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Molecular Mechanism of Salinity Stress Tolerance in Barley (*Hordeum vulgare* L.) via Meta-Analysis of Transcriptome Data

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ABSTRACT

Salt stress, as the most important abiotic stress, limits growth of plants and causes extensive damage to agricultural production worldwide. Therefore, it is necessary to identify genes that play a key role in tolerance to salt stress in plants through the analysis of transcriptome data such as microarray and High-Throughput Sequencing (HTS or NGS). In the present research, the combined analysis of microarray data by R packages for *Hordeum vulgare* L. under salinity stress identified 685 upregulated meta-DEGs (differentially expressed genes) and 766 downregulated meta-DEGs. The upregulated genes mostly belong to abiotic stress tolerance and hormone biosynthesis, and the downregulated genes pertain to late embryogenesis abundant protein and salinity stress response. GO terms in the upregulated genes are mostly associated with response to external and internal stresses; and in the downregulated genes, they are mostly associated with cellular metabolism. In the up and down meta-DEGs by KEGG, most of the genes connected to salinity stress included PP2C, ABF, AGT, and ChiB and F-box connected to the downregulated genes. Moreover, Transcription Factors (TFs) in the up and downregulated meta-DEGs with high frequency included AP2, ERF, bZIP, and bHLH. Most of the hub upregulated genes acquired from this research were metabolite biosynthesis and photosynthesis-related; and the hub downregulated genes were mainly the tricarboxylic acid cycle and glycolysis processes-related. Finally, a comparison was made between this meta-analysis and data obtained from other investigations. The findings validated their up and down expression. Our results give a new understanding about the molecular mechanism and present many TFs and candidate genes for salt stress tolerance in barley breeding programs.

Keywords: Bioinformatics, Gene ontology, Hub genes, Salt stress.

INTRODUCTION

Salt stress enhanced by climate change and management of resources such as water (i.e. irrigation) and biodiversity in agriculture threatens staple crop production globally (Arzani and Ashraf, 2016). Barley with high salt tolerance properties is used as a model plant for small grain cereals to decipher mechanisms of salt tolerance (Gharaghanipor *et al*., 2022).

According to Colmer *et al*. (2005), salt tolerance has a positive relationship with Na+ ion exclusion under saline conditions during growth. Adem *et al*. (2014) conducted a comparative study and concluded that during the salinity treatment, salt-tolerant genotypes maintained a stable concentration of K+ and accumulated less Na+ in the root. Researchers cloned and expressed HvAKT1, HvAKT2, and HvKCO1 differentially as the genes that code K+ channels, in the roots and leaves of barley (Boscari *et al*., 2009). The plant parts in which HvAKT1 and HvAKT2

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were mainly expressed were the roots and the leaves, the latter being almost 20 times higher. According to Roslyakova *et al*. (2011), the NHX1-3 isoforms was enhanced by salinity stress on tonoplast (150 mM NaCl) in the tissues of leaf and root. Transgenic barley plants that overexpress AtCIPK16 demonstrated salinity tolerance; they also had a higher biomass in comparison to the control plants, 20–45%, under exposure to 300 mM NaCl (Roy *et al*., 2013).

"Salt tolerance" is the capability of plant genotype by which it decreases ion toxicity, resultant oxidative stress, and osmotic stress to hinder the detrimental salt impacts, thereby causes a decrease in the product (Munns and Tester, 2008; Arzani and Ashraf, 2016). Figuring out plants' molecular responses to abiotic stress conditions was made possible through transcriptional profiling by microarray technology (Kilian *et al*., 2012). In recent years, DNA microarrays have been extensively applied in the investigations of expression levels of regulatory genes related to abiotic stresses in model crops and led to identification of responsive genes with salt stress at the transcriptional level (Ozturk *et al*., 2002; Ueda *et al*., 2004; Narimani *et al*., 2022).

Transcription Factors (TFs) are a group of proteins that regulate the target genes expression, including the salt stress-responsive genes through binding to DNA in specific cis-elements in promoter regions. Walia *et al*. (2005) aimed to identify the transcripts under salt stress, utilizing Affymetrix 22K Barley1 GeneChip Array. Serine/Arginine (SR)-rich protein and 12-oxophytodienoate reductase were recognized as up and downregulated genes under, respectively, 3, 8 and 27 hours 100 mM NaCl treatments. Another study related to leaf transcripts of barley under abiotic stress showed lipid transfer protein, MT2, allene oxide synthase, early responsive to dehydration, glutathione s-transferase and P5CS induced (Ozturk *et al*., 2002). According to a report by Ueda *et al*. (2004), genes related to Plasma Membrane Protein 3 (PMP3), receptor-like protein, and cytochrome P450 family and the genes with a role in the amino acids biosynthesis were a number of upregulated genes. Moreover, Salt-responsive protein (SalT), phospholipase C, DNA-binding protein CCA1, the water channel protein 2's transcript levels, and water channel protein 1's mRNA abundance were a number of downregulated genes under osmotic stress in barley.

Due to a broad range of experimental conditions, a meta-analysis can add a new perspective to data analysis of gene expression (Moreau *et al*., 2003). Accordingly, to report precise results on mechanisms of salinity tolerance in barley, considering the results of various individual experiments is essential. Based on the literature, no meta-analysis has been conducted regarding microarray data of barley under salt stress. In fact, the outcomes of integration of various individual researches can lead to the identification of key genes that are associated with the mechanisms of barley salt tolerance. Through conducting a meta-analysis, this study aimed to find out the key genes connected to TFs, metabolic pathways, and core genes under salt stress.

MATERIALS AND METHODS

In the present study, the Gene Expression Omnibus was used as the source of the microarray expression data (GPL1340 and GPL16130) related to the barley aerial tissue studied under salinity stress. Four series (GSE6325, GSE5605, GSE3097, and GSE41517) with those in which the aerial tissue was tested in the control and salinity stress (shoot samples) were chosen and used in this study.

Meta-Analysis and Differential Gene Expression (DEG)

The expression data of microarray was pre-analyzed using the R language package, as a separate dataset. PCA plot, boxplot, and CorHeatmap controlled the data quality. The

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treated and control data under salt stress were classified based on PCA. The quantile normalization and background correction were carried out on unnormalized data; then, the good quality data were selected for additional analysis applying AgiMicroRna package. The Limma package was applied to perform the selected data's difference analysis, and a linear model was fitted to the acquired data; and a simple Bayesian experimental model was used to correct the standard errors. The moderated t-statistic and log2fold change for each contrast/gene were measured. The genes with meaningful P value were attained (FDR≤ 0.01) and additional analysis was regarded for the DEG and log2fold-change values of each dataset (de Abreu Neto and Frei, 2016).

