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Comparative Analysis of Salinity Responsive Expression Pattern of Putative Candidate Genes in Rice and Finger Millet

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ABSTRACT: *Rice (*Oryza sativa*), a salt-sensitive species, whereas finger millet (*Eleusine coracana *L.) another member of poaceae family closely related to rice is a resilient cereal known for its superior level of tolerance against drought and salinity. In this study comparative physiological and molecular analysis was done to see the effects of NaCI stress on contrasting genotypes of rice and finger millet. Gas exchange parameters measured with LICOR64 has shown higher photosynthetic rate, stomatal conductance and transpiration rate under salinity stress conditions in tolerant finger millet genotype as compared to susceptible finger millet and both the rice genotypes. Northern blot analysis using heterologous probes of rice shown salinity specific upregulation of* AKT *1 like potassium channel protein in finger millet genotypes.* DREB1A *transcription factors was found to be upregulated in response to salinity stress specifically in finger millet genotypes whereas* DREB 2 *was upregulated in both susceptible rice and finger millet genotypes than the tolerant ones under stress condition. Heterologous probes for genes encoding Group 1 and Group 3* LEA *protein and glycosyl transferase were found to be upregulated specifically in salinity tolerant finger millet genotype where as it was found to be downregulated in both rice and susceptible finger millet genotype. The study gives an insight into the existence of similar orthologous molecular responses in finger millet and rice under salinity stress.*

Keywords: Finger Millet, Salinity, Northern Blotting, Heterologous Probes, Photosynthesis, Gas Exchange.

INTRODUCTION

Salinity represents a strong limitation for agricultural production worldwide, especially in arid and semi-arid areas and restricts efficient utilization of available land resources. Around 45 million hectares (M ha) of irrigated land, accounting 20% of total land area have been affected by salinity worldwide and approximately 1.5 M ha are taken out of production each year due to high salinity levels in the soil (Pitman, and Läuchli 2002; Munns, and Tester 2008). Most of the cereal crops are sensitive to salinity and have limited amount of genetic variation for salinity tolerance in their germplasm. Rice a staple food crop for half of the world population has been rated as a salt sensitive crop (Shannon, *et al.*, 1998). Hence genetic improvement of crops for their tolerance against salinity will be helpful in achieving targeted food production to meet the demands of growing population. Conventional plant breeding approaches have resulted in limited success in developing salt tolerant crop varieties due to limited understanding on the complexity of salt tolerance mechanisms, difficulties involved in selection of component traits and presence of low genetic variation in major crops. Reproductive barriers and linkage drag in turn adds the stumbling block in transferring genes from wild relatives into domesticated cultivar through conventional breeding.

Responses against salinity stress involve many molecular processes such as ion homeostasis (membrane proteins involved in ionic transport), osmotic adjustment and water regime regulation (osmolytes) and scavenging of toxic compounds (Munns, and Tester 2008). During recent years, considerable attention has been given towards elucidating the molecular basis of salt tolerance in crop plants. Utilization of modern genetic approaches and high-throughput methods of functional genomics have resulted in elucidation of salt tolerance mechanisms, especially salt ion signaling and

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transport, as well as several important pathways involved in salinity tolerance have been identified in model plants like *Arabidopsis* and rice (Zhu 2003; Walia, *et al.,* 2005; Cotsaftis, *et al.,* 2011). The significant progress in the area of functional genomics paved the way for identifying candidate genes, understanding their expression patterns in response to salt stress and determination of their potential functions in salt stress adaptation which provided the basis for effective genetic engineering strategies to enhance tolerance against salt stress (Cushman, and Bohnert 2000). Transgenic approaches through genetic engineering strategies targeting various metabolic pathways *viz*., accumulation of osmolytes, antioxidant enzymes and up regulation of genes involved in stress responses like ion transporters, ion channels, transcriptional factors and various signaling pathway components resulted in the production of genetically modified crop plants exhibiting improved level of salinity tolerance (Zhang, *et al*., 2001; Turan, *et al.,* 2012; Apse, and Blumwald 2002; Bohnert, and Jensen 1996). Recently through genetic engineering approach transgenic plants have been developed by transferring genes or transcription factors from halophytes, distantly related tolerant/resilient crops or wild progenitors of cereal food crops exhibiting superior levels of salinity tolerance (Yadav, *et al.,* 2012; Ganesan, *et al.,* 2012; Rajan, *et al.,* 2015). Through several studies, it has been hypothesized that exploitation of highly saline tolerant "halophytes" or wild germplasm may serve as an excellent source for understanding physiological/molecular mechanisms underlying salinity tolerance and thereby leading to identification of novel candidate gene(s) for engineering salinity tolerance in agriculturally important crop plants (Mehta, *et al.,* 2005; Mishra, *et al.,* 2007; Garg, *et al.,* 2013)]. Finger millet (*Eleusine coracana* L.) is one of the resilient cereal crops belonging to the family, Poaceae and genetically close to rice (Davos and Gale, 1997) which is known for its high degree of tolerance against drought, salinity and blast disease (Shailaja, and Thirumeni 2007; Agarwal, *et al.,* 2011; Rahman, *et al.,* 2014).

