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Comparative Analysis of Salinity Responsive Candidate Gene Expression in Selected Sri Lankan Rice Varieties

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ABSTRACT

Studying of expression dynamics of salt responsive candidate genes under different genetic background of rice is important to develop resilient salt tolerant rice varieties. The present study was focused to assess the comparative expression of salinity responsive genes which are involved in Na^+/K^+ homeostasis (*OsNHX1*), signal transduction pathway (*OsMAPK5*, *OsCDPK7*) and synthesis of osmoprotectant (*OsTPS1*). Comparative expression of selected genes were assessed in *At354* (improved), *Godawee* (traditional) and *Kuruwi Perunel* (traditional), salinity tolerant varieties and *Suwandel*, a salinity susceptible traditional variety. The assessment was done before salinization (at 0 h) and after salinization at 24 h and 72 h using semi-quantitative Reverse Transcriptase PCR (RT-PCR). RT-PCR products were analysed using 1.5% agarose gel electrophoresis. Resulted gel profiles were assessed and relative level of gene expression was measured based on the intensity of the PCR product in terms of average pixel values. Relative level of gene expression in four varieties was plotted against the exposure time to salinity. Accordingly, marked variation in expression pattern of the selected genes was detected in four varieties over the tested in *Godawee* and *Suwandel* compared to *At354* and *Kuruwi Perunel*. At 24 h, all four genes were up-regulated in *At354* and *Godawee* while they were down-regulated in *Suwandel*. In *Kuruwi Perunel*, *OsNHX1*, *OsMAPK5* and *OsCDPK7* genes were up-regulated at 24 h while *OsTPS1* was up-regulated at 72 h. At 72 h, continuous expression of all genes was observed in *Kuruwi Perunel* and *Godawee*, whereas expression was comparatively down-regulated in *At354* approximately to the same level under non-stress condition. It could be suggested that *At354* might have been adapted to the stress relatively earlier compared to *Godawee* and *Kuruwi Perunel*, where expressions of genes were less pronounced. Further experiments with various time period of salinity exposure should be carried out to make detailed inference regarding the expression dynamics in tested rice varieties.

Keywords: Gene expression, Rice, RT-PCR, Salinity tolerance

INTRODUCTION

Soil salinity is considered as the second most widespread soil problem causing the significant reduction in productivity of global rice cultivation (Gregorio *et al.,* 1997; Ashraf and O'Leary, 1996; Munns *et al.,* 2006). Degree of salinity tolerance in rice varies with the stage of development. Rice is relatively tolerant to salinity during germination, active tillering, and maturity period whereas it is highly sensitive to salinity during early seedling and reproduction stages (Lafitte *et al.,* 2004; Pearson and Bernstein, 1959). Growth-stage-dependent salinity tolerance in rice is

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mainly associated with salt exclusion or low uptake, compartmentalization of toxic ions in structural and older tissues, higher tissue tolerance, responsive stomata that close faster upon exposure to salt stress, up-regulation of antioxidant systems for protection against reactive oxygen species generated during stress and vigorous growth to dilute salt concentration in plant tissue (Yeo and Flowers, 1986; Yeo *et al.,* 1990; Peng and Ismail, 2004; Moradi and Ismail, 2007). Accordingly, salinity tolerance in rice is a complex mixture of mopho-physiological traits, which is controlled by multiple genes (Chinnusamy *et al.,* 2005). Ismail *et al.* (2007) has reported that in rice most salt-tolerant genotypes are superior in only one or a few of these traits and therefore there is a potentiality to pyramid superior alleles for all useful mechanisms into one rice genotype towards producing resilient salt tolerant varieties (Ismail *et al.,* 2007). Therefore, it is essential to understand the fundamental molecular mechanisms behind stress tolerance in plants. Considerable progress towards understanding the molecular basis of salt tolerance in rice has been made during the last two decades and number of genes involved in signal transduction, transcription regulation, ion transporters and metabolic pathways, conferring salt tolerance in rice have been isolated (Ismail *et al.,* 2007; Kumar *et al.,* 2013).

Response to salinity stress in rice begins with the acquisition and transmission of the stress signals through signalling components, which would activate the stress-related genes leading to synthesis of diverse functional proteins responsible for various physiological and metabolic responses (Kumar *et al.,* 2013; Basu and Roychoudhury, 2014). The comprehensive understanding of the biological functions of these candidate genes is one of the greatest challenges in functional genomics research, which has to be revealed through the determination of their expression profiles (Basu and Roychoudhury, 2014). Transcriptomic analysis provides detailed knowledge about the gene expression at mRNA level, which is widely used to screen candidate genes involved in stress responses. Genomic approaches play a significant role in encoding, cloning, and characterization of important genes. The vast number of salt-responsive candidate genes which are either up-regulated or down-regulated in response to salinity stress have been identified and characterized using transcriptomic and genomic approaches such as microarrays, RNAseq , quantitative real time PCR (qRT-PCR) and semi-quantitative RT-PCR expression analysis methods (Ismail *et al.,* 2007; Negrao *et al.,* 2011; Gupta and Huang, 2014). These include signalling genes (Boonburapong and Buaboocha, 2007; Chen *et al.,* 2006; Martinez-Atienza *et al.*, 2007; Wan *et al.,* 2007), genes involved in ion homeostasis (Garciadeblas *et al.,* 2003; Horie *et al.,* 2007; Fukuda *et al.,* 2010) and in the synthesis of osmoprotective proteins (Wang *et al.,* 2007), as well as transcription factors (Liu *et al.,* 2007; Matsukura *et al.,* 2010) and genes involved in the rapid post-translational regulation of cell proteomes

(Martinez-Atienza *et al.,* 2007; Khan *et al.,* 2005).

