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RESEARCH ARTICLE

Identification of differentially-expressed genes in response to salt stress in the salt-tolerant Sri Lankan rice variety At354

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Abstract: Rice is highly sensitive to salt stress, an expanding abiotic stress factor that limits rice yield improvement. Development of salt tolerant rice varieties based on molecular breeding methods requires identification of genes responsible for various mechanisms and responses that contribute to salt tolerance. The objective of the present work was to identify genes, which are differentially-expressed in response to salt stress in the salt-tolerant Sri Lankan rice variety, At354. Two cDNA libraries were constructed from mRNA of shoot samples of salt-stressed (100 mM NaCl) At354 at Phase I and Phase II (24 hours and 10 days, respectively after increasing salt stress up to 100 mM) of salt stress development. A total of 3192 and 960 cDNA clones respectively were screened from Phase I and II libraries. Differential hybridization of the cDNA clones with probes prepared from salt-stressed and unstressed At354 shoot samples enabled identification of up and down-regulated genes in response to salt stress in Phase I and Phase II. The identified, differentially-expressed cDNA clones were re-confirmed by another round of differential hybridization and through Northern hybridization by the RNA dot blot method. Relative reverse transcription polymerase chain reaction (RT-PCR) was performed to compare the expression levels of selected differentially-expressed genes. Sequencing and subsequent homology search in databases identified 14 up-regulated genes and 17 down-regulated genes during Phase I in At354. Similarly, 11 up-regulated genes and 2 down-regulated genes were identified during Phase II. Possible functions of the identified, differentially-expressed genes in conferring salt tolerance in At354 is discussed extensively. These genes may enable exploration of newer avenues for engineering salt tolerance in rice.

Keywords: Differential hybridization, gene expression, *Oryza sativa*, salt stress, salt tolerant genes.

INTRODUCTION

Rice is one of the most sensitive crops to salt stress (Munns & Tester, 2008). Salinity in crop lands has been observed to be spreading because of poor quality of irrigation water and global warming-induced sea level rise. As reclamation of salt affected agricultural lands is difficult and expensive, breeding of salt tolerant rice varieties becomes the only option available to counter increasing salinity. While development of salt tolerant crop varieties is possible through conventional plant breeding strategies (Schubert *et al.*, 2009), molecular based plant breeding strategies offer a more focused, efficient and effective approach to develop salt tolerant varieties (Ashraf & Akram, 2009). Molecular based breeding strategies could be especially useful in rice, where the entire genome has been sequenced. Such strategies require identification of physiological mechanisms that confer salt tolerance and the putative genes that are responsible for such mechanisms of tolerance (Munns & Tester, 2008; Rajendran *et al.*, 2009).

The two-phase growth response of plants to salinity as developed by Munns (2002) forms the physiological basis of a plants' response to salt stress. The two-phase model postulates that plants respond in two sequential phases to increasing salinity. The first phase of salt stress development (Phase I) occurs immediately upon exposure to a salt ion concentration above a threshold ECe of 4 dS m^{-1} (1.9 dS m^{-1} for rice), which is equivalent to 40 mM NaCl (19 mM NaCl for rice), for most plant species (Munns & Tester, 2008). During Phase I, excess salt ions in the external soil solution decreases its osmotic potential thereby decreasing the ability of plant roots to absorb water. In this phase of 'osmotic stress', plants show the typical responses to drought stress. With increasing salt concentrations in the external soil solution and/or with increasing duration spent in a saline soil, salt ion concentrations in the meristematic tissues increase

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beyond the capacity of cells to compartmentalize the excess into the vacuole. As a result, the plant enters the second phase of salt stress development (Phase II) in which salt concentration increases in the cytoplasm, where all the important physiological processes take place, to toxic levels resulting in gradual injury and death of tissue.

The two-phase model forms the basis for identification of mechanisms that are responsible for salt tolerance. Accordingly, three major mechanisms can be identified. Salt tolerance during Phase I can be achieved primarily by tolerance to osmotic stress (Mechanism No. 1), which involves maintenance of shoot and root growth even under salt stress. Hence, a tolerant genotype may show a lower growth reduction than a sensitive genotype. Tolerance during Phase II may be achieved by either salt exclusion from photosynthesizing leaves (Mechanism No. 2) and/or by increasing the tissue tolerance to excess salt (Mechanism No. 3). As sodium chloride is the most abundant and soluble salt that is released from weathering of rocks (Szabolcs, 1989), salt-stressed plants have to develop tolerance mechanisms primarily against Na^+ and Cl^- ions. Out of these two ions, Na^+ toxicity often develops earlier than Cl^- toxicity (Munns & Tester, 2008). Consequently, the majority of research on tolerance to salt ion toxicity has focused on mechanisms of exclusion of and tissue tolerance to Na^+.

At354 is the popular salt tolerant improved rice variety, developed through incorporation of salt tolerant genes from 'Pokkali', a traditional salt tolerant rice variety in Sri Lanka. However, genes that are responsible for salt tolerance in At354 have not been identified.

Determination of the gene expression patterns under salt stress in identified salt tolerant varieties is one approach to identify putative genes of salt tolerance (Kawasaki *et al.*, 2001). Altering the expression of identified putative genes of salt tolerance has been shown to improve tolerance (Zhang *et al.*, 1999). Thus, the objective of the present work was to identify genes, which are differentially-expressed in response to salt stress in the salt-tolerant Sri Lankan rice variety At354.

METHODS AND MATERIALS

Plant culture, salt treatments and sample collection

Rice variety At354 was grown hydroponically in a rain-sheltered planthouse at the Agricultural Biotechnology Centre, University of Peradeniya, Peradeniya. A specific nutrient solution for hydroponic culture of rice (Yoshida *et al.*, 1972) was used to grow the plants. Composition of the full-strength nutrient solution was: (in gL^{-1}) NH_4NO_3, 91.4; $NaH_2PO_2.2H_2O$, 40.3; K_2SO_4, 71.4; $MgSO_4.7H_2O$, 324; $CaCl_2$, 88.6; $MnCl_2.4H_2O$, 1.5; $(NH_4)_6.Mo_7O_{24}.4H_2O$, 0.074; H_3BO_3, $ZnSO_4.7H_2O$, 0.035; $CuSO_4.5H_2O$, 0.031; $FeCl_3.6H_2O$, 7.7; Citric acid, 11.9. Micronutrient salts were dissolved separately and combined with 50 mL conc. H_2SO_4 and the volume was adjusted to 1 L by distilled water (final pH = 5.00). The seeds were germinated on moist filter papers and transferred to ¼ strength nutrient solutions in 3 L pots 1 wk after germination. Strength of the nutrient solution was increased in 25 % steps up to full strength at 2 d intervals. Salt treatments started a week after giving the full strength nutrient solution. The salt-stress treatment started at 25 mM NaCl and was increased up to a final concentration of 100 mM in 25 mM steps at 2 d intervals. The unstressed control treatment was maintained at 1 mM NaCl.

Plants were harvested for Phases I and II at 24 h and 10 d, respectively after increasing the salinity up to 100 mM NaCl as determined by a separate experiment (De Costa *et al.*, 2012). Immediately after harvesting, the shoots and roots were separated, snap frozen and ground in liquid nitrogen to be used for extraction of RNA.

Extraction of total RNA and mRNA from rice shoots and construction of cDNA libraries

RNA was extracted from shoot tissues of both Phase I and II by modifying the method of Suzuki *et al.* (2001) with Trizol method (Invitrogen, USA). Quality and integrity of the extracted RNA samples were determined spectrophotometrically and by running in a denaturing agarose gel. Samples of mRNA were extracted from the total RNA samples isolated from the shoot tissues at Phase I and II by the paramagnetic particle technique using Dynal beads (Dynal Biotech ASA, Norway) according to the manufacturer's instructions. Two cDNA libraries were prepared, one each from mRNA of salt treated shoot tissues harvested at Phase I (OsPHI) and Phase II (OsPHII). The cDNA library was constructed using CloneMiner™ cDNA Library Construction kit (Catalogue No. 18249-029, Invitrogen, USA) based on Gateway® Technology according to the manufacturer's instructions. Individual cDNA clones were designated with code numbers and stored in LB medium supplemented with kanamycin (50 µg/mL).

