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EFFECT OF TEMPERATURE STRESS ON PHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS OF RICE TRANSGENICS

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ABSTRACT
Abiotic stresses are often interrelated, either individually or in combination; they cause morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity and ultimately yield. Heat, drought, cold, high temperature and salinity are the major abiotic stresses that induce severe cellular damage in plant species, including crop plants. Among abiotic stresses, high temperature stress affects a broad spectrum of cellular components of living organisms and its severity depends on the rate of temperature change, intensity and duration. To counteract the stress effect, plants have evolved various mechanisms (changing leaf orientation, stress avoidance and acclimation mechanisms) to ensure survival under high temperatures stress. Among general stress tolerance mechanisms, stress proteins, osmo-protectants, free-radical scavengers, ion transporters and factors involved in signaling cascades and transcriptional control are essential to counteract stress effects. In the present study, rice transgenics co-expressing HSF4, *p68* and *pg47* was screened for thermo tolerance in order to assess the variation in intrinsic tolerance at cellular level by using TIR technique. TIR protocol standardized for rice was used to assess the variation in cellular level tolerance in the selected transgenic lines. The results of this study revealed that, transgenics are capable of withstanding high temperature by reducing electrolyte leakage through some molecular mechanisms and also showed high recovery growth during alleviation from high temperature stress. The best performing high temperature tolerant lines also showed more total soluble protein content compared to wild type. Identified transgenic lines are characterized further to understand the basic mechanism of high temperature tolerance through molecular mechanism.

KEYWORDS: HSF4, *P68* and *Pg47*

INTRODUCTION

Rice is the staple food of about 3 billion people and its demand is expected to continue to grow as the population increases. Globally, rice is grown over an area of about 149 million ha with an annual production of 600 million tonnes (http://www.fao.org). About 80% of the world's rice is grown under irrigated and rainfed lowland ecosystem, both of which depend on fresh water resources. Increasing scarcity of fresh water resources in the world´s leading rice-producing countries *i.e*., China and India has threatened the production of the flood-irrigated rice crop. According to Tao *et al*. (2006), rice is the most unproductive crop in terms of water loss. On average, about 2,500 liters of water need to be supplied to a rice field to produce 1 kg of rice. This 2,500 liters account for all the outflow of water through evapotranspiration, seepage and percolation (Bouman, 2009). Atlin *et al*., (2004) stated that the new upland adapted varieties (aerobic rice) have improved lodging resistance, as well as highest harvest index and input responsiveness. Aerobic rice can achieve yields of 4–6 tons per hectare and does not require flooded wetland (50-70% less water compared to lowland rice). Generally, irrigated rice tends to become stressed when water is reduced; therefore aerobic rice is the strategy of water saving agriculture. Bouman *et al*. (2002) reported that, in the recent decades, international and national rice institutes have tested various new techniques for growing rice such as aerobic rice (alternate wet and dry systems) which partially or totally suppress the need for ponding at the field level (Zeng *et al*., 2002; Peng *et al*., 2005). However, continuously non-flooded rice cultivation leads to moisture stress and inturn leads to less stable productivity and lower grain yields (Kamoshita and Abe, 2007; Sikuku *et al*., 2010; Wei *et al*., 2011). Therefore, improvement of abiotic stress tolerance might increase actual yields.

Abiotic stresses are the principal causes of crop yield loss worldwide. Drought, high temperature, cold and salinity are among the major abiotic stresses that adversely affect plant growth and productivity. Among abiotic stresses, high temperature stress has a wide range of effects on plants in terms of physiology, biochemistry and gene regulation pathways. The susceptibility to high temperatures in plants varies with the stage of plant development. The observed effects of temperature depend on species and genotype, with abundant inter- and intra-specific variations (Barnabás *et al*., 2008; Sakata and Higashitani, 2008). Various physiological injuries have been observed under elevated temperatures, such as scorching of leaves and stems, leaf abscission and senescence, shoot and root growth inhibition, which consequently lead to decreased plant productivity (Vollenweider and Günthardt-Goerg, 2005). Generally, tolerance to heat is characterized by a lesser effect on essential processes such as photosynthesis and by consistent increases of transcripts involved in the biosynthesis of protective components. As photosynthesis and reproductive development are the most sensitive

