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Screening for seedling stage salinity tolerance and comparative transcriptome analysis in Rice, *Oryza sativa* L.

A Mary Roshni*, KR Saravanan, M Harikrishnan, R Anandan & M Prakash*

Department of Genetics and Plant Breeding, Faculty of Agriculture, Annamalai University, Annamalai Nagar - 608 002, Tamil Nadu, India

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Soil salinity is one of the major abiotic stresses which affect crop productivity including rice, *Oryza sativa* L. Developing salt tolerant varieties gained considerable attention accordingly. Here, we studied salinity tolerance in rice crop. We screened ten rice genotypes for saline tolerance at different concentrations of 0 dSm^{-1}, 2 dSm^{-1}, 4 dSm^{-1}, 6 dSm^{-1}, 8 dSm^{-1}, 10 dSm^{-1}, 12 dSm^{-1} and 14 dSm^{-1}, respectively. Among the ten genotypes studied, CARIDhan-7 tolerated salt stress up to 6 dSm^{-1} of irrigated water. Comparative transcriptome analysis was done with the genotypes by treating with saline water at 0 dSm^{-1} and 6 dSm^{-1}. A total of 1013 genes were differentially expressed under simulated stress conditions. Out of which, 551 genes were upregulated and 462 genes were downregulated. Based on the metabolic pathway analysis, the MAPK signaling pathway, along with the other 9 pathways were found to be enriched in the stressed sample. Nine ion transporter genes, 1 potassium channel, 1 protein phosphatase gene, IAA homologs OsIAA9, two ROS scavenging-related genes, and 4 stress-regulated genes identified were found to be significantly up-regulated along with some functional proteins previously reported under salt stress. An AP2-like ethylene-responsive transcription factor PLETHORA 2 was found to be downregulated. The results suggested that the CARIDhan-7 genotype undergoes various saline tolerant mechanisms and pathways in response to the stress imposed when compared to non-stressed seedlings.

Keywords: Abiotic stress, Paddy

Soil salinity, serious abiotic stress, is one of the major environmental constraints of crop production, particularly rice, *Oryza sativa* L., mainly due to by high concentrations of NaCl along with other compounds of mineral salts like Ca, Mg, K, B, Fe, CO_3^{2-}, SO_4^{2-} and CHO[3] that is expected to increase further due to climate change. It includes all problems due to salts, primarily due to the abundance of sodium chloride from natural accumulation or irrigation[1]. Global status of salt affected area is estimated to be 1,128 million ha· In India, nearly 5% of net cultivated area is being affected by soil salinity, which accounts for nearly 6.727 million ha[2]. Flood water with an electrical conductivity of more than 2 dSm^{-1} leads to a loss of up to 1 t/ha in rice[3]. Ability to tolerate salinity is a key factor in plant productivity[4]. It takes place at the whole plant, cellular and molecular levels of the organization to contribute to saline tolerance[5]. This detrimental effect often degrades the root cells under soil salinization. Previous studies at the seedling stage of cereal crops stated that salt exclusion from leaves is the most important stress tolerance mechanism[6-9]. Maintenance of the whole plant and shoot water status, as well as mechanisms such as Na^+ exclusion or maintenance of potassium in developing tissues and rapidly growing leaves, contribute to the tolerance of salt in rice varieties[10]. Rice has the ability to compartmentalize the ions which are harmful, in the older leaves and structural tissues, thereby providing a way for plant survival. The salt in the plant enhances the senescence of old leaves. Matured leaves have larger vacuole when compared to younger leaf cells which have only dispersed miniature vacuoles. Old leaves accumulate much higher levels of Na^+, Cl^- and NO_3^- than young leaves under salinity[11].

Growth differences between different genotypes in response to salinity depend on salt concentration and the degree of salt tolerance[12]. A proper understanding of the mechanism of high salinity stress and the subsequent development of salinity-tolerant crops can be a solution to increase food production. New varieties with higher yield potential and stability across environments, climates, and geographical locations also need to be developed. This can be done by accelerating the discovery of genes and alleles, and delivery of marker-assisted selection and genetic modification to crops. The availability of the whole-

*Correspondence:
E-Mail: geeth_prakash@yahoo.co.in, roshni2395@gmail.com

genome sequence of rice contributed to the rapid development of the functional genomics of salt tolerance in rice, which paved the way for the identification of new genes and pathways. Comparative transcriptomics, with the widespread growth in genomic platforms and NGS, can be used to compare any genomes thereby ending with knowledge of the gene expression pattern. As mRNA will focus only on the exons of the genome, it can be widely used to avoid discrepancies among the complexity of results. Expressed genes may vary with time, environment, age of the plant, etc. Thus, comparative transcriptomics could eventually provide us with the differentially expressed genes (DEGs) between the two plants taken under consideration.

