours the growth of YRG2-OsLti reached nearly the level of cells grown at 30°C. Although the YRG2-BD began growth after 3 hours of chilling, the growth was slow and by 48 hours the OD of the colonies was only 60% of the same strain grown at 30°C (Fig. 2).

YRG2-OsLti and YRG2-BD strain had significant reductions in growth within the first 3 hours of osmotic (1 M mannitol) or salt (0.75 M NaCl) stress (Fig. 2c, 2d). Both strains reached the exponential growth phase within 6 to 12 hours while control yeast grown at 30°C began this phase after 3 hours (Fig. 2a). In medium containing 1 M mannitol, the growth of YRG2-OsLti was consistently higher than that of YRG2-BD (Fig 2c). On the other hand, there was little difference in the growth of the two strains in medium containing 0.75M salt (Fig. 2d).

To evaluate the effect of over-expression of the OsLti6a protein on membrane stability during stress, the percentages of electrolyte leakage (EL) and cell viability were measured for YRG2-OsLti and YRG2-BD cell suspensions. The FUN-1 viability stain provided a quantitative visualization of the effect of stress on viability and membrane integrity. The percentages of EL and dead cells for both strains under control conditions were similar, however, the YRG2-OsLti strain showed lower EL and dead cells under stress conditions compared to YRG2-BD (Fig. 3).

Over-expression of OsLti6a fusion protein in yeast increased survival and growth under chilling stress. Moreover, the OsLti6a fusion protein decreased electrolyte leakage and increased cell viability compared to the yeast strain expressing BD without OsLti6. These results provide indirect evidence of enhanced membrane integrity, and suggest that OsLti6a has a role in stress tolerance.

Reports (Dunn et al., 1994; Capel et al., 1997) indicate that OsLti6a and its homolog are induced by chilling, salt- and water-deficit stresses in rice and other plants, suggesting a role in each of these stresses. Previously (Morsy et al., 2005), the induction pattern of the OsLti6 gene family in a chilling-tolerant rice genotype was compared to that in a chilling-sensitive genotype, and electrolyte leakage from leaves and tolerance to chilling were correlated. The expression pattern and electrolyte leakage data in rice suggested a role of the OsLti6a protein in protecting the cellular membrane from dam-
age in the early stages of stress. Interestingly, over-expression of the OsLti6a protein in yeast increased the survival, growth, and regrowth of the yeast strain not only under chilling stress but also under osmotic stress. We used FUN-1 stain, a dye that provided an indication of membrane permeability in yeast (Roth et al., 1995), to identify the effect of the OsLti6a protein on plasma membrane integrity. The percentage of cells with intact cellular membrane was higher in yeast expressing the OsLti6a-BD fusion protein compared to yeast expressing only BD. These results provide indirect evidence that OsLti6a enhances membrane integrity during stress. Further confirmation was obtained by measurement of membrane leakiness of suspension cultures of yeast expressing OsLti6a and control-yeast strains. Expression of OsLti6a was inversely correlated with the level of membrane injury, as measured by electrolyte leakage, compared to yeast expressing the BD protein only. Cell viability, as measured with FUN1, and decreased electrolyte leakage of suspension cultures of yeast, suggest that OsLti6a increases membrane integrity during stress.

**SIGNIFICANCE OF FINDINGS**

In view of the correlation between OsLti6 gene expression and decreased membrane leakiness in rice and in yeast in this study, it is reasonable to hypothesize that OsLti6 is important in increasing membrane integrity during stress. Moreover, considering the increased expression of OsLti6 genes in a chilling-tolerant rice genotype compared to a chilling-intolerant rice genotype, as well as increased survival and growth rate during chilling stress, it seems that OsLti6 genes are intimately involved in the mechanisms that protect plants from chilling stress. The OsLti6 proteins seem to play a role in the protective machinery of chilling tolerance, and to some extent in osmotic stress tolerance, but their role in salt tolerance is minimal. The OsLts6 may be an excellent candidate for selection in a molecular breeding program to enhance chilling tolerance.

**ACKNOWLEDGMENT**

The authors thank the Rice Research and Promotion Board for its financial support of this research.

**LITERATURE CITED**


Fig. 1. Survival of YRG2-OsLt (open bars) compared to YRG2-BD (solid bars) yeast cells under different stresses. Survival was measured as number of colonies that grew under chilling stress, or in media containing NaCl for salt stress, or mannitol for osmotic stress. (a) The effect of 12°C on the survival of yeast strains compared to those grown at 30°C. (b) The effect of osmotic stress induced by different concentrations of mannitol added to the media on yeast strains grown at 30°C. (c) The effect of salt stress as NaCl at different concentrations on yeast strains grown under 30°C. Different letters indicate significantly different means between treatments or strains (n = 5; α ≤ 0.05).
Fig. 2. Growth rate of YRG2-OsLti (dashed line, closed circles) compared to YRG2-BD (solid line, open circles) under different stresses. Growth rate was measured as optical density of cell suspension at OD$_{600}$ under chilling stress; media contained NaCl for salt stress or mannitol for osmotic stress. (a) Yeast strains grown at 30°C in optimum medium. (b) The effect of 12°C temperature on the growth of yeast cells. (c) The effect of osmotic stress (1 M mannitol) on yeast cells grown at 30°C. (d) The effect of salt stress (0.75 M NaCl) on yeast cells grown at 30°C.
Fig. 3. Percentage of dead cells and electrolyte leakage of *S. cerevisiae* YRG2-OsLti and YRG2-BD. (a) Percentage of dead cells stained with FUN-1 and observed with a fluorescence microscope equipped with a long-pass filter set with excitation emission of 530 nm for visualization of fluorescein. (b) Percentage of electrolyte leakage in cell suspension cultures grown under stress conditions. White bars represent yeast expressing OsLti6 fusion protein and black bars represent control yeast. (c) Microscopic field illustrating the appearance of live and dead yeast cells stained with FUN-1. The cylindrical intravacuolar structures (CIVS) appear as rod-shaped inclusions indicating viable cells with functional membranes (arrows) while the dead yeast cells have very bright fluorescence and do not contain discrete fluorescent structures (arrowheads).