physiological processes to stress (Prasad *et al*., 2008), a heat-tolerant variety will be usually characterized by higher photosynthetic rates reflected in stay-green leaves, increased membrane-thermostability and successful seed germination and fruit set under high temperature conditions (Nagarajan *et al*., 2010; Scafaro *et al*., 2010). Higher plants exposed to excess heat, atleast 5°C above their optimal growing conditions exhibit a characteristic set of cellular and metabolic responses required for the plants to survive under the high temperature conditions (Guy, 1999) such as, changes in the organization of cellular structures, including organelles and the cytoskeleton, and membrane functions (Weis and Berry, 1988), accompanied by a decrease in the synthesis of normal proteins and accelerated the transcription and translation of heat shock proteins (HSPs; Bray *et al*., 2000), the production of phytohormones such as abscisic acid (ABA) and activation of antioxidant machinery and other protective molecules (Maestri *et al*., 2002). However, multiple strategies exist to crop improvement for heat stress tolerance. In the present study, transgenic approach was used and developed rice transgenic by co-expressing HSF4, *p68* and *pg47* in rice cultivar (AC39029) with superior water relation traits like water mining and water use efficiency. HSF4 is an important transcription factor associated in up regulation of several of HSPs which function as chaperones to stabilize the proteins (Krishna, 2004). The *p68* RNA helicase involved in pre mRNA processing. The other RNA helicases *pg47* plays an important role in removing the RNA secondary structure and facilitates translation processes (Sahoo *et al*., 2012). The main objective of the present investigation was to characterize transgenic lines to identify superior events with improved tolerance under temperature stress. To achieve this objective, diverse physiological, biochemical and molecular approach has been employed to identify the tolerant lines.

MATERIALS & METHODS

Temperature induction response at seedling level

Two days old and uniform sized seedlings of rice of selected transgenic lines were taken in aluminum trays plated with blotting paper. Before transferring the seedlings to the aluminum trays having wet blotting paper, the initial measurement of seedlings were taken. Three replications for each transgenic line were maintained with each replication having 10 seedlings. Once the measured seedlings were transferred to aluminium trays, the trays were kept inside the TIR chamber/ growth chamber where the temperature and relative humidity (RH) are regulated. Initially, the seedlings were exposed to a standardized induction protocol (32 to 44°C at 2° interval) for a known period of time followed by high stringency temperature (54°C) for a known period of time inside the growth chamber and end of which, the aluminum trays containing seedlings were kept at room temperature for 72 hours for recovery. At the end of recovery period, the number of seedlings survived in each transgenic line was assessed. Similarly, the final seedling growth was also measured and based on the final and initial growth of the seedlings, recovery growth of seedlings was determined (Senthil-kumar *et al*., 2003).

Recovery growth = Final growth - Initial growth

For comparison, one set of seedlings of the same size were exposed directly to the high stringency temperature and one more set after their initial measurements were kept at room temperature all though the experiment period which served as absolute control. The recovery growth measured in the absolute control was used to calculate the % reduction in recovery growth of the selected transgenic lines.

$$\text{Per cent survival of seedlings} = \frac{\text{Number of seedlings survived at the end of recovery}}{\text{Total number of seedlings}}$$

Temperature stress at plant level

Germinated seedlings were raised in small plastic pots and allowed for grow up to 20 days. 20 days old seedlings were exposed to 50°C temperature for 10 hours. After 10 hours of stress treatments, samples were collected for estimating electrolyte leakage and protein content.

Estimation electrolyte leakage

Percent leakage, which reflects loss of membrane integrity, was quantified according to (Hoekstra *et al*., 2001).The leaf samples were collected from the wild type and transgenic plants. Leaf segments were incubated in 20 ml of water for 2 hours. Initial electrical conductivity (EC) was taken using EC-TDS analyzer (ELICO-CM183). Then the leaf segments were boiled for 30 minutes and final EC was taken. The cell leakage was computed using the formula.

Percent Leakage= (Initial EC/Final EC) *100, where, EC = Electrical Conductivity.

Estimation of total soluble protein content

Leaf material from plants were frozen in liquid nitrogen and ground in 100 mM Tris HCl buffer (pH 7.8) containing 1 mM phenyl methyl sulfonyl fluoride (PMSF) and 5 mM benzamidine and the solution was centrifuged at 12000 rpm for 10 min at 4°C. The supernatant was used for quantification of protein by following Bradford method (Bradford, 1976). BSA standard curve was prepared (0.1-1.0 mg/ml) and determined the protein concentration of the plant extract was determined from the regression curve.