Detection of salt-responsive clones in OsPHI and OsPHII libraries by differential-hybridization

A total of 3192 cDNA clones of OsPHI library and 960 cDNA clones of OsPHII were arrayed separately onto sterilized nylon membranes (Hybond N^+, Amersham

Pharmacia, USA). Membranes with well grown colonies were lysed, neutralized, washed and fixed to be used for hybridization. Probes for hybridization were prepared using total RNA extracted from salt treated rice shoot samples (treated probe) and shoot tissues of non-salt treated plants (control probe) collected at Phase I and II. Probes were labelled with digoxygenin according to the instructions of Roche, Germany (Cat. No. 11093657910). The cDNA colonies of OsPHI and OsPHII libraries fixed on membranes were subjected to differential hybridization separately, according to the method described by the manufacturer (Cat. No. 11093657910, Roche, Germany).

Briefly, and with reference to membranes containing colonies of OsPHI library, the initial hybridization was done with the control probe. The colonies with positive signals were detected on X-ray films by chemeluminescence and according to the instructions of Roche, Germany (Cat. No.11093657910). After the positive clones were recorded by their designation codes, the control probe on the membrane was stripped off and the same membrane was subjected to hybridization with the treated probes. Once again, the positive clones for treated probes (i.e. clones giving higher signal intensity) were selected by their designation codes upon detection by chemiluminescence. Based on the signal intensity given on X-ray film, cDNA clones which were having up-regulated and down-regulated genes under salt stress were detected as described by Xiong *et al.* (2001). Briefly, a cDNA clone, which is having an up-regulated gene in response to salt stress would show higher signal intensity when hybridized with the treated probe than the signal intensity given by the control probe. In contrast, cDNA clones, which are having down-regulated genes in response to salt stress would show lower signal intensity to the treated probe relative to the control probe.

The same differential hybridization procedure was employed for the membrane blots having cDNA clones of OsPHII library to detect the clones having up - and down-regulated genes at Phase II of salt stress. The selected cDNA clones with up - and down-regulated genes at Phases I and II were reconfirmed with another round of differential hybridization, conducted only for those selected clones.

Identification of candidate genes in cDNA clones with up- and down-regulated genes

For identification of putative genes, which are up- or down-regulated due to salt stress at Phases I and II, sequencing and subsequent homology search were conducted. To this end, plasmid extraction was done by alkaline SDS method (Sambrook *et al*., 1989) from selected cDNA clones by differential hybridization. The plasmid pDONR^{TM} 222 harboured by the cDNA clones contain cDNA fragments of different genes expressed as up- or down-regulated genes under salt stress. Plasmid extraction was done for all the selected cDNA clones of both OsPHI and OsPHII libraries. The cDNA fragments harboured in extracted plasmids were amplified by M13 universal primers (M13F- 5'GTAAAACGACGGCCAG3' and M13R-5'CAGGAAACAGCTATGAC3') for 30 repeated cycles having the following PCR reaction conditions: denaturing step at 94 ^{o}C for 30 s; annealing step at 50 ^{o}C for 2 min and extension step at 72 ^{o}C for 30 s. The PCR products obtained were subjected to DNA sequencing at GeneTech, Sri Lanka. DNA sequence information obtained for each PCR product was subjected to homology search using available DNA and protein databases (i.e. BLASTN and BLASTP of NCBI, respectively).

Confirmation of differential expression of identified candidate genes of OsPHI and OsPHII libraries by dot blot analysis

RNA dot blot method

RNA dot blot method was conducted as described by Kafatos *et al.* (1979) and Rapley (2000). Briefly, a piece of Hybond N^{+} membrane was soaked in DEPC treated water for 1 min. The membrane was transferred to a solution of 20 x SSC, soaked for 2 min and allowed to air dry for 20 min. Equal concentrations of total RNA samples extracted separately from salt-treated and control plants were spotted on to the dried membrane, side by side. The membranes were air-dried for 30 min, covered with two Whatman filter papers and oven-baked for 2 h at 80 ^{o}C. The membranes were stored at -20 ^{o}C, under dark conditions until they were used for Northern hybridization.

Probes needed for RNA dot blot hybridization were prepared as follows: several cDNA clones carrying the identified up- and down-regulated genes at both phases were subjected to PCR amplification using standard M13 primers. The amplified PCR products of each selected cDNA clone were labelled with Dig DNA labelling and detection kit as instructed by the manufacturer (Roche, Germany, Cat. No.11093657910). As a reference gene for comparison of gene expression, α-tubulin gene (i.e. a housekeeping gene) was amplified using total RNA extracted from shoot tissues of salt-treated and non salt-treated plants using the primer pair 5' TACCGTGCCCTTACTGTTCC3' and 5'CGGTGGAATGTCACAGACAC3'. The PCR

programme consisted of 30 cycles, each having a denaturing step of 94 °C for 30 s, an annealing step of 50 °C for 30 s and an extension step of 72 °C for 30 s. Independent hybridization procedures were conducted for each probe prepared from each selected cDNA clone (up- or down-regulated). Positively-hybridized clones were detected by the colorimetric method using Dig detection kit (Roche, Germany, Cat. No.11093657910). Intensity of signals produced on RNA blots of salt stressed and control plants was quantified by scanning the Northern blots and analyzing the signal intensity using the UN-SCAN-IT software, which converts scanned images into data (Silk Scientific Inc, USA). Relative signal intensity values were calculated by comparing the intensities of salt treated samples with control (i.e. non salt treated) samples according to the method described by Wilen *et al.* (1993).

Comparative expression analysis of some selected genes of OsPHI library during Phase I and Phase II by relative RT-PCR

Relative RT-PCR, as suggested by Gause and Adamovicz (1995) was performed to compare the expression levels of selected genes of salt tolerance of OsPHI library during Phases I and II of salt stress as follows:

Total RNA was extracted from shoots of salt stressed and control plants harvested at the two phases, and genomic DNA was eliminated by treating with DNase 1 (Promega, USA). DNase I treated RNA samples were subjected to cDNA synthesis by SuperScript™ First-Strand synthesis system (Invitrogen, USA) as recommended by the manufacturer. Synthesized cDNA from total RNA samples extracted from salt stressed and unstressed control plants harvested at Phases I and II were quantified using Biospec–Nano Spectrophotometer (Shimadzu, Japan) and samples having the same concentration were taken to perform relative RT-PCR.

RT-PCR reaction was performed with gene specific oligonucleotide primers designed for three up-regulated genes identified from Phase I of salt treated plants. Designation codes of cDNA clones carrying those up-regulated genes of Phase I library (OsPHI) were OsPHI-30, OsPHI-147 and OsPHI-928 and the cDNA fragments carried by those clones were also designated by the same codes. Based on the DNA sequence information available for those three cDNA clones, the following sequence specific primers were designed for RT-PCR amplifications:OSPH1-30F -5'-GGCTCCTTCCTCCACTACC-3' / OSPH1-30R - 5'-CCAAAGCTTCACCCTCCAC-3', OSPH1-147F-5'-GGAGCTTTGTGTTGTTGTC-3'OSPH1-147R-5'-CTTAACTGTAATATCCTGACG-3', OSPH1-147F- 5'-GTTGGATTATATGTTCGTTC-3'/ OSPH1-147R- 5'-CATTTGTAGAGTTGTACAAC-3'.

RT-PCR was performed with cDNA of salt stressed and unstressed plants obtained at Phases I and II, targeting the up-regulated genes and the reference gene (α-tubulin). For amplification of the tubulin gene, specific primers were used (TubF-5'- TACCGTGCCCTTACTGTTCC -3'/ TubR-5'- CGGTGGAATGTCACAGACAC -3'). All PCR amplifications were performed using thermocycler (Techne, USA), having 35 cycles at 94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min.