Thus, present study was mainly focused on four selected salt responsive candidate genes and to study their expression in relation to up or down regulation in selected Sri Lankan rice varieties under salt stress condition, aiming at using them in future breeding purpose.

MATERIALS AND METHODS

Plant Material and Stress Treatment

The study was conducted at the laboratory of International Rice Research Institute (IRRI), Philippines. Two traditional salt tolerant local rice varieties, i.e. *Kuruwi Perunel* (IRGC Ac No: 8994) and *Godawe*e (IRGC Ac No 15653), one traditional salt susceptible local variety, *Suwandel* (IRGC Ac No 31552) obtained from IRRI and salt tolerant improved variety, *At354* collected from Rice Research and Development Institute (RRDI), Batalagoda, Sri Lanka were used for the present study based on the assessment of salt tolerance in our previous studies.

Surface sterilized seeds thoroughly rinsed with distilled water were placed on moistened filter papers in Petri dishes and incubated at 30 ºC for 48 h. Pre-germinated seeds were sown in holes on Styrofoam floats with a nylon net bottom suspended on trays filled with water. After 2 days, when seedlings were well established, water was replaced with Peter's nutrient solution (Perter's 1 g/L) (Scotts Peters (20-20-20) Professional water-soluble fertilizer) supplemented with $FeSO_4.7H_2O$ (300 mg/L)). Seedlings were grown inside the growth chamber under the controlled conditions of 12 h light with 29 °C temperature and 12 h dark with 21 °C temperature, 80% Relative humidity (RH) and the photoperiod of 200 µmol photons m^{-2} s^{-1} photo intensity at the IRRI. The pH of the nutrient solution was adjusted to 5.0 every other day by adding 1 M NaOH or 1 M HCl. After 4 days, nutrient solution was salinized upto 100 mM (EC 12 dS/m) by adding appropriate amount of analytical grade NaCl.

Sampling of Tissues

Shoot and root samples of each variety were harvested just before the salinization to serve as a control, 24 h after salinization and 72 h (3 days) after salinization. Pre-determined weight of shoot and root samples were harvested quickly into the eppendorf tubes with a hole and immersed in liquid nitrogen within less than 1 minute to minimize the expression of new mRNAs because of tissue wounding or detachment from the plant. After harvesting all the samples were stored in the -80 °C freezer until use for the RNA extractions.

Extraction of Total Plant RNA

Total RNA was isolated from frozen shoot and root samples together using TRIzol Reagent (Invitrogen, Carlsbad, USA) according to the manufactures instructions and Sambrook *et al.* (1989). RNA samples were treated with RNase free DNase 1 (Promega, USA) to remove genomic DNA

contaminations in the extracted RNA samples according to manufactures instructions. Quality and quantity of the RNA preparations were assessed using both 2% non-denaturing agarose gel electrophoresis and by NanoDrop 8000 UV spectrophotometer (Thermo Scientific, USA) with respect to the absorbance at 260 nm, 280 nm and 230 nm.

Designing of Candidate Gene Specific Primers for Semi-Quantitative Reverse Transcriptase - PCR (RT-PCR)

In present study, four candidate genes were selected based on the previous studies regarding salinity responsive candidate genes. Triosephosphate isomerise (TPI) housekeeping gene was taken as the reference gene for normalization of transcripts (Table 1). Candidate gene specific primers for RT- PCR was designed using Roche Universal Probe Library Assay design center with ProbeFinder software version 2.49 for rice, which is based on Primer3 software. The Coding sequences of the selected candidate genes were retrieved from the sequence databases of National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) and Rice genome annotation project (http:// rapdb.dna.affrc.go.jp/) and fed to Roche Universal Probe Library Assay design center as input sequences. Primers were designed according to the method of intron-spanning assay in order to eliminate the false positive signals from residual genomic DNA, with the default optimized settings of the software. Details of the designed RT-PCR primers for four candidate genes and TPI housekeeping gene are mentioned in the Table 2.