The present inquiry is aimed to identify and cull out the saline tolerant genotype from the ten genotypes of interest during the seedling stage. In addition, we did transcriptomic analysis and compared transcriptomes of the CARIDhan 7 genotype at two different salinity levels *viz*., 0 dSm^{-1} (control) and 6 dSm^{-1} (treated samples) to get an insight into the molecular mechanisms through which the genotypes respond to the salinity environment.

Materials and Methods

Plant materials and growth conditions

Ten rice genotypes *viz.*, IRLON GSR-9, IRLON GSR-5, CSR-2016-IR-18-18, CR-3878-245-2-4-1, CSR-11-143, CARIDhan-7, CSR-2748-441-195, CR-3437-1*200-83, JK-58 and NDRK-11-20 were investigated for their tolerance to salinity at seedling stage under laboratory condition in the Faculty of Agriculture, Annamalai University. The experiment was conducted by adopting CRD with three replications. Different saline concentrations were prepared by using four salts *viz.*, NaCl, $Na_2SO_4^{2-}$, $CaCl^2$ and $MgSO_4^{2-}$ (by mixing NaCl - 5 meq, $Na_2SO_4^{2-}$ 1.75 meq, $CaCl^{2-}$ 2.5 meq, and $MgSO_4^{2-}$ - 0.75 meq), as per the protocol outlined by Sehrawat *et al.*[13].

After pre-soaking of seeds in water for 24 hrs, the seeds were exposed to various saline concentrations *viz*, 0, 2, 4, 6, 8, 10, 12, and 14 dSm^{-1} with control, with seeds at EC 0 dSm^{-1} maintained in distilled water. The well-filled seeds of the above-mentioned genotypes were allowed to germinate in Petri plates of 10 cm diameter with two soaked germination papers of corresponding saline solutions to study each genotype's *in vitro* tolerance potential. The covered Petri plates were allowed to germinate under a 14:10 hour ratio of light (300µmol m^{-2} s^{-1}): dark in a growth chamber. Out of 10 seeds, the number of seeds germinated was recorded on the fifth day after sowing. The growth of plumule and radicle above 2 mm were considered to be germinated.

For screening under the seedling stage, well-filled plumpy and sound seeds of the above-mentioned genotypes after 24 hrs of imbibition in RO water, were sown separately in eight aluminum trays filled with sand (obtained from the Cauvery river basin, Mettur of Tamilnadu, India which had a pH of 7.1 and EC of 0.05 dSm^{-1}), saturated with Hoagland's nutrient solution. After four days, each tray was supplied with corresponding saline solutions and the experiment was conducted with three replications under CRD. They were maintained under a temperature of 28±1°C and relative humidity of 80 percent in a greenhouse. Salinity concentrations were maintained by occasional checking of corresponding EC for the entire treatment period of 14 DAS. The germination percentage, root length, shoot length, fresh weight, dry weight, and seedling vigour were recorded 15 days after sowing. The best tolerant genotype as taken for further transcriptome analysis to find out the differential expression of genes of the same genotype at different salinity concentrations.

Total RNA extraction, cDNA library synthesis, and mRNA sequencing

For total RNA extraction, 100 mg of whole plant material at 15 DAS was ground for the two samples. The extraction was made with an RNeasy plant mini kit (Qiagen). The purity and integrity of RNA were confirmed with a Nanodrop spectrometer and quantified using a Qubit BR assay. The samples were checked for degradation using Agligent Bioanalyzer RNA 6000 nano kit. The samples with an RNA integrity value (RIN) of more than 7 were used for RNA sequencing. The mRNA sequencing libraries were prepared using NEBNext® Ultra™ II RNA Library Prep Kit for Illumina®. Illumina HiSeq™ 2500 sequencing platform was used to generate reads of 151 bp length.

RNA-Seq Data QC

The sequence data quality was checked using FastQC and MultiQC software. The quality of the raw sequence was checked by running the FastQC program which generated an output file of sequence data with quality scores along with all bases of the

paired-end sequencing process. Thus, the quality was checked for all the four raw data sequences generated. The data was checked for base call quality distribution, percent bases above Q20, Q30, percent GC, and sequencing adapter contamination. All the samples have passed the QC threshold (Q30>80 percent). Raw sequence reads were processed to remove adapter sequences and low-quality bases using fastp. Blastx was done for functional annotation of the assembled transcripts.

Alignment and indexing

The high-quality paired end reads were mapped into the indexed rice reference genome (MSU 7) using STAR2 RNA-Seq aligner. On average, 95.44 per cent of the reads aligned onto the reference genome. Gene level expression values were obtained as read counts using feature counts software.

Expression profiling analyses in the tolerant and sensitive genotypes in response to salt stress

For comparing the transcriptomes of the two samples, differential expression analysis, DESeq2 package was used. Genes with less than 5 reads in any one of the samples were removed from further analysis. The read counts were normalized (variance stabilized normalized counts) using DESeq2 and differential expression analysis was performed. Sample treated EC6 (Test) was compared to sample Control (Reference). Genes with absolute log2 fold change = 2 and p-value greater than or equal to 0.05 were considered significant. The expression profile of the differentially expressed genes across the samples is presented in volcano plot (Fig. 1).