PCR products were separated on 1.2 % agarose gels and the gel was stained with ethidium bromide to visualize the amplified PCR products. Thereafter, photographic images were obtained with a gel documentation system. Densitometric analyses were performed with the Gel UN-SCAN-IT software (Silk Scientific, USA) as described by Dean *et al*. (2002). The relative intensity was determined based on the intensities of the reference gene and the genes of interest. The average pixel values were calculated for the reference gene and each of the genes of interest by Gel UN-SCAN-IT software (Silk Scientific, USA). To identify the relative expression, the ratio of the gene of interest band intensity to reference/ control gene band intensity was calculated in salt treated and non salt treated plants.

RESULTS

Identification of salt stress responsive genes, which are expressed during Phase I of rice variety At354

Based on differential hybridization signals, 48 and 66 different cDNA clones were identified as clones carrying up- and down-regulated genes, respectively at Phase I of salt stress. These up- and down-regulated clones were reconfirmed for the up- or down-regulated gene expression by another round of differential hybridization on separately prepared nylon membranes. The second round of differential hybridization confirmed 22 cDNA clones as up-regulated and 30 cDNA clones as down-regulated at Phase I (Figures 1.a & 1.b).

Sequencing the PCR products of OsPHI cDNA library and homology search

Among the 52 PCR products sent for DNA sequencing, successful results were obtained for 31 cDNA clones in which 14 were for up-regulated clones and 17 for

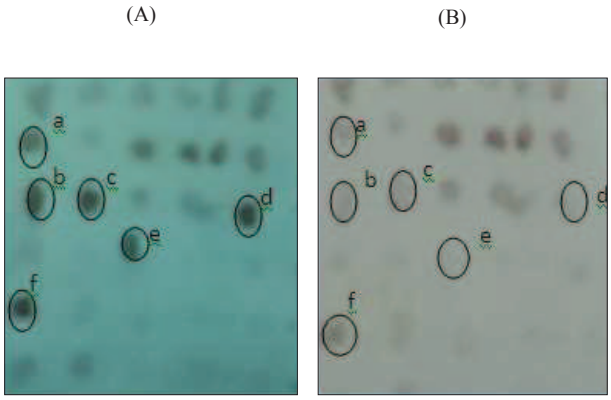


Figure 1.a: Identified up-regulated clones at Phase I of salt stress development. Signals developed by salt-treated cDNA probe (A) and control cDNA probe (B) when the selected cDNA clones were subjected to differential hybridization. Six differentially hybridized clones corresponding to clones (a) 30, (b) 32, (c) 147, (d) 194, (e) 928 and (f) 1271 are indicated by circles.

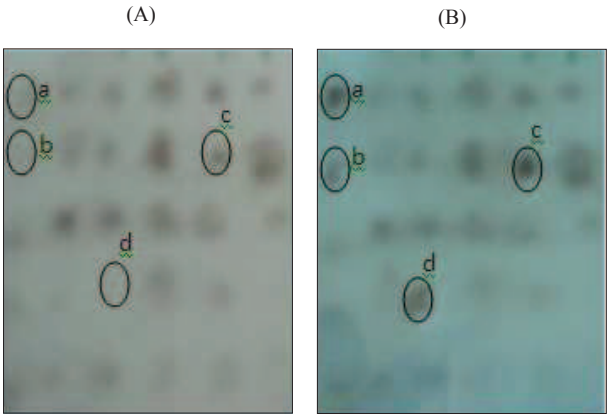


Figure 1.b: Identified down -regulated clones at Phase I of salt stress development. Signals developed by salt treated cDNA probe (A) and control cDNA probe (B) when the selected cDNA clones were subjected to differential hybridization. Four differentially hybridized clones corresponding to clones (a) 230, (b) 2792, (c) 2805 and (d) 2855 are indicated by circles.

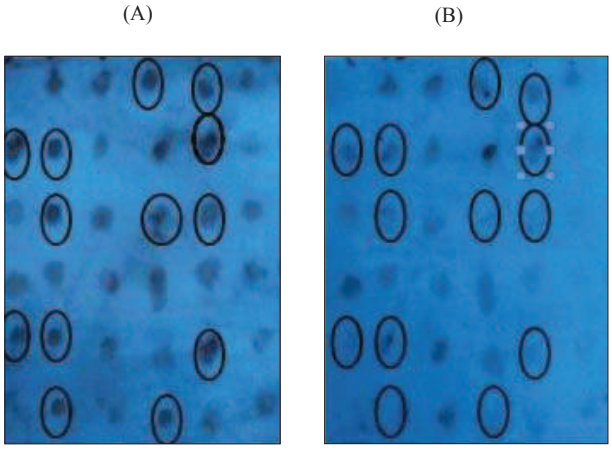


Figure 2.a: Identified up-regulated clones at Phase II of salt stress development. Signals developed by salt treated cDNA probe (A) and control cDNA probe (B) when the selected cDNA clones were subjected to differential hybridization. Thirteen up-regulated clones are indicated by circles.

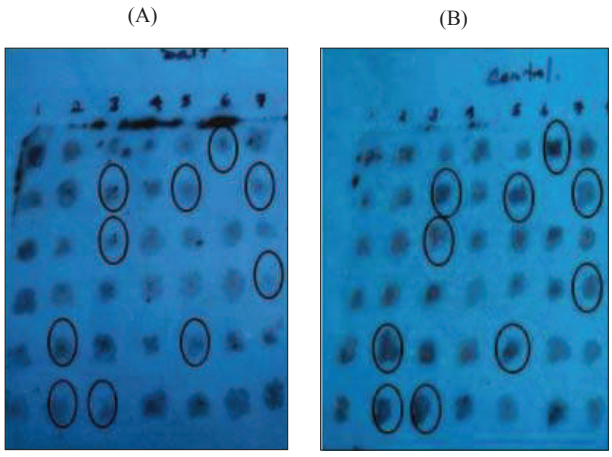


Figure 2.b: Identified down-regulated clones at Phase II of salt stress development. Signals developed by salt treated cDNA probe (A) and control cDNA probe (B) when the selected cDNA clones were subjected to differential hybridization. Ten down-regulated clones are indicated by circles.

down-regulated clones. Homology search was done for the DNA sequences of the 31 cDNA fragments and Tables 1 and 2 summarize the putative genes and their possible functions of the 31 cDNA clones, which were differentially-expressed in At354 in response to salt stress during Phase I.

Identification of salt stress responsive genes, which are expressed during Phase II of rice variety At354

Differential screening of 960 clones from salt stress-induced Phase II cDNA library identified 48 cDNA

Table 1: Putative genes and probable proteins produced by the up-regulated cDNA clones of the salt tolerant Sri Lankan rice variety At354 during Phase I of salt stress development

Clone No.	Homology information	Identity	Probable function under salt stress
OsPHI-30	Putatively expressed salt tolerant gene of rice identified previously from *Oryza sativa* Japonica (variety Nipponbare)	D 75	No information on the physiological function is available. May be a novel unreported gene
OsPHI-32	Homologous to eukaryotic translation initiation factor eIF5A	D 96	Protection of older leaves from salt ion toxicity; Exclusion of Na^+ from younger leaves
OsPHI-33	Homologous to cytochrome oxidase I of mitochondria	D 87	Activation of mitochondria to adapt to osmotic stress
OsPHI-147	Homologous to a gene of *Oryza sativa* which produces a putative serine decarboxylase	D 97	Increased biosynthesis of glycinebetaine, an osmoprotectant
OsPHI-194	Homologous to a gene similar to LSTK-1-like kinase of *Oryza sativa* Japonica (variety Nipponbare)	D 99	Osmotic stress/drought tolerance
OsPHI-240	Homologous to WD40-like domain containing protein	D 91	Function *via* regulatory pathway to tolerate osmotic stress
OsPHI-928	Homologous to a putative gene of *Oryza sativa* that produces AP domain DRE binding factor	D 100	Osmotic stress/drought tolerance
OsPHI-941	Homology to Zinc finger protein 1-like isoform	D 81	Enhance the activities of reactive oxygen species-scavenging enzymes under stress conditions and increased tolerance of plants to oxidative stress
OsPHI-1271	Homologous to a putative gene which produces a chromosomal replication initiator protein	D 96	No information is available on physiological function. May be a novel unreported gene
OsPHI-2277	Homology to EST of water-stressed durum wheat	D 100	Osmotic stress/drought tolerance
OsPHI-2465	Homology to EST of water-stressed durum wheat	D 95	Osmotic stress/drought tolerance
OsPHI-2468	Homology to a hypothetical protein of *Oryza sativa*	D 89	No information is available on physiological function. May be a novel unreported gene
OsPHI-2470	Similar to receptor-like kinase protein of *Oryza sativa*	D 88	Salt and drought stress tolerance in rice, through the activation of antioxidant systems
OsPHI-2487	Homology to SET domain protein	D 86	Osmotic stress/drought tolerance

