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IN SILICO IDENTIFICATION OF BARLEY (*HORDEUM VULGARE* L.) GENE-ORTHOLOGS AGAINST ABIOTIC-STRESS TOLERANCE BY USING ARABIDOPSIS

MUHAMMAD NAVEED SHAHID[1*], AREEJ ARSHAD[1], MUHAMMAD SARFRAZ KIANI[2], ADIL JAMAL[3], SEEMAL ABBAS[1] AND MAFIA MUSHTAQ[1]

[1]Department of Botany, Division of Science and Technology, University of Education, Lahore, Pakistan
[2]Department of Biological Sciences, Superior University, Raiwind Road, Lahore, Pakistan
[3]Department of Biochemistry and Biotechnology, Faculty of Sciences, The University of Faisalabad, Faisalabad, Pakistan

*Corresponding author's email: naveed.shahid@ue.edu.pk

Abstract

Crop health and yield are both affected severely by abiotic strains globally. One of the most tolerant crops to abiotic stresses, barley (*Hordeum vulgare*) is grown in every continent. The current study finds gene orthologs responsible for salt-stress tolerance in barley. It seems possible to identify orthologs in other plants using the available information. For this purpose, 89 genes were selected from the scientific literature that had been peer-reviewed. To find the genes associated with abiotic stress, the *Arabidopsis thaliana* genome-wide gene expression database was examined. A total of 519 *A. thaliana* genes associated with salt-stress were shown using the STRING database and co-expression analysis. Fourteen clusters connected to 138 genes were discovered through cluster analysis. TAIR database was used to obtain the amino acid sequences of the *Arabidopsis* gene. Comparative analyses between the non-redundant *Hordeum vulgare* protein sequence and the *Arabidopsis* network-associated protein sequence identified 14 transcription factors and 9 *A. thaliana* domains with unclear functions. In comparison, barley had five domains with unknown functions and 9 transcription factors. The enrichment tool helped us to recognize six molecular functions that were crucial to the salt-stress response of plants. The orthologs data may be used to increase productivity and reduce the loss of barley yield on account of abiotic stresses.

Key words: *Hordeum vulgare*; Abiotic stress; Gene orthologs; Transcription factor; STRING; TAIR

Introduction

Harmful effects by any kind of abiotic stress element (drought, heat, salinity or climate susceptibility) are visible on plants grown within a specific setting, which leads to a multitude of bad responses pertaining to cell metabolism and gene expression, together with growth and fruit (Zhang *et al*., 2023). Additionally, the frequency and intensity of abiotic stresses may increase due to global climate change, indicating that cultivating improved stress-tolerant cultivars is essential for sustainable crop production in the future (Yoon *et al*., 2020). Plants can reprogram their transcriptomes, proteomes, and metabolomes to respond to a broad range of biotic (bacterial, fungal, and insect pests) and abiotic (salinity, drought, and temperature extremes) stresses. Global climate change, salinity, and drought effect grain output and agricultural development in several regions (Ashraf, 2010). Plants' ability to tolerate both abiotic and biotic stresses is mediated by complex regulatory networks, according to several omics studies (Megha *et al*., 2018; Summanwar *et al*., 2019).

The most extensively studied plant model organism for decades is *Arabidopsis thaliana*, and comparative genomic studies have also been conducted on several other closely related species (Koenig & Weigel, 2015). Owing to the recent advances in microarray technology several stress-responsive genes in *Arabidopsis* have been identified (Sahoo *et al*., 2020). As the most salt-stress tolerant cereal, barley (*Hordeum vulgare* L.) makes a great model plant for studying salt-stress (Yu *et al*., 2020). Barley and cereals are used in the malting and brewing industries. Due to its inherent resistance to drought, salt, and fungi-caused illness, barley is used as a main plant in stress related studies (Bray *et al*., 2000). Barley genes and orthologs can be identified with the help of data from an *Arabidopsis* microarray.

A key component of comparative genomics is gene orthology that is an important idea in evolutionary biology (Bult & Sternberg, 2023). Ortholog-based gene identification is feasible and efficient in today's genomic age (Barthelson *et al*., 2010). Important tasks of modern genome analysis are the determination of orthologous genes and the computation of comparative analyses for sets of genomes (Dieckmann *et al*., 2021). Individual genome sequence studies revealed a lot about the structure of the genome but little about how it works (Venter *et al*., 2001). An important development in the biological sciences was the identification of human and model organism genome sequences (Miller *et al*., 2004). The augmented absorption of NaC by the root system, coupled with elevated concentrations of NaC within the xylem sap as facilitated by HvHKT2, resulted in enhanced translocation of NaC to the aerial parts; in contrast to the species of wheat and rice, the increased incorporation of saline conditions could represent an advantageous strategy for tolerance in barley (Mian *et al*., 2011 Mohamed *et al.*, 2024). Utilizing Arabidopsis as a reference, researchers can identify barley orthologs associated with abiotic stress tolerance, enabling

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the development of more resilient barley varieties through targeted breeding and genetic engineering approaches (Mahmood, 2011; Saleem *et al.,* 2014).

Plants can prioritize their responses to salinity under situation of combined salinity as well as pathogen stress by activating ion transporters that help to maintain ionic equilibrium, such as NHX (Na+/H+ antiporters) along with SOS (salt overly sensitive) pathways (Saleem *et al.,* 2014; Maach *et al*., 2024). However, when exposed to biotic pressures like animal attacks, other plant species, like those in the *Brassica* genus, display cross-tolerance mechanism that improve their adaptability to abiotic stresses, such as heat or drought (Cantila *et al*., 2024; Yoo *et al*., 2024).

The research on rice's ability to withstand against salt-stress has been greatly improved by the application of genomic technologies, which have revealed complex genetic and biochemical processes that control this trait. The role of candidate genes in salt-stress signaling has been highlighted, including calmodulin-binding transcription factors and MIKC-type MADS domain proteins (Leawtrakun *et al*., 2024). In addition to ionic processes, rice, wheat, and maize have well-established phytohormone-mediated salt tolerance pathways. For example, rice has jasmonic acid, salicylic acid, and abscisic acid (Kumar *et al*., 2022). Advances in genomics have identified salt-tolerance genes, enabling development of more resilient barley varieties.

The current study is completely dependent on computational methods for predicting salt-stress tolerant genes that could be used to improve barley cultivars in the future. The study of how species have changed through time and the discovery of the genes that make each creature unique will be done using comparative genomics. Different search tools such as; PubMed, ATTED-II, String, Cytoscape, ShinyGO and CELLO2GO were used to study Barley's salt-stress tolerant genes and transcription factors, which will help to reduce its losses.

Research Methodology

Data retrieval: *Arabidopsis* genes associated with salt-stress were collected using PubMed database. Search keywords given in the PubMed database were "*Arabidopsis* + salt-stress + gene" to find the salt-stress responsive genes in *A. thaliana*.

Co-expression analysis: The main bait-gene list carrying all stress-responsive genes was established and ATTED-II database (http://atted.jp) was used to retrieve co-expression networks for each enlisted gene. To understand the structural basis of gene co-expression, a network of connected and unconnected genes was used to characterize the interactions between co-expressed genes.