Gene Ontology enrichment

The genes that showed significant differential expression were used for Gene Ontology (GO) and pathway enrichment analysis. Enrichment analysis for the biological process, molecular function and cellular component was performed using the agriGO tool using the significantly differentiated genes obtained as input in the above process. Multiple tests were adjusted by Benjamini and Hochberg false discovery rate. Significantly enriched top 10 GO terms (BH *P* value <0.05) were found.

Biological pathway assessment

Enrichment analysis for KEGG Pathway was performed using Cluster Profiler R package. Pathway terms with multiple tests adjusted p-value greater than or equal to 0.05 are considered significant. The pathways were visualized using Path view package to check the differential expression level of the genes in the pathway.

Results

Well plumpy and sound seeds of ten rice genotypes were investigated for their performance under-stimulated stress conditions in Petri dishes as well as in aluminium trays filled with sandy soil. It was observed that high salt concentration affected the seedling growth and development. The salt-affected plants showed yellowing of leaves with subsequent senescence and death. The shoot and root growth were reduced with the increasing concentrations of salt. The culm was lean and lanky with the higher concentrations of salt. However, a slight stimulating effect was also observed at low salt concentrations.

Phenotypic variation for salinity tolerance in rice

Ten genotypes of rice seedlings were screened for salinity tolerance after five days of salinization under *in vitro* conditions and simultaneously for fourteen days using aluminium trays *in vivo*. Salt injuries were observed under severe stress due to salinized circumstances. The symptoms include yellowing of leaves with subsequent senescence, death, and shoot, root development was also highly hampered with an increase in the concentration of salt. The seedling biomass was very much reduced with increased concentrations of salt. Salt tolerant seedlings were differentiated from delicate seedlings with phenotypic appearance. The tolerant salinity lines showed low salt injury symptoms. They were greenish-yellow with delayed symptoms of senescence.

Variance analysis (ANOVA)

Analysis of variance indicated that the genotypes differed significantly among themselves for all the traits of interest, indicating that the genotypes selected for this study were genetically different. Hence, further analysis was appropriate (Suppl. Table S1. *All supplementary data are available only online along with the respective paper at NOPR repository at http://nopr.res.in*).

***In vitro* screening**

Germination percentage

Ten rice genotypes were screened to assess their level of salinity tolerance under different levels of salinity concentrations. The germination percentage under EC 2 ranged from 70.5 percent (CSR 2016-IR-18-18) to 93.3 percent (IRLON GSR-9) in Petri plates. The stimulative effect was higher i.e., 97.56 at

EC 2 level with CSR 11-143, followed by CR 3878-245-2-4-1 (96.12) and CSR 2748-44-195 (92.81). The reduction percentage was higher at EC 14 in CARIDhan-7 (38.62) followed by CR 3878-245-2-4-1 (37.98) and CSR 2748-44-195 (37.41) respectively. The germination percentage was found to be reduced in all the treatments under *in vitro* conditions (Suppl. Table S2).

Root length

The root length at EC 2 ranged from 4.01 cm (NDRK 11-20) to 4.85 cm (CARIDhan-7). The deleterious effect was higher in CR 3878-245-2-4-1 (51.10), followed by CARIDhan-7 (49.90) and JK-58 (49.48) at EC 14. The reduction in root length was corresponding with the increase in salt concentrations (Suppl. Table S3).

Shoot length

The shoot length at EC 2 ranged from 0.75 cm (NDRK 11-20) to 1.34 cm (CARIDhan-7). A higher stimulative effect (113.78) was recorded at EC 2 concentration with IRLON GSR-9 followed by NDRK 11-20 (103.18) and CSR 2748-44-195 (102.21). The genotype CARIDhan-7 (66.67) followed by CSR 278-44-195 (64.09) and CR 3878-245-2-4-1 (53.93) recorded a higher reduction percentage at EC 14 (Suppl. Table S4).

***In vivo* screening**

All ten rice genotypes were evaluated for their response to salinity stress during vegetative stage under laboratory conditions. Most of the rice genotypes were found susceptible to 0.6 mM salinity treatment within a week. Among the genotypes, CARIDhan-7 and IRLON GSR-9 were found to be promising under different salinity concentrations and the genotype CARIDhan-7 (tolerant) was taken up for further studies.

