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Revealing Potential Regulatory Genes and Modules Involved in Salt Tolerance using a Comparative Microarray Analysis of Rice at Seedling Stage

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ABSTRACT: Deciphering the regulatory mechanisms by which plants cope with salt stress is an indispensable prerequisite for the identification of the regulators to engineer salinity tolerance. To this end, a comparative transcriptome analysis of the extremely tolerant and extremely sensitive recombinant inbred lines bulks derived from a cross between salt-tolerant variety CSR 27 and salt-sensitive variety MI 48was carried out using microarray data analysis, Gene Ontology (GO) enrichment and network analysis. According to the results, 50 and 286 genes showed differential expression in the tolerant bulk as compared with the sensitive bulk under control and salt stress conditions, respectively. The most enriched GO terms of differentially expressed genes (DEGs) under salt stress were the metabolic process, the cellular process, localization (transport) and response to the stimulus. Network analysis of DEGs under salt stress, revealed 14 genes as critical genes; among them, three transcription factors (TFs) were notable. Additionally, according to gene module analysis, these TFs interact with each other and make a distinct gene module (cluster 7) which significantly enriched "plant hormone signal transduction" pathway. To the best of our knowledge, the role of these TFs in salt tolerance in rice has not been reported previously. The fundamental knowledge gained from this research can be further exploited toward enhancing salt tolerance of plants.

Keywords: network analysis, *Oryza sativa*, salt stress, transcription factors

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INTRODUCTION

Rice (*Oryza sativa* L.) being the most salt sensitive monocot, the accumulation of salt in the soil adversely affects the yield production across the world (Wang *et al.,* 2012). The salinity tolerance in rice is the quantitative traits, and a myriad of molecular, biochemical, and physiological processes are involved in the mechanism of salt tolerance. Identification of key genes and clarifying molecular mechanisms of complex salt tolerance trait in rice are essential prerequisites for improving the worldwide food production. Despite tremendous research efforts on salt tolerance of rice, a few studies have addressed the molecular mechanism responsible for the tolerance of rice plants to salt stress (Li *et al.,* 2017). However, comparative transcriptome analysis of genotypes with contrasting salt tolerance is an effective strategy for identifying factors such as metabolites, proteins, and genes which can be exploited by traditional breeding and genetic engineering approaches for improvement of salinity tolerance in plants (Hossain *et al.,* 2016).

Gene expression analysis based on microarray technology is considered as a high-throughput and tremendous approach for discovering many suitable candidate genes which played a critical role in tolerance mechanisms at the molecular level (Zinati, 2017; Mishra *et al.,* 2013). In the current study, as a first step, the differential expression analysis was performed to detect the genes that are significantly differentially expressed in the tolerant bulk as compared with the sensitive bulk under salt stress and control conditions. Then, aiming to and explore the biological significance of differentially expressed genes (DEGs) and some key genes and gene modules, DEGs were subjected to Gene Ontology (GO) enrichment and network analysis.

Some key genes and pathways closely associated with salt tolerance were screened by conducting a series of bioinformatics analysis on DEGs. Given the important roles of transcription factors (TFs) and protein kinases (PKs) as master regulators of a large spectrum of downstream stress-responsive genes (Wang *et al.,* 2016), we focused on genes which encodethem. This comparative transcriptome analysis has provided new insights on genes and regulatory mechanisms controlling salinity tolerance in rice at the seedling stage.

MATERIALS AND METHODS

A. Microarray analysis

Microarray dataset of the extremely tolerant and extremely sensitive recombinant inbred lines (RILs) bulks derived from a cross between salt-tolerant variety CSR 27 and salt-sensitive variety MI 48 submitted by Pandit *et al.* (2010) (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ftwlhksemcguepa&acc=GSE16108) was utilized to screen DEGs between the extremely tolerant and extremely sensitive RILs bulks.

Gene vestigator was applied to differential expression analysis. Genevestigator database contains thousands of public microarray and RNAseq experiments that are manually curated and well described. Genevestigator visualizes gene expression profiles in various biological contexts such as nutrients, genotypes, diseases, chemicals, tissues, or biotic and abiotic stress conditions (https://genevestigator.com/gv/doc/intro_plant.jsp).

Affymetrix CEL files in Genevestigator are normalized by the Bioconductor Robust Multi-array Averaging (RMA) implementation (Gentleman *et al*., 2004) for the experiment level, and are also adjusted with an inter-experiment correction. The *Diff-Expression* tool embedded in Genevestigator enables to find the significantly differentially expressed genes between two conditions. In the Data Selection dialogue box, "GSE16108: Transcription profiling of parental lines and bulked salt sensitive and salt tolerant RILs derived from 2 rice varieties" was searched in experiments part and selected. In the Define Comparison dialogue box, the samples for groups X and Y was defined as "tolerant RILs stress/ sensitive RILs stress" as well "tolerant RILs control/ sensitive RILs control". Then, the upregulated genes, with a log2 fold change greater than 0.6 and false discovery rate (FDR) less than 0.01, and down regulated genes, with a log2 fold change less than 0.6 and FDR less than 0.01, were screened for further analyses.