D- Percent identity in nucleotide sequence

clones as salt-responsive, out of which 34 were up-regulated and 14 were down-regulated. Figures 2.a and 2.b present 13 of the up-regulated and 10 of the down-regulated genes indicated above. These selected clones were reconfirmed as up- and down-regulated clones by subjecting to another round of differential hybridization.

Sequencing of the PCR products of OsPHII cDNA library and homology search

Among the 48 PCR products sent for DNA sequencing successful results were obtained only for 13 cDNA clones consisting of 11 up- and 2 down-regulated clones. Tables 3 and 4 summarize the putative genes of 13 cDNA clones by homology search and their possible functions, which were differentially-expressed in At354 in response to salt stress during Phase II of salt stress.

Confirmation of differential expression of identified candidate genes of OsPHI and OsPHII libraries by dot blot analysis

When northern hybridization was done by the dot blot method using α-tubulin and gene specific probes, visible signals were obtained in the dot blots. As shown in Tables 5 and 6, the average pixel values of the signals of the

Table 2: Putative genes and probable proteins produced by the down-regulated cDNA clones of the salt tolerant Sri Lankan rice variety At354 during Phase I of salt stress development

Clone No.	Homology information	Identity	Probable function under salt stress
OsPHI-42	Aldo keto reductase family protein of *Oryza sativa*	D 84	Function via metabolic pathway to tolerate salt stress
OsPHI-54	Homologous to a gene similar to enolase (2-phosphoglycerate dehydratase)	D 100	This enzyme may play a role in decreasing energy metabolism rates to conserve energy and limit further generation of reactive oxygen species (ROS)
OsPHI-154	Homologous to a hypothetical protein of *Arabiodopsis lyrata* sub sp.	D 92	No information is available on physiological function
OsPHI-172	Homology to a hypothetical protein of Nipponbare cultivar of *Oryza sativa*	D 86	No information is available on physiological function. May be a novel unreported gene
OsPHI-181	Homologous to a gene similar to beta-amyrin synthase of *Oryza sativa*	D 94	No information is available on physiological function
OsPHI-183	Similar to 30 S ribosomal protein S18	D 87	Regulation of gene expression by suppressing bulk protein synthesis
OsPHI -230	Homologous to amine oxidases	D 89	Reduced degradation of polyamines and ensures tolerance of osmotic stress and ion toxicity; Reduced production of H_2O_2
OsPHI-719	Similar to photosystem II P680 chlorophyll A apoprotein (CP-47 protein)	D 90	May prevent photoinhibitory damage due to the formation of reactive oxygen species (ROS)
OsPHI-841	Homologous to a hypothetical protein of *Arabiodopsis lyrata* sub sp.	D 81	No information is available on physiological function
OsPHI-864	Homology to a hypothetical protein of Nipponbare cultivar of *Oryza sativa*	D 86	No information is available on physiological function. May be a novel unreported gene
OsPHI-937	Homologous to EST found in male and female panicle of *Pistacia vera*	D 83	No information is available on physiological function. May be a novel unreported gene
OsPHI -939	Similar to 30 S Ribosomal protein S18	D 87	Regulation of gene expression by suppressing bulk protein synthesis
OsPHI-1142	Homology to a hypothetical protein of Nipponbare cultivar of *Oryza sativa*	D 91	No information is available on physiological function. May be a novel unreported gene
OsPHI-2792	Homologous to amine oxidases	D 70	Reduced degradation of Polyamines and ensures tolerance of osmotic stress and ion toxicity; Reduced production of H_2O_2
OsPHI-2805	Homology to protein product of rap-GTPase-activating protein	P 100	Stomatal closure through down-regulation of cyclic neucleotide pathway; Cytoplasmic ion homeostasis
OsPHI-2806	Homology to a hypothetical protein of Nipponbare cultivar of *Oryza sativa*	D 90	No information is available on physiological function. May be a novel unreported gene
OsPHI-2855	Homology to agmatine deiminase protein product	P 100	Reduced production of putrescine to increase osmotic tolerance

D- Percent identity in nucleotide sequence; P- percent identity in amino-acid sequence

two samples of α-tubulin (i.e. control and salt treated), which were hybridized with gene specific probes were more or less similar to each other. In contrast, the average pixel values of the salt treated sample were higher in comparison to that of salt non-treated samples when the samples were hybridized with probes OSPHI-30, OSPHI-147, OSPHI-194 and OSPHI-928 of OSPHI cDNA library and OSPHII-1, OSPHII-51, OSPHII-311,

Table 3: Putative genes and probable proteins produced by the up-regulated cDNA clones of the salt-tolerant Sri Lankan rice variety At354 during Phase II of salt-stress development

Clone No.	Homology information	Identity	Probable function under salt stress
OsPHII-1	Similar to dicarboxylate transporters	D 92	Transports malate to vacuole and regulates stomatal opening
OsPHII-11	Dehydration responsive element binding protein of *Zea mays*	D 92	Transcriptional regulation of stress gene expression
OsPHII-51	Similar to heat shock protein (HSP81-1) / (HSP83) of *Zea mays*	D 95	Hsps/chaperones play a crucial role in protecting plants against stress and in the re-establishment of cellular homeostasis
OsPHII-77	Homology to a hypothetical protein of *Oryza sativa* (variety Nipponbare)	D 92	No information is available on physiological function. May be novel unreported genes
OsPHII-133	Similar to heat shock protein (HSP81-1) / (HSP83) of *Zea mays*	D 95	Hsps/chaperones play a crucial role in protecting plants against stress and in the re-establishment of cellular homeostasis
OsPHII-151	Similar to peroxidase precursor of *Oryza sativa* Japonica (variety Nipponbare)	D 95	Detoxification of free oxygen radicals
OsPHII-311	Similar to MADs box protein of *Oryza sativa* (variety Nipponbare)	D 88	Transcriptional regulation of stress gene expression
OsPHII-331	Ubiquitin-protein ligase of *Arabidopsis thaliana*	D 92	Protein turnover
OsPHII-370	Homology to a hypothetical protein of *Oryza sativa* (variety Nipponbare)	D 96	No information is available on physiological function. May be novel unreported genes
OsPHII-373	EF-Hand type domain containing protein of *Oryza sativa* (variety Nipponbare)	D 90	Stress signal transduction and gene expression
OsPHII-590	Harpin inducing protein	D 87	Activation of ethylene-response factor 5 (SlERF5) to tolerate salt stress

D- Percent identity in nucleotide sequence

Table 4: Putative genes and probable proteins produced by the down-regulated cDNA clones of the salt-tolerant Sri Lankan rice variety At354 during Phase II of salt-stress development

Clone No.	Homology information	Identity	Probable function under salt stress
OsPHII-159	Homology to a hypothetical protein of *Oryza sativa*	D 88	No information is available on physiological function. May be a novel unreported gene
OsPHII-333	Similar to KN motif and ankyrin repeat domains protein of *Ciona intestinalis*	D 88	May function as negative regulator of gluthathione S-transferase and thereby contributes to antioxidation function