Interacting genes/proteins determination: Genes involved in co-expression analysis and main bait genes were employed in STRING to identify the genes (http://string.db.org/). After choosing a high confidence level in the parameters, disconnected genes were eliminated, and their network was downloaded once again with only the linked genes.

Analysis through cluster: Database of ClusterViz Cytoscape (https://cytoscape.org/download.html) was accessed to develop a co-expression network by examining the network interactions in clusters. The genes' networks were categorized using cluster analysis, while network visualization was performed using the STRING database.

Arabidopsis gene protein sequence retrieval: The genes associated with the *Arabidopsis* network were compiled into a list and the protein sequences for all enlisted genes were retrieved using TAIR (The *Arabidopsis* Information Resource, https://www.arabidopsis.org).

Barley orthologs estimation: Using NCBI protein blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), barley orthologs were identified. *Arabidopsis* protein downloaded sequences were copied individually in FASTA format, and placed into the protein blast search bar against non-redundant protein sequences (nr) from *Hordeum vulgare* L.

Analysis of functional barley genes: Barley orthologs in FASTA format were used to perform research on Gene Ontology (GO) enrichment using STRING and ShinyGO v0.741 (http://bioinformatics.sdstate.edu/go/) platforms. To manage further functional annotation, CELLO2GO platform (http://cello.life.nctu.edu.tw/cello2go/) was accessed.

Results

Retrieval of data: After experimental validation, a total of 89 stress-tolerant genes were retrieved using PubMed database. For analysis of co-expression and identification of orthologs, these primary genes were used (Table 1).

Analysis of co-expression: Using each of the 89 genes, ATTED-II database was accessed to compile the directly related genes based on MR values. Following co-expression analysis, 519 genes with both primary and co-expressed genes were included in a new pooled gene list.

Interacting genes/proteins determination: A co-expression network of 519 genes was established by using the STRING database. All connected & unconnected genes were present in that network (Fig. 1). A more complex co-expression network was produced with a high degree of confidence using the STRING database. The final gene list was chosen based on a newly acquired set of genes, which revealed a unique network clustering pattern (Fig. 2).

Cluster analysis: The interaction data was displayed using Cytoscape and the Cluster Viz was used to investigate and depict network interactions. The entire network was subjected to cluster analysis using the MCMODE method, making it possible to identify even overlapping clusters (Fig. 3). Following were the parameters for MCMODE algorithm: - K-Core Threshold: 2, Degree threshold: 2, NodeScore Threshold: 0.2, and MaxDepth: 100. A total of 14 sub-network clusters were recognized. The existence of a 'conn' was discovered by Cluster analysis (Table 2).

Table 1. Curated using 89 genes. The genes linked to salt stress, as determined through experiments, were collected from peer-reviewed literature.

S. No.	Gene ID	Citation used for current work	S. No.	Gene ID	Citation used for current work
1.	*AT1G02400*	Magome *et al*., 2008	46.	*AT3G29090*	Yan *et al*., 2018
2.	*AT1G02730*	Gu *et al*., 2016	47.	*AT3G45410*	He *et al*., 2004
3.	*AT1G03060*	Steffens *et al*., 2015	48.	*AT3G45700*	Li *et al*., 2016
4.	*AT1G05850*	Sanchez *et al*., 2012	49.	*AT3G46550*	Basu *et al*., 2016
5.	*AT1G06040*	Datta *et al*., 2007	50.	*AT3G47950*	Vitart *et al*., 2001
6.	*AT1G10940*	Julkowska *et al*., 2015	51.	*AT3G48850*	Zhu *et al*., 2012
7.	*AT1G18890*	Cheng *et al*., 2002	52.	*AT3G50500*	Mogami *et al*., 2015
8.	*AT1G24460*	Roy & Bassham, 2015	53.	*AT3G53530*	Luo *et al*., 2016
9.	*AT1G27760*	Park *et al*., 2009	54.	*AT3G55530*	Zhang *et al*., 2015
10.	*AT1G29060*	Tarte *et al*., 2015	55.	*AT3G55610*	Szekely *et al*., 2008
11.	*AT1G32230*	Li *et al*., 2018	56.	*AT3G55980*	Sun *et al*., 2007
12.	*AT1G35670*	Urao *et al*., 1994	57.	*AT3G59900*	Kuluev *et al*., 2019
13.	*AT1G35720*	Laohavisit *et al*., 2013	58.	*AT4G01420*	Saito *et al*., 2018
14.	*AT1G45249*	Uno *et al*., 2000	59.	*AT4G16830*	Ambrosone *et al*., 2015
15.	*AT1G45688*	Endler *et al*., 2015	60.	*AT4G17615*	Cheong *et al*., 2003
16.	*AT1G50960*	Magome *et al*., 2008	61.	*AT4G22330*	Wu *et al*., 2015
17.	*AT1G60940*	MeLoughlin *et al*., 2012	62.	*AT4G22820*	Adai *et al*., 2005
18.	*AT1G69270*	Shi *et al*., 2014	63.	*AT4G30960*	Guo *et al*., 2001
19.	*AT1G73660*	Gao *et al*., 2008	64.	*AT4G33000*	Lin *et al*., 2009
20.	*AT1G78290*	Kim *et al*., 2012	65.	*AT4G33730*	Chien *et al*., 2015
21.	*AT2G01450*	Frick & Strader, 2017	66.	*AT4G33950*	Boudsocq *et al*., 2004
22.	*AT2G01980*	Yue *et al*., 2012	67.	*AT4G34890*	Zarepour *et al*., 2010
23.	*AT2G03150*	Guan *et al*., 2013	68.	*AT4G35100*	Pou *et al*., 2016
24.	*AT2G04240*	Ko *et al*., 2006	69.	*AT4G40010*	Boudsocq *et al*., 2004
25.	*AT2G17270*	Zhu *et al*., 2012	70.	*AT5G05410*	Nakashima *et al*., 2000
26.	*AT2G26980*	Tang *et al*., 2015	71.	*AT5G08590*	Boudsocq *et al*., 2004
27.	*AT2G38470*	Jiang *et al*., 2006	72.	*AT5G13170*	Seo *et al*., 2010
28.	*AT2G39800*	Abraham *et al*., 2003	73.	*AT5G14040*	Zhu *et al*., 2012
29.	*AT2G40140*	Sun *et al*., 2007	74.	*AT5G17850*	Cai & Lytton, 2004
30.	*AT2G40950*	Liu *et al*., 2007, 2008	75.	*AT5G19660*	Liu *et al*., 2008
31.	*AT2G41010*	Perruc, 2004	76.	*AT5G19690*	Koiwa *et al*., 2003
32.	*AT2G41560*	Geisler *et al*., 2000	77.	*AT5G24270*	Ishitani *et al*., 2000
33.	*AT2G41900*	Jinget *et al*., 2019	78.	*AT5G27150*	Apse *et al*., 1999
34.	*AT2G45640*	Song & Galbraith, 2006	79.	*AT5G35410*	Liu *et al*., 2000
35.	*AT2G46400*	Ding *et al*., 2013	80.	*AT5G37850*	Gonzalez *et al*., 2007
36.	*AT2G47770*	Balsemao *et al*., 2011	81.	*AT5G42860*	Endler *et al*., 2015
37.	*AT3G02140*	Garcia *et al*., 2008	82.	*AT5G51110*	Zhang *et al*., 2015
38.	*AT3G05880*	Liu *et al*., 2012	83.	*AT5G52300*	Narusaka *et al*., 2003
39.	*AT3G08720*	Mizoguchi *et al*., 1995	84.	*AT5G52310*	Yamaguchi & Shinozaki, 1994
40.	*AT3G08730*	Mizoguchi *et al*., 1995	85.	*AT5G57630*	Pandey *et al*., 2015
41.	*AT3G11020*	Nakashima *et al*., 2000	86.	*AT5G58580*	Tian *et al*., 2015
42.	*AT3G12360*	Sakamoto *et al*., 2008	87.	*AT5G63650*	Boudsocq *et al*., 2004
43.	*AT3G16890*	Zsigmond *et al*., 2008	88.	*AT5G63980*	Quintero *et al*., 1996
44.	*AT3G19290*	Uno *et al*., 2000	89.	*AT5G66880*	Boudsocq *et al*., 2004
45.	*AT3G26520*	Schussler *et al*., 2008			