B. Discovery of transcription factors and kinase genes among DEGs

With the goal of specifying those genes encoding TFs and PKs the DEGs were searched against the plant genes in the Plant Transcription factor & Protein Kinase Identifier and Classifier, iTAK (http://bioinfo.bti.cornell.edu/cgi-bin/itak/index.cgi) and the Plant Transcription Factor Database (PlantTFDB) (http://plntfdb.bio.uni-potsdam.de/v3.0/).

C. GO enrichment analysis

To make some meaningful biological inference from DEGs, GO enrichment analysis was undertaken using the AgriGO web-based tool (Tian *et al.,* 2017), available from http://bioinfo.cau.edu.cn/agriGO/index.php. The Singular Enrichment Analysis (SEA) was done setting 'Rice TIGR gene model' as a reference, 'Bonferroni' as multi-test adjustment method, 'Chi-square as a statistical test method, 0.05 as p-value cut-off and '10' as the minimum number of mapping entries. After that, the DEGs were annotated and defined according to the GO terms under the biological process, molecular function and cellular component categories.

D. Gene network analysis

The Search Tool for the Retrieval of Interacting Genes/Proteins, STRING 10.5 (http://www.mybiosoftware.com/string-9-0-search-tool-retrieval-interacting-genesproteins.html) (Szklarczyk *et al.,* 2014) database was used to acquire PPIs of DEGs. The database makes relations according to several lines of evidences: co-expression data from the NCBI Gene Expression Omnibus database, the empirical evidence from protein–protein interaction assays, coexistence of the genes in the same organisms, conserved gene neighborhood in known genomes, the extraction of information from other databases, pathway annotation from other resources such as the Kyoto Encyclopedia of Genes and Genomes or GO databases, gene fusion events, and automated text-mining tools (Szklarczyk *et al.,* 2014). STRING calculates a confidence value for those interactions based on the pieces of evidences from above 0.4 to 0.9, as the medium to highest score, respectively. In the current study, protein-protein interactions (PPIs) were predicted with a minimum required interaction score of 0.7 (high confidence).

E. Discovery of critical genes and gene modules

All PPIs in the network were loadedinto Cytoscape software, version 2.5.0 (Shannon *et al.* 2003). Then, Cyto-Hubba, a Cytoscape Plug-in for Hub Object Analysis in Network, used to find key genes by a variety of topological analysis algorithms, *i.e.*, Bottleneck(BN), Closeness centrality, Clustering coefficient, Betweenness centrality, Degree, EcCentricity, Edge Percolated Component (EPC), Maximal Clique Centrality (MCC), Density of Maximum Neighborhood Component (DMNC), Maximum Neighborhood Component (MNC), Stress centrality, Radiality centrality, (Chin, *et al.,* 2014).

At last, to extract important gene modules with similar expression patterns, module analysis was conducted using plug-in Molecular Complex Detection (MCODE) in Cytoscape software. Then, GO term and pathway enrichment analysis were carried out to probe the biological significance of the detected gene modules.

RESULTS AND DISCUSSION

A. Microarray analysis

Under control conditions, there were 50DEGs consisting 46 significantly upregulated DEGs and 4 down regulated DEGs in the tolerant bulk as compared with the sensitive bulk, while the corresponding numbers of up regulated and down regulated genes under salt stress were 199 and 87, respectively.

The transcriptome profiles of bulked RNA extracted from ten most tolerant and ten most sensitive RILs were used in order to reduce background and consequently the number of DEGs (Pachauri *et al.,* 2014). Microarray analysis revealed that more number of genes were differentially expressed in the tolerant bulk as compared with the sensitive bulk upon exposure to stress compared to that of under control condition. These findings are implying that salt tolerance is very complicated trait and is a result of organized expression of a high number of genes. Tolerant RILs bulk utilizes its adaptive mechanism via a very large number of genes which are induced or repressed under salt stress while they are not activated under other conditions (Hossain *et al.,* 2016).

B. Discovery of transcription factors and kinase genes

Discovery of transcription factors and kinase genes among the DEGs under salt stress condition. Eight DEGs including LOC_Os10g25230 (ZIM domain containing protein), LOC_Os03g08330 (ZIM domain containing protein), LOC_Os04g23550 (basic helix-loop-helix family protein), LOC_Os08g42470 (BEE 1), LOC_Os07g31450 (CHR4/MI-2-LIKE, putative, expressed), LOC_Os08g40430 (mTERF domain containing protein, expressed), LOC_Os02g43790 (ethylene-responsive transcription factor), and LOC_Os03g06630 (heat stress transcription factor) showed similarity to transcription factors falling under various families such as Tify, bHLH, PHD, AP2/ERF-ERF, mTERF, HSF. Four DEGs including LOC_Os11g46860 (protein kinase domain containing protein), LOC_Os01g61620 (protein kinase family protein, putative), LOC_Os01g10890 (CBL-interacting protein kinase 5), and LOC_Os09g18594 (Serine/threonine-specific receptor protein kinase-like) showed similarity to kinase genes belonging to RLK-Pelle_WAK, CMGC_DYRK-PRP4, CAMK_CAMKL-CHK1, RLK-Pelle_LRR-I-1 groups. Under salt stress, all the identified transcription factors except LOC_Os03g06630 were upregulated. Also, among detected kinase genes, LOC_Os11g46860 and LOC_Os01g61620 were upregulated while LOC_Os01g10890 and LOC_Os09g18594 were down regulated under salt stress.