Investigating the mechanisms and pathways involved in salt-tolerance of finger millet could facilitate better understanding of the molecular basis of salt tolerance and therefore enable the effective use of genetic and genomic approaches to improve salt tolerance in major cultivated crops like rice. Although a wide range of significant physiological mechanisms and genetic adaptations to salinity stress has been observed, the underlying mechanisms of salt-tolerance in plants are still poorly understood. The best possible approach to explore tolerance mechanisms is to compare the components involved in stress response in tolerant and sensitive plants. The other alternative to overcome this limitation would be to pick up some putative candidate genes which may be used to perform limited transcriptome analysis among the contrasting genotypes of sensitive and tolerant crop.

With this background, we planned to understand the physiological and molecular basis of salinity responsiveness in finger millet in comparison to the major cereal food crop, rice. Two finger millet and rice genotypes exhibiting contrasting levels of salt tolerance were selected for the study. With the non-availability of genome information in finger millet, the molecular response of finger millet genotype under salinity stress was analyzed by monitoring the mRNA levels using heterologous gene information of selected salt responsive candidate genes from rice. This is the first comparative study conducted to understand physiological and molecular basis for superiority of finger millet over rice in terms salinity tolerance.

MATERIALS AND METHODS

Genetic materials used

Seeds of two contrasting genotypes of rice (FL478-tolerant, White Ponni-Susceptible) and finger millet (Trichy 1-tolerant, CO12 -Susceptible) in terms of salinity tolerance were evaluated for their responses against salinity stress under greenhouse conditions. Nucleus seeds of rice genotypes were obtained from Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore, India and finger millet genotypes were obtained from Millet Breeding Station of Tamil Nadu Agricultural University, Coimbatore, India.

Effect of salinity stress during vegetative stage

Imposition of salinity stress

Contrasting genotypes of rice and finger millet genotypes (three seedlings per pot) were grown in perforated pots of 15 cm diameter and 20 cm height (having 3–5 mm holes on the side walls and bottom) as described by Rahman et al. (2014). Salinity stress was imposed on 21st day when plant has reached to 5 leaf stage by adding desired concentrations of NaCl viz. 150 mM and 300 mM along with suitable control pots irrigated with normal water.

Physiological responses of contrasting finger millet genotypes to salinity stress

Gas exchange parameters were recorded using a portable infrared gas analyzer (LI-6400, LI-COR, Lincoln, Nebraska, USA) in the completely expanded healthy third leaf (from top) of control and salinity stressed plants of rice and finger millet genotypes between 9030 hours to 1130 hours at 7 DAS (days after stress) when susceptible rice genotype (White Ponni) started showing salinity symptoms. The instrument was set with the following conditions: photo-synthetically active radiation 1,500 µmol of photon $m^{-2}s^{-1}$; ambient levels of CO_2 and temperature; leaf area 3 cm^2 and flow rate of 500 µmol s^{-1}.