Table 1. Selected salt responsive candidate genes

Candidate gene	Locus ID	Ch[a].	Description of gene	References
OsNHX1	Os07g0666900	7	Intracellular NHX proteins are Na+, K+/H+ antiporters involved in K+ homeostasis (specially compartmentalization of Na+ and K+) NHX1- NHX isoform localized in tonoplast is essential for compartmentalization of Na+ into the vacuoles under salinity stress	Ahmadi *et al.,*2011; Gupta and Huang, 2014; Kumar *et al.,* 2013; Ismail *et al.,* 2007; Chen *et al.,* 2007; Fukuda *et al.,* 2010
OSTPS1	Os05g0518600	5	Trehalose-6-phospate synthase	Kumar *et al.,* 2013; Li *et al.,* 2011
OsMAPK5	Os03g0285800	3	Mitogen-activated protein kinase (MAPK)	Kumar *et al.,* 2013, Xiong and Yang, 2003

OsCDPK7	Os04g0584600	4	Ca^{2+} dependent protein kinases	Kumar *et al.,* 2013, Saijo *et al.,* 2000
OsTPI	Os01g0147900	1	Triosephosphate isomerise (housekeeping gene)	Maksup *et al.,* 2013

[a]*Chromosome*

Semi-Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

First-strand cDNA was synthesized from 4 µg of total RNA using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, USA) according to manufacturer's protocol. All the cDNA samples prepared from the *At354*, *Godawee*, *Kuruwi Perunel* and *Suwandel* harvested in 0 h (just before salinization), 24 h and 72 h after salinization were diluted to equal concentrations of 200 ng/ µl and amplified using selected 4 salt responsive candidate gene specific primers along with the *OsTPI* housekeeping gene (Table 2). The PCR mixture (20 µl) consisted of 1 µl of cDNA (200 ng/ µl), 2 µl of 10X $MgCl_2$ free PCR buffer, 1.2 µl of $MgCl_2$ (25 mM), 0.4 µl of dNTPs (2.5 mM of each dNTP), 0.4 µl of forward primer (10 µM), 0.4 µl of Reverse primer (10 µM) (Invitrogen, USA) and 0.16 µl of Taq DNA polymerase (5 U/µl) (iNtRON Biotechnology, Inc.). Final volume of the mixture was adjusted to 20 µl by adding sterile nano pure water. PCR amplification was performed using G-Storm Thermal Cycler (Model GS1, Gene Technologies, Essex, UK) and amplification profile consisted of initial denaturing at 94 °C for 2 minutes followed by 35 cycles of 15 seconds at 94 °C, 45 seconds at 55 °C seconds at 55 °C, 1 minute at 72 °C and final extraction cycle of 8 minutes at 72 °C.

Semi-quantitative RT-PCR experiment was conducted in duplicate for each primer in order to confirm the reproducibility of the specific expression profiles in each variety at different time intervals under salinity stress.

Assessment of Differential Expression of Candidate Genes

The PCR products (20 µl) were mixed with 2 µl of 6X loading buffer and separated on 1.5% agarose gel. After SYBR-Safe (Invitrogen, Carlsbad, USA) staining (5 µl of SYBR-Safe per 50 ml of nanopure water), gel was visualized under UV light using Alpha Innotech gel documentation system. Photographic images were obtained using the gel documentation system and differential level of expression in candidate genes was assessed based on the relative intensity of fragments. Intensity of fragments was measured using ImageJ [version 1.49] (Schneider *et al.,* 2012) software in terms of average pixel values. Estimated level of expression of each gene in terms of average pixel values in 4 varieties was plotted against the exposure

time to salinity, in order to make inferences regarding the differential expression of genes over the tested time period under salinity stress.

RESULTS

In present study, all of these gene specific RT-PCR primers designed according to intron- spanning assay were able to produce positive amplification with the expected size of product indicating the specificity and efficacy of all the designed RT- PCR primers (Table 2; Figure 1). In addition, present results revealed the differential level of expression with respect to four selected salt responsive candidate genes, in 4 varieties under salinity stress and non-saline conditions (Figure 1 and Figure 2).

Expression of OsTPI Housekeeping Gene

According to the semi-quantitative RT-PCR analysis, all 4 varieties *viz*., *At354*, *Kuruwi Perunel*, *Godawee* and *Suwandel* exhibited equal level of expression under salinity stress and non-saline conditions with respect to the *OsTPI* housekeeping gene exhibiting constitutive expression of *OSTPI* housekeeping gene irrespective of the different genetic background, developmental and environmental conditions (Figure 1).

Expression of OsNHX1 Gene

With respect to the expression of *OsNHX1* gene, *At354* and *Kuruwi Perunel* exhibited relatively low level of gene expression under non-stress condition compared to *Godawee* and *Suwandel* (Figure 1 and Figure 2). After 24 h of salinity stress, *OsNHX1* gene expression was relatively up-regulated in *At354* and *Kuruwi Perunel* (Figure 1 and Figure 2). At this time compared to *Kuruwi Perunel*, *At354* exhibited relatively higher number transcript abundance. And also, 24 h after salinity stress, *OsNHX1* gene expression in *Godawee* was slightly up-regulated compared to non-stress condition, whereas in *Suwandel* gene expression was drastically down-regulated (Figure 1 and Figure 2). With the extension of salinity stress for 72 h, it was noted that in At354, gene expression was again declined to the level comparable to the non-stress condition. In contrast, *Kuruwi Perunel* exhibited comparatively higher abundance of transcripts at 72 h after salinity stress condition (Figure 1 and Figure 2). At this time, *Godawee* again showed the same amount of transcripts of *OsNHX1* gene comparable to non-stress condition while *OsNHX1* gene expression was not observed in *Suwandel* (Figure 1 and Figure 2).

