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ORIGINAL ARTICLE

Identification of Key Genes Related to Salinity Stress in Rice (*Oryza sativa*) Using Bioinformatics Analysis

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ABSTRACT
Salinity stress is the main cause of crop failure. In this study, we explored and identified the key genes related to the salt stress of Oryza sativa L. by computational bioinformatics analysis of gene expression. A diverse no. of differential expression genes (DEGs) has been identified from GSE58603, P 0.05 and]log FC > 1.0. Gene ontology (GO) and pathway enrichment analysis were performed using ShinyGO 0.80, a gene data set analysis tool. GO theories are divided into molecular function (MF), cellular components (CC), signalling pathways, and biological processes (BP). In addition, 10 hub genes have been identified that can play a role in the development of salt stress. Therefore, we hope that our research will inspire interest and encourage further studies into genes and pathways that may contribute to rice salt tolerance.
Keywords: *Differential expression gene (DEG), Hub Genes, Interactome analysis, KEGG, Rice plant, Salinity Stress.*

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INTRODUCTION

Plant growth is notably altered by salt stress, a critical abiotic stress factor. Rice (*Oryza sativa* L.), a moderately salt-sensitive crop, serves as a primary food source for over half of the world's population [1, 2]. The effects of salt stress on rice include reduced germination, decreased growth rates, and diminished tillering, which subsequently impact biomass and plant height. Salt stress also results in lower grain yield by reducing thousand-grain weight, panicle number, and setting percentage. Furthermore, rice grade is adversely affected by salt stress, as evidenced by diminished tasseling of grains, quality, and elemental makeup [3,4]. Consequently, elucidating the molecular mechanisms underlying salt tolerance in rice and identifying key salt-tolerance genes are crucial steps in developing robust, salt-tolerant rice varieties through breeding programs. At the molecular level, saline conditions induce ionic toxicity, osmotic stress, and oxidative damage. Ionic toxicity typically results from the excessive accumulation of sodium and chloride ions under high-salt conditions. This phenomenon leads to sodium accumulation in the cytoplasm, which disrupts membrane potential and facilitates potassium efflux from the cell, ultimately resulting in plant mortality [5]. Research has demonstrated that the potassium transporter OsHAK1 enhances potassium uptake and improves the potassium-to-sodium ratio under salt stress, rendering it crucial for increased salt resistance in rice plants [6]. In reaction to osmotic stress induced by elevated salinity levels, plants synthesize significant amounts of osmotic adjustment substances, including proline, betaine, glycerol, and a range of sugars along with their derivatives [7]. The oxidative stress that arises from salt exposure increases the activity of various antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX). Additionally, this stress stimulates the synthesis of non-enzymatic antioxidants, including ascorbic acid, glutathione, and carotenoids [8]. As a result, the mechanisms that confer salt tolerance are characterized by a complex regulatory framework that integrates the diverse range of metabolic pathways. The ongoing advancement of sequencing technology has enabled research utilizing transcriptome sequencing data to investigate the metabolic pathways linked to plants reactions to salt stress, as well as to pinpoint candidate genes

associated with salt tolerance. Additionally, the mechanisms of salt tolerance in rice have been examined through the application of transcriptome sequencing technology. So, in this study, we aimed to explore and identify the key genes connected to salinity stress of *Oryza* by using the computational bioinformatics analysis of gene expression. GSE58603[9], a gene expression profile data has been acquired from the "Gene Expression Omnibus (GEO)". The collected data comprises of root tissue of 4 days old seedlings treated with NaCL (140mM), 4 days old seedlings treated with NaCL (140mM) along with ABA (10μM), and the control set of no treatment. The seedlings were 3 leaf stage and sourced from salinity tolerant introgression line under IR64 genetic background. The reason behind choosing IR 64 is it is widely consumed rice and several neo salt tolerant breed have been developed and more popular than the wild type. Since the initiation and accumulation of salt ions happen to the roots first than other parts of the plants, we investigate the root tissue. The marking of 'Differential Expression Genes (DEGs)'' between the treated samples and control has been carried out using GEO2R tool. The DEGS result indicates more and less similar amount of upregulated and down regulated genes, having only very few numbers of overlapping genes. The upregulated genes and their protein-protein interactions further reveals the significant hub genes. The 10 hub genes are identified and their corresponding metabolic pathways exhibited significant enrichment and likely involved in the endurance towards salinity stress in *Oryza sativa* L.