For merging unadjusted P-values, sum logs of Fisher method was carried out. Employing 'FDR' method, to set the combined P-values for multiple experiments, the p.adjust function in metaRNASeq was used. Provided that the adjusted P-values were ≤0.01 and log2-fold change value >1 and <-1, the genes were obtained to have different expression in the meta-analysis (supplementary file Figure 1) (Soltanpour *et al*., 2022).

Gene Ontology and KEGG Pathways

The Gene Ontology (GO) analysis was performed for down- and upregulated genes, and g.profiler as source was used. The common GO down- and upregulated genes were detected using web tool Venny v2.1. GO annotation's biological process classes were specified under salt stress and applied annotation of down- and upregulated genes were acquired via Kyoto Genes and Genomes Encyclopedia (KEGG) .

Investigation of TFs through Promoter Analysis

The sequences of the down and upregulated genes (1,500 bp upstream) were obtained from the EnsemblPlants and PlantPAN 3.0 and used for analysis of TFs (Bhargava *et al*., 2013) . The TFs frequency in the down and upregulated genes' promoters and percentage of TFs up to down regulated genes were determined.

Identification of Hub Genes

The information acquired from Cytoscape software and the STRING website were employed to draw the network of Protein-Protein Interaction (PPI). The CytoHubba plugin in Cytoscape 3.6.1 characterized the most linked genes as core genes, in the biological network (Chin *et al*., 2014) with EPC, Closeness, Bottle Neck, DMNC, Degree, EcCentricity, Betweenness, MNC, Clustering Coefficient, Radiality, Stress, and MCC methods.

Approval of Downregulated and Upregulated Hub Genes by Other Studies

For validation, seven hub genes were selected from the obtained down and upregulated genes and were compared to qRT-PCR data of other studies.

RESULTS

The results showed 766 downregulated and 685 upregulated genes associated with salt stress being significant at p-values ≤ 0.01 with log2-fold change> 1 and < -1. Down and upregulated genes that were recorded as the top 15 genes are presented in Table 1. The upregulated genes mostly related to abiotic stress, bZIP TF, glycoside hydrolase and biosynthesis of hormones including auxin and ethylene. On the other hand, the downregulated genes mainly related to Late Embryogenesis Abundant (LEA) protein and salinity stress response.

All core salt-responsive DEGs underwent GO enrichment analysis according to the g:

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profiler. The central salt-responsive down and upregulated genes were analyzed to identify meaningful (with P< 0.05) GO terms for biological process in salt response (Table 2). Also, 20 and 27 terms were enriched in up and downregulated genes, respectively (Figure 1). GO terms in the upregulated genes mainly related to the response to external and internal stresses; and, in the downregulated genes, primarily associated with metabolic and catabolic processes. In the up and downregulated genes, Common GO terms included GO:0008150 (biological process), GO:0044248 (cellular catabolic process), GO:0044036 (cell wall metabolic process), GO:1901071 (glucosamine metabolic process), and GO:0006040 (amino sugar metabolic process).

Accordingly, the up and down meta-DEGs via KEGG pathways mostly belonged to metabolic pathways, MAPK signaling pathway-plant, secondary metabolites biosynthesis, ubiquitin mediated proteolysis, proline metabolism, starch and sucrose metabolism, plant-pathogen interaction, glutathione metabolism, carbon metabolism, glycolysis and gluconeogenesis, plant hormone signal transduction, mRNA surveillance pathway, amino acids biosynthesis, protein export, and peroxisome. According to the biological pathways, most of the genes related to salinity stress including PP2C, ABF, AGT, ChiB, CPSF5 and F-box mainly associated to the downregulated genes. The up and down regulation meta-DEGs in the signaling pathway of MAPK, ubiquitin mediated proteolysis, peroxisome, pathway of mRNA surveillance, and signaling transduction of plant hormone of *H. vulgare* L under salt stress are shown in Figure 2.

The meta-DEGs' promoter sequence (1,500 bp) was obtained from barley genome, and TF binding site was scanned in the promoter sequence. The 40 families of TF were grouped: 56.99% of the downregulated TFs and 54.74% of the upregulated TFs related to ERF, AP2, Dof SBP, bZIP, and bHLH, (Figure 3-A). The

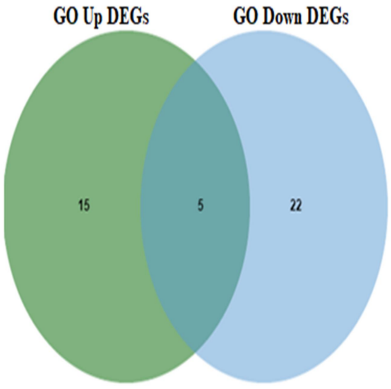

Figure 1. Venn diagrams illustrating common genes of meta-DEGs. Five common GO terms are observed in the comparison between some Go up-regulated and down-regulated DEGs of *H. vulgare* L.

highest percentage of TF in the up and downregulated meta-DEGs related to AP2, ERF, bZIP and bHLH. TF up to down frequency examination demonstrated that AP2, ERF under salt stress enables 1,293% more up genes and GRF enables 171% more down genes (Figure 3B).

To identify the biological communication between the genes, the PPI network was illustrated separately for up and downregulated genes, applying Cytoscape STRING website and version 3.6.1 (Supplementary file Figures 2 and 3). HORVU. MOREX.r3.1HG0055890 (proline oxidase), HORVU. MOREX.r3.5HG0493330 (alike nucleotide sugar epimerase), HORVU. MOREX.r3.7HG0670200 [NAD (P)-binding domain], HORVU. MOREX.r3.4HG0345500 (pyruvate kinase), HORVU. MOREX.r3.6HG0613490 (aldolase-type TIM barrel domain), HORVU. MOREX.r3.1HG0090740 (CTP synthase 1), HORVU. MOREX.r3.2HG0182010 (phenylalanine ammonia-lyase), and HORVU. MOREX.r3.1HG0071210 (U-box E3ubiquitin ligase) were as top commonly upregulated core genes, which are recorded in Table 3. Accordingly, the upregulated DEGs hub genes are mainly related to photosynthesis.