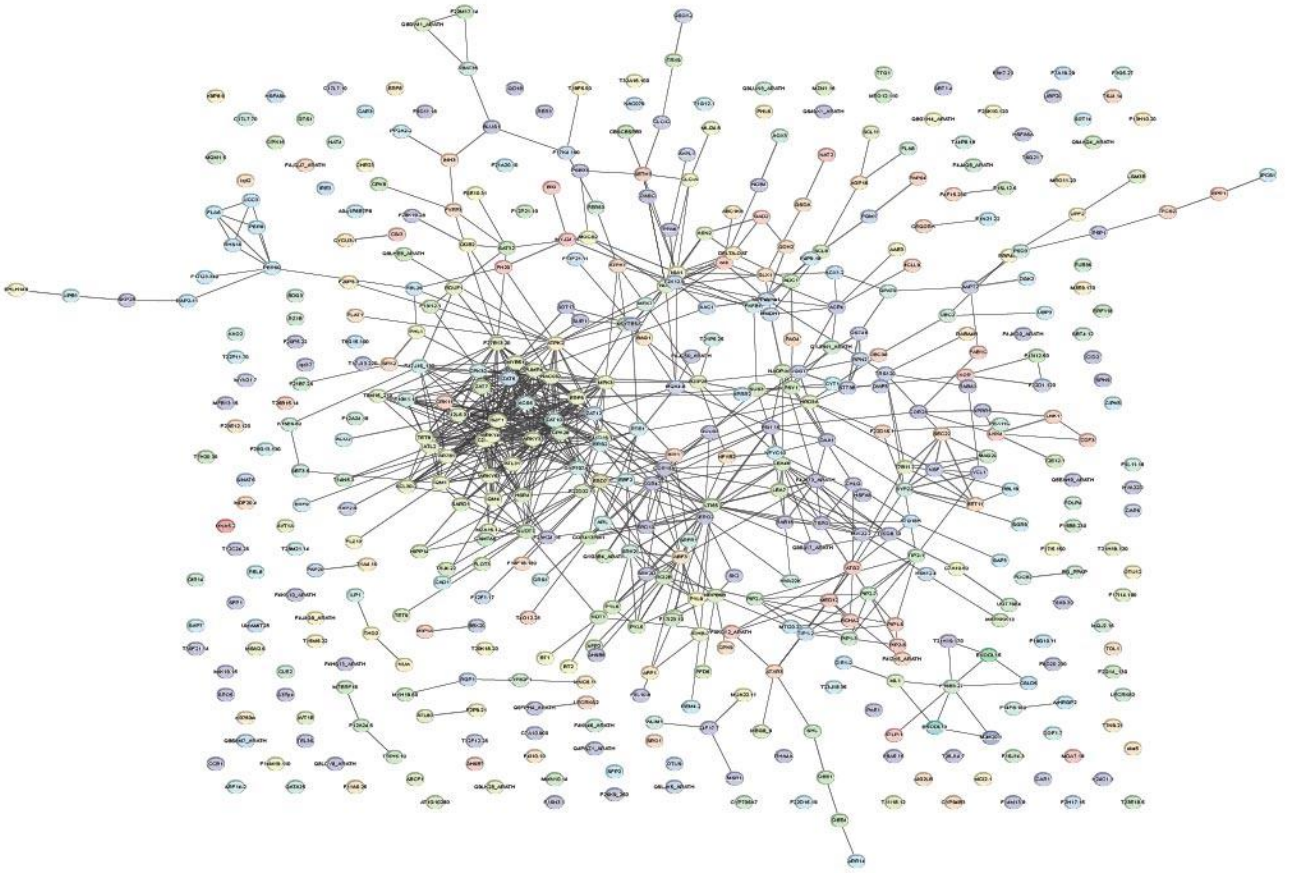

Fig. 1. Indicating two types of gene networks: those with disconnected genes and those with co-expression. The STRING database built the network with 519 primary and co-expressed genes. All connected and unconnected genes were present in the network.