Identification of transcription factor and protein kinase genes as key modulators of signaling pathway among DEGs *may* open a new vista for a better understanding of the regulatory mechanism of salt tolerance. Additionally, due to the importance of TFs and kinase genes as a valuable means for the engineering of salinity-tolerant crop plants (Huang *et al.,* 2015; Hong *et al*., 2016; Li *et al*., 2016; Hichri *et al*., 2017) identification of the genes encoding these proteins would be of great significance for transferring them into plants to boost their stress tolerance.

Eight DEGs showed similarity to transcription factors falling under various families such as Tify, bHLH, PHD, AP2/ERF-ERF, mTERF, HSF. In accordance with these results, the meta-analysis of *potential candidate salinity tolerance associated genes* in rice uncovered differentially expressed transcription factors belonging to gene families including AP2-EREBP, AUX/IAA, bZIP, C2H2, bHLH, C3H, HB, HSF, MYB, MYB-related, NAC (Kaur *et al*., 2016).

Six out of eight detected transcription factors including LOC_Os10g25230 (ZIM domain containing protein), LOC_Os03g08330 (ZIM domain containing protein), LOC_Os04g23550 (basic helix-loop-helix family protein), LOC_Os07g31450 (CHR4/MI-2-LIKE, putative, expressed), LOC_Os02g43790 (ethylene-responsive transcription factor, putative, expressed), LOC_Os03g06630 (heat stress transcription factor) and three out of four detected kinase genes including LOC_Os11g46860, LOC_Os01g61620, LOC_Os01g10890 were involved in the PPIs network.

Moreover, all detected kinase genes and one transcription factor, LOC_Os02g43790 (ethylene-responsive transcription factor, putative, expressed), were also among DEGs which enriched biological process category ‘signal transduction’. Transcription factors belonging to the ERF family have been also reported to participate in biotic and abiotic (Cheng *et al.* 2013) stress responses (Dey *et al*., 2015). It has been reported that LOC_Os02g43790 (ERF 91) displayed a reduction in the expression along the time of exposure to salt stress conditions, however at 24 h a slight increase was observed (Pegoraro *et al.,* 2013).

Discovery of transcription factors and kinase genes among the DEGs under control condition. Three DEGs including LOC_Os01g01470 (no apical meristem protein) belonging to NAM, ATAF, and CUC transcription factors (NAC) and LOC_Os10g28340 (heat stress transcription factor) and LOC_Os03g06630 (heat stress transcription factor) belonging to heat shock factor (HSF) showed similarity to transcription factors.

All identified transcription factors except LOC_Os01g01470 were down regulated. No DEGs showed homology to kinase genes. LOC_Os03g06630 (heat stress transcription factor) was a common transcription factor under both salt stress and control conditions showing differential expression in the tolerant bulk as compared with the sensitive bulk. Based on our microarray data analysis, LOC_Os03g06630 expression level was decreased in the tolerant bulk as compared with the sensitive bulk under both salt and control conditions. However, the amount of decrease in the expression level of Os03g06630 under salt condition was lower than control condition, supporting its role in conferring the drought tolerance.

C. GO enrichment analysis

GO enrichment analysis was utilized to gain a deeper insight into the meanings of underlined biological, molecular and cellular components from DEGs in the tolerant bulk as compared with the sensitive bulk, under salt stress and control conditions.

GO enrichment analysis of the DEGs under salt stress condition. The major biological processes overrepresented under stress conditions are response to endogenous stimulus (GO:0009719) (16 genes), secondary metabolic process (GO:0019748) (12 genes), generation of precursor metabolites and energy (GO:0006091) (30 genes), response to abiotic stimulus (GO:0009628) (12 genes), translation (GO:0006412) (44 genes), response to stimulus (GO:0050896) (41 genes), response to stress (GO:0006950) (33 genes), photosynthesis (GO:0015979) (11 genes), gene expression (GO:0010467) (61 genes), biosynthetic process (GO:0009058) (90 genes) cellular biosynthetic process (GO:0044249) (76 genes), cellular protein metabolic process (GO:0044267) (49 genes), cellular process (GO:0009987) (135 genes), signal transduction (GO:0007165) (10 genes), metabolic process (GO:0008152) (149 genes), cellular metabolic process (GO:0044237) (119 genes), transport (GO:0006810) (29 genes), establishment of localization (GO:0051234) (29 genes), localization (GO:0051179) (29 genes), significantly enriched by DEGs under salt stress condition in the tolerant bulk as compared with the sensitive bulk (Fig. 1). A number of genes belonging to significant biological processes will be further discussed in more details. As determined by the singular enrichment analysis, significant DEGs under salt stress enriched molecular functions including structural molecule activity (GO:0005198) (41 genes), and transporter activity (GO:0005215) (23 genes) (Fig. 2). Furthermore, to determine the location of the DEGs under salt stress condition, cellular component enrichment analysis raced them mainly in cytoplasmic part (GO: 0044444) (81 genes), plastid (GO:0009536) (17 genes), cytoplasm (GO: 0005737) (88 genes), thylakoid (GO: 0009579) (20 genes), ribosome (GO:0005840) (39 genes), mitochondrion (GO: 0005739) (20 genes), plasma membrane (GO: 0005886) (10 genes), ribonucleoprotein complex (GO: 0030529) (39 genes), macromolecular complex (GO: 0032991) (65 genes), intracellular part (GO: 0044424) (114 genes), organelle (GO: 0043226)(97), intracellular organelle (GO:0043229) (97), intracellular (GO : 0005622) (114 genes), cell (GO : 0005623) (139 genes), cell part (GO : 0044464) (139 genes). Cell wall (GO : 0005618) (11 genes), external encapsulating structure (GO:0030312) (11 genes), intracellular non-membrane-bounded organelle (GO:0043232) (43 genes), non-membrane-bounded organelle (GO:0043228) (43 genes), membrane (GO:0016020) (55 genes), membrane-bounded organelle (GO:0043227) (59 genes), intracellular membrane-bounded organelle (GO:0043231) (55 genes) (Fig. 3).