Germination percentage

All ten rice genotypes were tested for salinity tolerance under *in vivo* germination. Sterilized rice seeds were germinated in Petri plates containing different levels of NaCl along with control plants after 14 days of stress. From the results, it was found that germination percentage significantly varied among the rice genotypes under different levels of saline concentration. The germination percentage at salinity of EC 2 ranged from 78.12 percent (NDRK 11-20) to 91.4 percent (CARIDhan-7). The stimulative effect was higher with 102.12 percent at EC 2 concentration with CARIDhan 7 genotype followed by CSR 11-143 (101.22 percent) and CSR 2748-44-195 (101.08 percent). The germination percentage was found to decrease with the increasing salt concentrations. CARIDhan 7, CSR 2748-44-195, and CR 3878-245-2-4-1 recorded lower germination percentages with different levels of saline concentration. The genotype CARIDhan 7 recorded the highest germination percentages with 89.5, 91.4, 90.2, 83.5, 72.4, 70.2, 65.6 and 65.8 per cent at 0, 2, 4, 6, 8, 10, 12 and 14 dSm^{-1} respectively. Similarly, CSR 2748-44-195 recorded 88.5, 89.5, 90.1, 82.5, 71.3, 69.3, 64.3 and 64.99 percentages and CR 3878-245-2-4-1recorded 86.4, 86.9, 87.2, 81.5, 69.1, 64.2 and 64.85 percentages at 0, 2, 4, 6, 8, 10, 12 and 14 dSm^{-1} respectively. The reduction in germination percentages was found rapidly increased with the increasing NaCl concentrations for CSR 2748-44-195 (Suppl. Table S5).

Root Length

The root length varied significantly among rice genotypes under different salt concentrations. The genotypes CARIDhan-7, CSR 2748-44-195, and CR 3878-245-2-4-1 recorded low reduction against increasing salt concentrations. The root length for EC 2 ranged from 6.25 cm (NDRK 11-20) to 7.24 cm (CARIDhan-7). The stimulative effect was higher with 105.66 at EC 2 concentration with IRLON GSR-5 genotype followed by NDRK 11-20 (104.52) and CSR 2016-IR 18-18 (103.35), whereas the reduction percentage was higher at EC 14 with genotype CR 3878-245-2-4-1 (68.99), followed by IRLON GSR-9 (66.72) and NDRK 11 20 (66.56) respectively (Suppl. Table S6).

Shoot length

Shoot length is an important trait to evaluate the performance of rice genotypes for salt tolerance. The shoot length ranged from 8.55 cm (NDRK 11-20) to10.12 cm (CARIDhan-7) at EC 2. The genotype IRLON GSR-9 recorded a higher stimulative effect (100.00) followed by CSR 2748-44-195 (99.89) and IRLON GSR-5 (99.01) at EC 2. The reduction percentage was higher in CSR 2748-44-195 (65.96) followed by CSR 2016- IR 18-18 (64.65) and NDRK 11-20 (64.16) at EC 14 and the highest reduction percentage was noted in JK 58 (60.62 percent), followed by CARIDhan-7 (61.81) and CSR-11-143(61.98) (Suppl. Table S7).

Seedling vigour index

Salinity levels significantly affected vigour index in all the rice genotypes. It ranged from 17.25 at EC 2 to 18.84 at EC 12. The stimulative effect was higher

with 101.98 at EC 2 concentration with CR 3878-245-2-4-1, followed by CSR 11-143 (100.60) and CARIDhan-7 (100.51). A higher reduction percentage was noted at EC 14 with genotype CARIDhan-7 (42.35), followed by CSR 2748-44-195 (41.86) and CR 3878-245-2-4-1(41.60) (Suppl. Table S8).

Seedling fresh weight

Salinity level had a high influence on the fresh weight of root and shoot in all the genotypes. The fresh weight of root and shoot ranged from 0.24 g (NDRK 11-20) to 0.7 g (CARIDhan-7). The stimulative effect was higher *i.e.*, 109.76 at EC 2 concentration with the genotype IRLON GSR-5. The reduction percentage was found higher at EC 14 in NDRK 11-20 (56 percent) which was followed by IRLON GSR-9 (55.17) and CSR 2748- 44-195 (53.13). The coefficient of variation was higher with the EC 14 (6.12), followed by EC 12 (5.44) (Suppl. Table S9).

Seedling dry weight

The dry weight of rice genotypes was severely affected by different salt concentrations. The seedling's dry weight ranged from 0.6 mg (NDRK 11-20) to 1.7 mg (CARIDhan-7). The reduction percentage was found higher at EC 14 in JK-58 (77.78) followed by IRLON GSR-5 (75.00) and CSR 2748-44-195 (72.73). The seedling dry weight was drastically reduced at EC 14 and EC 12 in NDRK-11-20 (50%), followed by IRLON GSR-9 (60%), respectively (Suppl. Table S10).

RNA-Seq Data QC

A total of 61,392,182 and 20,290,464 reads were generated from control and saline-treated samples respectively. Out of this, 52,208,700 and 17,292,918 reads were obtained after quality trimming. An average QC threshold of Q30 >80% is needed for good quality. It was found that 82.71 and 81.74 percentages were found for Q30% before quality trimming and 95.98 and 94.11 percentages were obtained after quality trimming for control and treated samples respectively. Further, the data was processed to remove the adapter sequences using FastP (Table 1).