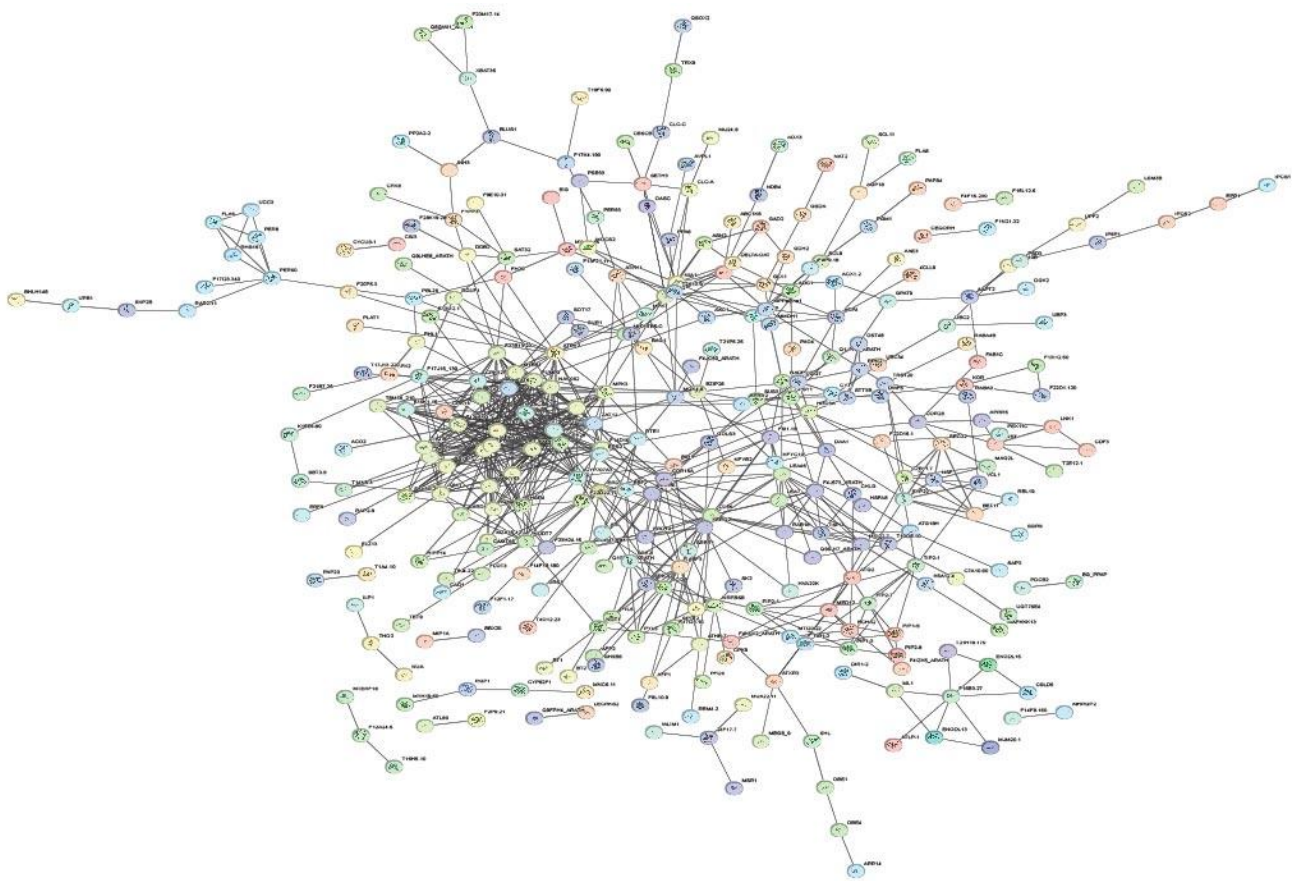

Fig. 2. Genes arranged in a network with only direct connections. Through the STRING database, the network was rebuilt with a high degree of confidence in order to remove the disconnected genes.

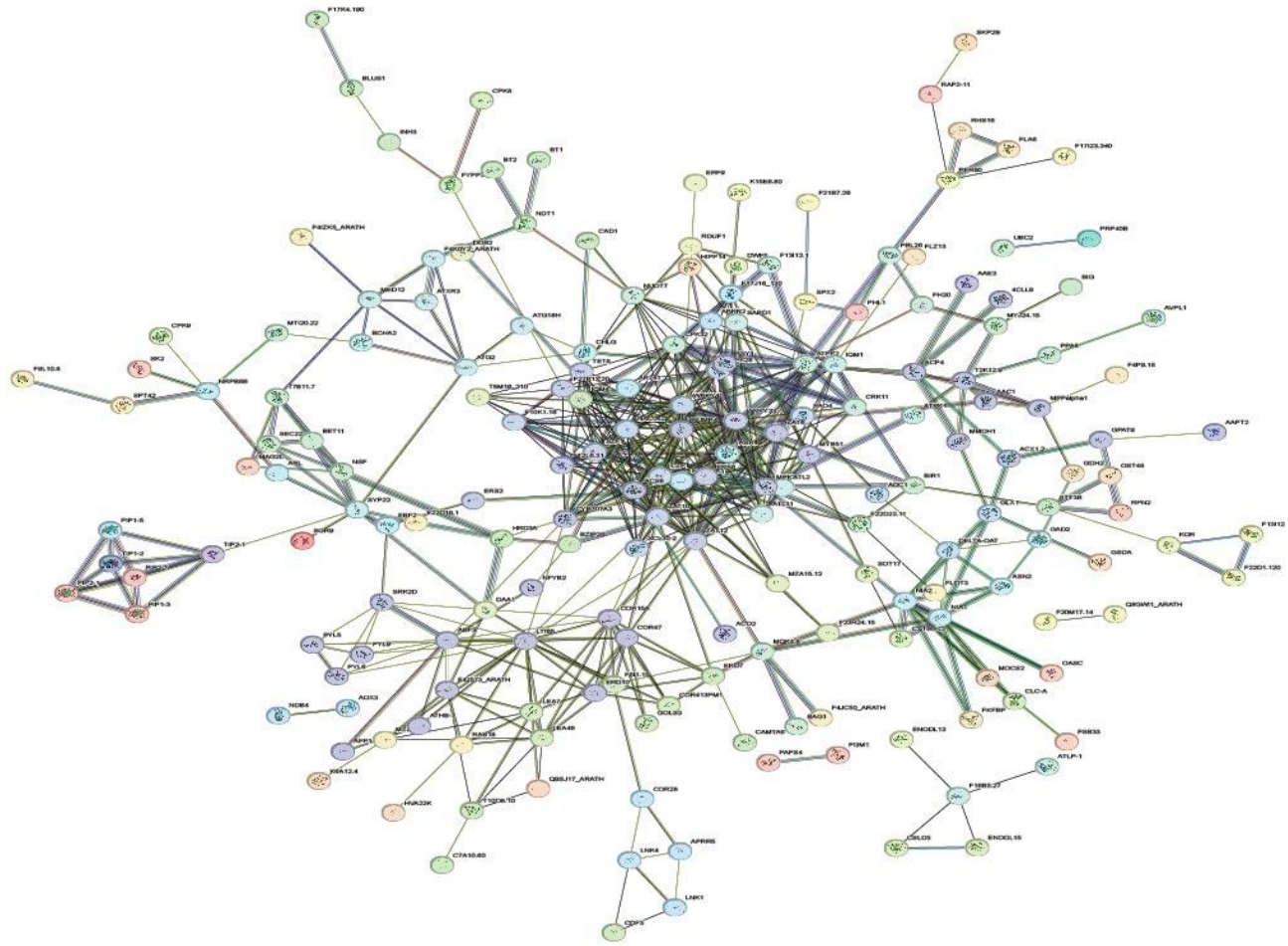

Fig. 3. A network of gene co-expression with unique clusters inside its sub-network. The entire network was subjected to cluster analysis using the MCMODE technique, which made it possible to identify overlapping clusters.