MATERIAL AND METHODS

Retrieval of Gene Expression Datasets

The gene expression datasets were obtained from the GEO database (https://www.ncbi.nlm.nih.gov/geo). Salinity stressed *Oryza* sample were chosen as specimens for evaluation on the basis of their ability to more precisely reflect the actual variance in expression of genes in case of salinity stress. Following careful assessment, the series GSE58603 was selected. The data were readily accessible on the GEO database.

Data Analysis of DEGs

The GEO2R an online analytic tool was implemented to identify DEGs between the three samples from salt stressed *O. sativa* seedlings and the three samples from healthy individuals. The tool identified the P value and absolute log fold change (|log FC|) of each gene. Genes which met the specified criteria, with a significance level P-value< 0.05 and |log FC| ≥ 1.0, were classified as DEGs. Upregulated Genes were identified by P-value< 0.05 &|log FC| ≥ 1.0. Conversely, downregulated genes are identified by P-value< 0.05 & |log FC| ≤1.0.

Gene Functional and Pathway Enrichment Analysis

For the gene functional and pathway enrichment analysis, the ShinyGO 0.80 [10] (http://ge-lab.org/go/), a gene data set analyser tool was used to perform "Gene Ontology (GO)'' analysis. The further pathway enrichment was done using "Kyoto Encyclopedia of Genes and Genomes (KEGG)''. This analysis reveals the genes involved in biological processes as well as in the cellular functions.

Building A PPI Network

We utilised the STRING (https://string-db.org/) [11] to analyse PPI data. In order to evaluate the potential PPI link for our unique goals, we mapped the DEGs which were identified in the STRING database. PPI pairings were extracted selecting combined score threshold of >0.7. Resulting PPI network was visualised by the Cytoscape tools [12].

Detection of Key Genes

The centrality metrics suggested above may recognize the genes in a complex PPI network that have the most impact. These genes are able to swiftly pass along and receive information, and are highly responsive to both local and global changes. frequently it can also serve as a means for recognizing key genes. The computation of centrality parameters was performed using Cytoscape plugins, specifically cytoHubba and Network Analyzer.

RESULT AND DISCUSSION

Differential Gene Expression Analysis

GSE58603 consists of three samples from 4 days old salt stressed *O. sativa* seedlings and three samples from control and three samples from 4 days old salt-ABA stressed *O. sativa* seedlings and three samples from control with no treatment. A total of 2456 DEGs were discovered from control vs salt treated samples, whereas 7074 DEGs have been found from control vs salt-ABA treated samples, both analysed with P < 0.05 and |log FC| > 1.0 (Table 1). At least 18% of highly expressed genes during salt stressed are getting down regulated during the ABA combined saline condition (Figure 1). Among these DEGs, 250 were key upregulated and 134 were key downregulated in case of salt treatment found, rests were

overlapping or non-significant (Figure 2(a)). The salt along with ABA treated samples showed 172 upregulated gene and 334 down regulated key genes (Table 2) along with large no of nonsignificant other DEGS (Figure 2(b)). The DEGs involved analogized samples obtained from salt stressed *O. sativa* seedlings to samples obtained from healthy seedlings. The study revealed there is a peculiarly that higher amount of key upregulated genes in case of only salt treatment than the salt-ABA combined treatment, whereas the result of down regulated genes is completely contrasting.