Expression of OsCDPK7 gene

Relatively low level of expression of the *OsCDPK7* gene was also observed in *At354* and *Kuruwi Perunel* under non-saline conditions whereas *Godawee* and *Suwandel* exhibited relatively higher level of gene expression under non-saline condition (Figure 1 and Figure 2). After 24 h of salinity treatment, it was noted the up-regulation of gene expression in *At354*, *Kuruwi Perunel* and *Godawee* (Figure 1 and Figure 2). Of them highest amount of

transcript abundance was observed in *Godawee*. However, expression of *OsCDPK7* was drastically down regulated compared to non-saline condition in *Suwandel* (Figure 1 and Figure 2). With the extension of salinity stress until 72 h, expression of *OsCDPK7* was comparatively declined both in *At354* and *Godawee*. However, compared to *At354*, *Godawee* exhibited relatively higher transcript abundance (Figure 1 and Figure 2). In contrast to that, *Suwandel* exhibited relatively higher up-regulation of gene expression while level of gene expression was slightly up-regulated in *Kuruwi Perunel* compared to 24 h after salinity stress (Figure 1 and Figure 2).

Expression of OsMAPK5 gene

According to the expression of *OsMAPK5* gene, relatively higher abundance of transcripts of *OsMAPK5* was detected in *Godawee* and *Suwandel* compared to *At354* and *Kuruwi Perunel* under non-saline conditions (Figure 1 and Figure 2). Expression of gene was up-regulated after 24 h of salinity stress in *At354*, *Kuruwi Perunel* and *Godawee*. Of them, highest level of transcripts of *OsMAPK5* gene was detected in *Godawee* (Figure 1 and Figure 2). However, expression of *OsMAPK5* gene was drastically down-regulated in *Suwandel* compared non-saline condition (Figure 1 and Figure 2). After 72 h of sanity stress, level of gene expression was down-regulated to the level comparable to non-saline condition in *At354* (Figure 1 and Figure 2). However, *Kuruwi Perunel* exhibited progressively up-regulation of expression in *OsMAPK5* gene with the extension of salinity stress period (Figure 1 and Figure 2). In contrast to that, transcript abundance of *OsMAPK5* gene was slightly lowered in *Godawee* after 72 h of salinity treatment compared to 24 h after salinity treatment. In addition, expression of *OsMAPK5* gene was progressively down-regulated in *Suwandel* with the extension of time period under saline condition (Figure 1 and Figure 2).

Expression of OsTPS1 gene

Under non-saline condition, *OsTPS1* gene expression was not observed in *At354* and *Kuruwi Perunel* whereas gene expression was detected in *Godawee* and *Suwandel* (Figure 1 and Figure 2). Compared to *Suwande*l, *Godawee* exhibited relatively high amount of transcripts of *OsTPS1* gene (Figure 1 and Figure 2). After 24 h of salinity stress, up-regulation of *OsTPS1* gene expression was detected in both *At354* and *Godawee* whereas expression was not detected in *Suwandel* and *Kuruwi Perunel* (Figure 1 and Figure 2). However, level of of gene expression was relatively high in Godawee compared to the expression in At354. It was noted that after 72 h of salinity stress condition, again *OsTPS1* gene was not expressed in At354 while expression of *OsTPS1* gene was observed in Kuruwi Perunel and Suwandel (Figure 1 and Figure 2). However, compared to Kuruwi Perunel, Suwandel exhibited relatively low amount of transcripts of *OsTPS1* gene. At this time, level of gene expression in Godawee was slightly lowered compared to the gene expression level at 24 h after salinity stress (Figure 1 and Figure 2).

Table 2. Details of designed candidate gene specific RT-PCR primers

Gene	Primer F 5'-3' (Left)	Primer R 5'3' (Right)	T_m ºC	Size of amplicon (nt)	spanned intron size (nt)	Total if genomic DNA (nt)	Reference Gene sequence
OsNHX1	CTGTCGTTCTTTTTAGCACTATGG	GGTGACAGGATGGCCTGA	55	89	571	660	*O.sativa* Japonica Group OsNHX1 mRNA, (complete cds.)
OsTPS1	GTTTCAAGCGAGCATTGGAG	TCGGTCAACACCAAGCATTA	55	98	659	757	*O.sativa* Indica Group trehalose-6-phosphate synthase 1 (TPS1) mRNA, complete cds (gi|328864180|gb|HM050424.1)|
OsMAPK5	CCGCTGCAGAGAATCACAG	TGCTCGAAGTCGAAGGAGA	55	113	524	637	*O. sativa* MAP kinase MAPK5a (MAPK5) mRNA, complete cds, alternatively spliced.(AF479883.1|AF479883:EMBL)
OsCDPK7	TCTCGCTCAAGGCCATAGAT	CAACAACACTTCTGGAGCTACG	55	107	523	630	*O. sativa* Japonica Group mRNA for OsCDPK7, complete cds.(AB042550.1|AB042550:EMBL)
OsTPI	TGC TGC ACA AAC AAA AGC A	CTT CTT GTG CTT GAT CTG GTG T	55	122	480	602	*O. sativa* Japonica Group Os01g0147900 (Os01g0147900) mRNA, complete cds (ref|NM_001048551.1|)