Alignment and indexing

After indexing to the rice reference genome (MSU7), a total of 52,208,700 and 17,292,918 reads of control and treated samples were mapped onto the reference genome, out of which 49,940,826 and 16,468,032 reads were mapped onto the genome. Around 48,290,536 and 15,950,686 reads were uniquely mapped on the reference genome, which accounted for around 92.5 and 92.24 percentages respectively. Gene level expression values showed that 55,986 and 55,986 genes were obtained from control and treated samples and the expressed genes ranged around 30,451 and 26,437 respectively (Table 2).

Expression profiling analyses intolerant and sensitive genotypes in response to salt stress

In the analysis of 27,472 genes, 1013 genes were significantly expressed, 551 genes were upregulated and 462 genes were downregulated. Genes with absolute log2 fold change = 2 and p-value = 0.05 were considered significant. The expression profile of the differentially expressed genes across the samples is presented in the volcano plot (Fig. 1).

Table 1 — Comparative results of raw reads before and after quality trimming

Sample-ID	No. of reads	Read Length	GC%	% Bases	
				> Q20	> Q30
Summary of Raw sequence data and quality.					
Control	61,392,182	151	55	99.63	82.71
TreatedEC6	20,290,464	151	57	99.37	81.74
Summary of QC passed sequence data.					
Control	52,208,700	50-145	56	99.8	95.98
TreatedEC6	17,292,918	50-145	58	99.2	94.11

Table 2 — Number of expressed genes in each sample (genes with at least 1 mapped read)

Sample-ID	Total genes	No. of expressed genes
Control	55986	30451
TreatedEC6	55986	26437

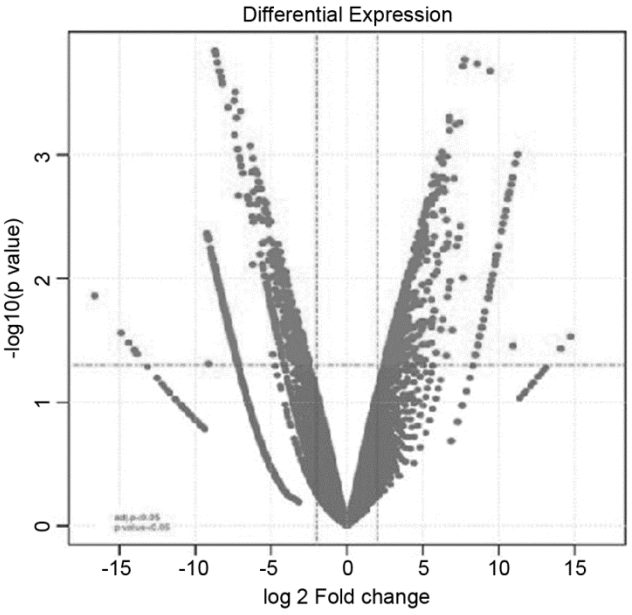

Fig. 1 — Volcano plot: Expression profile of differentially expressed genes across the sample.

Gene Ontology enrichment

Multiple tests in the analysis were adjusted by Benjamini and Hochberg's false discovery rate (FDR) (Suppl. Table S11). Around 27 genes were significant under biological processes (BP) (Fig. 2A), 16 genes under molecular functions (MF) (Fig. 2B), and 6 genes under cellular components (CC) (Fig. 2C) which were given in table along with their p and FDR values.

Biological pathway analysis

The significant pathways from the analysis which are below the p- value of 0.05 are presented here (Table 3). The pathways like diterpenoid biosynthesis (plant growth inhibition), MAPK signaling pathway (intra and extra-cellular stress signaling), cutin, suberin and wax biosynthesis (reduction in transpiration), linoleic acid metabolism (cell metabolism and signaling), phenylpropanoid biosynthesis (generate forms of suberin and lignin) and plant hormone signal transduction were found to be playing a major role in inducing stress tolerance to plants under salinity conditions. The pathways were visualized using the path view package to check the differential expression level of the genes in the pathway (Suppl. Fig. S1 A-J).

Discussion

Screening for saline tolerance

The overall screening procedure provides a clear view of the dose-dependent reduction in terms of germination percentage, root length, shoot length, fresh weight, dry weight and seedling vigour under both *in vitro* and *in vivo* conditions. As the concentration of salts increased, the reduction percentage also increased invariably in all the genotypes. The increase in saline concentrations showed a nonlinear pattern of inhibitory effect over the genotypes under higher concentrations. The