Functional Enrichment Analyses of DEGs

ShinyGO 0.80, was utilised for conducting KEGG pathway enrichment and GO function studies for DEGs. The graphical representation of GO Function enrichment was plotted using the TBtool online tool. The enhanced GO theories are categorised into "Biological Process" (BP) (Figure 3), "Cellular Component (CC)" (Figure 4), "Molecular Function" (MF) (Figure 5), and "Signalling pathways" (Figure 6). The upregulated genes in combined treatment showed significant response to jasmonic acid pathway but there is no such response with only salt treatment. The molecular function involves ferredoxin NADP reductase activity during combined stress but lacks completely during only saline condition. Also, during combination stress the upregulated genes get involved in nitrogen metabolism, whereas single treatment resulting genes get involved more in biosynthesis of secondary metabolites. All these evidences indicate combination of stress is more detrimental for the system so the genes get involved in more practical activities to thrive.

Constructing A PPI Network and Identifying Hub Genes

The PPI of the DEGs were predicted and retrieved using the STRING online too. The PPI network included nodes, as shown in Figure 7. The top 10 hub genes for salt treatment only, identified by their degree of connectedness in the PPI network, are listed in Table 3. CytoHubba, a plugin for Cytoscape, aids in identifying these genes by quantifying their degree in the PPI network (Figure 8(a)) and arranging them in order of connectivity. The findings indicated that CCR1 exhibited the highest gene connection degree of 6.

Another top 10 hub genes for salt-ABA combination treatment have been identified by their degree of connectedness in the PPI network, are listed in Table 4. With the help of CytoHubba, a plugin for Cytoscape, aids in identifying these genes by quantifying their degree in the PPI network (Figure 8(b)) and arranging them in order of connectivity. The findings indicated that NIA1 exhibited the highest gene connection degree of 10.

The study reveals the more interacting genes are involved in case combination than single treatment.