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Table 1. Top 15 up and downregulated meta-DEGs of H. *vulgare* L.

Upregulated genes				
Gene stable ID	LogFC	Meta-p.value	UniProtKB/TrEMBL ID	Description
HORVU.MOREX.r3.2HG0135060	3.31	2.84E-14	Q0D5H2	Similar to carbohydrate transporter/Sugar porter
HORVU.MOREX.r3.1HG0055890	2.37	2.74E-11	F2E6C0	Proline oxidase
HORVU.MOREX.r3.7HG0717870	2.21	1.85E-08	Q0DBI8	Abiotic stress tolerance
HORVU.MOREX.r3.5HG0508610	1.79	7.83 E-04	F2D3Z8	Patatin family protein
HORVU.MOREX.r3.7HG0746640	1.56	2.86 E-03	A0A287XXT8	Ethylene biosynthesis
HORVU.MOREX.r3.2HG0138660	1.54	5.29 E-04	Q6YVU7	Glycoside hydrolase
HORVU.MOREX.r3.5HG0433250	1.46	7.97 E-05	A0A0N7KUB6	bZIP TF
HORVU.MOREX.r3.5HG0430940	1.46	8.03E-11	A0A287QDF2	Glycoside hydrolase
HORVU.MOREX.r3.7HG0739110	1.42	4.40E-06	F2DBS4	bZIP TF, and stress response
HORVU.MOREX.r3.2HG0112610	1.41	8.25E-05	F2CV55	Peroxidase
HORVU.MOREX.r3.5HG0535950	1.38	3.82 E-04	Q7XH16	Similar to SRK5 protein
HORVU.MOREX.r3.3HG0311560	1.36	1.05 E-03	M0X8R7	Auxin responsive SAUR protein
HORVU.MOREX.r3.2HG0166450	1.33	1.18E-05	Q8GT50	Cell-wall invertase
HORVU.MOREX.r3.1HG0071210	1.25	5.18 E-04	F2DFA0	U-box E3 ubiquitin ligase, and abiotic stress response
HORVU.MOREX.r3.5HG0464580	1.15	8.62 E-03	M0XRX0	Protein kinase of serine/threonine, and abiotic stress
Downregulated genes				
Gene stable ID	LogFC	Meta-p.value	UniProtKB/TrEMBL ID	Description
HORVU.MOREX.r3.7HG0658900	-2.61	0	A0A287VX26	Similar to MtN3 protein precursor
HORVU.MOREX.r3.1HG0080000	-2.61	2.20E-07	F2DW12	Late embryogenesis abundant protein
HORVU.MOREX.r3.2HG0167060	-2.49	0	A0A0P0WA35	Similar to OSIGBa0092M08.2 protein
HORVU.MOREX.r3.4HG0343130	-2.35	1.15E-05	F2EBH8	Isopenicillin N synthase
HORVU.MOREX.r3.1HG0080000	-2.35	5.48 E-04	F2DW12	LEA
HORVU.MOREX.r3.2HG0194770	-2.32	6.04E-11	C5IYF2	Pectinesterase inhibitor
HORVU.MOREX.r3.3HG0302060	-2.30	0	A0A287G754	Similar to P5CS
HORVU.MOREX.r3.2HG0172200	-2.28	3.90E-08	F2DH67	Similar to H0818E04.21 protein
HORVU.MOREX.r3.1HG0080020	-2.02	9.37 E-03	B5TWD0	Late embryogenesis abundant protein
HORVU.MOREX.r3.5HG0495360	-1.91	1.03E-06	A0A0P0XPM4	Glycoside hydrolase
HORVU.MOREX.r3.7HG0639230	-1.76	2.09 E-04	A0A0P0YB83	Lipoxygenase
HORVU.MOREX.r3.3HG0308770	-1.70	1.14 E-03	M0WAY2	Conserved hypothetical protein
HORVU.MOREX.r3.5HG0508860	-1.66	1.02E-12	F2CRE4	Similar to major facilitator superfamily protein
HORVU.MOREX.r3.1HG0093680	-1.34	6.75E-05	A0A2R9IMA9	S-like ribonuclease, and salinity tolerance
HORVU.MOREX.r3.5HG0526120	-1.14	5.46 E-04	F2CRE4	Salinity stress response

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Table 2. Top 10 GO annotation's biological process classification of core salt-responsive regulated *H. vulgare* L genes.

Upregulated genes				
GO	Gene Stable ID	UniProtKB/TrEMBL ID	Adjusted P value	Description
GO:0008150	HORVU.MOREX.r3.2HG0215250	A9ZPL2	1.967E-06	Biological process
GO:0050896	HORVU.MOREX.r3.3HG0304380	M0YCC3	5.29E-05	Response to stimulus
GO:0004601	HORVU.MOREX.r3.2HG0112580	M0WYK6	4.88 E-08	H2O2 catabolic process
GO:0042221	HORVU.MOREX.r3.3HG0307420	A0A287M9A8	5.73 E-04	Response to chemical
GO:0072593	HORVU.MOREX.r3.1HG0044290	F2DMT6	6.15 E-04	Metabolic process of ROS
GO:0005515	HORVU.MOREX.r3.3HG0330590	Q8LPD7	1.55 E-03	Response to toxic substance
GO:0005975	HORVU.MOREX.r3.2HG0166450	Q8GT50	2.29 E-03	Metabolic process of carbohydrate
GO:0097237	HORVU.MOREX.r3.4HG0409010	M0WZ37	3.03 E-03	Toxic substance response
GO:0004601	HORVU.MOREX.r3.2HG0112580	M0WYK6	1.67 E-03	Oxidative stress response
GO:0005975	HORVU.MOREX.r3.2HG0138660	Q6YVU7	3.72 E-02	Metabolic process
Down-regulated genes				
GO term	Gene stable ID	UniProtKB/TrEMBL ID	Adjusted P value	Description
GO:0006869	HORVU.MOREX.r3.1HG0083750	Q6L4G9	1.16E-07	Lipid transport
GO:0005975	HORVU.MOREX.r3.3HG0281620	F2CSS7	7.54E-06	Chitin catabolic process
GO:0006022	HORVU.MOREX.r3.7HG0668560	F2DKD7	1.25 E-04	Aminoglycan metabolic process
GO:1901575	HORVU.MOREX.r3.7HG0744930	F2DGL9	3.50 E-04	Organic substance catabolic process
GO:0044248	HORVU.MOREX.r3.7HG0678990	F2D4I5	9.41 E-04	Cellular catabolic process
GO:0016998	HORVU.MOREX.r3.5HG0508860	M0Z2Y3	1.37 E-03	Cell wall macromolecule catabolic process
GO:0005524	HORVU.MOREX.r3.4HG0414960	M0V098	6.87 E-03	Nucleoside diphosphate catabolic process
GO:0005975	HORVU.MOREX.r3.1HG0054950	F2DJR4	9.17 E-03	Defense response to fungus
GO:0016052	HORVU.MOREX.r3.5HG0429390	Q9FYY1	2.17 E-02	Carbohydrate catabolic process
GO:0046872	HORVU.MOREX.r3.7HG0639230	Q2QNN5	4.28 E-02	Lipid oxidation