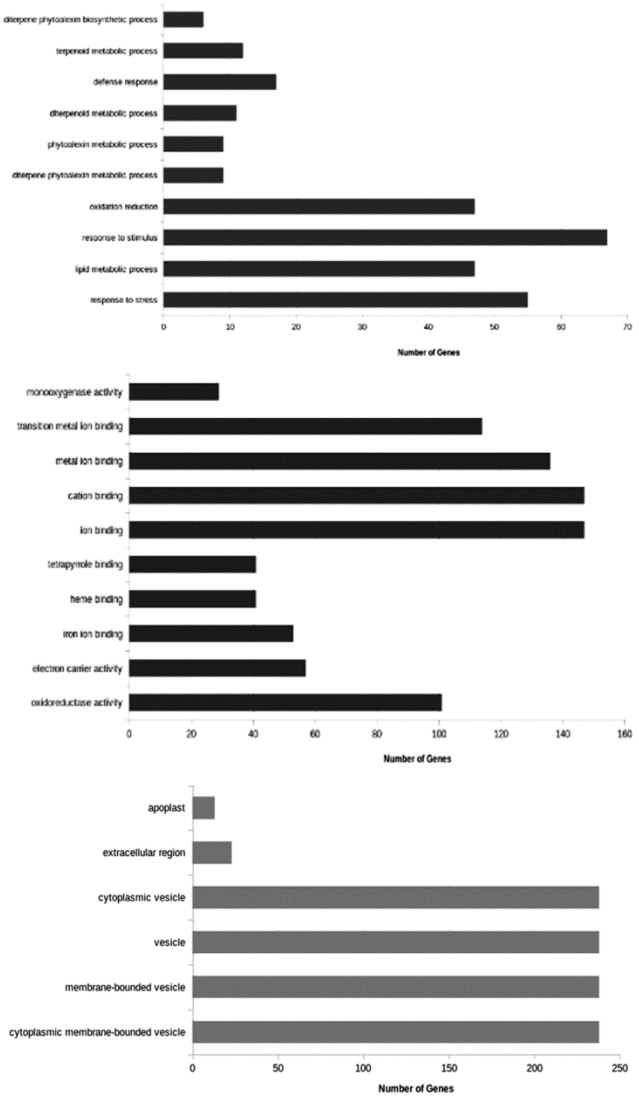

Fig. 2 — Significant mechanisms: Bar graph represents the number of genes involved under each component during differential expression of genes. (A) Number of genes under biological process; (B) Number of genes under molecular function; and (C) Number of genes under cellular components.

Table 3 — Significant genes and their respective pathways

ID	Description	Count	GeneRatio	BgRatio	pvalue	p.adjust
osa00904	Diterpenoid biosynthesis	13	13/165	35/4200	2.3E-10	1.9E-08
osa04016	MAPK signaling pathway - plant	14	14/165	124/4200	0.0003	0.01267
osa00592	alpha-Linolenic acid metabolism	7	7/165	43/4200	0.00124	0.03471
osa00350	Tyrosine metabolism	6	6/165	37/4200	0.00283	0.05951
osa00061	Fatty acid biosynthesis	6	6/165	41/4200	0.00481	0.08088
osa00073	Cutin, suberine and wax biosynthesis	4	4/165	20/4200	0.0068	0.09514
osa00500	Starch and sucrose metabolism	12	12/165	149/4200	0.01335	0.16026
osa00591	Linoleic acid metabolism	3	3/165	15/4200	0.01912	0.18623
osa00940	Phenylpropanoid biosynthesis	14	14/165	195/4200	0.01995	0.18623
osa00902	Monoterpenoid biosynthesis	2	2/165	7/4200	0.02829	0.2236
osa04075	Plant hormone signal transduction	14	14/165	205/4200	0.02928	0.2236
osa00780	Biotin metabolism	3	3/165	19/4200	0.03632	0.25426
osa01212	Fatty acid metabolism	6	6/165	65/4200	0.04097	0.26471

enhanced coefficient of variation showed that with varying salt concentrations, distinct genotypes reacted differently and, on the whole, it was higher with EC 14 followed by EC 12. The results clearly indicated stimulative effect at lower concentrations of salt and ill effects at a higher concentration at both *in vivo* and *in vitro* conditions.

Transcriptome sequencing of *Oryza sativa* under salinity stress

In order to study the underlying mechanism and genes involved in salt tolerance, transcriptome profiling of leaves and root from control and salt-treated (14 days at 8 dSm^{-1}) rice plants were carried out. Comparative transcriptome sequencing resulted in 52.2 million high-quality reads in the control plant and 17.29 million reads in the salt-treated plants. Of the clean read data, 99.63 and 99.37 percent of reads had deciphered quality scores at the Q20 level (an error probability of 0.01) in control and salt-treated respectively. An average of 95.44 percent of the reads aligned onto the reference genome, out of which 92.5 percent reads in control and 92.24 percent reads in the salt-treated plant were uniquely mapped. Assembling the reads generated a total of 55,986 unigenes, of which 30,451 expressed in control and 26,437 in salt-treated. Similar transcriptome profiling was studied in other cultivars of *Oryza sativa*[14,15], *Triticum aestivum*[16], *Zea mays*[14] and *Spartina alterniflora*[17]. The difference in the read counts could account for the differential expression of genes.