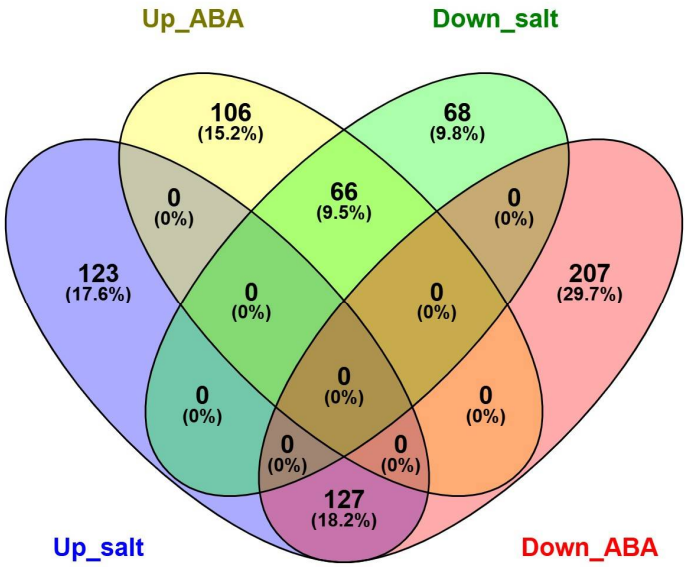

Figure 1: Venn diagram showing both up and down regulated genes

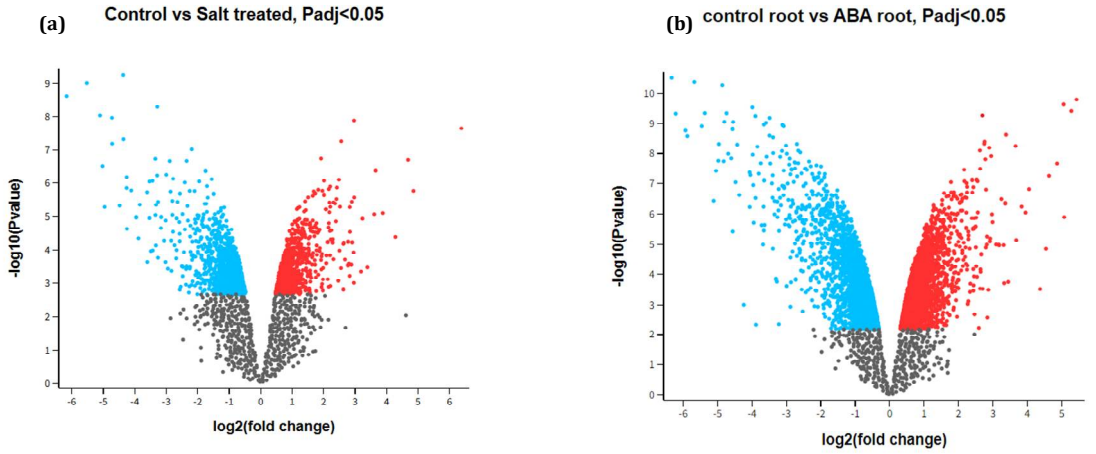

Figure 2: Volcano plot displaying all Differentially Expressed Genes (DEGS) related to salinity stress. Colour codes: Red: Upregulated genes; Blue: Downregulated genes; Grey: Overlapping genes

(a) Volcano plot of control vs salt treated samples

(b) Volcano plot of control vs salt-ABA treated samples

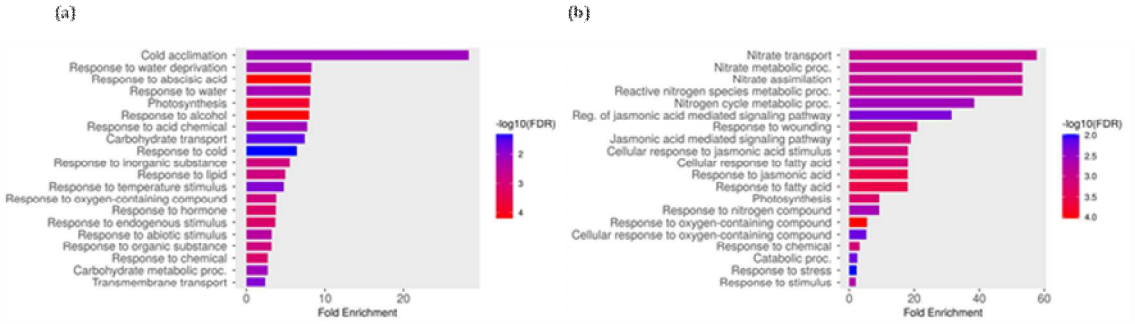

Figure 3: Involved DEGs in the biological function; x axis- biological function, y axis-fold enrichment

(a) control vs salt treated samples

(b) control vs salt-ABA treated samples

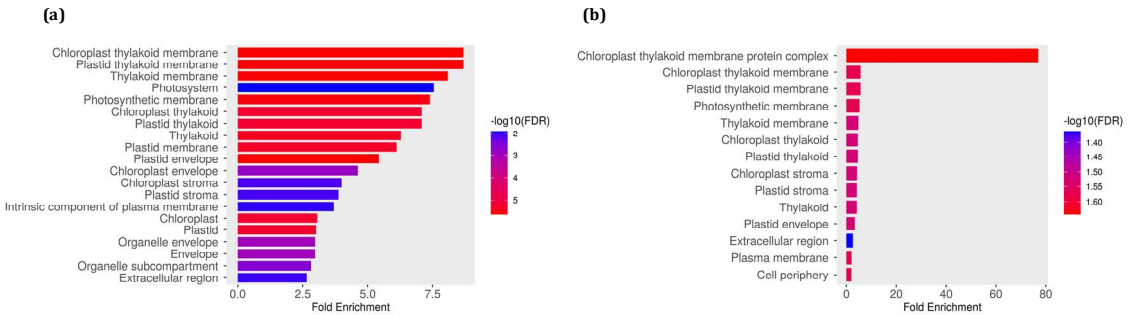

Figure 4: Involved DEGs in the cellular component; x axis- cellular component, y axis-fold enrichment