The significantly enriched terms and pathways could help us a lot to further understand the role that DEGs play in salt tolerance. GO enrichment analysis of DEGs under salt stress condition showed that the greatest percent of genes were related to the cellular process (>82% genes), metabolic process (>45% genes), response to stimulus (>20%), and localization (transport) (7% genes). In accordance with these GO enrichment results, these biological categories have been previously reported in a study conducted by Hossain and coworkers (2016) on salt stress responsive genes in diverse rice genotypes.

To be more specific, 29 DEGs significantly enriched in transport, among which LOC_Os01g70490 (potassium transporter, putative, expressed) and LOC_Os01g34850 (OsHKT2;3 - Na^+ transporter, expressed) are notable. These results are consistent with the previous studies that K^+ and Na^+ homeostasis are important in salt tolerance (Qi *et al*., 2004; Almeida *et al*., 2017). Also, according to gene module analysis results, 8 of 29 DEGs including 4351757 (LOC_Os12g10570.1), LOC_Os04g16748.1, atpB (LOC_Os10g21266.1), 4335236 (LOC_Os10g21238.1), LOC_Os05g35320.1, LOC_Os06g39740.1, LOC_Os01g25065.1, LOC_Os10g38272.1 were found to form gene module 6. This gene module was significantly enriched in ATP synthesis coupled proton transport (GO: 0015986). Several membrane intrinsic and ion channel related genes having putative roles in ion homeostasis were also found to be significantly expressed e.g., aquaporin (LOC_Os10g36924). Four genes were found to be involved in lipid transfer proteins (LTPs) such as LOC_Os02g44310 (LTPL112), LOC_Os06g07220 (LTPL128), LOC_Os04g46810 (LTPL120), and LOC_Os03g57970 (LTPL73). Lipid-transfer proteins were reported to modulate plant tolerance to salt, drought, cold stress and also fungal pathogens and bacterial infections (Sarowar *et al*., 2009; Choi *et al*., 2012; Guo *et al*., 2013; Gangadhar *et al*., 2016).