D- Percent identity in nucleotide sequence

Table 5: RNA dot blot results and average pixel values quantified with a computer aided software for differentially-expressed genes of OsPHI cDNA library

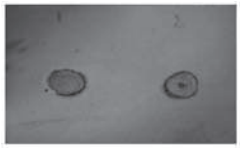
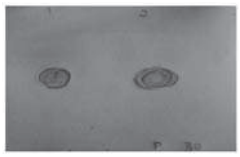
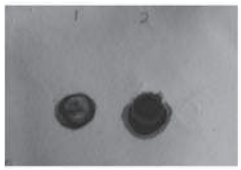
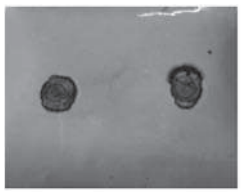
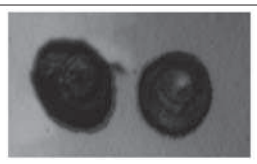
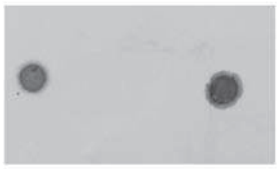
Probe	Sample description		Average pixel value		Relative expression of treatment compared to control
	Control	Treatment	Control	Treatment	
Tubulin			156.41	156.91	Similar
OSPHI 30			149.78	151.17	High
OSPHI 147			183.37	206.38	High
OSPHI 194			179.58	188.31	High
OSPHI 928			150.06	171.17	High
OSPHI 939			227.55	212.8	Low
OSPHI 2855			233.43	225.18	Low
OSPHI 172			173.88	153.27	Low

Table 6: RNA dot blot results and average pixel values quantified with a computer aided software for differentially-expressed genes of OsPHII cDNA library

Probe	Sample description		Average pixel value		Relative expression of treatment compared to control
	Control	Treatment	Control	Treatment	
Tubulin			165.32	165.74	Similar
OSPHII 1			183.43	210.52	High
OSPHII 51			165.39	217.53	High
OSPHII 311			199.32	229.28	High
OSPHII 370			111.95	147.76	High
OSPHII 590			163.16	167.74	High
OSPHII 333			167.88	144.91	Low

OSPHII-370, OSPHII-590 of OSPHII cDNA library. All these were up-regulated cDNA clones. In contrast, the average pixel values of the salt treated sample were lower in comparison to non-treated samples when the samples were hybridized with probes OSPHI-939, OSPHI-2855, OSPHI-172 of OSPHI cDNA library and OSPHII-333 of OSPHII cDNA library. All these were down-regulated cDNA clones.

Comparative expression analysis of some selected genes of OsPHI library at Phase I and Phase II by relative RT-PCR

The relative RT-PCR gels of selected genes of salt tolerance for Phase I and II are shown in Figure 3. As shown in Table 7, during Phase I, relative expression of OsPHI-30, OsPHI-147, OsPHI-928 were higher in

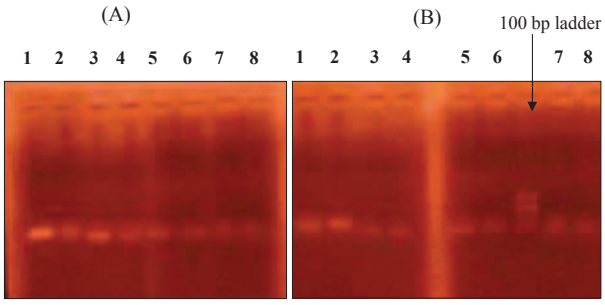


Figure 3: (A) Relative RT-PCR gel of Phase I plant samples; (B) Relative RT-PCR gel of Phase II plant samples. 1 - Tubulin gene of control plant; 2 - Tubulin gene of salt-treated plant; 3 - OsPHI-30 gene of control plant; 4 - OsPHI-30 gene of salt-treated plant; 5 - OsPHI-147 gene of control plant; 6 - OsPHI-147 gene of salt-treated plant; 7 - OsPHI-928 gene of control plant; 8 - OsPHI-928 gene of salt-treated plant

Table 8: Relative expression of some identified up-regulated genes of OsPHI library at Phase II of salt-stress development

Genes amplified from control and salt treated plants of Phase II	Average pixel value	Expression relative to reference gene (Tubulin)
Control –Tubulin	81.27	1
Salt stress - Tubulin	86.47	1
Control - OsPHI-30	69.27	0.85
Salt stress - OsPHI-30	71.98	0.83
Control - OsPHI-147	72.37	0.89
Salt stress - OsPHI-147	61.06	0.70
Control - OsPHI-928	64.31	0.79
Salt stress - OsPHI-928	63.60	0.73

salt treated plants than in untreated plants. Therefore, this experiment and the experiment on dot blot analysis revealed that OsPHI-30, OsPHI-147 and OsPHI-928 are contributing as up-regulating genes to tolerate osmotic stress in variety At354. The existence and level of expression of these genes were checked during Phase II as well. The results showed that none of the three genes that were tested showed up-regulation during Phase II (Table 8).

Table 7: Relative expression of some identified up-regulated genes of OsPHI library at Phase I of salt-stress development

Genes amplified from control and salt-treated plants of Phase I	Average pixel value	Expression relative to reference gene (Tubulin)
Control –Tubulin	105.24	1
Salt stress –Tubulin	82.90	1
Control - OsPHI-30	86.9	0.82
Salt stress - OsPHI-30	72.91	0.88
Control - OsPHI-147	74.03	0.70
Salt stress - OsPHI-147	69.8	0.84
Control - OsPHI-928	67.08	0.64
Salt stress - OsPHI-928	64.47	0.78

DISCUSSION

Probable involvement of the up- and down-regulated genes in the salt tolerance of variety At354 during phase I

Genes up-regulated in At354 in response to salt stress during Phase I

The cDNA Clone No.OsPHI-30 showed 75 % identity with a nucleotide sequence, which has been identified from the Japonica rice variety Nipponbare, and expressed under salt stress. At present, no further information is available in databases on this gene to deduce its possible physiological role in conferring salt tolerance.

The cDNA fragment of Clone No.OsPHI-32 from salt-stressed At354 is homologous to an eukaryotic translation initiation factor eIF5A. This factor, formerly called eIF-4D, is the only cellular protein known to contain the unusual amino acid hypusine and is known to accumulate in rice in response to salt and heavy metal stress (Chou *et al.*, 2004). During salt stress development, salt ion toxicity is initially experienced by the older leaves, which are unable to dilute incoming salts by expanding. Hence, OseIF5A expressed in salt stressed At354 may play a role in protecting rice leaves from salt ion toxicity. Although in the present study, up-regulation of OseIF5A has been observed during Phase I of salinity

where salt toxicity is not expected to be significant, the high salt sensitivity of rice (Grattan *et al.*, 2002; Munns and Tester, 2008) may mean that salt toxicity has already begun in Phase I. This was confirmed by measurements of tissue Na^+ concentrations in old and young leaf tissues in the present experiment (data not shown). Being more abundant in older leaves, OseIF5A may be contributing to Na^+ exclusion from younger leaves by helping in the transport of excess Na^+ to the vacuole of older leaves. Alternatively, through its expression in panicles and older leaves, OseIF5A may be contributing to a protective function from salinity. OseIF5A plays an important role in the determination of cell proliferation and death in eukaryotic cells (Thompson *et al.*, 2004). As expression of eIF5A in rice is high in older leaves (Chou *et al.*, 2004), it could contribute to keep older leaves alive under salt stress by increasing their tissue tolerance to salt ion toxicity.

The cDNA Clone No. OsPHI-33 gave homology to cytochrome oxidase I of mitochondria. The mitochondria are sensitive to salt stress. Mitochondrial function is required for proper salt and osmotic stress adaptation because mutants with defects in many different mitochondrial components show hypersensitivity to increased NaCl concentrations. Cytochrome oxidase I is needed to activate mitochondria to adapt for osmotic stress (Pastor *et al.*, 2009). It can function *via* general metabolism to reduce salt stress in rice plants (Sahi *et al.*, 2006a) and hence its up-regulation in At354 during Phase I of salt stress may activate mitochondria to adapt to osmotic stress.