HORVU. MOREX.r3.4HG0410550 [alike glyceraldehyde-3-phosphate dehydrogenase (GAPDH)], HORVU. MOREX.r3.7HG0720730 (alike fructose-bisphosphate aldolase), HORVU. MOREX.r3.1HG0068880 (alike 3-phosphoglycerate kinase), HORVU. MOREX.r3.5HG0513810 (alike malate dehydrogenase), HORVU. MOREX.r3.6HG0564980 (alike quinone oxidoreductase), HORVU. MOREX.r3.2HG0104740 (alike rubisco), HORVU. MOREX.r3.2HG0106830 (transketolase), HORVU. MOREX.r3.7HG0658050 (Acyl-CoA oxidase), HORVU. MOREX.r3.5HG0514560 (similar to citrate synthase), HORVU. MOREX.r3.5HG0471620 [chloroplast precursor (AK-HD 2)], and HORVU. MOREX.r3.1HG0093680 (salinity tolerance) shown in Table 4 are as top commonly downregulated core genes. Accordingly, the downregulated DEGs hub genes are mainly related to tricarboxylic acid cycle and glycolysis processes.

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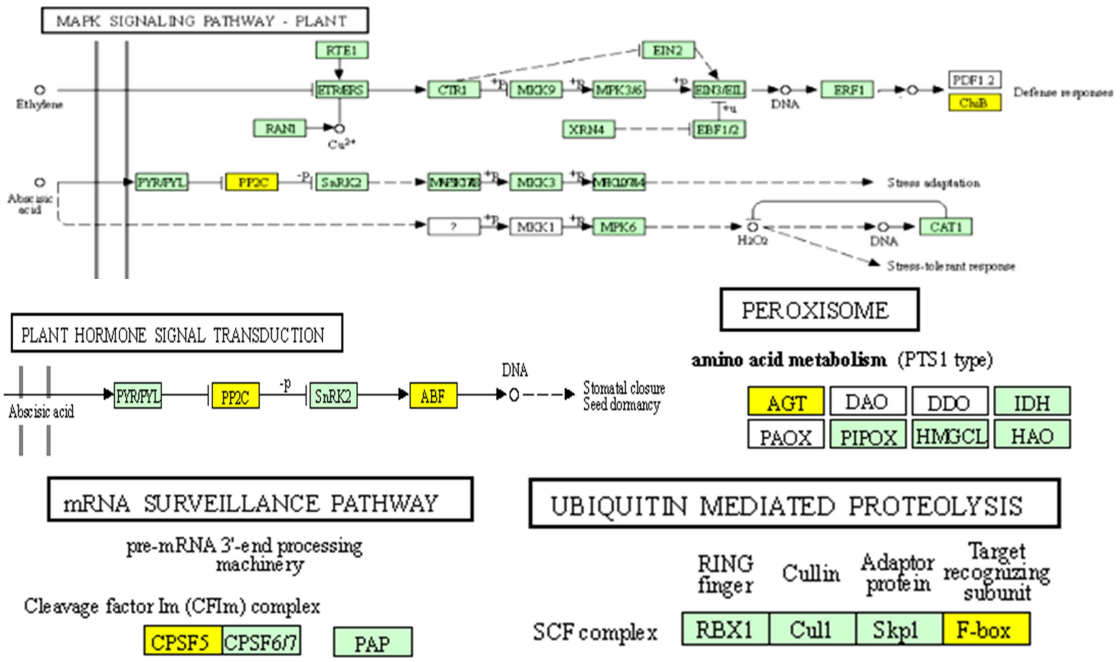

Figure 2. Up and down regulation meta-DEG in the signaling pathway of MAPK, ubiquitin mediated proteolysis, peroxisome, pathway of mRNA surveillance, and signaling transduction of plant hormone of *H. vulgare* L under salt stress. The yellow box color shows down-regulated genes.