Differential expression analysis of unigenes

Further, out of the 27,472 unigenes tested for differential expression between the Control and Salt-treated, 1,013 genes were found significantly expressed. In this research, 462 genes were discovered to be down-regulated, and up-regulated were discovered to be 551 DEUs (Differentially Expressed unigenes). It has been discovered among the DEGs that in past research, a number of genes have been shown to be involved in reacting to salt stress. DEGs characterized as magnesium ion binding (GO:0000287), and peroxidase activity (GO:0004601) were differentiated under salinity stress in our study. The differentially expressed genes identified could also provide references in rice pre-breeding processes for the selection of salt-tolerant breeding materials.

Gene ontology and KEGG pathway enrichment

From the differentially expressed unigenes, a total of 14,407 unigenes were assigned Gene Ontology, mapped with 3,787 genes under Biological process, 3,417 genes under Molecular Function and 7,203 genes under Cellular component. GO enrichment could reflect altered biological processes and molecular functions because of the changes in the expression of DEGs. Among them, 756 genes were found to be significantly enriched in biological processes, 5,950 genes in cellular components and 1,668 genes in molecular function. The majority of unigenes in the Biological process were mapped to "response to stimulus", "response to stress", "oxidation-reduction" and "lipid metabolic process". The majority of unigenes in Molecular Function were mapped to "catalytic activity", "ion binding", "cation binding" and "metal ion binding" and unigenes in Cellular Component were mainly mapped to "cell part", "cell", "intracellular part" and "intracellular organelle", which suggested that these functions were more active under salt stress.

As salinity is a form of abiotic stress, nearly 55 unigenes were found to be classified under "response to stress" and 9 unigenes under "response to abiotic stimulus". In biological processes, "metabolic processes", "cellular processes" and "single-organism processes" were the most abundant entries and under molecular functions, "binding" and "catalytic activity" were the most two abundant catalogs. To understand better the biological function of the genes, an enrichment analysis with KEGG pathway was carried out which assigned 277 genes to 84 biological pathways with the majority of them mapped under "MAPK signaling pathway-plant", "Phenylpropanoid biosynthesis", "Plant hormone signal transduction", "Diterpenoid biosynthesis" and "Starch and sucrose metabolism". Among the KEGG pathways, significantly enriched DEGs were found to map under "Diterpenoid biosynthesis", "MAPK signaling pathway-plant" and "alpha-Linolenic acid metabolism". These pathways were reported to be playing a major role in inducing stress tolerance in plants under salinity conditions. All these results added a note to the saline tolerance mechanism which is prevalent in crops; ion exclusion, tissue tolerance, and somatic tolerance. Almost most of the pathways mentioned had a wider degree of up and down regulations, which may account for energy conversion mechanisms prominent during stress conditions, to provide a dormant state to mitigate the stress conditions.

Identification of salt-stress adaptability and responsive genes

By two primary processes, ion exclusion, and osmotic tolerance, rice plants usually tolerate salt[18]. It

is also possible to further classify these processes into ion exclusion, osmotic tolerance, and tissue tolerance[19]. Ion exclusion mainly involves Na^+ and Cl^- transport processes in roots, which prevent their excess accumulation in leaves. High shoot/root proportions, inherent growth rates, and the lack of an apoplastic root pathway[20] decrease the rate at which salts enter and accumulate in the shoot. Roots exclude most of the Na^+ and Cl^- dissolved in the soil solution, to prevent the salt accumulation in the shoot which becomes so high that it can kill the plants. Osmotic effects due to high salt concentration can reduce the relative growth rate of the shoot. This can lead to a rise in the concentration of shoot ions with rising in salinity that is not due to the rise in the rate of ion uptake. For the same uptake rate, a fast-growing plant usually has a lower concentration in the shoot than a slow-growing plant. Roots operate mainly to protect crops from excessive salt intake and filter most of the salts in the soil while bringing up water[18]. This process is facilitated by ion transporters such as vacuolar Na^+/H^+-antiporters and high- and low-affinity K^+ channels. Osmotic tolerance includes the capacity of the plant to tolerate the salinity stress and preserve leaf development and stomatal behavior[21]. Tissue tolerance involves Na^+ sequestration in the vacuole, synthesis of compatible solutes, and production of enzymes catalyzing reactive oxygen species detoxification. Some serine/threonine protein kinases could be activated by a salt-stress-elicited calcium signal, and then it results in the activation of various ion transporters, such as the plasma membrane Na^+/H^+ antiporter. Three HKT (high-affinity potassium transporter) genes OsHKT1;5, OsHKT1;1, OsHKT2;4, were significantly up-regulated in RNA-Seq analyses. Other Ion transporter families like Ca_2^+ATPases (calcium ATPases), CAXs (cation hydrogen exchangers), and ABC transporter were also up-regulated. Also, a unique potassium channel SKOR (LOC_Os06g14030) was up-regulated. This showed that there was a regulation of stress adaption by means of storing the excess ions in older leaves and maintaining younger leaves at low salt concentrations.