One effective mechanism to reduce damage from salt stress is the accumulation of high intracellular levels of osmoprotectant compounds (Munns, 2005). The cDNA Clone No.OsPHI-147 from salt stressed At354 was homologous to a gene of *Oryza sativa* which produces a putative serine decarboxylase, an enzyme needed to produce ethanolamine, a precursor of choline (McNeil *et al.*, 2001). Choline is needed to produce glycinebetaine (Huang *et al.*, 2000), which acts as an osmoprotectant conferring salt tolerance in plants (Blumwald *et al.*, 2004). Hence, the gene products of this cDNA clone could be providing a protective function, primarily from osmotic stress that occurs during Phase I.

DNA and protein homology search showed that cDNA Clone No. OsPHI-194 from salt stressed At354 is homologous to a gene similar to LSTK-1-like kinase of *Oryza sativa* Japonica Nipponbare rice variety. LSTK-1-like kinase enzyme is expressed in maize plants exposed to drought stress (Li *et al.*, 2009). As drought, induced by osmotic stress, is the major stress that operates during Phase I of salt stress development, up-regulation of clone 194 in At354 provides evidence that it may be coding for a gene product that is responsible for osmotic stress tolerance.

The cDNA Clone No OsPHI-240 from salt-stressed At354 gave homology to WD40-like domain containing protein. This protein functions *via* regulatory pathway to tolerate osmotic stress (Lee *et al.*, 2010).

The cDNA Clone No. OsPHI- 928 from salt-stressed At354 gave homology to a putative gene of *Oryza sativa* that produces the AP domain DRE (dehydration responsive elements) binding factor. The DRE-related motifs are at promoter regions of many cold and drought inducible genes (Dubouzet *et al.*, 2003). Overexpression of these regulatory elements, which are transcription factors, increases drought (Chen *et al.*, 2008; Kumashiro & Yamaguchi-Shinozaki, 2008), salt and low temperature tolerance (Wang *et al.*, 2008). Promoter regions of most genes induced by drought, salinity and cold in rice are enriched with two DRE transcription factors, the ABA-independent DRE and the ABA-responsive ABRE (Zhou *et al.*, 2007). Comparison of expression profiles for several stresses showed that there was considerable overlap between profiles induced by drought, salinity and ABA treatment. Therefore, it is logical that the gene product of this particular cDNA clone is up-regulated in the salt-tolerant rice genotype At354 during Phase I of salt stress, which is dominated by osmotic stress-induced drought.

Homology search elucidated that the cDNA Clone No. OsPHI- 941 is similar to zinc finger protein 1- like isoform. Several zinc finger proteins have previously been identified in rice (Huang *et al.*, 2005; Huang *et al.*, 2007; Xu *et al.*, 2008) under different abiotic stresses. It enhances the activities of reactive oxygen species-scavenging enzymes under stress conditions and increases the tolerance of plants to oxidative stress, which can be caused by the osmotic stress that prevails during Phase I of salt stress.

OsPHI-1271 was identified as an up-regulated cDNA clone due to salt-stress in At 354. The transcripts carried by this clone were homologous to a putative gene, which produces a chromosomal replication initiator protein of *Pectobacterium wasabiae*. There have been no reports on the possible involvement of this gene product in stress tolerance in plants.

Homology search on the cDNA Clone Nos. OsPHI- 2277 and OsPHI-2465 showed more than 95 % identity with ESTs of water stressed durum wheat. Thus, they may also be involved in osmotic tolerance of variety At 354.

The cDNA Clone No. OsPHI-2468 gave homology to a hypothetical protein of *Oryza sativa*. There have been no reports of the possible involvement of these protein products in salt stress tolerance in plants.

Activating antioxidative systems to avoid salt stress is a mechanism of salt tolerance. Clone OsPHI-2470 gave homology to a gene product, which is similar to receptor-like kinase protein (RLK) of *Oryza sativa.* RLKs play essential roles in plant growth, development and responses to environmental stresses and are activated during salt- and drought stress tolerance in rice, through the activation of antioxidant systems (Ouyang *et al.*, 2010). Thus, the protein, which is similar to RLK in At354 may activate antioxidative systems to tolerate osmotic stress.

The cDNA Clone No. OsPHI- 2487, which was up-regulated gave homology to a gene that produces a SET domain-containing protein expressed in roots of drought tolerant upland rice variety Prata Ligeiro (Rabello *et al.*, 2008). As drought is the principal stress factor operating during Phase I of salt stress, it is possible that this protein may be involved in salt tolerance of At354 during Phase I.

Genes down-regulated in At354 in response to salt stress during Phase 1

The cDNA Clone No. OsPHI-42 gave homology to an aldo-keto reductase family protein of *Oryza sativa.* Kim *et al.* (2011) found that aldo-keto reductase was down-regulated in the rice variety Nipponbare, 6 days after experiencing a salt-stress of 130 mM. Down-regulating a metabolic pathway, which causes damage due to osmotic stress by this gene is possible. Hence, its down-regulation increases tolerance to osmotic stress during Phase I of salt-stress development.

Under NaCl stress, plants decrease energy metabolism rates to conserve energy and limit further generation of reactive oxygen species (ROS) (Moller, 2001), which can cause damage to cellular components during abiotic stress. The cDNA Clone No. OsPHI-54 obtained from salt-stressed At354 was homologous to a gene of *Oryza sativa*, which produces a protein similar to enolase. Jiang *et al.* (2007) found that phosphopyruvate hydratase/enolase (LOS2, At2g36530), which catalyses the formation of high-energy phosphoenol pyruvate from 2-phosphoglycerate in the glycolytic pathway, decreased in response to NaCl stress in roots of Arabidopsis. Therefore, the observation in the present study that enolase is down-regulated during early phase of salt stress in variety At354 indicates that this enzyme may play a role in decreasing energy metabolism rates to conserve energy and limit further generation of ROS and thereby tolerate osmotic stress during Phase I of salt-stress development.

The cDNA Clone Nos. OsPHI-154 and *OsPHI-*841 gave more than 80 % identity with a hypothetical protein of *Arabiodopsis lyrata* sub sp. There have been no reports of the possible involvement of these protein products in salt stress tolerance in plants. This may show the involvement of novel genes, whose functions are yet to be elucidated.

The cDNA fragments of Clone Nos. OsPHI-172, OsPHI-864, OsPHI-1142 and OsPHI-2806 were identified as down-regulated genes during Phase I of salt-stress. These were found to have more than 85 % identity to a hypothetical protein of the cultivar Nipponbare of *Oryza sativa*. There have been no reports of the possible involvement of these gene products in stress tolerance in rice. Therefore, it is possible that these genes are coding for novel proteins responsible for osmotic stress tolerance in rice.

Homology search elucidated that the cDNA Clone No OsPHI- 181 is similar to Beta-amyrin synthase of *Oryza sativa.* No information is available on physiological functions of this gene product in stress tolerance in rice.

The cDNA Clone Nos. OsPHI-183 and OsPHI-939 gave homology to a protein similar to 30S Ribosomal protein S18. A decrease in bulk *de novo* protein synthesis following NaCl treatment has been detected in *Arabidopsis* by Ndimba *et al.* (2005). Also, Jiang *et al.* (2007) showed that the synthesis of three ribosomal proteins was decreased by NaCl treatment in *Arabidopsis*. They suggested that short-term NaCl stress may repress protein synthesis *in vivo.* Thus, down-regulation of ribosomal proteins in variety At354 during Phase I of salt-stress may also contribute to regulated gene expression by suppressing bulk protein synthesis.