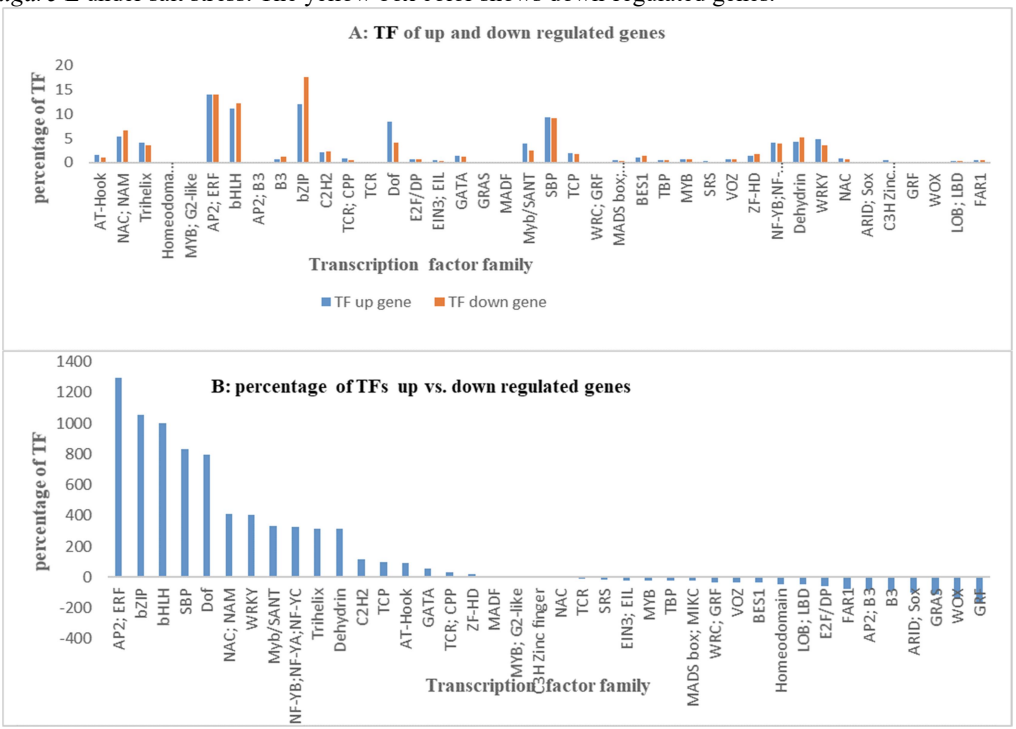

Figure 3. The up and down salt-responsive DEGs related TF Diagram according to the PlantPAN 3.0 in barley. A: TF of up and down regulated genes; B: Percentage of TF up versus down regulated genes.

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Table 3. List of 20 top upregulated meta-DEG, scored by MCC approach in cytohubba.

Name	Gene stable ID	Score	Description
Q336U3	HORVU.MOREX.r3.1HG0055890	12	proline oxidase domain containing protein
Q0J0N3	HORVU.MOREX.r3.5HG0493330	7	similar to nucleotide sugar epimerase-like protein
Q75II7	HORVU.MOREX.r3.1HG0077280	6	HAD-superfamily hydrolase subfamily IIB protein
Q6ERD9	HORVU.MOREX.r3.5HG0478850	6	HAD-superfamily hydrolase subfamily IIB protein
Q5JNJ1	HORVU.MOREX.r3.3HG0288880	6	HAD-superfamily hydrolase subfamily IIB protein
Q652S1	HORVU.MOREX.r3.7HG0670200	5	NAD(P)-binding domain containing protein
Q6Z662	HORVU.MOREX.r3.6HG0610730	4	similar to Gpi-anchored protein
Q2R8U5	HORVU.MOREX.r3.4HG0345500	4	pyruvate kinase family protein
Q6ZHG2	HORVU.MOREX.r3.6HG0618650	4	similar to 1-aminocyclopropane-1-carboxylate oxidase
Q69Q88	HORVU.MOREX.r3.5HG0483830	4	PDZ/DHR/GLGF domain containing protein
Q0DJU1	HORVU.MOREX.r3.3HG0293790	4	similar to eukaryotic translation initiation factor 3 subunit 6-interacting protein
Q0DXY9	HORVU.MOREX.r3.6HG0611090	4	tRNA methyltransferase
Q6Z744	HORVU.MOREX.r3.6HG0613490	4	aldolase-type TIM barrel domain containing protein
A0A0N7KSD1	HORVU.MOREX.r3.2HG0097950	3	similar to GDA1/CD39 family protein
Q65XK2	HORVU.MOREX.r3.1HG0090740	3	similar to CTP synthase 1
Q69RP3	HORVU.MOREX.r3.2HG0145360	3	similar to peroxidase 7 precursor
Q6F3B7	HORVU.MOREX.r3.4HG0388500	3	N2,N2-dimethylguanosine tRNA methyltransferase family protein
Q2QLN8	HORVU.MOREX.r3.5HG0420730	3	protein of unknown function NUC189
Q7X8V3	HORVU.MOREX.r3.2HG0182010	3	similar to phenylalanine ammonia-lyase
Q75GN8	HORVU.MOREX.r3.1HG0071210	3	U-box E3ubiquitin ligase, and positive regulation of abiotic stress response

Then, Cytohubba plugin in Cytoscape 3.6.1 was used to validate the hub genes, with twelve ranking methods. Because the genomic information of barley has not yet been fully reported, rice orthologues from Ensembl plants were used (https://plants.ensembl.org). Accordingly, HORVU. MOREX.r3.7HG0670200 (Q652S1) and HORVU. MOREX.r3.1HG0055890 (Q336U3) in eleven methods; HORVU. MOREX.r3.6HG0613490 (Q6Z744), HORVU. MOREX.r3.6HG0548940 (Q6Z730), and HORVU. MOREX.r3.4HG0345500 (Q2R8U5), in five approaches, HORVU. MOREX.r3.5HG0493330 (Q0J0N3) in eight ways were detected as upregulated core genes (Figure 4).

Then, HORVU. MOREX.r3.7HG0720730 (Q69V57), HORVU. MOREX.r3.4HG0410550 (Q9SNK3), HORVU. MOREX.r3.2HG0104740 (Q2QTJ1), and HORVU. MOREX.r3.1HG0068880 (A0A0P0WP33) in ten methods; HORVU. MOREX.r3.5HG0513810 (Q7XZW5) and HORVU. MOREX.r3.7HG0645350 (Q0DEU8) in eight methods; HORVU. MOREX.r3.6HG0564980 (Q7EYM8) and HORVU. MOREX.r3.5HG0511800 (A0A0P0W399) in sex methods, and HORVU. MOREX.r3.7HG0678990 (Q6ZFI6) in eleven ways were detected as downregulated core genes (Figure 5). Supplementary file Tables 1 and 2 represents the list of the down and upregulated top genes through several ways.

The selected genes based on the reported fold changes by qRT-PCR result of other

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Table 4. List of 20 top downregulated meta-DEGs, scored by MCC approach in cytohubba.