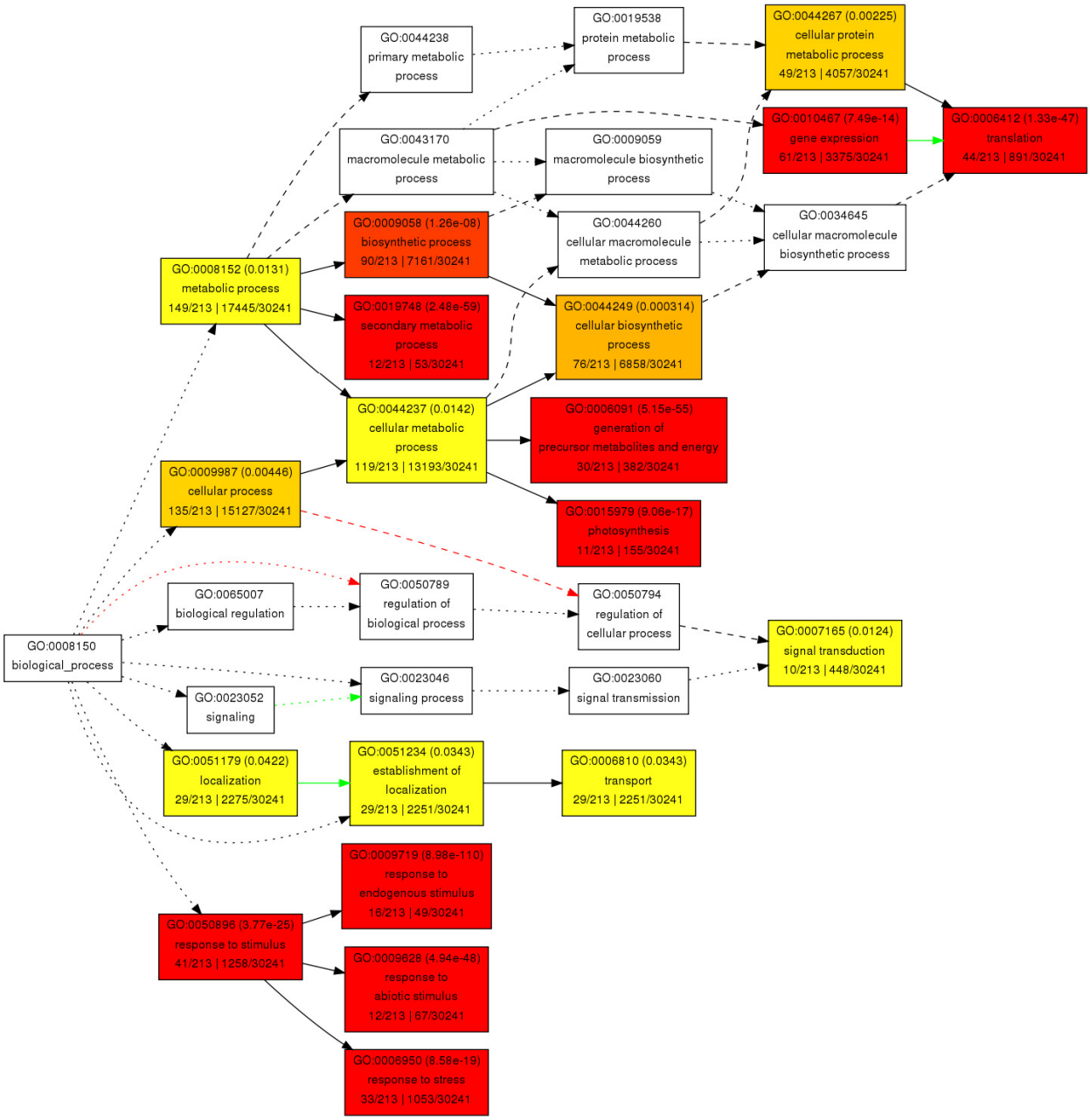

Fig. 1. Biological process enrichment analysis of the DEGs under salt stress condition.

Additionally, 30 DEGs significantly enriched in the generation of precursor metabolites and energy (GO: 0006091), a subcategory of the metabolic process and cellular process. On the basis of gene module analysis results, Cluster 3 with 11 genes was enriched in "generation of precursor metabolites and energy (GO: 0006091).

GO enrichment analysis of the DEGs under control condition. Gene ontology enrichment of DEGs under control condition revealed that response to stimulus (GO:0050896) (16 genes), and response to stress (GO:0006950) (14 genes) are highly overrepresented in the tolerant bulk as compared with the sensitive bulk under control condition (Fig. 4). These significant biological processes may help tolerant bulk to better cope with salt stress when exposed to salinity condition. According to the results of comparative genomics in salt tolerance between Arabidopsis and Arabidopsis-related halophyte *Thellungiella halophila*, a large number of *T. halophila* homologs of Arabidopsis stress-inducible genes were overexpressed under normal growth conditions (Taji *et al.*, 2004).

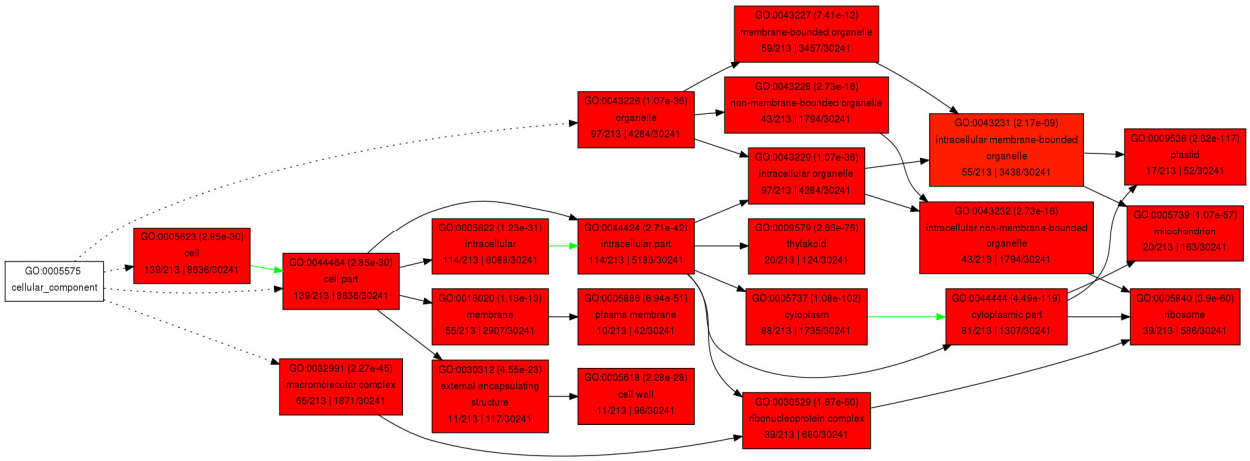

Fig. 2. Cellular component enrichment analysis of the DEGs under salt stress condition.

Table 1: The list of critical genes identified based on applying different algorithms embedded in Cyto-Hubba along with their expression fold change in salt tolerant RILs bulk under salt stress.