(a) control vs salt treated samples

(b) control vs salt-ABA treated samples

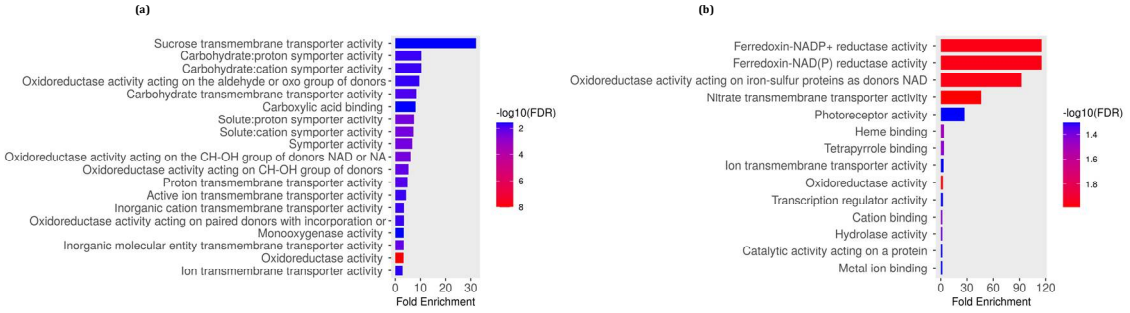

Figure 5: Involved DEGs in the molecular function; x axis- molecular function, y axis-fold enrichment

(a) control vs salt treated samples

(b) control vs salt-ABA treated samples

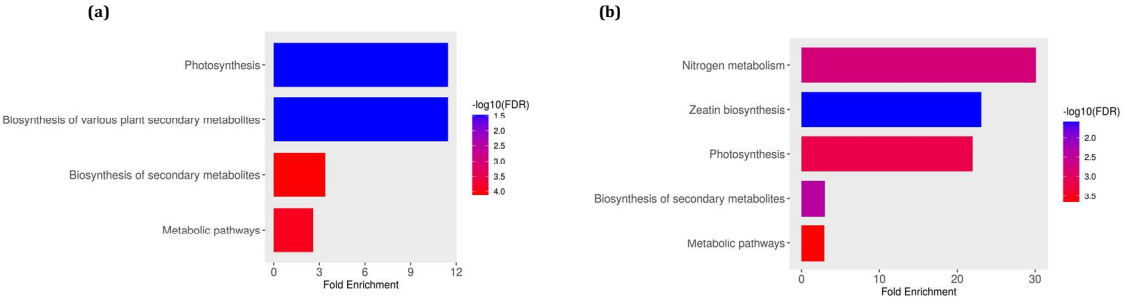

Figure 6: Involved DEGs in the KEGG signalling pathway; x axis- KEGG signalling pathway, y axis-fold enrichment

