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Salt Stress-Responsive Protein Interaction in *Hordeum vulgare*

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Abstract

Salt stress affects crop productivity by altering the biology of the plant and limiting productivity. *Hordeum vulgare* is the most tolerant cereal crop, with rich genetic resources. The underlying molecular mechanism involved in salt stress response is yet to be comprehensively addressed. A total of 305 proteins are involved in the network. We attempted to find relationships between a few representative stress-responsive proteins of osmotic (pip1), ionic (K^+/Na^+ ratio in the leaf sheath, HvHAK, HAK4, NHX1 and Ha1), and oxidative stress (APX, CAT1, SOD1) from the public protein database to identify the most influential protein in the network. Further, the salt response proteins were analyzed for their enriched protein domains, Kyoto Encyclopedia of Genes and Genomes pathways, molecular functions, and cell localization. The graph theory analysis of the large data could provide clues for the identification of potential biomarkers for salt stress in barley. An experiment was performed in three accessions of *H. vulgare* to identify the reliability of the theoretical network relationship in biological systems. The expression of the above-mentioned proteins was further experimentally proven based on the expression and assay.

Keywords: Salinity stress, salt-responsive protein, protein-protein interaction network, barley

Introduction

Salt stress is one of limiting factors in the productivity of crops. Salt tolerance is a complex mechanism, in which plants undergo oxidative, ionic, and osmotic stresses. Salt stress eventually leads to major biochemical and physiological changes in the plants [1]. In order to understand such a complex system, it is necessary to investigate all the specific components, especially proteins involved in system biology. This can further elicit the relationship between even the ignored, elementary, yet important constituents. The relationships between them can be considered as a network and can be represented as a graph. The network will be highly complex, with many nodes and vertices as higher numbers of proteins are involved in stress tolerance. *Hordeum vulgare* (barley) is the most tolerant cereal crop, with rich genetic resources. The underlying molecular mechanism involved in salt stress response is yet to be comprehensively addressed. In this paper, we approach salt tolerance in *H. vulgare* using graph theory by analyzing the expression of the key salt-responsive proteins and their interactions. Further, the protein interaction network can be analyzed using cytoscape, an open source, to reveal the most important protein(s) involved in the salt tolerance mechanism and their biological significance to the system.

Barley accessions ELS 6302-5, STB 51 and machine barley obtained from the United States Department of Agriculture (USDA) were used for study. Plants were exposed to salt stress. The oxidative stress was evaluated based on the salt stress-responsive proteins, such as APX, CAT1, and SOD1. Ionic

stress was analyzed on the basis of K^+/Na^+ ratio. The expression levels of HvHAK, HAK4, NHX1, and Ha1 were evaluated based on PCR amplification of DNA. The osmotic stress was examined on the basis of plant-water relations.

Materials and methods

A field experiment was laid out in BITS Pilani, Dubai Campus, to evaluate *H. vulgare* accessions ELS 6302-5, STB 51 and machine barley on salt stress. The pre-germinated seedlings were transferred to the field and propagated using fresh water (ECe 0.3 dSm^{-1}). The plants were given four levels of salt treatment, viz. 0.3, 5, 10, and 15 dSm^{-1} from a three leaf stage. Growth performance and anti-oxidant defense mechanisms were studied one week after the commencement of salt treatment.

Immediately after harvesting, the fresh weight of the leaf and leaf sheath were determined. One set of leaves were placed in 10 ml of millipore water at room temperature in dark conditions for 20 h to find the turgor weight. The other set of plant parts were dried in a natural convection oven at 80 °C to determine the dry weight. The plant-water relations were calculated by the below mentioned formula;

$$\text{Water content} = \frac{(\text{FW} - \text{DW})}{\text{DW}} \quad (1)$$

$$Succulance\ \left({}^{g}/_{cm^2}\right) = \frac{(\text{FW} - \text{DW})}{\text{Area}} \quad (2)$$

$$\text{Relative Water Content}(\%) = \frac{(\text{FW} - \text{DW})}{(\text{TW} - \text{DW})} * 100 \quad (3)$$

$$\text{Salt tolerance index}\ (\%) = \frac{\text{DW}}{\text{FW}} * 100 \quad (4)$$

(FW- fresh weight; DW- dry weight; TW- turgor weight) Area of leaf is measured by using Image J software.

Mineralization and Na^+ and K^+ analysis

The oven dried plant samples were ground using a ceramic mortar and pestle and sieved through ASTM No 30 BSS Standard Test Sieve to get 500 µm partials. 50 mg of the powder was digested with 25 ml of 3 % HNO_3 at 50 °C for 72 h, then filtered through ash-free filter paper no. 44. The filtrate was analyzed for Na^+ and K^+ content using ICP OES.