Critical genes	Algorithms that identified critical genes	Description	Fold change
LOC_Os06g02980.1	Betweenness, Closeness, Degree, MNC, EcCentricity, Radiality, Stress	ATP synthase F1, epsilon subunit family protein	1.4019537
LOC_Os03g55874.1	Bottle Neck	ATP synthase subunit beta, putative	1.4019537
LOC_Os08g15322.1	Clustering Coefficient	Cytochrome b559 subunit alpha, putative,	0.99531364
LOC_Os10g21298.1	Clustering Coefficient	Cytochrome b559 subunit alpha, putative	0.99531364
LOC_Os10g25230.1, 4348531	Clustering Coefficient	ZIM domain containing protein, putative, expressed; Repressor of jasmonate responses	1.8476067
LOC_Os04g23550.1, 4335417	Clustering Coefficient	Basic helix-loop-helix family protein, putative, expressed	1.207386
LOC_Os03g08330.1, 4331834	Clustering Coefficient	ZIM domain containing protein, putative, expressed; Repressor of jasmonate responses	1.5495434
LOC_Os04g16728.1	DMNC	Chloroplast 30S ribosomal protein S15, putative	0.85575485
LOC_Os12g34550.1	DMNC	Ribosomal protein S15 containing protein	0.85575485
LOC_Os10g21212.1	EcCentricity	Photosystem II 44 kDa reaction center protein, putative	0.9105263
LOC_Os02g24632.1	EcCentricity	Photosystem II 44 kDa reaction center protein, putative, expressed	0.9105263
LOC_Os03g55874.1	EcCentricity	ATP synthase subunit beta, putative	1.4019537
LOC_Os10g21290.1	EcCentricity	Apocytochrome f precursor, putative	1.392808
LOC_Os09g07910.1	EPC, MCC	DNA-directed RNA polymerase subunit alpha, putative	1.24263

This finding strongly supports our hypothesis that a greater tolerance of salt-tolerant genotypes may be due to higher initial capacity for adaptive response. Also, no molecular functions were significantly enriched by the DEGs. For cellular component, the DGEs significantly enriched in cytoplasmic part (GO:0044444) (11 genes), cytoplasm (GO:0005737) (11 genes), membrane (GO:0016020) (13 genes), intracellular organelle (GO:0043229) (13 genes), organelle (GO:0043226) (13 genes), intracellular part (GO:0044424) (13 genes), intracellular membrane-bounded organelle (GO:0043231) (10 genes), membrane-Bounded organelle (GO:0043227) (10 genes), cell (GO:0005623) (17 genes), cell part (GO:0044464) (17 genes) (Fig. 5).

D. Gene network analysis of the DEGs

The network of the DEGs under salt stress contained 100nodes, 1047 edges, whilst under control condition contained 11 nodes, 15 edges.

Since the number of connected node in the network of the DEGs under control condition was 11, which made it very simple, further network analysis was focused on the network of the DEGs under salt stress. Based on the Cyto-Hubba analysis results, 14 genes were considered critical in the network. These critical genes along with their fold-changes were presented in Table 1.

Moreover, module analysis of PPI network revealed 7 distinct gene modules (Table 2). According to GO ontology and pathway enrichment analysis on these gene modules, 32 DEGs in the largest gene modules (Cluster 1) in PPI network enriched in translation and transcription processes. There were no significant pathway and terms enrichments in Cluster 2 with 8 DEGs. 11 DEGs in Cluster 3 enriched in "generation of precursor metabolites and energy (GO:0006091)". 10 DEGs in Cluster 4 enriched in translation (GO:0006412) and gene expression (GO:0010467). 5 DEGs in Cluster 5 enriched in NADH dehydrogenase (ubiquinone) activity (GO:0008137) and quinone binding (GO:0048038). 8 DEGs in Cluster 6 significantly enriched ATP synthesis coupled proton transport (GO:0015986). 3 DEGs in Cluster 7 significantly enriched two pathways "Plant-pathogen interaction (04626) and plant hormone signal transduction (04075). Network analysis paves the way for identifying hub (critical) genes, potential candidate genes which can be exploited as biomarkers and enlighten tightly coexpressed modules of genes (Ficklin *et al.,* 2010; Allen *et al.,* 2012; Zinati and Barati, 2017).

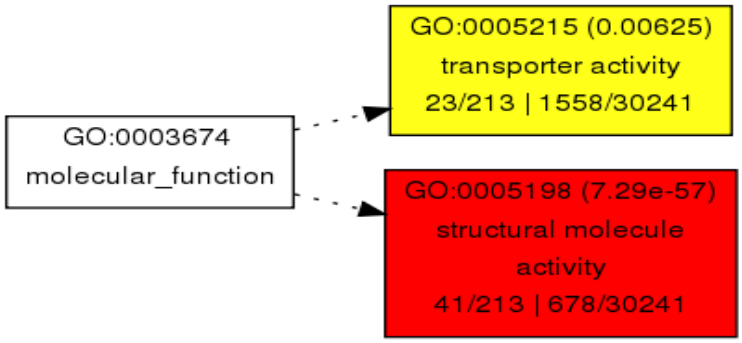

Fig. 3. Molecular function enrichment analysis of the DEGs under salt stress condition.