In living organisms, one PP2C gene (LOC Os01g62760) catalyzing proline biosynthesis was discovered, which has been shown to be involved in abiotic stress tolerance[17]. On exposure to salinity, plants exhibit an enhanced level of reactive oxygen species (ROS), superoxide radicals (O^{-2}), hydrogen peroxide (H_2O_2), and hydroxyl radicals (-OH) that can disturb cellular homeostasis resulting in oxidative damage to cellular structures which leads to cell death. Reactive oxygen species scavenging systems may play an important role in alleviating salt stress at the early seedling stage in rice[22]. Also, up-regulated in our research was the unigene connected with ROS scavenging related gene "glutathione S- transferases" (LOC Os01g27210, LOC Os06g12290). The present research discovered up-regulated genes such as the UDP-glucosyl transferase gene (LOC Os02g11640, LOC Os04g12960, LOC Os04g12970) and the endo-1,3-beta-glucosidase gene (LOC Os03g27980). In reaction to salt stress, the IAA homologs were also articulated differently. In our study, the homolog OsIAA9 was found to be significantly upregulated and OsIAA14 was found to be down-regulated.

Identification of functional proteins under salinity stress

A number of functional proteins are expressed differently under salt stress and play the main role in controlling the salt tolerance of plants[23]. Among them AQPs (aquaporins) (LOC_Os01g74450), HSPs (heat shock proteins), LEA (late embryogenesis abundant group 1) (LOC_Os06g21910) proteins, F-box proteins (OsFBX322, OsFBX384, OsFBX19, OsFBX40, OsFBX62), sugar transporter (LOC_Os02g17500), electron transport (LOC_Os04g51150) and nucleotide sugar inter-conversion (LOC_Os05g51670) are important. Other stress-responsive proteins like OsRCI2-5 (low temperature and salt responsive protein), and sucrose phosphate synthase (LOC_Os01g69030) were also found to be upregulated.

Identification of significantly different transcription factors

Transcription factors are integral to linking salt sensory pathways to several tolerant responses since they regulate the level of expression of different genes that ultimately affect the level of salt tolerance of plants[24]. In this study, MYB-related TFs, WRKY115, bHLH, C_2H_2 zinc finger protein (ZOS3-10, ZOS3-21), zinc C3HC4 family protein, and WD-40[16] are known to be up-regulated in the salt stress response, whereas AP2-like ethylene-responsive transcription factor PLETHORA 2 was down-regulated[14]. Significant transcription factors including homeobox-associated leucine zipper (LOC_Os04g45810) and HSF-type DNA-binding domain-containing protein (LOC_Os01g39020, LOC_Os01g53220, LOC_Os08g43334) also were found to be upregulated in the present study.

In the present study, many TFs previously identified as important candidate genes for rice salt stress breeding, such as two HD-IP genes (LOC Os06g46740 and LOC Os02g43330), two MYB genes (LOC Os01g54030 and LOC Os08g39730) were found highly up-regulated. Other proteins like AWPM-19-like membrane family protein (LOC_Os05g31670), pre-mRNA-splicing factor SF2 (LOC_Os01g21420), and eukaryotic peptide chain release factor subunit 1-1 (LOC_Os05g31020), found with significant up-regulation under salt stress in the present study, were also reported to serve as good candidate genes for future salt stress tolerance breeding. Rice plant susceptibility or tolerance to elevated salinity is a coordinated action of various stress-responsive genes that also interact with other stress signal transduction pathways elements.

Conclusion

Salt tolerant varieties can be produced by marker-assisted selection or genetic engineering through the introduction of genes for salt tolerance. Since salinity stress tolerance is regulated by various genes, it is necessary to introgress genes that can operate in tandem at both root and leaf concentrations. Field assessment of varieties that were produced at the laboratory is needed, particularly in varying salt levels and distinct areas as varieties tend to perform differently under controlled circumstances rather than under field circumstances. The perfect variety tolerant for salinity should have high Na^+ tolerance, be able to regulate Na^+ uptake and maintain high K^+ uptake, excellent original vigor, agronomically superior with elevated yield potential. The study indicated that most of the pathways and gene expressions were genotype-specific. The major mechanism of saline tolerance exhibited by the genotype under study was through ion exclusion through activation various ion transportation related genes. The data mobilized in the present study can support future studies in the understanding of perspectives of gene ontology and transcriptional regulation and networking pathways for salinity tolerance. Hence, this genotype could well be utilized to impart saline tolerant genes during the seedling stage, which is normal saline susceptible to the agronomically superior rice genotypes, which can also assist in transferring saline tolerance to high yielding rice varieties.

Conflicts of interest

Authors declare no competing interests.

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