Molecular characterization

Extraction of plant genomic DNA

Genomic DNA was extracted by CTAB method (Sambrook *et al*., 1989). For genomic DNA extraction, leaf sample (0.1) was ground in liquid nitrogen. Finely powdered leaf sample was then transferred to 750 µl of preheated 4% (w/v) CTAB buffer with 10 µl beta-mercaptoethanol and incubated for 30 min at 65°C in water bath (Eppendorf, Germany). After incubation, samples were centrifuged at 6000 rpm for 20 min. The supernatant was mixed with 600 µl chloroform: isoamyl alcohol (24:1) and centrifuged at 6000 rpm for 20 min.

The aqueous phase was collected and precipitated with 700 µl of isopropanol at 70^0C for 2 hours and the mixture was later centrifuged at 8000 rpm for 15 minutes at 20 °C and the pellet was washed with 50 µl of 70 % ethanol and centrifuged again at 8000rpm for 10 minutes. The supernatant was carefully discarded and the pellet was air dried and re-suspended in 40 µl Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH-8.0) and stored at room temperature.

Polymerase chain reaction

The DNA fragments were amplified from various plasmid DNA/genomic DNA templates in a 20 µl reaction volume containing 100 ng of template DNA, 2 µl PCR buffer (10X), 2 µl dNTPs (2 mM), 1 µl forward primer (5 pmol/µl), 1 µl reverse primer (5 pmol/µl), 1 U Taq DNA polymerase and volume made up to 20 µl with sterile water. PCR was performed in Master cycler Gradient (Eppendorf AG, Germany). The optimal number of PCR cycles and the annealing temperature (T_a) was determined empirically for each PCR programme. A standard PCR programme followed is as follows:

Step 1: 94°C for 4 min (initial denaturation)
Step 2: 94°C for 1 min (denaturation)
Step 3: T_a for 30 sec (primer annealing)
Step 4: 72°C for 1 min/kb fragment (DNA synthesis)
Step 5: Go to step 2; repeat 25 cycles
Step 6: 72°C for 10 min (final extension)
Step 7: Hold at 4°C; End

The PCR samples were analyzed by Agarose gel electrophoresis. The presence of the expected PCR products was determined with the help of DNA ladders (Gene Ruler DNA ladders, MBI-Fermentas).

RESULTS & DISCUSSION

Temperature induction response at seedling level

One of the important drought adoptive traits which impart tolerance to the plants under stress condition is the ability of the plants to tolerate the stress effects at cell level called as cellular level tolerance. Any genotype which has ability to survive under stress and put on growth after stress alleviation (recovery growth) is considered as tolerant genotype. In the present study, the per cent seedling survivability and recovery growth was measured in few selected transgenics by subjecting them for temperature induction response technique. The results of the experiment revealed that, there is marked variation in seedling survival and recovery growth among the transgenics tested to indicate variations in cellular level tolerance (CLT).

In induction treatment, mean per cent seedling survival was 100 per cent both in transgenic and wild type. Mean recovery growth of transgenics was 1.75 cm and the range was in between 0.5-3cm whereas mean recovery growth of wild type was 1.2cm and the range was in between 0.9-1.6cm. Mean percent reduction in recovery growth over control of transgenic was 33.99 and range was in between 27.9-40.1 whereas wild type was 37.63 and range was in between 35.01-40.2 (Table 1).

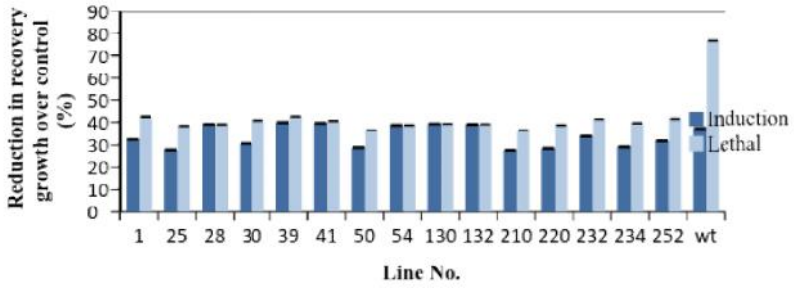
In lethal treatment, mean per cent seedling survival of transgenic was 60% and the range was in between 50-70 per cent whereas, in wild type it was 20 per cent and the range was in between 10-30%. Mean recovery growth of transgenics was 0.74cm and the range was in between 0.5-0.97cm whereas wild type was 0.25cm and the range was in between 0.1-0.4cm. Mean per cent in reduction recovery growth over control of transgenic was 39.50 and range was in between 36.5-42.51 whereas wild type was 76.68 and range was in between 71.01-82.35 (Table 1).