(a) control vs salt treated samples

(b) control vs salt-ABA treated samples

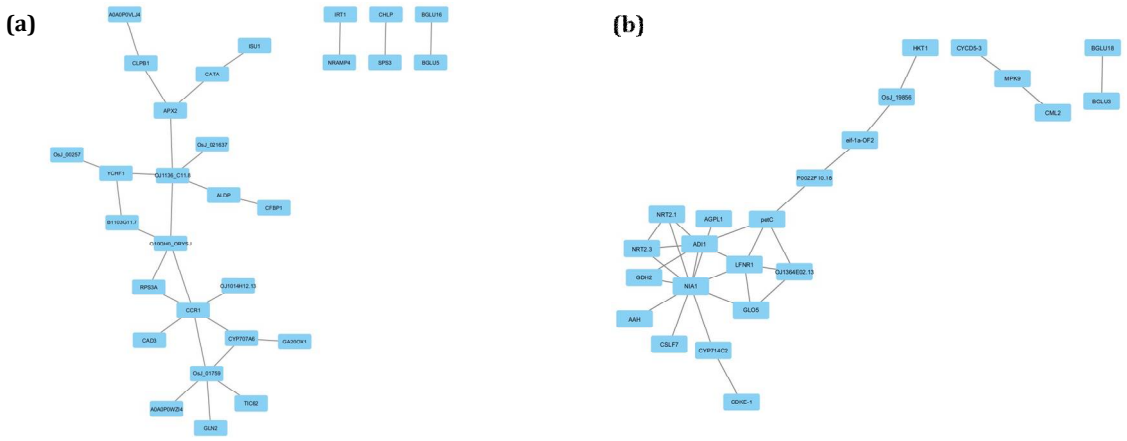

Figure 7: PPI Network of up regulated DEGs: Cytoscape image

(a) control vs salt treated samples

(b) control vs salt-ABA treated samples

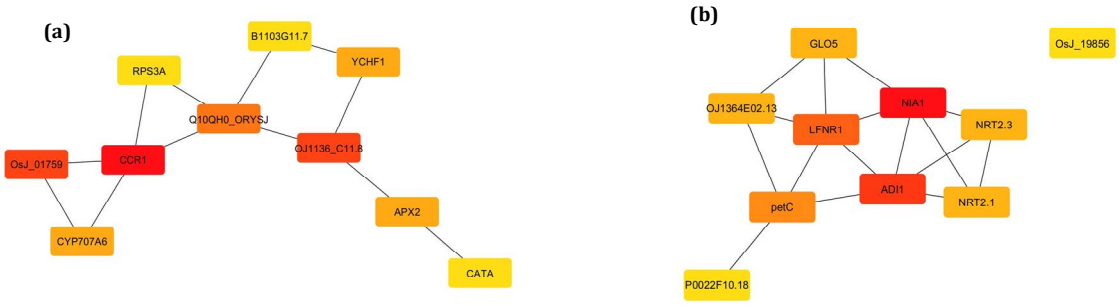

Figure 8: Top 10 upregulated hub genes with highest connectivity degree in PPI network
(a) control vs salt treated samples
(b) control vs salt-ABA treated samples

Table 1: Top 10 upregulated and down regulated genes respectively from control vs salt treated samples

ID	Gene title	Log FC	Gene Expression
Os.56004.1.S1_at	bidirectional sugar transporter SWEET12	6.162566	Up-Regulated
Os.12703.1.S1_at	polyol transporter 5	5.521799	Up-Regulated
Os.3280.1.S1_at	tetratricopeptide repeat protein SKI3	4.955772	Up-Regulated
Os.10497.1.S1_s_at	protein TsetseEP	4.716642	Up-Regulated
Os.9820.1.S1_at	dehydrin Rab16D	4.477715	Up-Regulated
Os.6274.1.S1_at	non-specific lipid-transfer protein 2	4.368443	Up-Regulated
Os.54579.1.S1_x_at	protein TsetseEP	4.358486	Up-Regulated
Os.12633.1.S1_at	water stress-inducible protein Rab21	4.255734	Up-Regulated
Os.12633.1.S1_s_at	water stress-inducible protein Rab21	4.110065	Up-Regulated
Os.16615.1.S1_at	Os10g0569800	3.881434	Up-Regulated
Os.54812.1.S1_at	endoglucanase 23	-1.00189	Down-Regulated
Os.7934.2.S1_at	AT-hook motif nuclear-localized protein 9	-1.00351	Down-Regulated
Os.9533.1.S1_at	subtilisin-like protease SBT1.2	-1.00647	Down-Regulated
Os.54508.1.S1_s_at	protein IQ-DOMAIN 1	-1.00661	Down-Regulated
Os.49544.1.S1_at	fasciclin-like arabinogalactan protein 2	-1.00738	Down-Regulated
Os.15501.1.S1_at	Putative disease resistance protein RGA3	-1.01311	Down-Regulated
Os.9637.1.S1_at	proteoglycan 4	-1.0152	Down-Regulated
Os.17180.1.S1_at	cytochrome P450 78A9	-1.01979	Down-Regulated
Os.14530.2.S1_x_at	GDSL esterase/lipase EXL3	-1.02655	Down-Regulated
Os.23030.1.S1_at	NAC domain-containing protein 83	-1.02883	Down-Regulated