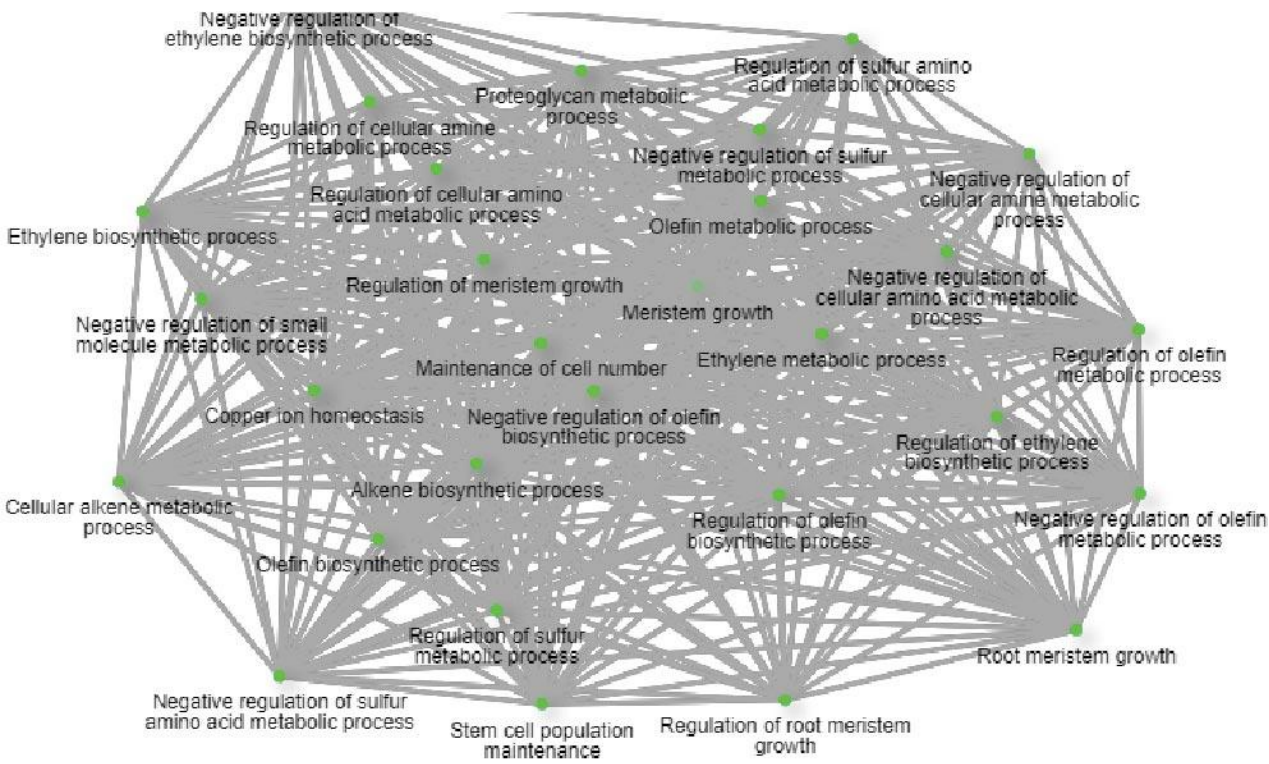

Fig. 4. Showing interaction of biological processes. Interactions between biological processes were demonstrated by an interactive enrichment network that revealed genes involved in plants' response to salt-stress and were related to 13 biological processes.

Table 2. Showing the nodes, edges and clusters. Fourteen clusters were achieved in 138 genes' network. All the genes contained in these fourteen clusters are listed in table 2 showing nodes, edge numbers and clusters.

Cluster No.	Nodes No.	Edges No.	Node ID (Genes)
1	24	219	AR781, ERF5, AT1G07135, ATL2, AT1G72900, CZF1, MPK3, SZF1, AT2G39650, TCH3, BCS1, WRKY40, WRKY33, RHL41, NUDT7, AT1G19020, CNI1, AT5G52760, EDA39, CBP60G, WRKY46, WRKY53, AT5G39670, XLG2
2	15	61	ACS6, ERF6, AT4G29780, DIC2, AT2G26190, MYB51, STZ, AT3G59080, HSFA4A, SYP122, CRK11, S6K2, AT3G01830, AT5G42050, BIR1
3	20	57	SOS3, CDPK1, SOS1, NHX1, COR15A, ABF3, RCAR1, SNRK2.3, ABF4, OST1, ABF2, SNRK2.2, DREB2B, GDH2, NIA1, DELTA-OAT, P5CS2, NIA2, ASN2
4	6	14	RVE7, CDF3, AT5G64170, STO, AT5G06980, CCA1
5	12	29	AFP1, AFP2, TMAC2, PP2CA, ATAF1, HIK, AT3G02640, AT4G15830, CYCA1;1, CSLD5, SYP111, ENODL14
6	13	29	COR413-PM1, KIN2, COR47, ERD7, ERD10, GolS3, AT1G16850, LEA4-5, LEA14, LEA7, AT5G50360, TSPO, AT3G17520
7	25	52	LTI65, SOS2, SOS4, SOS5, P5CS1, AAC1, GAD2, AT1G51980, mMDH1, ASP1, NADP-ME1, CCB1, ACP4, G4, AT5G51110, AT1G74730, AT1G71500, SEC22, NSF, BS14A, AT2G34250, STT3B, DGL1, AT2G01720, HAP6
8	4	6	SOT17, SAL1, AKN2, SUR1
9	3	3	AT5G18940, CIPK15, AT4G14480,
10	3	3	S1P, EBS1, BZIP17
11	3	3	PK1, MPK17, AT4G02450
12	3	3	ARL, ORS1, ARGOS
13	3	3	SBT4.12, AT3G45700, CYP83F1
14	4	4	BIG, AT4G02660, AT1G73660, SPI

Table 3. Showing identified DUFs (domains of unknown functions) and TFs (transcription factors) of *A. thaliana*.

DUFs	TFs
1. AR781 (Pheromone receptor-like protein, DUF1645, unknown function)	1. WRKY46 (Probable WRKY transcription factor)
2. AT1G19020 (Unknown protein)	2. MYB51 (Transcription factor myb)
3. AT4G15830 (Uncharacterized protein)	3. CBP60G (Transcription activator that binds DNA in a sequence-specific manner)
4. AT1G74730(DUF11187, protein of unknown function)	4. WRKY40 (Probable WRKY transcription factor)
5. AT5G50360 (A domain protein, unknown protein)	5. WRKY33 (Probable WRKY transcription factor)
6. AT4G29780 (Uncharacterized protein)	6. ATAF1 (NAC domain transcriptional regulator superfamily protein)
7. AT3G02640 (uncharacterized protein)	7. RVE7 (Transcription factor)
8. AT2G39650 (DUF506, unknown protein)	8. BZIP17 (Transcription factor)
9. AT1G16850 (Uncharacterized protein)	9. CCA1 (Transcriptional repressor that performs overlapping functions with LHY)
	10. WRKY53 (Transcription factor interacts specifically with the W box)
	11. HSFA4A (Heat stress transcription factor)
	12 ERF5 (Ethylene responsive element binding factor)
	13. ERF6 (Ethylene responsive element binding factor, transcriptional activator)
	14. AT5G51110 (Transcriptional co-activator)

Table 4. Showing identified DUFs (domains of unknown functions) and TFs (transcription factors) of *H. vulgare* L.