Name	Gene stable ID	Score	Description
Q9SNK3	HORVU.MOREX.r3.4HG0410550	166	Alike GAPDH
Q69V57	HORVU.MOREX.r3.7HG0720730	165	Alike fructose-bisphosphate aldolase
A0A0P0WP33	HORVU.MOREX.r3.1HG0068880	158	Alike 3-phosphoglycerate kinase
Q7XZW5	HORVU.MOREX.r3.5HG0513810	92	Similar to malate dehydrogenase
Q6ZFI6	HORVU.MOREX.r3.7HG0678990	67	Pyridoxal phosphate-dependent transferase
Q7EYM8	HORVU.MOREX.r3.6HG0564980	61	Alike quinone oxidoreductase
Q0DEU8	HORVU.MOREX.r3.7HG0645350	58	Alike transferase
Q2QTJ1	HORVU.MOREX.r3.2HG0104740	52	Alike rubisco
Q7XWP9	HORVU.MOREX.r3.2HG0106830	34	Transketolase
Q2R1G8	HORVU.MOREX.r3.7HG0658050	18	Acyl-CoA oxidase
Q6EUF8	HORVU.MOREX.r3.5HG0514560	18	Similar to citrate synthase
Q69LG7	HORVU.MOREX.r3.5HG0471620	13	Chloroplast precursor (AK-HD 2)
Q10PU4	HORVU.MOREX.r3.4HG0395940	12	Similar to homocysteine s-methyltransferase 1
Q7F2G3	HORVU.MOREX.r3.3HG0274780	10	Beta-carbonic anhydrase
C7IY87	HORVU.MOREX.r3.6HG0580230	10	Similar to cysteine synthase
SBP	HORVU.MOREX.r3.1HG0093680	10	Salinity tolerance
Q2QRV3	HORVU.MOREX.r3.7HG0637280	9	Fatty acid alpha-dioxygenase family
Q7XCS3	HORVU.MOREX.r3.1HG0050330	9	Pyridoxal phosphate-dependent enzyme domain containing protein
Q8GT01	HORVU.MOREX.r3.3HG0319810	8	Similar to pyrroline-5-carboxylate reductase
Q0IZW8	HORVU.MOREX.r3.5HG0502000	8	MaoC-like dehydratase domain containing protein

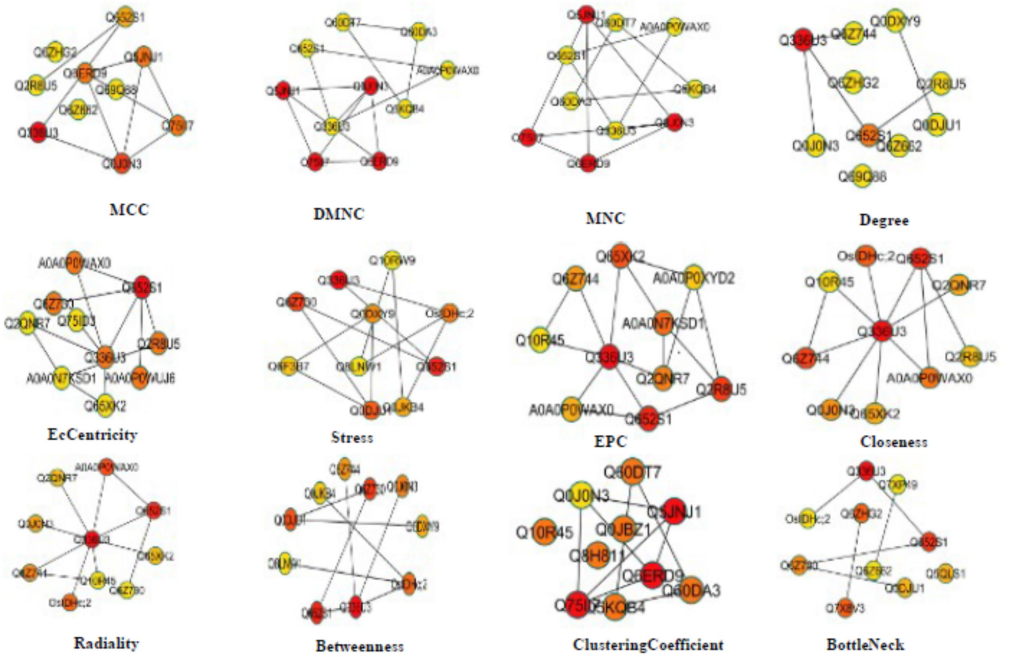

Figure 4. PPI network construction and identification of upregulated core genes using Cytoscape 3.6.1 by twelve ways. The nodes with red to yellow color display the most necessary to the least necessary.

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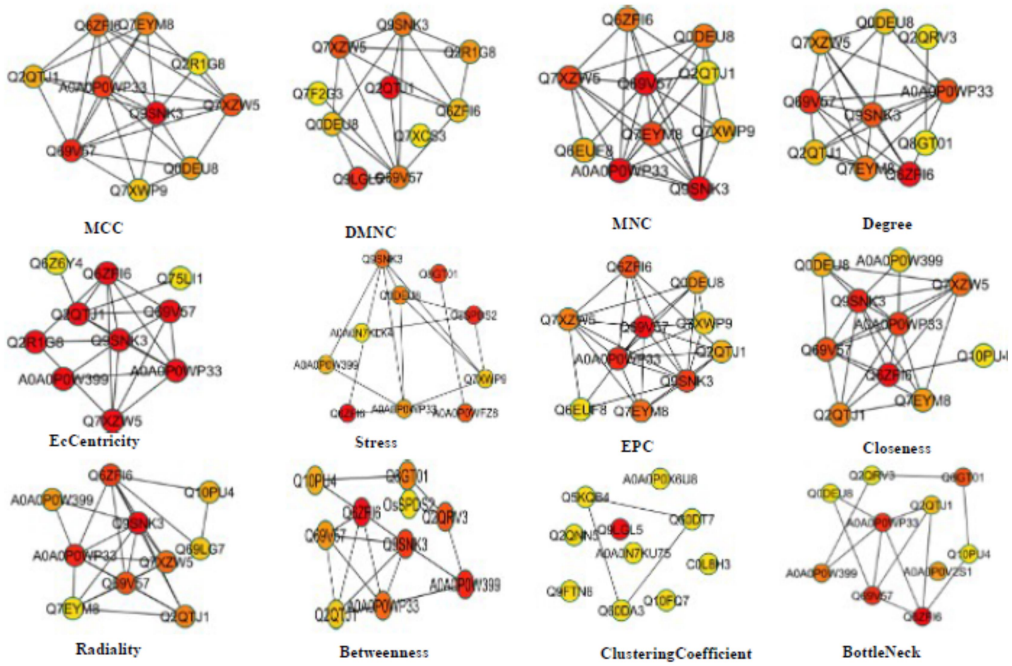

Figure 5. PPI network construction and identification of downregulated core genes using Cytoscape 3.6.1 by twelve ways. The nodes with red to yellow color display the most necessary to the least necessary.

HORVU. MOREX.r3.7HG0645350 (Os06g0133800), and HORVU. MOREX.r3.5HG0420500 (Os12g0559200) (Figure 6). Our results were compared with other research using Ensembl Plants' rice genome results (https://plants.ensembl.org).