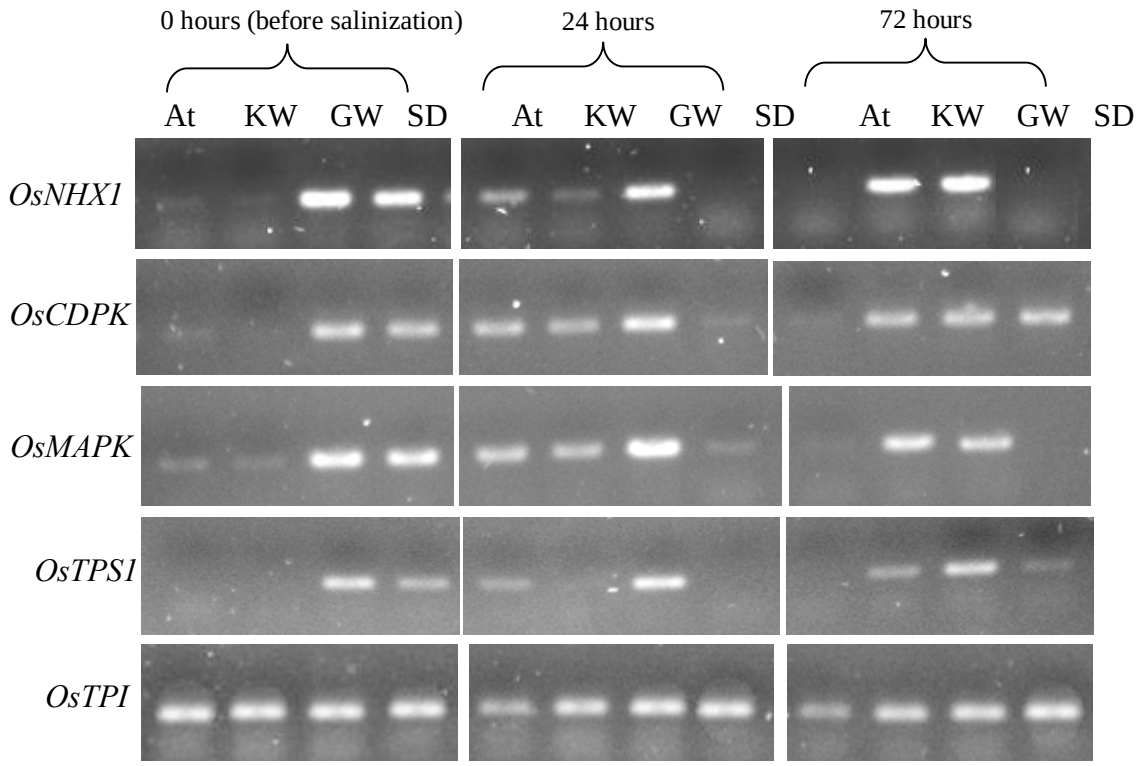

Figure 1. 1.5 % agarose gel electrophoresis of amplified cDNA from *At354* (At), *Kuruwi perunel* (KW), *Godawee* (GW) and *Suwandel* (SD) in 0 h (before salinization), 24 h after salinization and 72 h after salinization with *OsNHX1*, *OsCDPK7, OsMAPK5*, *OsTPS1* and *OsTPI* (housekeeping gene) gene specific primers.

DISCUSSION

Even though rice is considered to be generally salt sensitive, there is a wide spectrum of genetic variation for the salt tolerant mechanisms at critical stages i.e. early seedling and reproductive stages, in the rice gene pool (Yeo and Flowers, 1986; Moradi *et al.*, 2003). Therefore, identification and functional characterization of salt responsive candidate genes would be important to understand the salinity tolerant mechanisms for the genetic improvement of rice tolerance to salinity (Wu *et al.*, 2005).

There are two major phases of salinity stress as osmotic stress and ionic stress. Plants usually achieve osmotic homeostasis during several hours or at least within the first day following salt stress (Munns, 2002). The ionic stress component of salinity stress, as a second phase, becomes gradually more severe, typically beginning after 1–3 days of salinity stress (NaCl application) despite rapid influx of Na^+ ions and transport to the shoots. This is because the concentration of Na^+ must reach a certain toxic level in cell protoplasts of shoots and this process requires some days (Munns, 2002; Munns, 2005; Roshandel