DUFs	TFs
1. XP_044978670.1 (Uncharacterized protein LOC123445710)	1. XP_044954199.1 (WRKY transcription factor WRKY71-like)
2. XP_044959024.1 (Uncharacterized protein LOC12341019)	2. XP_044977846.1 (WRKY transcription factor WRKY24-like)
3. XP_044974129.1 (Uncharacterized protein LOC123442091)	3. ABI13388.1 (WRKY transcription factor 22)
4. XP_044972474.1 (Uncharacterized protein LOC123439925)	4. XP_044972580.1 (transcription factor WRKY19-like)
5. XP_044980473.1 (Uncharacterized protein LOC123447843)	5. XP_044970181.1 (transcription factor MYB16-like)
	6. XP_044960061.1 (bZIP transcription factor 46-like)
	7. XP_044951040.1 (bZIP transcription factor 23 isoform X1)
	8. KAE8809518.1 (NAC transcription factor)
	9. XP_044974543.1 (bZIP transcription factor 39-like)

Table 5. Showing the GO-biological processes. The table shows individual GO-IDs, p-values and the details of salt-stress responsive genes controlling various process.

GO ID	P Value	Description
GO:0010364	1.1e-02	Regulation of ethylene biosynthetic process
GO:0031335	1.1e-02	Sulfur amino acid metabolic process regulation
GO:0006029	1.1e-02	Proteoglycan metabolic process
GO:1900908	1.1e-02	Regulation of olefin metabolic process
GO:1900911	1.1e-02	Regulation of olefin biosynthetic process
GO:0009692	1.1e-02	Ethylene metabolic process
GO:0009693	1.1e-02	Ethylene biosynthetic process
GO:0043449	1.1e-02	Cellular alkene metabolic process
GO:0043450	1.1e-02	Alkene biosynthetic process
GO:1900673	1.1e-02	Olefin metabolic process
GO:1900674	1.1e-02	Olefin biosynthetic process
GO:0033238	1.1e-02	Regulation of cellular amine metabolic process
GO:0006521	1.1e-02	Regulation of cellular amino acid metabolic process

Table 6. Showing the GO-molecular function. The table shows individual GO-IDs, p-values and the details of molecular response of salt-stress responsive genes.

GO ID	P Value	Description
GO:0005484	3.4e-02	SNAP receptor activity
GO:0016782	3.4e-02	Transferase activity, transferring sulfur-containing groups
GO:0030674	3.4e-02	Protein-macromolecule adaptor activity
GO:0000149	3.4e-02	SNARE binding
GO:0060090	3.4e-02	Molecular adaptor activity
GO:0008146	3.4e-02	Sulfotransferase activity

Retrieval of Arabidopsis protein sequences: Through co-expression network analysis, 138 genes associated with salt-stress were exposed. TAIR was then used to determine the protein sequences of these genes.

Identification of barley orthologs: Using NCBI Blastp program, network-associated genes' protein sequences in *Arabidopsis* were aligned with a set of non-redundant barley genes' protein sequences. It was revealed that DUFs (domains of unknown functions) were present. The DUFs information is given in tables 3 and 4. There were 9 domains in *A. thaliana* with unidentified functions as well as 9 transcription factors, compared to 5 domains in barley with unidentified functions along with 9 transcription factors. These findings enabled the discovery of novel genes that provide information on the way that plants react to abiotic stresses.

Analysis of gene ontology enrichment: ShinyGO v0.741 was used for the study of gene ontology enrichment. While certain genes were discovered to be related to molecular processes, others were determined to be involved in biological functions. Table 7 and figure 5 include the list of GO terminology for biological processes.

Biological functions: By utilizing the gene-enrichment tool, ShinyGO v0.741, it was revealed that 13 biological processes were connected to the genes that influence the plants' reaction to salt stress. These biological processes were the regulation of sulfur amino-acid metabolism, olefin metabolism, regulation of alkene biosynthesis, regulation of ethylene biosynthesis, and cellular alkene metabolism (Table 5). On the y-axis, FDR (false degree rate) was shown as -log10 (FDR), while the fold enrichment of relevant pathways was shown on the x-axis (Fig. 3, Table 6, 7).

Molecular functions of genes: The genes associated with how plants react to salt stress were shown to be linked to a total of 6 molecular processes by the enrichment method. Barley genes were also subjected to hierarchical clustering with –log 10 false discovery rates (FDR) given on y-axis; while on x-axis, FDR related to enhancement of each unique route are displayed (Fig. 4). Functional GO annotation displayed the proteins engaged in several molecular processes and biological functions (Table 7, Figs. 5, 6, 7).

Gene annotation and subcellular organization: A FASTA format list of barley orthologs was copied and pasted into CELLO2GO. Salinity stress genes were subsequently categorized based on their biological and molecular roles. Most of these proteins were involved in various biosynthetic processes, such as, signal transduction, cellular protein modification, stress adaptation, formation of cellular structures and cell differentiation. The molecular roles of these proteins were ion binding, oxidoreductase activity, and transcription control.

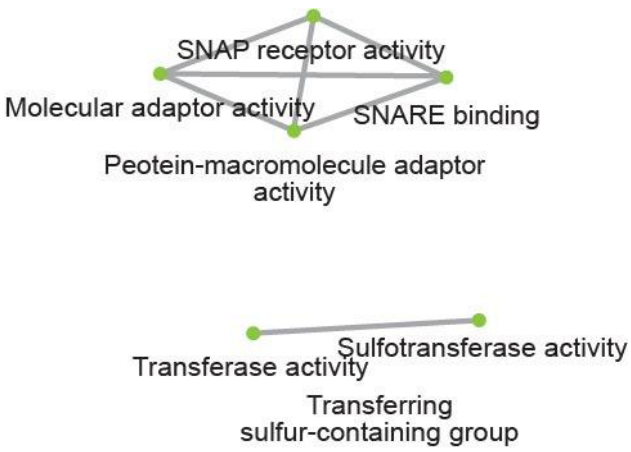

Fig. 5. Showing the molecular functions interaction. An collaborative enhancement network illustrating the relationships between various molecular-processes. Six molecular activities were shown to be linked with genes involved in the plants' reaction to salt-stress by enrichment technique.

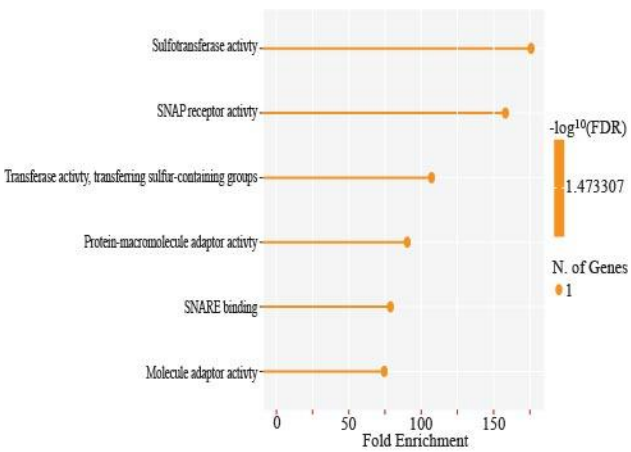

Fig. 7. Genes connected to salt stress by pathway enrichment analysis (GO Molecular Function). Barley ortholog genes were subjected to hierarchical clustering in order to conduct pathway enrichment analysis. The y-axis displayed the assigned routes according to FDR enrichment. The FDR values for each pathway's enrichment were displayed on the x-axis.