DISCUSSION

Transcriptome analysis deciphers the plant response's molecular mechanisms to stresses through search for DEGs and biological

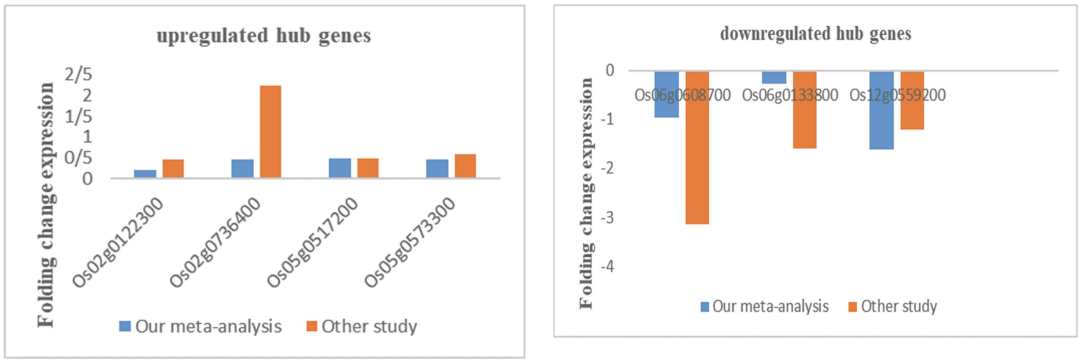

Figure 6. Confirmation of the downregulated and upregulated hub genes by other research. The fold changes in expression level from the upregulated hub genes including Os02g0122300 (Rodrigues *et al.,* 2009), Os02g0736400 (You *et al*., 2017), and Os05g0517200 and Os05g0573300 (Kottapalli *et al.,* 2007); and the downregulated core gene including O s06g0608700 (Chen *et al*., 2019), Os06g0133800 (Dai *et al*., 2013), and Os12g0559200 (Kadotani *et al*., 2016) strongly confirmed the expression levels acquired from other qRT-PCR studies.

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processes performances joined in the stress tolerance mechanisms and, thus, to link the genotype to the phenotype of a plant species at a certain developmental stage (Gharaghanipor *et al*., 2022). Plants show different physiological responses to various environments that can be identified using DEGs. In this study, the upregulated genes are mostly related to abiotic stress, bZIP TFs, glycoside hydrolase and biosynthesis of auxin and ethylene hormones; and the downregulated genes are mainly associated with salinity stress response and LEA protein. Based on other reports, MtN3 family protein, pyrroline-5-carboxylate synthetase (Walia *et al*., 2007), LEA protein (Tsuda *et al*., 2000), and serine/threonine protein kinase (Ueda *et al*., 2004) were reported as upregulated genes under salt stress in barley. The common GO terms in downregulated and upregulated genes were related to biological process, cellular catabolic process, and the metabolic processes of glucosamine-containing compound, cell wall macromolecule, and amino sugar.

In this research, the TFs including bHLH, bZIP, EIN3, WRKY, NF-YB/A/C, NAC; NAM, Myb/SANT, AP2; ERF, Dof, SBP, Dehydrin, and LOB: LBD exhibited with higher frequency in response to salt stress. Regulation of different biological processes is done by bZIP family TFs, which might be involved in abiotic stress and morphogenesis responses through connecting to promoters of particular target genes aimed at up- or downregulation of their expression (Li *et al*., 2015). bZIP-TFs are downstream factors in the Abscisic acid (ABA) signal pathway according to Jakoby *et al*. (2002), and in the salt stress response, they are also the pivotal cell process' regulators. The Lateral Organ Boundaries (LOB) TFs are of importance for lateral root formation modulated by ABA under salt stress and driven by auxin signaling, (Ariel *et al*., 2010). Nakashima *et al*. (2012) believes that bHLH are able to regulate the plants adaptive response to abiotic stresses besides having a vital role in secondary metabolism. The large family of MYB has also important roles that include primary and secondary metabolism, and their response to abiotic stresses (Dou *et al*., 2016). According to a report by Ying *et al*. (2004), the expression of Ethylene Insensitive 3 (EIN3) was induced by ethylene and salinity treatments.

Based on this study, the ABA signaling pathway includes two key genes (i.e., ABF and PP2C) as downregulated genes affecting stomatal closure. Protein Phosphatase 2C (PP2C), a stress responsive gene in eukaryotes including plants, was differentially in both cultivated and wild barley (Gharaghanipor *et al*., 2022). Since ABRE-Binding Factors (ABFs) are ABA-dependent controllers of gene expression and responders to abiotic stresses (Liu *et al*., 2017), ChiB and PP2C are considered as downregulated genes, in the signaling pathway of MAPK, which can affect the defense response through influencing ethylene biosynthesis and the stress adaption through influencing ABA biosynthesis, respectively. EIN2 (transcriptional activator) acts as regulator of ChiB expression related to the pathways of hormone response (Potuschak *et al*., 2003).

In the present research, CPSF5 was recognized as a CFIm complex's downregulated gene in pre-mRNA 3′-end processing machinery of mRNA surveillance pathway. There are two essential steps in mRNA maturation: the 3′-end cleavage and succeeding polyadenylation. CFIm is a tetramer with two 25 kDa (CF Im25) subunits and two proteins of either 59 or 68 kDa; and CFIm25 is encoded by CPSF5 (Yang *et al*., 2010). Moreover, *AGT* gene related to amino acid metabolism in peroxisome targeting signal was identified as a downregulated gene. Plant peroxisomes as ubiquitous small cell organelles are of importance in oxidative metabolic processes and reactive oxygen species metabolism (del Rio *et al*., 2002). The photorespiratory enzyme of Ala-Glyoxylate aminotransferase (AGT) was identified by Igarashi *et al*. (2003). Moreover, *F-box* gene of SCF complex was

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detected as downregulated gene in the ubiquitin mediated proteolysis, which are considered important in controlling biological functions under environmental stresses (Gagne *et al*., 2002).