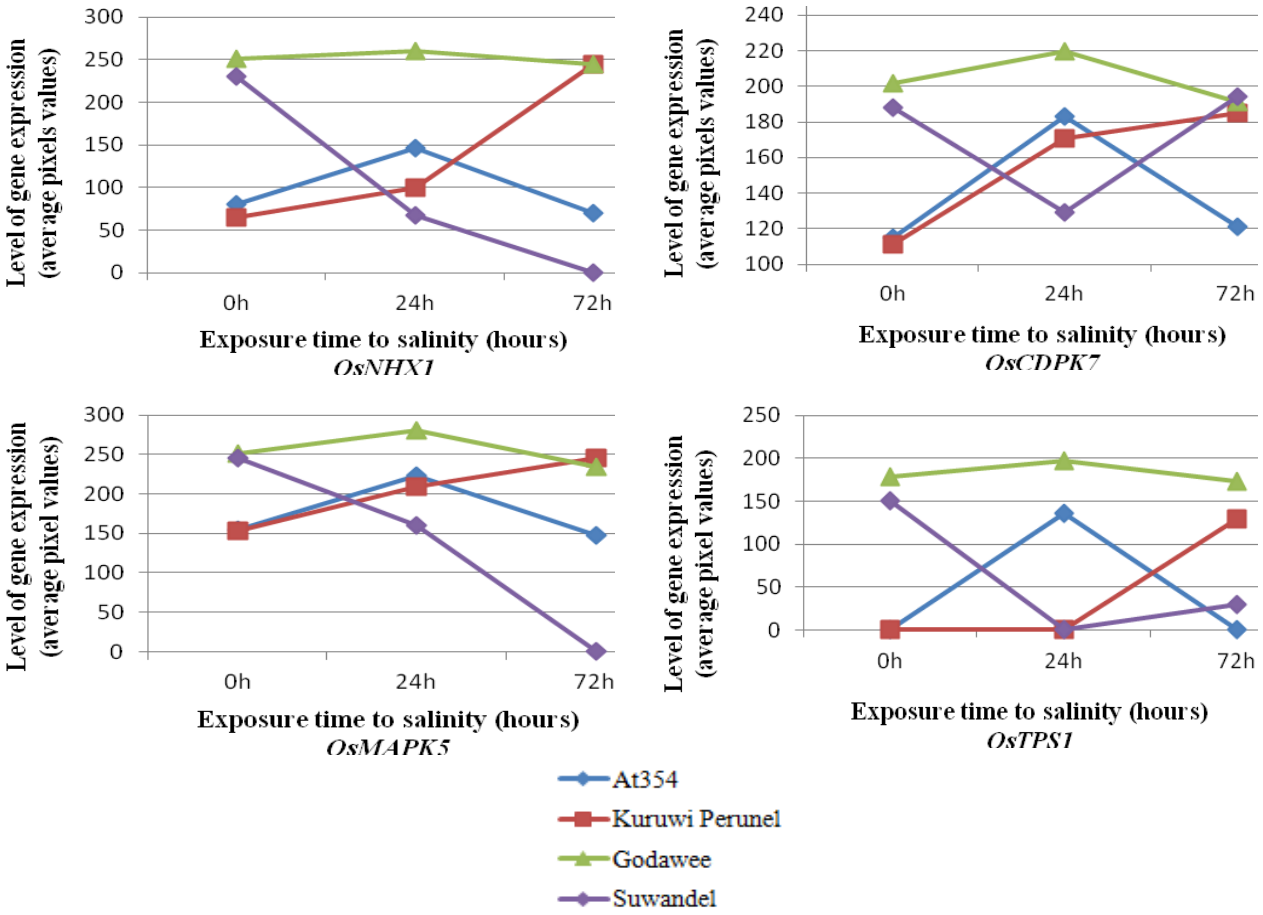

Figure 2. Differential expression of *OsNHX1, OsCDPK7, OsMAPK5* and *OsTPS1* candidate genes in *At354*, *Kuruwi Perunel*, *Godawee* and *Suwande*

and Flowers, 2009). After 24–72 h of salinity stress, the concentration of Na+ in cytoplasm may be close to toxic level and, therefore, plants react to the ionic stress progressively. Na^+ exclusion, Na^+ compartmentalization and Na^+ reabsorption are common mechanisms of tolerance to the ionic component of salt stress, where one or all of these mechanisms are employed by tolerant plant types (Munns and Tester, 2008). Genes showing altered expression are primarily responsible for osmotic adjustment and osmolyte production. The second, ionic phase of salt stress occurs slightly later, with minimum overlap of changes in gene expression between genes associated with the ionic and osmotic phases of salt stress. There are a large number of genes identified as being associated with the ionic phase, many of which are involved in responses to toxic cellular concentrations of Na^+ ions (Shavrukov, 2013).

In present study, a few of the salt responsive genes were selected with the evidences of involving in both of these stress components of salinity stress.

Accordingly, *OsNHX1 OsTPS1*, *OsMAPK5* and *OsCDPK7* candidate genes were selected to study the differential expression of these genes in four local rice varieties viz., *At354* (salinity tolerant improved variety), *Godawee, Kuruwi Perunel* (salinity tolerant traditional varieties), and *Suwandel* (salinity susceptible variety) at the whole plant level under salinity stress with the aim of understanding the expression dynamics under different genetic backgrounds exhibiting differential pattern of salinity tolerant mechanisms. In order to assess the candidate gene specific expression, gene specific RT-PCR primers were designed according to intron-spanning assay as the PCR products derived from the cDNA could be easily distinguished from the larger intron-containing products derived from contaminating genomic DNA. Resulted positive amplification in all of these designed primers with the expected size of PCR product derived from cDNA indicated the success of designing gene specific RT-PCR primers for the assessment of gene expression using semi-quantitative PCR. Present results revealed the constitutive expression of *OsTPI* housekeeping gene in all four varieties irrespective of the different genetic background, developmental and environmental conditions indicating the successful synthesis of cDNA in all the samples harvested from four varieties at three different time intervals (before salinization, 24 h after salinization and 72 h after salinization).