The protein homology search showed that cDNA fragments harboured by the Clone Nos. OsPHI-230 and OsPHI-2792, which have been identified as down-regulating genes in the present study encode for amine oxidases. Amine oxidases catalyze the oxidative de-amination of polyamines (Cona *et al.*, 2006), which are an important group of compounds regulating

plant responses to various stresses such as salinity, drought, low temperature and ozone (Liu *et al.*, 2007). Overexpression of polyamine biosynthesis could lead to abiotic stress tolerance *via* stabilization of negatively-charged molecules such as proteins and membrane lipids, reduction of free radicals by anti-oxidant properties and improvement of ionic balance (Liu *et al.*, 2007). By down-regulating genes encoding for polyamine degradation, gene products of cDNA Clone Nos. 230 and 2792 obtained from salt-stressed At354 are likely to reduce polyamine degradation during salt stress, thereby allowing the stress tolerance functions of polyamines to operate. This could contribute to the salt tolerance of At354 in Phase I of salinity. Furthermore, polyamine degradation produces H_2O_2, which mediates cell death and thereby increases stress sensitivity. Down-regulation of genes that trigger the above process would also contribute to the stress tolerance of At354.

The cDNA Clone No. OsPHI- 719 was down-regulated during Phase I of salt stress. This clone gave homology to a protein, which is similar to photosystem II P680 chlorophyll A apoprotein (CP-47 protein). Photosystem II is responsible for capture of radiation for photosynthesis. However, osmotic stress during Phase I of salt-stress probably reduces the demand for radiation energy because of reduced photosynthesis under osmotic stress. Hence, down-regulation of the expression of a gene, which is responsible for a protein linked to photosystem II may indicate a response to reduce radiation absorption and reduce the energy load in the chloroplasts. Such a reduction in the energy load would prevent photoinhibitory damage due to the formation of reactive oxygen species (ROS).

The cDNA Clone No. OsPHI-937 showed 83 % identity to an EST of the panicle of *Pistacia vera*. There have been no reports of the possible involvement of these ESTs in stress tolerance in plants. However, it is possible that down-regulation of this gene in salt-stressed At354 could be responsible for delaying of panicle development during the period of osmotic stress. Such a delay in panicle development would be advantageous to the rice plant because water stress during Phase I of salt-stress development would have adverse effects on several sub-processes of panicle development leading to the formation of sterile grains. Therefore, down-regulation of this gene could be part of a mechanism of stress avoidance, which probably contributes to the salt tolerance of At354.

The cDNA fragment harboured by Clone No. OsPHI- 2805 from salt-stressed At354 was homologous to a protein product of rap-GTPase-activating protein, which is needed to activate the cyclic neucleotide pathway in mammals. Although the presence of cyclic neucleotides (i.e. 3',5'-cyclic adenyl monophosphate, cAMP, and 3',5'-cyclic guanyl monophosphate, cGMP) and their functional roles have been well-established in animal cells, their presence in plant cells have been confirmed only recently (Newton & Smith, 2004; Martinez-Atienza *et al.*, 2007). Cyclic neucleotides have been implicated in the signalling and regulation of many important plant processes such as stomatal functioning, cation fluxes, chloroplast development, gibberellic acid signalling, pathogen response and gene transcription (Martinez-Atienza *et al.*, 2007). There are several reports of evidence indicating a role for cAMP and/or cGMP in stomatal opening (Newton & Smith, 2004). Results of the present study have shown a down-regulation of a gene product (i.e. rap-GTPase-activating protein), which is responsible for activation of the cyclic neucleotide pathway in At354 during Phase I of salt stress. As osmotic stress induced water deficits are the main effect of salinity during Phase I, partial stomatal closure by down-regulating the cyclic neucleotide pathway may help alleviate adverse effects of water stress and thereby contribute to salt-tolerance of At354 in Phase I.

There is evidence of direct effects of cyclic neucleotides on intra-cellular cation fluxes raising the possibility of a role in regulating the homeostasis of K^+, Ca^{2+} and Na^+ ions (Maathuis, 2006). Ion homeostasis involves maintaining cytoplasmic ratios of K^+:Na^+ and Ca^{2+}:Na^+ within optimum ranges. This may require both increases and decreases of the uptake of these cations in plants experiencing salt stress. Therefore, it is possible that down-regulation of the cyclic neucleotide pathway in salt-stressed At354 during Phase I may contribute to maintenance of cytoplasmic ion homeostasis.

The cDNA Clone No. OsPHI-2855 from salt-stressed At354 was homologous to agmatine deiminase protein product, which is an enzyme in the biosynthesis pathway of putrescine, an important member of the polyamine group (Gill & Tuteja, 2010). It has already been noted that polyamines can play an important role in stress tolerance as stress messengers in plant responses to different stress signals (Alcázar *et al.*, 2006, 2010). Interestingly, Zapata *et al.* (2003, 2004) observed on a range of vegetable crops that salt stress decreased tissue putrescine (Put) levels while increasing the levels of the other two polyamines, spermidine (Spd) and spermine (Spm) so that the ratio (Spd + Spm)/Put was increased. They further observed that salt tolerance increased with the above concentration changes in different polyamines. In a comparative study with six crop species differing in salt tolerance, Zapata *et al.* (2008) showed that Put

concentration in shoot was negatively correlated with salt tolerance of the species, with the most tolerant species (*Beta vulgaris*) having the lowest shoot Put concentration. Similarly, in a screening of nine rice cultivars differing in salt tolrance, Krishnamurthy and Bhagwat (1989) observed significant increases in Spd and Spm in salt tolerant cultivars, but no significant alteration in Put when subjected to salt stress. In contrast, the salt-sensitive cultivars showed an excessive accumulation of Put under salt stress but Spd and Spm concentrations remained low. Therefore, the observed down-regulation of the gene product responsible for biosynthesis of Put in the salt tolerant At354 under salinity in the present study probably contributed to its salt tolerance. This finding is in agreement with the observations of Zapata *et al.* (2004) and Krishnamurthy and Bhagwat (1989).

Probable involvement of the up-and down- regulated genes in the salt tolerance of variety At354 during Phase II

Genes up-regulated in At354 in response to salt stress during Phase II

In the present study, the cDNA Clone No. OsPHII-1 from OsPHII library was similar to dicarboxylate transporters, which are needed to mediate the counter-exchange of malate, glutamate and aspartate (Flügge *et al.*, 1988). Transport of malate into the vacuole and its subsequent storage are essential in stomatal opening (Emmerlich *et al.*, 2003). Thus, up-regulation of the above transporter gene during ionic stress, which is the principal stress during Phase II of salt-stress development may increase malate transport ability to the vacuole and thereby maintain the osmotic pressure, charge balance and regulate stomatal aperture to maintain photosynthesis under salt stress.

One important aspect of tolerance to ionic stress is the ability to transfer the toxic Na^+ and Cl^- ions to the vacuole. In order to maintain ionic balance in the vacuole, compatible solutes such as malate, glutamate and aspartate also have to be transferred to the vacuole. Therefore, the dicarboxylate transporters encoded by the cDNA Clone No. OsPHII-1 probably play an important role in intracellular compartmentation of excess salts in the vacuole. As such, up-regulation of the genes responsible for dicarboxylate transporters probably contributes to the salt-tolerance of At354 in Phase II of salt stress development.

The cDNA fragment of Clone No. OsPHII-11 gave homology to DRE-binding protein of *Zea mays*, whose role in salt tolerance was discussed earlier (Zhou *et al.*, 2007; Wang *et al.*, 2010). The cDNA Clone Nos. OsPHII-51 and OsPHII-133 showed identity with heat shock protein (HSP81-1) / (HSP83) of *Zea mays*. Many studies have shown that involvement of heat shock protein in regulatory mechanisms to tolerate salt stress (Sahi *et al.*, 2006b). Hence, up-regulation of this protein during Phase II probably contributed to the salt tolerance of At354.

The cDNA Clone Nos. OsPHII-77 and OsPHII-370 which have been identified as up-regulated genes of OsPHII library in our study, showed more than 92 % identity to a hypothetical protein of the cultivar Nipponbare of *Oryza sativa*. However, no reports are available of the possible involvement of these protein products in salt stress tolerance in plants.