TABLE1: induction treatment response at seedling level

Parameters	Induction treatment				Lethal treatment			
	Wild type		Transgenics (15)		Wild type		Transgenics (15)	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Seedling Survival (%)	100%	-	100%	-	20%	10-30%	60%	50-70%
Recovery growth (cm)	1.2	0.9-1.6	1.75	0.5-3	0.25	0.1-0.4	0.74	0.5-0.97
Per cent reduction in recovery growth over control	37.63	35.01- 40.2	33.99	27.9-40.1	76.78	71.01-82.35	39.50	36.5-42.51

In control condition, there is no considerable difference between transgenic and wild type in seedling growth, but in induction treatment there is a difference between transgenic and wild type in recovery growth but not significant, whereas in lethal treatment there is a significant difference between transgenic and wild type in recovery growth (Figure 1b). Out of 15 lines, many transgenics showed a significantly lesser percent reduction in recovery growth over control compared to wild type in lethal treatment, where as in induction treatment, transgenic not showed a significant reduction in recovery growth over control compared to wild type (Figure 1a). Upon induction, optimum expression of stress-responsive genes which brings about intrinsic differences in stress tolerance. Hahn and Li (1990) have shown that thermoprotection was phenomenally high in seedlings exposed to prior acclimation stress. The acclimation response studied in pearl millet and sorghum showed that the induced seedlings had significantly higher recovery growth after exposure to high temperature of 48 and 55°C (Howarth *et al*., 1997). To maintain cellular integrity against stress, cells control the quality and quantity of the proteins being synthesized. This mechanism is called protein homeostasis or proteostasis. One of the major adaptive responses is the heat shock response, which was first described by Ritossa (1962) who observed chromosome puffing in *Drosophila* after exposure to high temperature. Tissieres *et al*. (1974) showed that the appearance of this puffing was associated with the synthesis of a small number of new proteins, termed heat shock proteins (HSPs). After the finding in 1978 by Schlesinger, the high temperature induced the synthesis of similar proteins in cultured avian cells; comparable responses have been reported in all organisms. The heat shock response is characterized by the induction of a set of major HSPs such as HSP90 and HSP70, which act as molecular chaperones that facilitate the folding and assembly of proteins and inhibit their misfolding under

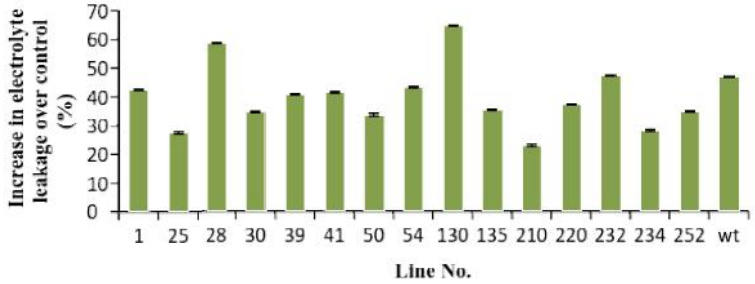
stress condition (Mohamed and Al-whaibi, 2010; Akira Nakai, 2010). In the present study, rice transgenic over-expressing HSF4 is involved in giving tolerance to high temperature by assisting protein refolding under stress condition.