Expression analysis of selected salinity responsive genes in contrasting genotypes of Finger millet and rice

RNA isolation, Northern blotting and hybridization

Expression analysis of already reported salinity responsive candidate genes in the leaves of contrasting rice and finger millet genotypes were studied by northern blotting. Top 3 leaves of both rice and finger millet genotypes exposed to 7 days of NaCl (300mM) stress along with the corresponding controls were collected and frozen immediately in liquid nitrogen. Total RNA was isolated from stressed and control leaf samples using One Step RNA Reagent (Biobasic Inc., Canada) as per manufacturer's protocol. The integrity of RNA was assessed by formaldehyde agarose gel electrophoresis. Total RNA was quantified using Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). Twenty microgram of RNA mixed with RNA loading dye (1:1) was denatured at 75°C for 10mins and separated on denaturing agarose gel as described by Streit et al. (2008). The gel was stained with ethidium bromide and photographed. Gel was processed and RNAs were transferred to positively charged nylon membrane (Pal Corporation, USA) using 20XSSC buffer. After capillary transfer to the membrane, RNAs were fixed by exposing the membrane to UV cross linker (Hoeffer, Piscataway). The membranes were hybridized using heterologous probes isolated from rice. The double-stranded probes were radioactively labelled (α-^{32}P) dCTP using DecaLabel DNA Labeling kit (Thermo Fisher Scientific, USA) and purified using IllustraMicroSpincolumns (Amersham Biosciences, USA). The Radiolabelled probes were denatured on boiling water bath snap cooled on ice and used for hybridization as described by Streit *et al.* (2008). RNA blots were pre-hybridized in ULTRAhyb® (Ambion, USA) at 45°C for 4–8 h. The hybridization was carried out 45°C for 12h. The blots were initially washed at 45°C with 2XSSC and 0.1% SDS followed by two washes with 1XSSC and 0.1%SDS at 45°C for 20 min each.

Hybridized membrane were dried on blotting paper and exposed to Kodak XAE-5 film with cassette having Kodak intensifying screen for 1–6 d. The resulting radiograms were scanned on ImageScanner-III (GE Healthcare, USA).

RESULTS AND DISCUSSION

Evaluation of rice and finger millet genotypes at vegetative stage

In our previous study (Rahman, *et al.*, 2014) evaluation of rice and finger millet genotypes for their salinity response during vegetative stage stress resulted in the identification of contrasting rice (White Ponni—susceptible; FL 478—tolerant) and finger millet (CO 12—susceptible; Trichy 1—tolerant) genotypes differing in their degree of salinity tolerance. Even tolerant rice genotype FL478 started showing salinity symptoms *viz.*, leaf tip drying and rolling symptoms within 5-6 days after imposing 300mM NaCl stress. Susceptible rice genotype *i.e.* White Ponni completely dried 12 days after 300mM salinity stress. All rice genotypes were completely dried after 17 days of 300mM NaCl stress whereas ragi genotypes were able to tolerate 300mM of NaCl stress upto 23 days. Trichy 1 was able to produce new leaf upto 30 days after imposing 300mM NaCl stress.

Gas exchange responses to salt stress

Salinity stress adversely affects photosynthesis in most plants by altering the ultrastructure of the organelles, impaired biosynthesis and/or accelerated chlorophyll degradation, disruption of metabolic enzymes and by stomatal regulation (Ashraf, and Harris 2013). Plant gas exchange provides a highly sensitive measure of the degree of stress to which a crop is exposed. In this study reduction in gas exchange parameters was detected under salinity stressed condition in both genotypes of rice and finger millet as compared to control. Photosynthetic rate reduced in both rice and finger millet genotypes but reduction was more in case of rice as compared to finger millet genotype. Tolerant finger millet genotype Trichy 1 recorded a reduction of 23.98% where as in susceptible finger millet genotype 45.59% reduction was observed in photosynthetic CO_2