DNA extraction and gene amplification

DNA was extracted by the modified CTAB method. 1 gram leaf sample was powdered using liquid nitrogen. 10ml of DNA extraction buffer (1.4 M NaCl, 100 mM Tris-Cl with pH 8 and 20 mM EDTA, and 1 % β mercapto ethanol) was added and incubated for 30 min at 65 °C, followed by centrifugation at 12,000 rpm for 10 min at 4 °C. To the supernatant, 6 ml of chloroform and isoamyl alcohol mixture (24:1) was added. The DNA was precipitated by isopropanol. Further, the DNA was washed with 70 % ethanol and air dried and stored in TE buffer.

Gene amplification by PCR was performed for HvHAK, HNX4, and Ha1. The primers for genes were designed based on the accession numbers DQ912169.1, AK359097.1, and AB032839.1 from the NCBI database [2] and used for the amplification using a thermal cycler. Denaturation and extension were done at 94 and 72 °C. The annealing temperature was 51, 47, and 45 °C for HvHAK, HNX4, and Ha1, respectively.

Enzyme assays

Fresh leaves (100 mg) were ground with a mortar and pestle in liquid nitrogen and homogenized with ice-cold 50 mM sodium phosphate buffer (pH 6.8) containing 1mM EDTA and 2 % (w/v) polyvinyl poly pyrrolidone and stored on ice. The homogenate was centrifuged at 10,000rpm for 30 min at 4 °C and supernatant was used for further analysis. Protein estimation was done by Lowry's method, using bovine serum albumin as standard. The following assays were carried out to find the activities of ascorbate peroxidase (APX) and catalase (CAT).

Native gel for superoxide dismutase (SOD) was performed using 10 µl (50 ng of protein) of crude extract loaded on 10 % resolving poly acrylamide gel electrophoresis. The gel was stained with 1M sodium bicarbonate, 200 mM methionine, 3 mM EDTA, 60 µM riboflavin, and nitro blue tetrazolium, then exposed to fluorescent light for 30 min. The appearance of white bands on blue gel indicated the expression of SOD.

APX activity was assayed by the Nakano and Asada method [3]. H_2O_2-dependent oxidation of ascorbic acid was the basis of the assay. Reaction mixture contained 1ml of the potassium phosphate buffer, 0.1 ml of 0.3 % H_2O_2 (v/v), 0.1 ml of 0.5 mM ascorbate, 3 mM of 0.1 M of EDTA, and 100 µl of the enzyme extract. APX activity was determined spectrophotometrically by monitoring the decrease in absorbance on ascorbate degradation at 290 nm (ε 02.8 mM^{-1} cm^{-1}).

In vitro activity of catalase was determined by the method of Aebi. The activity was determined by monitoring the disappearance of H_2O_2 by measuring a decrease in the absorbance at 240 nm. The reaction mixture contained 1.0 ml of the buffer, 0.1ml of the extract, and 0.1 ml of H_2O_2, and was allowed to run for 2 min. The enzyme activity was calculated using the extinction coefficient 0.036 $mM^{-1}cm^{-1}$.

Table 1 STRING analysis of annotated proteins

Name of the interacting proteins	Automated text mining	Co-expression	Combined score	Database annotated	Edge-betweenness	Experimentally determined interaction	Neighborhood on chromosome
pip1 (interacts with) MLOC_70866.1	0.52	0.16	0.97	-	44	0.93	-
CAT1 (interacts with) Sod1	0.64	0.12	0.91	0.75	28	-	-
MLOC_57408.2 (interacts with) NHX4	0.64	-	0.9	-	40	0.72	0.08
Ha1 (interacts with) MLOC_57408.2	0.51	-	0.79	-	40	0.54	0.14
CAT1 (interacts with) APX	0.29	-	0.73	0.63	28	-	-
APX (interacts with) Sod1	0.25	-	0.55	0.43	2	-	-
MLOC_70866.1 (interacts with) APX	0.54	-	0.54	-	52	-	-
MLOC_57408.2 (interacts with) HAK4	0.52	-	0.52	-	40	-	-
HvHAK1 (interacts with) MLOC_57408.2	0.52	-	0.52	-	40	-	-
pip1 (interacts with) MLOC_57408.2	0.51	-	0.51	-	40	-	-
MLOC_70866.1 (interacts with) HAK4	0.51	-	0.51	-	44	-	-
MLOC_70866.1 (interacts with) Sod1	0.51	-	0.51	-	52	-	-
HvHAK1 (interacts with) MLOC_70866.1	0.511	-	0.511	-	44	-	-

Cytoscape analysis

In order to understand the functional protein association network on salt stress-responsive proteins (Sod1, APX, HvHAK1, CAT, pip, HAK4, NHX, and Ha1), we used a comprehensive set of functional annotation tools called Search Tool for the Retrieval of Interacting Genes/Proteins (STRING).