Clone No. OsPHII-151 gave homology to a precursor of peroxidise, which has the ability to scavenge ROS such as H_2O_2 (Huang *et al.,* 2009), thus contributing to salt tolerance of At354. This finding reveals that variety At354 has the ability to eliminate ROS production by up-regulating ROS scavengers.

Protein degradation during stress is a highly conserved and regulated phenomenon in all the organisms reported so far (Vierstra & Callis, 1999). Genes encoding proteins like ubiquitin ligase, polyubiquitin, proteasome subunit, and protease inhibitor are known to be expressed in the cDNA libraries of stressed tissues (Mahalingam *et al.*, 2003). The up-regulated Clone No. OsPHII- 331 of the present study showed homology to ubiquitin-protein ligase, which probably contributed to the Phase II salt tolerance of At354 *via* protein turnover.

The cDNA fragment harboured by Clone No. OsPHII-311 obtained from OsPHII was homologous to MADs box protein of *Oryza sativa.* Cooper *et al.* (2003) showed that MADS box TFs are important components of salt stress networking in plants and their possible role during stress is transcriptional regulation of stress gene expression.

Different molecules have been shown to act as signals in the stress-associated signal transduction processes. Changes in cytosolic Ca^{2+} concentrations act as a second messenger in salt stress (Chinnusamy *et al.*, 2005). The present study revealed that up-regulation of the cDNA fragment from Clone No. OsPHII-373 encodes for EF-hand containing protein, which is likely to be one of the key transducers mediating Ca^{2+} action.

The cDNA fragment harboured by Clone No. OsPHII-590 gave identity to harpin-inducing protein,

which activates cellular events of transcription regulation, signal transduction, stress response, membrane transporting, photosynthesis and cell wall biosynthesis (Chuang *et al.*, 2010). Overexpression of ethylene-response factor 5 (designated as SlERF5), which is a harpin-induced protein, in *Arabidopsis thaliana* activated a large number of genes involved in signaling pathways of disease resistance, abiotic stress response and protein phosphorylation. Thus, harpin probably plays similar roles in At354 also and thereby contributes to its salt-tolerance during Phase II.

Genes down-regulated in At354 in response to salt stress during Phase II

The cDNA Clone No. OsPHII-159, which has been identified as a down-regulated gene of OsPHII library in the present study gave 88 % identity to a hypothetical protein of Nipponbare cultivar of *Oryza sativa*. There have been no reports of the possible involvement of these protein products in salt stress tolerance in plants. This may show the involvement of novel genes, whose functions are yet to be elucidated.

The cDNA fragment of Clone No. OsPHII-333 from OsPHII was down-regulated under salt-stress and it gave identity to KN motif and ankyrin repeat domains of *Ciona intestinalis*. Down-regulation of ankyrin repeat-containing protein AKR2 of *Arabidopsis* increased transcripts of genes encoding pathogen-induced protein PR-1 (pathogen-related protein 1) and stress-responsive protein glutathione S-transferase 6 (GST6) (Yan *et al.*, 2002). Glutathione peroxidase (GSH)-dependent peroxidase reactions protect cell components from oxidative damage by scavenging toxic organic hydroperoxides. Also, plant GSTs play a role in GSH-dependent thioltransferase that safeguards protein function from oxidative damage, and are also involved in dehydroascorbate reductase (DHAR) that functions in redox homeostasis. Moreover, plant GSTs play an indirect role in the regulation of apoptosis and in stress signaling pathways (Dixon *et al.*, 2002; Frova, 2003). Over-expression of the glutathione *S*-transferase gene contributes to the antioxidation function and thereby increases stress tolerance of plants (Katsuhara *et al.*, 2005; Diao *et al.*, 2010). Therefore down - regulation of the Clone No. OsPHII-333 encoding protein may increase gluthathione S-transferase activity during Phase II of At354 and contribute to its salt-tolerance.

Confirmation of differential expression of identified candidate genes of OsPHI and OsPHII libraries by dot blot analysis and comparative expression analysis of some selected genes by relative RT-PCR

Up-regulated salt tolerant genes are expected to produce more mRNA transcripts in order to produce high quantities of functional proteins under salt stress. Therefore, more mRNA transcripts can be expected within total cellular RNA of up-regulated genes than that of down-regulated genes that produce less transcripts. Hence, dot blot analysis confirmed the differential expression of the identified candidate genes of salt tolerance during Phases I and II.

Relative RT-PCR uses standard PCR techniques but permits the comparison of transcript quantities between samples by co-amplifying the gene of interest with a housekeeping gene that acts as an internal control (reference gene). In comparison to northern blots, relative RT–PCR is much more sensitive and requires much less RNA (Dean *et al.,* 2002). α-tubulin gene has been used as a reference gene for rice in several studies (Caldana *et al.,* 2007; Yadav *et al.,* 2012) and the homogeneity of α-tubulin gene expression under normal and salinity stress conditions has been tested previously (Yadav *et al.,* 2012). In the quantification of relative RT-PCR, expression of the gene of interest is compared with the α-tubulin gene that was expressed under the given treatment condition (i.e. salt treated or control condition). Hence, despite slight variations in the expression of some reference genes (which have been observed with some of the reference genes due to variations in template concentration and PCR amplification efficiency (Dean *et al.,* 2002), the normalization of gene expression at a given condition will not be affected. Although the three genes selected for the relative RT-PCR study contributed significantly to the osmotic tolerance of At354 during Phase I (Table 7), they did not contribute significantly to tolerance of salt ion toxicity during Phase II (Table 8).

Concluding remarks

Results of the present study clearly demonstrated that osmotic stress during Phase I and ionic stress during Phase II caused changes in the expression of several genes in the shoots of the salt tolerant Sri Lankan rice variety At354. In addition, 25 up-regulated and 19 down-regulated differentially - expressed genes in At354 belonging to a variety of functionality classes such as

Table 9: Summary of up-regulated genes/ probable proteins produced by variety At354 during Phase I and Phase II of salt-stress development to tolerate salt stress

Up-regulated candidate genes/ probable proteins responsible for salt tolerance during Phase I	Up-regulated candidate genes/ probable proteins responsible for salt tolerance during Phase II
Eukaryotic translation initiation factor eIF5A	Dicarboxylate transporters
Cytochrome oxidase I of mitochondria	Heat shock protein(HSP81-1) / (HSP83)
Putative serine decarboxylase	Peroxidise precursor
LSTK-1-like kinase	Ubiquitin-protein ligase
AP domain DRE binding factor	Hypothetical protein of *Oryza sativa*
WD40-like domain containing protein	MADs box protein
Chromosomal replication initiator protein	EF-Hand type domain containing protein
Similar to receptor-like kinase protein	Dehydration responsive element binding protein
Zinc finger protein 1- like isoform	Harpin inducing protein
SET domain protein	
Similar to EST of water stressed durum wheat	
Putatively expressed salt-tolerant gene	
Hypothetical protein of *Oryza sativa*	

Table 10: Summary of down-regulated genes/ probable proteins produced by variety At354 during Phase I and Phase II to tolerate salt stress

Down-regulated candidate genes/ probable proteins responsible for salt tolerance during Phase I	Down-regulated candidate genes/ probable proteins responsible for salt tolerance during Phase II
Enolase (2phosphoglycerate dehydratase)	KN motif and ankyrin repeat domains protein
Aldo keto reductase family protein	Hypothetical protein of *Oryza sativa*
Beta-amyrin synthase	
30 S Ribosomal protein S18	
Amine oxidases	
Photosystem II P680 chlorophyll A apoprotein (CP-47 protein)	
Similar to EST found in male and female panicle of *Pistacia vera*	
Agmatine deiminase	
Rap-GTPase-activating protein	
Hypothetical protein	

protein synthesis, folding and stabilization (4 genes), general metabolism (4 genes), transcription (4 genes), signal transduction (3 genes), cellular redox balance (5 genes), cellular transport/homeostasis (4 genes) and defence-related functions (3 genes) have been identified. Out of the remaining differentially - expressed genes, ten genes belonged to hypothetical proteins and seven genes are of unknown functions.

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