fixation rate due to 300mM NaCl stress. White Ponni recorded 45.91% whereas FL478 recorded 33.72% reduction in photosynthetic rate under salinity stressed condition (Fig. 1). Reduction in other gas exchange parameters like stomatal conductance (*gs*), transpiration rate and C_i/C_a (where; C_i is leaves internal CO_2 concentration and C_a is ambient/atm. CO_2 concentration) ratio was much higher in rice compared to finger millet. Reduction in stomatal conductance in case of rice genotypes ranged from 76.19% to 78.79% in White Ponni and FL478 and in finger millet genotype the reduction was 31.43% to 62.96% in Trichy 1 and CO12 respectively (Fig. 1). Reduction in transpiration rate in rice genotypes were 65.59% in White Ponni, and 67.67% in FL478 as compared to 22.16% in Trichy 1 and 56.16% in CO12 (Fig. 1). The stomatal control of transpiration is a mechanism used by different species to restrict loss of water during salinity and drought stress. Ci/Ca ratio reduced to 63.01% (FL478) to 75% (White Ponni) as compared to finger millet genotype where reduction was 48% in tolerant finger millet genotype Trichy 1 and 66.67% in susceptible finger millet genotype CO12 (Fig. 1). The impact of salinity on photosynthetic parameters in crop plants has been already reported (Noreen, *et al.,* 2012; Walia, *et al.,* 2005; Walia, *et al.,* 2007). Reduced net CO_2 assimilation rate/photosynthesis and transpiration rate under salinity stress might be due to a stomatal closure, which leads to a reduction in intracellular CO_2 partial pressure. Lesser reduction in stomatal conductance might be probable reason for lesser reduction in photosynthetic rate and Ci/Ca ratio in the leaves of salinity tolerant finger millet genotypes Trichy 1.

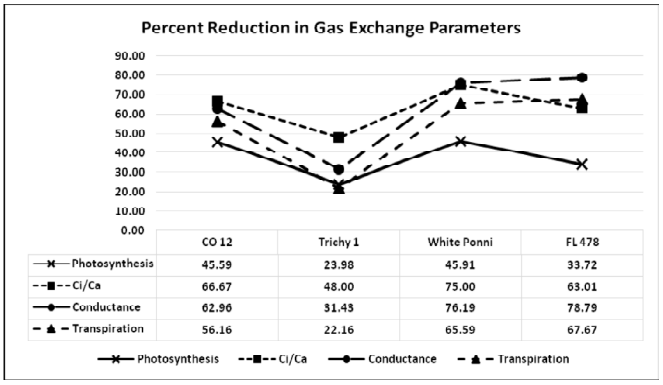

Figure 1: Effect of salinity stress on gas exchange parameters in the leaves of contrasting rice and finger millet genotypes.

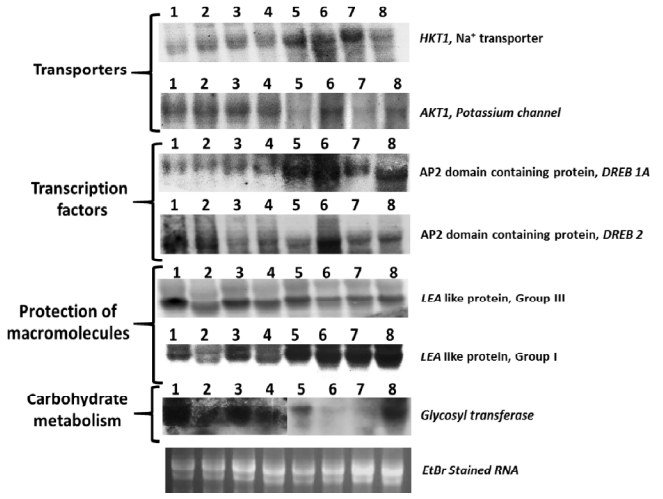

Figure 2: Northern blot analysis of salinity responsive genes in contrasting genotypes of rice and finger millet. Where 1 is White Ponni control, 2 is White Ponni stress, 3 is FL478 control, 4 is FL478 stress, 5 is CO12 control, 6 is CO12 stress, 7 is trichy1 control and 8 is Trichy 1 stress.

Differential expression of candidate genes in response to salinity stress

To glean an insight into comparative salinity stress tolerance mechanism in rice and finger millet genotype, representative genes of each class i.e., transcription factors, transporters, genes involved in sugar metabolism were picked up for northern analysis (Table 1). The selectively chosen heterologous probes showed a clear specific hybridization signal on northern blot indicating their usefulness in expression analysis in rice and finger millet genotypes (Fig. 2).

High salinity stress causes an imbalance in sodium ion (Na^+) homeostasis and the tolerant genotypes used to maintain the relatively lower level of sodium by using various ion pumps, resulting in either efflux of excess Na^+ ions and/or vacuolar compartmentalization thus avoiding toxic ion accumulation in the cytosol (Mahajan, and Tuteja 2005; Roy, *et al.,* 2014).