Gene ontologies, protein domains, and pathways of HvHAK, pip1, Ha1, HAK4, NHX4, CAT 1, APX, and SOD 1 in *H. vulgare* (*NCBI taxonomy Id: 4513*) were identified by STRING. As predicted, all the proteins of our interest interacted and formed a network; these were based on genomic context predictions, high-throughput lab experiments, conserved co-expression, automated text mining, and previous knowledge in databases (**Table 1**). The protein-protein interactions were either direct (physical) or indirect (functional) association. Two new proteins, namely, MLOC_57428.2 and MLOC_70866.1, were also present in the network, and occupied important positions in connecting the graphs.

The graph was then analyzed using Cytoscape v3.4.0 software. Cytoscape is an open-source software package used to integrate and visualize diverse data-sets in biology using graph theory. It helped to determine the relationships between the salt stress-responsive proteins in *H. vulgare* and to find the most influential proteins in the network. By envisaging the already-known information and interactions, it is possible to identify novel ways to perturb the system rapidly.

Result and discussion

Salinity is one of the major obstacles to increasing crop productivity. Salt-tolerant crop may help to increase productivity. *H. vulgare* is the most salt tolerant grain crop, widely grown in arid and semi-arid regions as forage and for grain [4,5]. When a plant is irrigated with salt water, the high concentration of salt causes ionic stress and ruins electron transportation. This in turn increases the formation of reactive oxygen species (ROS) such as singlet oxygen, hydrogen peroxide, superoxide, and hydroxyl radicals [6]. These disturb lipid peroxidation, protein oxidation, and DNA fragmentation in the cell and causes oxidative stress [7]. As a result, plants produce an array of enzymes, including SOD, Cat, and APX, to scavenge the ROS [8].

Table 2 Plant-water relations and enzyme activity of the barley accessions ELS 6302-5, STB 51 and machine barley

Plant ID	K^+/Na^+ ratio	Salt tolerance index (%)	Fresh weight of the leaf (gm)	Turgor weight of the leaf (gm)	Area of the leaf (cm^2)	Dry weight of the leaf (gm)	Water content	Succulence	Relative Water Content (%)	APX activity	CAT activity
ELS 6302-5	22.19	125	0.023	0.033	12.39	0.009	1.406	0.001	55.79	10.01	23.65
STB 51	30.58	100	0.101	0.152	14.28	0.038	1.599	0.004	54.59	7.185	1.828
Machine barley	1.770	220	0.078	0.091	7.030	0.009	7.255	0.010	83.99	6.159	2.587

(% - percentage; gm - gram; cm^2 square centimeter)

STRING analysis for the topological characterization and the interaction between the above mentioned salt-responsive proteins showed that they formed an interaction network. This had a clustering coefficient of 0.443, with a protein-protein interaction (PPI) enrichment p-value of 1.72×10^{-08}. The PPI network of these salt-responsive proteins revealed that the actual number of interactions between them (13) was much higher than expected (2) at medium confidence for an interaction score of 0.400. So, these proteins have significantly more interactions among themselves than what would be expected for a random set of proteins of similar size, drawn from the genome. Such an enrichment indicates these proteins are biologically connected as a group to some extent.

The three accessions of barley responded differently for salt stress. STB 51 maintained a very high K^+/Na^+ ratio compared to other two accessions, conferring it the ability to tolerate salt stress. This indicates that, in STB 51, the potassium transporters are efficient enough to maintain more K^+ content in the cell. This accession performed well and obtained high biomass in terms of fresh weight, dry weight, and leaf area. Concentration of catalase in terms of its activity was noted to be 1.8 EU/mg. Catalase enzyme play a crucial role in oxidative defense mechanism by disintegration of hydrogen peroxide into oxygen and water. We assume that, in STB 51, it was not necessary to elevate the expression of catalase.