According to previous studies it was reported that *OsNHX1 OsTPS1*, *OsMAPK5* and *OsCDPK7* genes exhibit different functional involvement in mediating salinity tolerant mechanisms under salinity stress (Ahmadi *et al.*,2011; Gupta and Huang, 2014; Kumar *et al.*,2013; Ismail *et al.*, 2007; Chen *et al.*, 2007; Xiong and Yang, 2003; Saijo *et al.*,2000; Maksup *et al.*, 2013).

OsNHX1 gene is one of the intracellular NHX proteins which are Na^+, K^+/H^+ antiporters involved in K+ homeostasis, endosomal pH regulation, and salt tolerance (Kumar *et al.*, 2013). In rice, 4 vacuolar Na^+/H^+ antiporters (*OsNHX1-4*) and one endosomal Na^+/H^+ antiporter (*OsNHX5*) have been reported (Bassil *et al.*, 2012). Of them, Chen *et al.*, (2007) reported that over-expression of *OsNHX1* in rice and in maize has improved salt tolerance by enhancing the compartmentalization of Na^+ into the vacuoles.

Calcium-dependent protein kinases (CDPKs) play important roles in regulating downstream components in calcium signalling pathways. (Asano *et al.*, 2011). Calcium plays an important role as a messenger in various signal transduction pathways (Trewavas and Malho 1998; Sanders *et al.*, 1999; Berridge *et al.*, 2000; Sanders *et al.*, 2002). It has been well established that high salt stress rapidly leads to cytosolic Ca^{2+} spiking. This event spontaneously initiates the stress signalling pathways for stress tolerance via stimulating various Ca^{2+} binding proteins including calcineurin B-like protein-CBL-interacting protein kinase (CBL-CIPKs),

Calcium-dependent protein kinases (CDPKs) and calmodulins (Mahajan *et al.*, 2008; Kader and Lindberg, 2010). In rice, the CDPKs constitute a large multigene family consisting of 29 genes, but the biological functions and functional divergence or redundancy of most of these genes remain unclear (Asano *et al.*, 2011). With regard to that Saijo *et al.* (2000) reported transgenic rice plants over-expressing *OsCDPK7* showed enhanced tolerance to cold, salt and drought stresses.

In addition, Mitogen activated protein kinase (MAPK) cascades too play a crucial role in salt stress signal transduction pathways in rice (Kumar *et al.*, 2013). This phosphorylation cascade typically consists of three functionally interlinked protein kinases: a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK), and a MAP kinase (MAPK). In this phosphorylation module, a MAPKKK phosphorylates and activates a particular MAPKK, which in turn phosphorylates and activates a MAPK. Activated MAPK often is imported into the nucleus, where it phosphorylates and activates specific downstream signalling components such as transcription factors (Khokhlatchev *et al.*, 1998). Several salt-inducible MAPKs have been reported in rice. Biotic and abiotic stress inducible *OsMAPK5* has been cloned and over-expressed in rice which subsequently exhibited increased tolerance to salt, drought and cold stresses with increased kinase activity (Xiong and Yang, 2003).

The osmoprotectants (organic solutes) are one of the most important factors to protect plant cells from dehydration and salinity (Rontein *et al.*, 2002; Yamaguchi-Shinozaki *et al.*, 2002). The organic solutes protect plants from abiotic stress by osmotic adjustment, detoxification of reactive oxygen species (ROS) and stabilization of the quaternary structure of proteins (Bohnert and Jensen, 1996). The most important plant osmoprotectants are proline, glycine betaine, trehalose and myo-inositol (Kumar *et al.*, 2013). In plants, the synthesis of this sugar occurs normally by the formation of the trehalose-6-phosphate (T6P) from the UDP-glucose and glucose-6-phosphate, a reaction catalyzed by the trehalose 6-phosphate synthase (TPS). Li *et al.* (2011) have shown that over-expression of *OsTPS1* gene encoding trehalose-6-phosphate synthase in rice improved the tolerance of rice to high salinity and other abiotic stresses. Over-expression of this gene in rice is associated with increased level of trehalose and proline along with upregulation of some of the stress inducible genes including *WSI18*, *RAB16C*, *HSP70* and *ELIP* (Turan *et al.*, 2012).