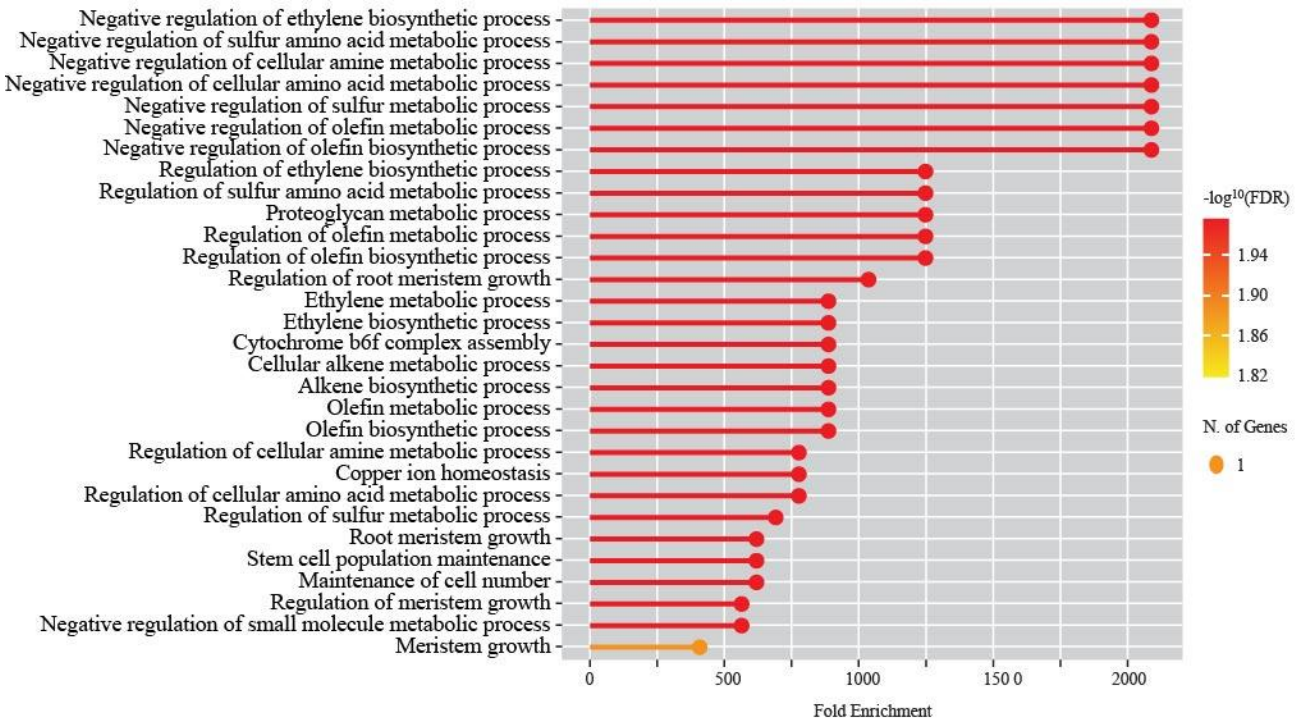

Fig. 6. Genes linked to salt stress by pathway enrichment analysis (GO Biological Processes). The top 30 biological activity categories were represented by functional enrichment. Hierarchical-cluster scrutiny was carried out on barley ortholog genes using ShinyGO program version 0.741. The y-axis displayed –log10 (FDR), while the x-axis displayed the false discovery rates (FDR) for each distinct pathway enrichment.

Table 7. Go-Biological process. The table shows GO-IDs, p-values and details of genes which have any role in the biological processes. Table shows salt-stress-related gene functions regulating various abiotic-stresses.

GO ID	P Value	Description
GO:0010366	1.1e-02	Negative regulation of ethylene biosynthetic process
GO:0031336	1.1e-02	Negative regulation of sulfur amino acid metabolic process
GO:0033239	1.1e-02	Negative regulation of cellular amine metabolic process
GO:0045763	1.1e-02	Negative regulation of cellular amino acid metabolic process
GO:0051175	1.1e-02	Negative regulation of sulfur metabolic process
GO:1900909	1.1e-02	Negative regulation of olefin metabolic process
GO:1900912	1.1e-02	Negative regulation of olefin biosynthetic process
GO:0010364	1.1e-02	Regulation of ethylene biosynthetic process
GO:0031335	1.1e-02	Regulation of sulfur amino acid metabolic process
GO:0006029	1.1e-02	Proteoglycan metabolic process
GO:1900908	1.1e-02	Regulation of olefin metabolic process
GO:1900911	1.1e-02	Regulation of olefin biosynthetic process
GO:0010082	1.1e-02	Regulation of root meristem growth
GO:0009692	1.1e-02	Ethylene metabolic process
GO:0009693	1.1e-02	Ethylene biosynthetic process
GO:0010190	1.1e-02	Cytochrome b6f complex assembly
GO:0043449	1.1e-02	Cellular alkene metabolic process
GO:0043450	1.1e-02	Alkene biosynthetic process
GO:1900673	1.1e-02	Olefin metabolic process
GO:1900674	1.1e-02	Olefin biosynthetic process
GO:0033238	1.1e-02	Regulation of cellular amine metabolic process
GO:0055070	1.1e-02	Copper ion homeostasis
GO:0006521	1.1e-02	Regulation of cellular amino acid metabolic process
GO:0042762	1.1e-02	Regulation of sulfur metabolic process
GO:0010449	1.1e-02	Root meristem growth
GO:0019827	1.1e-02	Stem cell population maintenance
GO:0098727	1.1e-02	Maintenance of cell number
GO:0010075	1.2e-02	Regulation of meristem growth
GO:0062014	1.2e-02	Negative regulation of small molecule metabolic process
GO:0035266	1.5e-02	Meristem growth

Supplementary Table S1.

Gene name	Symbol	Function	Reference
Vernalization 1	VRN1	MADS-box transcription factor promoting flowering after cold exposure	Yan *et al*., 2003
Vernalization 2	VRN2	Zinc-finger-CCT domain protein repressing flowering until vernalization	Yan *et al*., 2004
Sodium transporter	HvHKT1;5	Mediates Na^+ transport; contributes to salt stress tolerance	Mian *et al*., 2011
WRKY transcription factor 6	HvWRKY6	Required for resistance against *Pyrenophora teres* f. *teres* (net blotch disease)	Sharma *et al*., 2021
Drought-responsive MYB	HvMYB1	Transcription factor enhancing drought tolerance by regulating stress genes	Alexander *et al*., 2020
Basic Helix-Loop-Helix TF	HvbHLH56	Enhances low nitrogen tolerance by regulating nitrogen uptake genes	Yang *et al*., 2023
Pathogenesis-Related Protein 1	HvPR1	Involved in systemic acquired resistance against pathogens	Chen *et al*.,, 2023

Discussion

In current investigative study, 138 salt-stress tolerant barley gene orthologs were discovered using 89 genes that underwent experimental validation. In the beginning, 74 major bait-genes were analyzed to estimate the abiotic stress-tolerant gene orthologs of *Populus* (Kant, 2020). Later, a list of 519 genes was generated mutually containing co-expressed and primary genes by co-expression analysis. Co-expression analysis revealed 548 genes that are directly linked to the main genes. The network was rebuilt using ATTED-II, and 694 genes were found to be directly related (Yang *et al*., 2011).