Table 2: Top 10 upregulated and down regulated genes respectively from control vs salt-ABA treated samples

ID	Gene title	Log FC	Gene Expression
Os.49093.2.S1_x_at	high-affinity nitrate transporter 2.1	5.07289391	Up-Regulated
Os.27794.1.S1_at	alpha-humulene synthase	4.8655631	Up-Regulated
OsAffx.15973.1.S1_at	receptor like protein kinase S.2	4.63204142	Up-Regulated
Os.2881.1.S1_at	proline dehydrogenase 2, mitochondrial	3.83633766	Up-Regulated
Os.6043.1.S1_at	transcription factor bHLH35	3.68045666	Up-Regulated
Os.27297.1.A1_s_at	probable LRR receptor-like serine/threonine-protein kinase At1g56130	3.65975485	Up-Regulated
Os.36104.1.S1_at	glycerophosphodiester phosphodiesterase GDPD1, chloroplastic	3.38302368	Up-Regulated
Os.46849.1.S1_at	protein TIFY 11e	3.24941857	Up-Regulated
Os.49634.1.S1_x_at	dynein light chain LC6, flagellar outer arm	3.18221955	Up-Regulated
Os.42069.1.S1_x_at	dynein light chain LC6, flagellar outer arm	3.17229139	Up-Regulated
Os.32126.2.S1_at	protein TsetseEP	-1.00475	Down-Regulated
Os.18217.1.S1_s_at	pectin acetylesterase 12	-1.00678	Down-Regulated
Os.28079.1.S1_x_at	amino acid permease 6	-1.00812	Down-Regulated
Os.48013.1.A1_at	peroxygenase-like	-1.00928	Down-Regulated
Os.46533.1.S1_at	ent-cassadiene C2-hydroxylase-like	-1.01091	Down-Regulated
Os.51692.1.S1_at	protein ALTERED XYLOGLUCAN 4-like	-1.01817	Down-Regulated
Os.11553.1.S1_at	peroxidase 5	-1.02297	Down-Regulated
Os.53318.1.S1_at	7-deoxyloganetin glucosyltransferase	-1.03507	Down-Regulated
Os.37531.1.S1_at	protein trichome birefringence-like 14	-1.04636	Down-Regulated
Os.48076.1.S1_at	protein trichome birefringence-like 14	-1.0466	Down-Regulated

Table 3: 10 upregulated hub genes and their score for salt treated samples

Rank	Gene Name	Score
1	CCR1	6
2	OsJ_01759	5
2	OJ1136_C11.8	5
4	Q10QH0_ORYSJ	4
5	YCHF1	3
5	CYP707A6	3
5	APX2	3
8	CATA	2
8	B1103G11.7	2
8	RPS3A	2

Table 4: 10 upregulated hub genes and their score for salt-ABA treated samples

Rank	Gene Name	Score
1	NIA1	10
2	ADI1	6
3	LFNR1	5
4	petC	4
5	NRT2.1	3
5	GLO5	3
5	OJ1364E02.13	3
5	NRT2.3	3
9	P0022F10.18	2
9	OsJ_19856	2

CONCLUSION

This research investigates the distinct fundamental genes linked to the initiation of salinity stress. A total of ten hub genes respectively found both in combination and single treatment, have been identified, which may function key role in the development of salt stress. However, due to the limitations outlined in this study, further investigation is necessary. Consequently, we hope that our findings will inspire interest and encourage further studies into the identified genes and pathways as potential contributors to salinity stress in rice.

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