At plant level

Estimation of electrolyte leakage

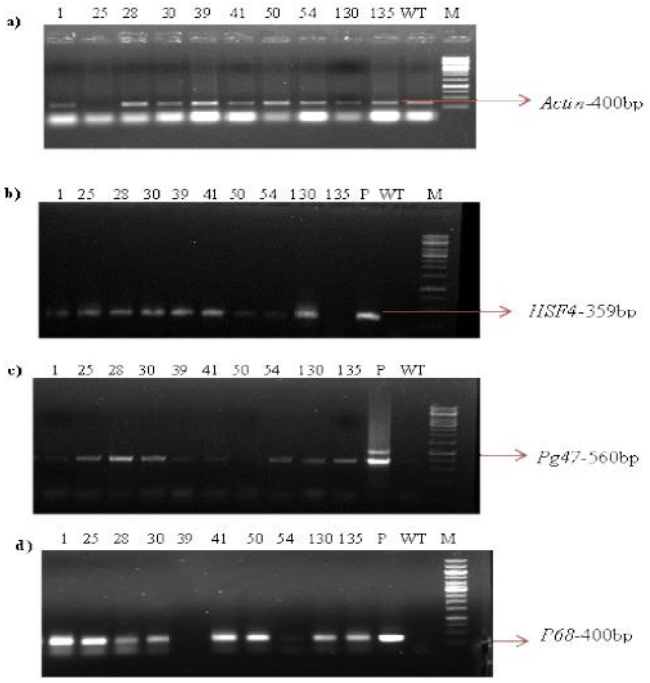
Cell membrane stability was determined by estimating the electrolyte leakage induced by High temperature. Many transgenics showed significantly lesser increase in electrolyte leakage content over the control compared to wild type (Figure 2). The result indicates that transgenics maintained higher cell membrane stability by reducing the electrolyte leakage induced by high temperature through some cellular tolerance mechanisms. The plasma membrane and cytoplasmic membranes of plants composed of lipids and proteins. There is some evidence to suggest that the composition, particularly of the lipid component, may change in response to environmental conditions such as temperature, water stress, *etc*. It is believed that these changes are required to adjust the physical characteristics of membrane structures so that they may perform their necessary physiological tasks when environmental factors change. If the environmental conditions are altered beyond the normal limits within which the plant survives, the cell membranes are often found to undergo gross structural changes. These structural perturbations cause leakage of the membrane constituents and are associated with characteristic disturbances of function such as loss of selective permeability and transport processes (Peter, 1988) Hence in the present study, overexpression of HSF4 could have positive effects on the membrane stability by reducing electrolyte leakage induced by high temperature through activating antioxidant machinery.



Molecular Characterization

Genomic DNA was isolated from both transgenics and wild type plants. Initially PCR amplification of genomic DNA with Actin primer as internal control was carried out for selected transgenics and wild type plants. The integration of genes was confirmed by amplifying the genomic DNA with different combinations of primers. The integration of *PgHSF4* in transgenics was confirmed using *2x35s* promoter specific forward primer and *PgHSF4* gene specific primers. The presence of 359bp amplicon on gel confirmed the presence of *PgHSF4* in transgenic plants (Plate 1b). The integration of *Pg47* in transgenics was confirmed using *RBCS* promoter specific forward primer and *Pg47* gene specific primers. The presence of 1200bp and 560bp amplicon on gel confirmed the presence of *Pg47* in transgenic plants (Plate 1c). The integration of *p68* in transgenics was confirmed using *ubiquitin* promoter forward and *p68* gene specific primers. The presence of 400bp amplicon on gel confirmed the presence of *p68* in transgenic plants (Plate 1d).

Based on seedlings growth, electrolyte leakage and molecular analysis, five transgenic lines were selected and total protein content was estimated for selected transgenic lines.



Estimation of total protein estimation

Transgenics showed less reduction in total protein content compared to wild type through some molecular mechanism (Figure 3).

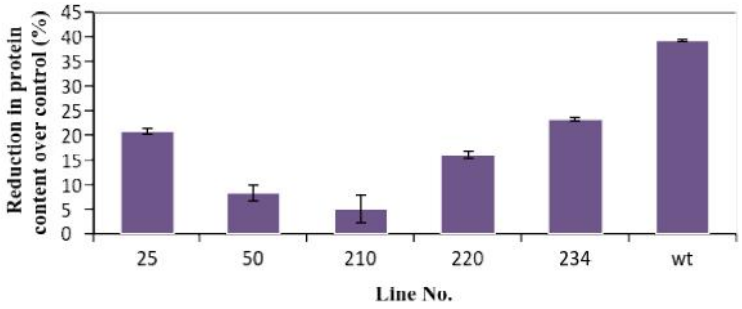


FIGURE 3: Reduction in protein content over control (%)

CONCLUSION

Rice transgenics co-expressing HSF4, *P68* and *Pg47* showed tolerance to high temperature stress by maintaining high seedlings recovery growth and by reducing electrolyte leakage through some molecular mechanism.

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