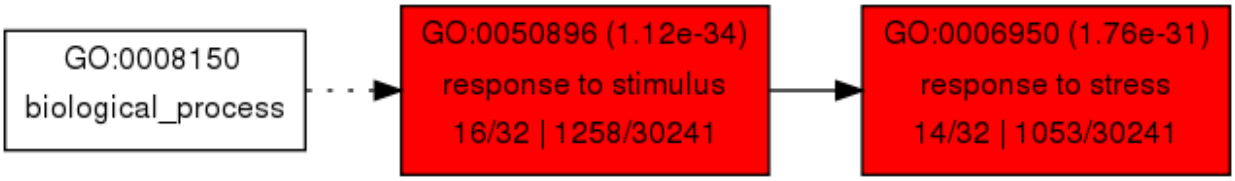

Fig. 4. Biological process enrichment analysis of the DEGs under control condition.

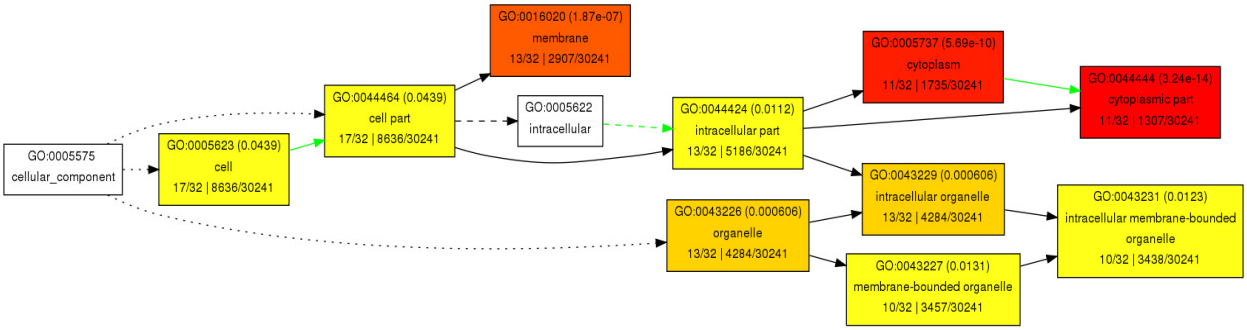

Fig. 5. Cellular component enrichment analysis of the DEGs under control condition.

E. Discovery of critical genes and gene modules

Because the multiple genes control salt tolerance, the interaction networks among related genes and the key factors that govern salt tolerance remain to be clarified. Along these lines, we also constructed a PPIs network using the DEGs under salt stress in order to enlighten key genes and gene modules.

Table 2: Module analysis of PPIs network using plug-in Molecular Complex Detection (MCODE) in Cytoscape software revealed 7 gene modules (clusters).

Cluster	Score (Density*#Nodes)	Nodes	Edges	Node IDs
1	26.581	32	412	LOC_Os08g15272.1, LOC_Os05g22716.1, LOC_Os09g24416.1, LOC_Os04g16834.1, LOC_Os01g57954.1, LOC_Os04g16832.1, LOC_Os10g21332.1, LOC_Os12g34092.1, 4335250, LOC_Os05g22712.1, LOC_Os12g34045.1, LOC_Os04g16838.1, LOC_Os09g24414.1, LOC_Os04g16820.1, LOC_Os06g02980.1, LOC_Os10g21328.1, LOC_Os08g15278.1, LOC_Os12g34154.1, LOC_Os04g16842.1, LOC_Os12g34550.1, LOC_Os10g21346.1, LOC_Os08g15288.1, 4338310, LOC_Os04g16728.1, 4325870, LOC_Os09g19952.1, LOC_Os08g15268.1, LOC_Os12g33954.1, LOC_Os05g22718.1, LOC_Os04g16822.1, LOC_Os09g07910.1, LOC_Os04g16824.1
2	6	8	21	LOC_Os02g24632.1, 4341439, LOC_Os10g21212.1, LOC_Os06g39728.1, LOC_Os01g65902.1, LOC_Os02g24596.1, LOC_Os08g15280.1, LOC_Os10g21290.1
3	4.8	11	24	LOC_Os04g16854.1, LOC_Os10g38212.1, LOC_Os01g58024.1, LOC_Os01g58000.1, LOC_Os07g25004.1, LOC_Os06g39756.1, LOC_Os10g21264.1, LOC_Os04g16742.1, LOC_Os12g19430.1, LOC_Os08g15248.1, LOC_Os10g21258.1
4	4.667	10	21	4126893, LOC_Os09g19954.1, 4126899, LOC_Os01g57958.1, LOC_Os06g39716.1, LOC_Os04g16826.1, LOC_Os12g34056.1, rpl16, LOC_Os10g21312.1, LOC_Os12g34128.1
5	4.5	5	9	LOC_Os01g58022.1, 4126907, LOC_Os06g22010.1, LOC_Os12g34094.1, LOC_Os10g21396.1
6	4.286	8	15	4351757, LOC_Os04g16748.1, atpB, 4335236, LOC_Os05g35320.1, LOC_Os06g39740.1, LOC_Os01g25065.1, LOC_Os10g38272.1
7	3	3	3	4335417, 4331834, 4348531

Module analysis of the network revealed 7 distinct gene modules which were strongly enriched in biological process and pathways such as translation and transcription, generation of precursor metabolites and energy, ATP synthesis coupled proton transport, and plant hormone signal transduction. All these gene modules were up regulated by salt stress in the tolerant bulk as compared with the sensitive bulk. Thereupon, these gene modules may underlie the mechanisms by which tolerant RILs bulk be able to tolerate salinity stress.