In machine barley, K^+/Na^+ ratio was 1.77. This indicates that machine barley did not have any control over the influx of highly toxic Na^+. The accession also showed lesser APX activity and catalase activity, which clearly indicates that this accession is highly sensitive to salt stress and vulnerable to ionic and oxidative stress. So, machine barley can be considered as a sensitive accession (**Table 2**).

ELS 6302-5, even though maintaining higher K^+/Na^+ ratio, accumulated less biomass and underwent oxidative stress. Enzyme expression was very high for catalase and APX. A prominent SOD band was also noted in native gel.

Enrichment analysis of *H. vulgare* salt-response proteins

Salt-tolerant proteins in *H. vulgare* have been studied for several years. Many proteins expressed in salt stress were still based on protein separation by two-dimensional protein gel electrophoresis [9,10]. In order to understand the significance of stress-responsive proteins, the interaction can be depicted as a network to unravel the possible relationships between them. In the present study, a systematic attempt was made to organize proteins involved in salt-tolerance. For the ten salt-responsive proteins in *H. vulgare*, pathways and biological processes involved in salt stress have been analyzed. Bioinformatic analysis indicates that the proteins could be clustered into different groups, so these proteins may participate in different physiological aspects.

Table 3 Summary of *Hordeum vulgare* salt-response proteins

Protein ID	Protein name with number of amino acids
Sod1	Cu/Zn superoxide dismutase; destroys radicals which are normally produced within the cells and which are toxic to biological systems (194 aa)
APX	Ascorbate peroxidase (301 aa)
HvHAK1	Putative high-affinity potassium transporter (775 aa)
CAT1	Catalase 1; occurs in almost all aerobically respiring organisms and serves to protect cells from the toxic effects of hydrogen peroxide (304 aa)
pip1	Aquaporin (288 aa)
MLOC_70866.1	Predicted protein (1515 aa)
HAK4	Predicted protein (621 aa)
NHX4	Sodium/hydrogen exchanger (534 aa)
MLOC_57408.2	Predicted protein (1107 aa)
Ha1	Plasma membrane P-type proton pump ATPase (951 aa)

Sod1 -super oxide dismutase; APX - ascorbate peroxidase; HAK4 and HvHAK1 - barley high-affinity K^+ transporter; CAT1 - catalase, pip1 - pyrophospatase; MLOC_70866.1; MLOC_57408.2 - predicted and unidentified proteins; and NHX4 - cation/proton transporters.

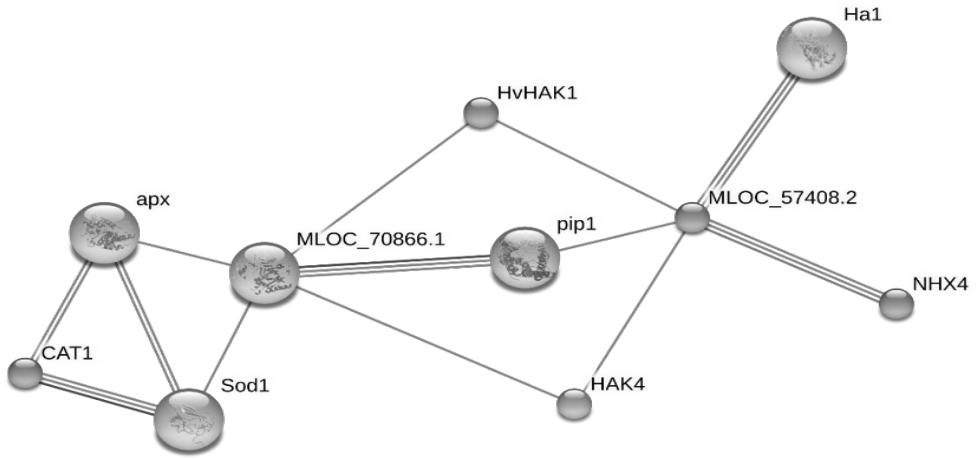

Figure 1 Protein interaction network generated by STRING

The ten annotated proteins (**Table 3**) have been analyzed in STRING and a protein interaction network is presented in **Figure 1**. In the *H. vulgare* salt-response proteins, enrichment analysis, including domain analysis, biological processes, molecular functions, and pathways, were performed. Functional enrichment in the network analysis revealed that 2 proteins were involved in oxidoreductase activity (GO: 0016491), and 2 proteins were involved in metal ion binding (GO: 0046872). Superoxide dismutase, copper/zinc binding domain (IPR001424) are ubiquitous metallo-proteins that prevent damage by oxygen-mediated free radicals by catalyzing the superoxide into molecular oxygen and hydrogen peroxide. Superoxide is a normal by-product of aerobic respiration and is produced by a number of reactions, including oxidative phosphorylation and photosynthesis. The dismutase enzymes have a very high catalytic efficiency due to the attraction of superoxide to the ions bound at the active site. The toxic effects of hydrogen peroxide are neutralized by Catalase 1, as it occurs in almost all aerobically respiring organisms, and thereby protects the cells. Ascorbate peroxidase takes part in L-ascorbate peroxidase activity and heme binding when there is oxidative stress.