The STRING database was used to build the network using the list of 519 genes. A complex co-expression network with disconnected genes was also built using a list of 397 genes. The 38 interlinked proteins in the *Arabidopsis* CAMTA protein network were found using the STRING database (confidence value = 0.5) (Rahman *et al*., 2016). The unconnected genes and inaccurate positive interactions were removed after applying the high confidence value. Cluster Viz was used to perform cluster analysis on network interactions using Cytoscape (Kant, 2020). Some genes in these clusters were found to serve as hub-genes, such as, MYB103 in gene-cluster 4, THE1 in gene-cluster 7 and SND3 in gene-cluster 11 (Yang *et al*., 2011).

Through the TAIR database, protein sequences of 138 *Arabidopsis* genes were gained as *A. thaliana* was the prime plant of molecular genetics and biology material (Kant, 2020). The NCBI blastp tool (nr) was used to match protein sequences associated with the *Arabidopsis* network to a non-redundant *Hordeum*, and 138 orthologs of the barley gene were discovered. When the *Populus* genome was queried using *Arabidopsis* genes, 817 *Populus* orthologs were found (Yang *et al*., 2011). The discovery of orthologous groups is helpful for annotation of genomes and comparative genomics research (Li *et al*., 2013). The research discovered 14 transcription factors and 9 DUFs in *A. thaliana*. Barley's 9 transcription factors and 5 DUFs were found. It was established through gene ontology-enrichment analysis certain genes among 138 barley gene-orthologs were engaged in certain biological processes; while others were involved in various molecular activities. The majority of *G. arboreum* homologs in the GO annotation were biological processes, including transcription, transport, reactions to biotic and abiotic stimuli, and biological processes that are not yet fully understood (Barozai & Husnain, 2012).

A variety of genes involved in metabolic and energy-related activities, particularly DNA metabolism, lipid metabolism, bioenergetics, amino acid metabolism, secondary metabolism, as well as nucleotide metabolism, are affected by salt stress. The peroxidase proteins, which are dispersed extensively throughout plants and have unique expression patterns, were compared to the contig 54. The reaction to salt stress was also linked to Contig 54 and peroxidase proteins (Amaya *et al*., 1999). The ATPase expression protein, which was linked to Contig 138, increased in both expression and reaction to salt stress in exercise (Golldack & Dietz, 2001). It was determined that contig 558 corresponds to ribosomal proteins that are well-established responsive elements against salinity-stress (Kawasaki *et al*., 2001).

The Arabidopsis SOS gene family contains six genes that have been linked in the past to salt tolerance, either directly or indirectly. Cytoplasmic SOS3 and SOS2 enhance salt resistance and can control K+ and Na+ ion homeostasis by controlling SOS1 on the plasma membrane. Within the SOS family, SOS1 is a gene directly linked to plant salt tolerance (Guo *et al*., 2009*)*. Studies have shown that transgenic plants that overexpress the tobacco osmotin protein are protected against a variety of stressors, including fungal infections and salt stress (Hao *et al*., 2021). Enhancing salt tolerance in Olives through overexpression of tobacco osmotin protein may be related to the transgenic plants' sulfur metabolism (Bashir *et al*., 2021).

In barley (Hordeum vulgare), the protein XP_044978670.1, designated as Uncharacterized protein LOC123445710, is not functionally characterized. The phenotypic effects of changing LOC123445710 expression can be ascertained by using methods like gene knockouts, overexpression studies, or RNA interference in barley or model plants (Campoli *et al*., 2012).

Members of the WRKY transcription factor 22 (WRKY22) family, which controls how plants react to different stresses and developmental processes, include the barley (Hordeum vulgare) protein ABI13388.1. Plant resistance to abiotic stressors like drought, salt, and

temperature extremes is increased by WRKY transcription factors, especially WRKY22. It may be possible to increase barley's resistance to environmental challenges by modifying WRKY22 expression (Zhang *et al*., 2023). In accordance with the phylogenetic associations, insights into the biological roles of barley WRKY genes have been derived through comparative analysis with the established functions of WRKY genes in A. thaliana (Grunewald *et al*., 2013). Four genes classified within subgroup II c (HvWRKY1, HvWRKY13, HvWRKY15, and HvWRKY83) exhibited significant up-regulation in response to simulated drought, cadmium (Cd), and salinity treatments. This observation implies that WRKY subgroup II c may play a crucial role in the positive modulation of the barley's response to these three environmental stressors (Zheng *et al*., 2021).

Annotated as a bZIP transcription factor 46-like, the barley (Hordeum vulgare) protein XP_044960061.1 belongs to the basic leucine zipper (bZIP) family of transcription factors. The basic functions of bZIP transcription factors in plants provide insights, notwithstanding the paucity of specific functional investigations on this specific protein in barley. To maintain yield stability in challenging environmental conditions, barley varieties with improved stress tolerance can be developed with the help of an understanding of the precise functions of bZIP transcription factors (Rahman *et al*., 2023).

The overexpression of SOS1 in transgenic Arabidopsis plants has been shown to enhance tolerance to salinity, while the levels of KC and NaC accumulation in these transgenic plants remained largely unchanged under salinity stress in comparison to control conditions (Yang *et al*., 2009). The researchers noted that transgenic plants that overexpress SOS3 exhibited similar improvements in salinity tolerance as those overexpressing SOS1 (Arzani & Ashraf, 2016). This suggests that the SOS1 protein may play a vital role in regulating the distribution of NaC from roots to shoots, which is crucial for salinity tolerance, as observed in barley (Bose *et al*., 2014; Saleem *et al.,* 2014) and Medicago spp. (Liu *et al*., 2015).

The current work, after thorough investigation, increases the understanding of reader regarding the barley gene equivalents that assist in protecting the crop against abiotic stresses like, salt-stress. Studying fitness-related features in wild populations has become possible by the discovery of genomic analytical tools with stronger evidence for positive selection (Ellegren & Sheldon, 2008).

Conclusion

Using 89 genes that had been experimentally verified, 138 salt-stress tolerant barley gene-orthologs were established in the current study. The network of 519 genes was constructed using a STRING database. In comparison to 5 domains and 9 transcription factors in barley, the research found 14 transcription factors and 9 domains in *A. thaliana* with unknown roles. Six molecular activities were identified with the enrichment tool essential to the salt-stress response. According to their biological and molecular functions, salt-stress tolerant genes were categorized by the CELLO2GO tool. The 138 barley orthologs reported in the current investigative work can be used and further evaluated by breeding specialists involved in barley breeding programs.

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