This research aimed to determine the network's central genes, and identified the highly connective hub genes. Accordingly, upregulated DEGs hub genes mainly related to proline oxidase, nucleotide sugar epimerase (UDP-d-glucuronate 4-epimerase), NAD(P)-binding domain, pyruvate kinase, Aldolase-type TIM barrel domain, and Phenylalanine Ammonia-Lyase (PAL). The more expression of P5CS in the barley leaf elevated proline's accumulation level (Janská *et al*., 2011). Abiotic stress-regulated genes were induced byCBF1/DREB1b and CBF3/DREB1a overexpression and caused proline accumulation. Then, this led to the salt tolerance increase (Gilmour *et al*., 2000). D-galacturonate is among the sugars found in plants cell wall where it is synthesized by a UDP-d-glucuronate 4-epimerase from a UDP-d-glucuronate (Usadel *et al*., 2004), and can have a role in stress tolerance.

Aligned with the present findings, Gharaghanipor *et al*. (2022) have shown the upregulation of PAL in salt-tolerant barley genotype. In plants, the primary reaction is catalyzed by PAL in the general phenylpropanoid pathway, producing the phenol compounds, which are nonenzymatic antioxidants with a role in the plant stress adaptation through ROS scavenging (Kiani *et al*., 2021), and are the most abundant plant phytochemicals that serve as a source of health-beneficial antioxidant in human diet (Soleimani *et al*., 2022). Next, downregulated DEGs hub genes were mainly related to pyridoxal phosphate-dependent transferase, fructose-bisphosphate aldolase cytoplasmic isozyme (FBA), rubisco, GAPDH, quinone oxidoreductase, phosphorylase, 3-phosphoglycerate kinase, and salinity tolerance. The active form of VB6 is Pyridoxal 5′-Phosphate (PLP), which is involved in cellular processes, and synthesized by two proteins (i.e., PDX1 and PDX2). Regulating during response to abiotic stresses, PLP deficient plants are found to be sensitive to abiotic stresses (Huang *et al*., 2013). GAPDH is involved in energy production, DNA repair, transcriptional regulation, ABA signal transduction, and embryo development. It can be classified into four subfamilies of GAPA/B/C/N. According to Jesús *et al*. (2009), at initial stages, *AtGAPCp1* gene expression in Arabidopsis shoots was caused by salinity stress. Cytosolic FBA is a part of gluconeogenesis and sucrose biosynthetic, and catalyzes fructose 1, 6-bisphosphate production. *FBA* genes regulate abiotic stress responses' development growth, and control (Fan *et al*., 2009). Generally, according to our results, the hub up and downregulated genes mostly belonged to biosynthesis of photosynthesis and glycolysis, respectively.

To accredit the meta-analysis results accuracy, a comparison was made between the fold changes in expression level, related to the four upregulated core genes, and levels acquired from other reports qRT-PCR, which included Os02g0122300 (Rodrigues *et al*., 2009), Os02g0736400 (You *et al*., 2017), and Os05g0517200 and Os05g0573300 (Kottapalli *et al*., 2007); and three downregulated core genes that included Os06g0608700 (Chen *et al*., 2019), Os06g0133800 (Dai *et al*., 2013) and Os12g0559200 (Kadotani *et al*., 2016). Thus, it can be concluded that by applying the acquired hub genes, the meta-analysis data will be sufficiently acceptable. This is the first study in which barley is considered as the model plant for detecting the genes participating in tolerance mechanism to salt stress. Briefly, in this study, a meta-analysis platform was chosen to recognize the most important TFs, genes and pathways, through integrating salt stress transcriptional data.

CONCLUSIONS

As more reliable results can be obtained by integrating information from multiple

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sources, meta-analysis is employed extensively. In spite of having some limitations, microarrays employment provides an opportunity to compare salt stress-induced transcriptome changes and to detect the main genes that are differentially expressed uniformly in studies. In the present study, some transcription factors and hub genes are found, which play a part in barley salt stress response. The findings can be used for understanding the mechanisms involved in salt stress response and introducing beneficial candidate genes in barley breeding programs.

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مکانیسم ملکولی تحمل به تنش شوری در جو (*Hordeum vulgare* L.) از طریق متاآنالیز داده های ترانسکریپتوم

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چکیده

تنش شوری به عنوان یکی از مهمترین تنش غیرزیستی رشد گیاه را محدود و خسارت جبران ناپذیری به تولیدات کشاورزی در سراسر جهان وارد می کند. بنابراین شناسایی ژن هایی که در تحمل به تنش شوری گیاهان نقش کلیدی ایفا نمایند از طریق آنالیز داده های ترانسکریپتوم نظیر ریزآرایه و توالی یابی با توان بالا (NGS) لازم و ضروری است. در این راستا، متاآنالیز داده‌های ریزآرایه جو توسط بسته‌های نرم افزارR تحت تنش شوری به ترتیب ٦٨٥ و ٧٦٦ ژن‌های با بیان متفاوت بالا و پایین را شناسایی کرد. ژن های با بیان بالا عمدتاً به تحمل استرس غیرزیستی و بیوسنتز هورمون و ژن‌های با بیان پایین مربوط به پروتئین‌های فراوان در اواخر جنین‌زایی (LEA) و پاسخ استرس شوری مرتبط هستند. ژن آنتولوژی، ژن‌های با بیان بالا بیشتر در پاسخ به استرس‌های خارجی و داخلی و ژن‌های با بیان پایین با متابولیسم سلولی مرتبط بودند. متاژن های حاصل از تحقیق حاضردر مسیر KEGG تحت تنش شوری شامل PP2C ، ABF، AGTبه ژن‌های با بیان بالا و ژنهای ChiB و F-box به ژن‌های با بیان پایین مرتبط بودند. فاکتورهای رونویسی (TFs) در متاژن های با بیان بالا و پایین شامل AP2 ، ERF، bZIPو bHLH بودند. بیشتر ژن‌های هاب با افزایش بیان به‌دست‌آمده از این تحقیق، به بیوسنتز متابولیت‌ها و فتوسنتز و ژن‌های هاب با کاهش بیان عمدتاً مربوط به چرخه اسید تری کربوکسیلیک و فرآیندهای گلیکولیز مرتبط بودند. در نهایت، مقایسه ای بین یافته های متاآنالیز حاضر و داده های به دست آمده از تحقیقات سایر محققین انجام و بیان بالا و پایین آنها را تایید نمود. نتایج حاصله درک جدیدی در مورد مکانیسم مولکولی تحمل به تنش شوری در جو ارائه می‌دهد و می توان بسیاری از فاکتورهای رونویسی و ژن‌های کاندید شناسایی شده در تحقیق حاضر را برای تحمل به تنش شوری در برنامه‌های اصلاحی جو استفاده نمود.

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