Potassium ion transmembrane transporter activity is the molecular function of HvHAK1 and HAK4. Both of them are said to be the putative high-affinity potassium transporter. NHX4 is a sodium/hydrogen exchanger and is involved in sodium: proton antiporter activity. Its biological process is to regulate the pH. High-affinity K^+ uptake transporter (HAK) is believed to regulate Na^+ concentration in the cell along with aquaporin [11]. Plasma membrane P-type proton pump ATPase, Ha 1, is involved in ATP binding, hydrogen exporting ATPase activity, and phosphorylation mechanisms. Biosynthesis of ATP is its biological process.

Aquaporin, pip1, acts as a transporter for water and physiological important substances across cell membranes, and plays a crucial role in plant-water relations, such as relative water content. It responds to stress related to water [12].

Ferredoxin-dependent glutamate synthase, MLOC_70866.1, is reduced by catalysis of oxidized ferredoxin in the presence of L-glutamate and is involved in nitrogen and energy metabolisms. MLOC_57408.2 is an uncharacterized protein.

H. vulgare salt-response proteins are involved in various cellular localizations. Based on the analysis of STRING GOTERM_CC_-FAT section of cellular components, cellular localization proteins had more than one cellular localization, such as a cell, cytoplasm, and cytoplasmic part, and intracellular membrane-bounded organelle (**Table 4**).

Table 4 Functional enrichment in the network based on cellular component

Pathway ID	Pathway description	Observed gene count	Matching proteins labels in the network
GO.0005622	Intracellular	2	CAT1,MLOC_70866.1
GO.0005623	Cell	2	CAT1,MLOC_70866.1
GO.0005737	Cytoplasm	2	CAT1,MLOC_70866.1
GO.0043231	Intracellular membrane-bounded organelle	2	CAT1,MLOC_70866.1
GO.0044444	Cytoplasmic part	2	CAT1,MLOC_70866.1
GO.0016491	Oxidoreductase activity	2	CAT1,MLOC_70866.1
GO.0046872	Metal ion binding	2	CAT1,MLOC_70866.1

Table 5 Network analysis of annotated protein using Centiscape2.1 in Cytoscape

Name of the protein	Betweenness	Closeness	Degree	Eccentricity	EigenVector	Stress
pip1	8	0.059	2	0.333	0.286	30
MLOC_70866.1	39	0.067	5	0.333	0.521	102
CAT1	0	0.037	2	0.200	0.241	0
Sod1	7	0.050	3	0.250	0.369	26
MLOC_57408.2	33	0.059	5	0.250	0.356	80
NHX4	0	0.040	1	0.200	0.116	0
Ha1	0	0.040	1	0.200	0.116	0
APX	7	0.050	3	0.250	0.369	26
HAK4	8	0.059	2	0.333	0.286	30
HvHAK1	8	0.059	2	0.333	0.286	30
Mean value	11	0.052	2.6	0.268	0.294	32.4

The mean value is considered as a threshold value for the measures (**Table 5**). This means that proteins which have more than the mean value are considered as the most important in the network. In our network, MLOC_70866.1, MLOC_57408.2, HAK4, HvHAK1, APX, and Sod1 scored above the threshold. The protein MLOC_70866.1, Ferredoxin-dependent glutamate synthase (FdGOGAT) had maximum value for all the measures considered. It involves both energy and nitrogen metabolism. A two-fold increase in the activity of FdGOGAT was also previously reported [13]. The role of FdGOGAT was fascinating, because this protein contains an FMN cofactor [14].

Based on our experimental results, each protein is independent, and the level of expression is also varied. Based on our insight into the interaction network, we tried to identify the critical protein in the network.

Conclusions

This work may urge people working on salt stress-responsive proteins to integrate the existing data to explore the significant functions and pathways involved. From our findings, salt-response proteins might become potential biomarkers. Cellular localization of the proteins under stress could reveal their roles and functions in unique locations. Further studies on more proteins with experimental proof are needed to understand their enriched functions and the pathways involved. This will help to understand the stress tolerance mechanism involved in plants.

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