Network analysis of DEG under salt stress, revealed 14 genes as critical genes. Based on our microarray data analysis, the expression level of all 14 critical genes was increased in the tolerant bulk as compared with the sensitive bulk, supporting their critical roles in conferring the salt tolerance. Transcriptional regulators associated with salt stress in crops could be attractive targets for controlling and enhancing plant tolerance.

It is worthy to note that there were three TFs among 14 critical genes detected by Cyto-Hubba; two ZIM domain containing protein, putative, expressed; repressor of jasmonate responses (LOC_Os10g25230.1and LOC_Os03g08330.1)and one basic helix-loop-helix family protein, putative, expressed (LOC_Os04g23550.1)were considered as critical genes by Clustering Coefficient algorithm.JAZ (JASMONATE ZIM-DOMAIN), a subfamily of TIFY, has been known as negative regulators of jasmonate-mediated response in Arabidopsis (Staswick 2008). According to gene module analysis, these transcription factors interact with each other and make a gene module 7. A functional study of JAZ10 conducted by Chung and Howe (2009) suggested that a highly conserved N-terminal ZIM domain (or TIFY motif)mediates homo- and heteromeric interactions between most Arabidopsis JAZs proteins (Chung and Howe 2009). Additionally, the Jas domain in the C-terminal part of JAZs is required for JAZ interaction with bHLH transcription factors (Song *et al*., 2011).It has been previously shown that JAZ proteins are repressors of bHLH transcription factors, MYC2 (chini *et al*., 2009),MYC3 (Cheng *et al*., 2011), and targets for SCF^{COI1}, which is likely jasmonate receptors. Upon hormone perception, JAZ repressors are degraded by the proteasome releasing MYC2 and MYC3 allowing the activation of JA responses. In the light of the result reported here, we can propose that two ZIM domain containing protein (LOC_Os10g25230.1 and LOC_Os03g08330.1) complexes could interact with Basic helix-loop-helix family protein (LOC_Os04g23550.1) and act as its repressors. This hypothesis needs to be validated by further experiments. Based on pieces of literatures reviewing, JAZ proteins have been well recognized as transcriptional repressors of jasmonate responses in Arabidopsis (Chini *et al*., 2007; Thines *et al*., 2007; Fernandez-Calvo *et al*., 2011; Song *et al*., 2011), however, rather limited studies of the function of JAZ proteins have been documented in economically significant crops (Wu *et al*., 2015).

There are 15 JAZ homologs in rice (Ye *et al*., 2009), but very few of them have been functionally characterized (Wu *et al*., 2015). Wu and colleagues (2015) revealed that overexpression of OsJAZ9conferred enhanced salt-tolerance mainly due to modulating K^+ transporters in rice. They suggested that OsJAZ9 interacts with several bHLHs including OsbHLH062 that may directly modulate the expression of ion transporter genes. Over-expression of a JAZ family gene from *Glycine soja* displayed increased tolerance to salt and alkali stresses (Zhu *et al*., 2012). Also, it has been reported that OsJAZ9 overexpression regulates JA level, signaling, root system architecture and physiology, improving tolerance to sheath blight disease and K deficiency tolerance in rice (Singh *et al*., 2018). As previously mentioned, the gene module 7 significantly enriched two pathways "Plant-pathogen interaction (04626)" and "plant hormone signal transduction (04075)". These results support that the detected JAZs in our study might play an important role in salt tolerance.

The result of the *Cyto-Hubba analysis* coupled with the module analysis has provided evidence indicating that these transcription factors may contribute to salt tolerance in rice, however, it is required to conduct overexpression and knockout experiments to determine the roles and importance of these genes in mechanisms of salinity tolerance. To the best of our knowledge, the role of these transcription factors in salt tolerance of rice has not been reported previously. This research may provide a foundation for functional genomics investigations to decipher the precise role of candidate genes in salt stress response in rice seedlings.

CONCLUSION

The comparative transcriptome analysis of stress-sensitive and –tolerant genotypes provides valuable information in elucidating the molecular mechanism mediating salt stress response in rice. This study was designed to identify key genes associated with salt tolerance mechanisms in rice with special emphasis on the role of transcription factors. Our study revealed candidate genes related to salt tolerance of rice at the seedling stage and exhibited the usefulness of using bioinformatics analyses on DEGs to better understanding the molecular systems that underlie salt tolerance. This research has laid a foundation for functional genomics investigations to decipher their precise role of candidate genes in salt stress response in rice seedlings and be targeted for genetic improvement of salinity tolerance in rice.

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