In this experiment as the amplification was repeated two times it was able to confirm the reproducibility of the specific expression profiles resulted in each variety at different time intervals under salinity stress. Under non-saline condition relatively higher expression of these genes was detected in *Godawee* and *Suwandel* compared to *At354* and *Kuruwi Perunel*. Out of four genes, expression of *OsNHX1*, *OsCDPK7* and *OsMAPK5* genes were up-regulated in *At354*, *Kuruwi Perunel* and

Godawee at 24 h after salinity stress. This indicates the comparative functional activation of these 3 genes under salinity stress mediating the salt tolerant mechanisms in *At354*, *Godawee* and *Kuruwi Perunel*, salinity tolerant varieties. Chen *et al.* (2007), Saijo *et al.* (2000), Kawasaki *et al.* (2001) and Xiong and Yang (2003) have reported that over-expression of these three genes could enhance the salinity tolerance showing an agreement with the present results. With the extension of time under salinity stress up to 72 h, continuous expression of these three genes was observed in *Kuruwi Perunel* and *Godawee*, whereas expression was comparatively down-regulated in *At354* to the approximately same level under non-stress condition. With this observed differential expression behavior in *At354* it could be speculated that *At354* might be adapted to stress relatively early compared to *Godawee* and *Kuruwi Perunel*, where expression of genes are less pronounced with the adaptation of plant. Kawasaki *et al.* (2001), was also suggested that declining of amount of transcripts with the time after up-regulation of a gene might be due to acquiring of early adaptation to stress in which abundance of transcript returned to the level under non- stress condition. On the other hand, Saijo *et al.* (2000), and Hirt, (1999) speculated that with respect to the regulation of expression in *OsCDPK7* and *OsMAPK5* there could be another post-translational mechanism(s) of regulating kinase activity of these genes in plant cell under salinity stress. One such mechanism is the degradation of activated *OsCDPK7* and *OsMAPK5* proteins immediately after it has transduced signal(s) and transcriptional up-regulation of the gene is to compensate for the loss of the *OsCDPK7* and *OsMAPK5* protein due to turnover of the relevant protein under stress. Therefore, it could be suggested that down-regulation of expression of *OsCDPK7* and *OsMAPK5* genes in *At354* at 72 h after salinity stress may be due to the comparatively less degradation rate of *OsCDPK7* and *OsMAPK5* proteins in *At354*.

In *Suwandel*, salinity susceptible variety, eventhough *OsNHX1* and *OsMAPK5* genes were expressed under non-saline condition, expression of these two genes were drastically down-regulated under salinity stress. This fact indicates the hindering of the expression of particular genes in *Suwandel* under salinity stress. However, expression of *OsCDPK7* was relatively down-regulated 24 after salinity stress and again it was noted the up-regulation of gene 72 h after salinity stress. Kawasaki *et al.* (2001) was also reported relative difference in the expression of *OsCDPK7* with the time under salinity stress in contrasting rice genotypes of *Pokkali* (salinity tolerant) and *IR29* (salinity susceptible). Accordingly, it was noted that up-regulation of expression in *OsCDPK7* gene in *Pokkali* during initial periods of salt stress specially 1 hour after salt stress but was not regulated significantly in *IR29*. Further, Kawasaki *et al.* (2001) reported that at the 3 h and 6 h time points, *OsCDPK* was up-regulated in both lines, suggesting a difference between the two lines in signal transduction at the early stages of stress and delay in the processing of signals could lead

to the ineffective response of *IR29* to salt stress. According to these previous evidences, it could be suggested that *Suwandel*, salinity susceptible variety of this study, exhibited delayed expression of *OsCDPK7* under salinity stress as in *IR 29*.

Similar to the expressional behaviour of *OsNHX1*, *OsCDPK7* and *OsMAPK5*, *OsTPS1* also exhibited comparative up-regulation of expression in *At354* and *Godawee* at 24 h after salinity stress indicating comparative functional activation of *OsTPS1* gene under salinity stress. In contrast, progressive down-regulation of gene expression throughout the studied time period under salinity stress, compared to non- stress condition in *Suwandel*, indicates the significant suppression of normal cellular functioning of *OsTPS1* gene in *Suwande*l under salinity stress. At the time of 72 h after salinity stress transcript abundance of *OsTPS1* gene in *At354* and *Godawee* were comparable to their non-stress condition suggesting both tolerant varieties might have early adaptation response to salinity stress with respect to the *OsTPS1* gene. In contrast to the regulation pattern of *OsTPS1* gene in *At354* and *Godawee*, *OsTPS1* gene was up-regulated in *Kuruwi Perunel* with a certain delay of time period i.e. 72 h after salinity stress. Due to delay in response with respect to *OSTPS1* gene, it could be suggested that *Kuruwi Perunel* may have some other osmoprotectants other than trehalose conferring similar functional mechanism under salinity stress.

In the present study, according to an overall expression of four selected salt responsive genes in the four varieties at different time period at whole plant level, exhibited differential level of expression over the tested time period indicating their expression dynamics with respect to these genes under non-saline and salinity stress condition. However, it should be noted that it was difficult to draw more precise inferences regarding the relative expression of genes using semi-quantitative RT-PCR analysis. Therefore, it is required to conduct further experiments utilizing more advanced technologies such as microarray, RNAseq, quantitative RT-PCR to reveal differential expression of salinity responsive genes in different rice varieties conferring tolerance to salinity in varying limits.

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