The heterologous probes for Na transporter (*HKT1*), potassium transporter (*AKT 1*) represents the genes involved in ion transport under salinity stress conditions and are having direct role in providing salinity stress tolerance (Roy, *et al.,* 2014). The basal level of transcripts for *AKT1* could be detected in rice genotypes even under control conditions but was found to be slightly upregulated under stress condition. Upregulation was more in case of susceptible finger millet genotype. High affinity potassium transporter (*HKT 1*) was found to be slightly upregulated under salinity stress in salt sensitive genotype of both rice and finger millet whereas it was downregulated in tolerant genotypes

Table 1
Details of heterologous probes and primers used for northern blot analysis.

Putative rice homolog (Locus_ID)	*Putative Name*	*Primer Sequence*	*Putative Function*	*Reference*
LOC_Os02g07830	*Os*HKT1;3 - Na+ transporter	F- GCAAACCGTATTGTTCCTCTC R- GTTATCTAAGCTTCCAGGCTC	Transport	Walia, *et al.,* (2007)
LOC_Os01g52070	Potassium channel *AKT1*	F- ATCCCACAGCCGTTCACCAT R- AGAATCCCTCTCGCTGCCTT	Transport	Walia, *et al.,* (2007)
LOC_Os09g35030	Dehydration-responsive element-binding protein (*DREB 1A*)	F- CGAAGATGTGCGGGATCAA R- CCATCCAATCTCTCGTCAC	Transcription factor	Flowers (2004)
LOC_Os02g45420	AP2 domain containing protein (*DREB 2*)	F- TAATGGTACATGGCTGACCTG R- ACCAAGACACGGCGATCGGT	Transcription factor	Matsukura, *et al.,* (2010)
LOC_Os08g23870	Late Embryogenesis Abundant Protein, Group 1	F- GTTCATCCAAATCCACAGCC R- CCCACAACATGATACCACTTC	Cellular protection	Taji *et al.,* (2004)
LOC_Os05g46480	Late Embryogenesis Abundant Protein, Group 3	F- GCTTAGGATCAATGGCTTCC R- ACGACCACCACTTCATACAG	Cellular protection	Xu, *et al.*, (1996)
LOC_Os03g20120	Glycosyltransferase 8 domain containing protein	F- GTACAAGCCGATCCCACTGA R- GCCTCGTTACATTTGTGAAGTG	Raffinose biosynthesis	Taji, *et al.*, (2004)

of both rice and finger millet. Transcription factors are the key regulators that modulate expression of other genes and thus help in providing tolerance to several abiotic stresses. In this study under long term of 300mM NaCl stress the transcript levels of *DREB 1A* was found to be upregulated in both the genotypes of finger millet whereas no change was observed in rice genotypes. *DREB2* transcription factor was not showing any change in susceptible rice genotype but was found to be upregulated in tolerant rice genotype FL478. Upregulation of *DREB2* was more prominent in both finger millet genotypes as compared to rice genotypes. Heterologous probes for two genes encoding group 1 and group 3 Late Embryogenesis Abundant protein (*LEA* protein) showed an higher induction in salinity tolerant genotype of finger millet where as in both rice genotype and susceptible finger millet genotype it was found to be slightly upregulated. The accumulation of soluble sugars had been widely reported in response to salinity or drought (Murakeözy, *et al.,* 2003; Garg, *et al.,* 2002). Glycosyl transferase are involved in biosynthesis of raffinose(Hopf, *et al.,* 1984; Lehle, and Tanner 1973) was found to be up-regulated in response to be specifically upregulated under salinity stress in tolerant finger millet genotype. Raffinose acts as an osmo-protectant as well as an antioxidant, thus protecting the plants during oxidative stress (Nishizawa, *et al*., 2008). Besides biosynthesis of raffinose,glycosyl transferases are also involved in glycosylation and plays a key role in maintaining cellular homeostasis during environmental stress (Wang, and Hou 2009).

Based on results obtained through this study and those published by others groups in various plant species, it may be inferred that maintenance of higher level of stress related transcripts in salinity tolerant genotypes than sensitive one appears to be a responsible for providing salinity tolerance in finger millet and can be used as good candidate genes for further functional validation through transgenic approach for developing salinity tolerant crops.

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