The RNA-seq transcriptomic analysis reveals genes mediating salt tolerance through rapid triggering of ion transporters in a mutant barley

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Abstract

Regarding the complexity of the mechanisms of salinity tolerance, the use of isogenic lines or mutants that have the same genetic background but show different tolerance to salinity is a suitable method to reduce the analytical complexity to study these mechanisms. In the current study, whole transcriptome analysis was evaluated using RNA-seq method between a salt-tolerant mutant line “73-M4-30” and its wild-type “Zarjou” cultivar at a seedling stage after six hours of exposure to salt stress (300 mM NaCl). Transcriptome sequencing yielded 20 million reads for each genotype. A total number of 7116 transcripts with differential expression were identified, 1586 and 1479 of which were obtained with significantly increased expression in the mutant and the wild-type, respectively. In addition, the families of WRKY, ERF, AP2/EREBP, NAC, CTR/DRE, AP2/ERF, MAD, MICK, HSF, and bZIP were identified as the important transcription factors with specific expression in the mutant genotype. The RNA-seq results were confirmed in several time points using qRT-PCR of some important salt-responsive genes. In general, the results revealed that the mutant compared to its wild-type via fast stomatal closure and consequently transpiration reduction under the salt stress, saved more sodium ion in the root and decreased its transfer to the shoot, and increased the amount of potassium ion leading to the maintenance a high ratio $[K^+]/[Na^+]$ in the shoot. Moreover, it caused a reduction in photosynthesis and respiration, resulting in the use of the stored energy and the carbon for maintaining the plant tissues, which is a mechanism of salt tolerance in plants. Up-regulation of catalase, peroxidase, and ascorbate peroxidase genes, which was probably due to the more accumulation of H_2O_2 in the wild-type compared to the mutant. Therefore, the wild-type initiated rapid ROS signals lead to less oxidative scavenging than the mutant. The mutant increased expression in the ion transporters and the channels related to the salinity to retain the ion homeostasis. Totally, the results demonstrated that the mutant responded better to the salt stress under both the osmotic and the ionic stress phases. Less damage was observed in the mutant compared to its wild-type under the salt stress.

Background

Soil salinity is known as a major environmental stress limiting the growth and development of plants, resulting in a considerable reduction of crop productivity and yield [100]. Therefore, understanding the mechanisms involved in salinity tolerance can be effective in improving cultivars. Among all cereal crops, barley (*Hordeum vulgare* L.) is a salt-tolerant crop with significant economic importance in the world [93]. Salinity tolerance in barley is a complex quantitative trait comprising more than a hundred genes that can affect each other in different pathways [58, 74].

Plant response to environmental stress occurs via a series of physiological, cellular, and molecular mechanisms [14]. Such mechanisms include alternations in morphology, anatomy, water relations, photosynthesis, hormones, toxic ion distribution, and biochemical adaptation such as the antioxidative metabolism [1, 8, 68]. Salt stress causes the first perception through the root system of plants by inducing osmotic stress in the short term and then leads ion toxicity effect due to a nutrient imbalance in the cytosol by inducing ionic stress in the long term [62].

Moreover, different plant responses have been observed in the long term. Some plants due to restraining a high K^+/Na^+ ratio in the cytosol by various salt-tolerance mechanisms show a higher tolerance in compared to salt-susceptible plants under salinity conditions. However, these mechanisms rely on the regulation and function of K^+ , and Na^+ transporters, as well as H^+ pumps, which generate the driving force for K^+ and Na^+ transport such as H^+ -ATPases and H^+ -PPases [93]. Some plants limit Na^+ uptake (by salt exclusion) and/or reduce its cytosolic Na^+ concentration (by sequestration of ions in the vacuoles via ion transporters), thus avoiding toxic effects on photosynthesis and other key metabolic processes [63]. In addition, salt stress contributes oxidative stress, resulting the generation of reactive oxygen species (ROS) (mainly hydrogen peroxide, H_2O_2) in various cell parts, such as chloroplast, mitochondria, and apoplast [2]. ROS production functions as a signal and regulator in plant development and common plant response to all different environmental stresses [90]. To cope with salt stress, the enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidases (POX) activate to ensure a balance between the rate of formation and removal of ROS [6]. Moreover, the synthesis and accumulation of compatible solutes, maintaining water uptake play a role in osmotic adjustment, membrane and protein protection or ROS scavenging [69]. These responses will lead to a restoration of cellular homeostasis, detoxification and therefore plant survival under salt stress [14]. Some main transporters involving in the regulation of Na^+ and K^+ homeostasis and salt tolerance include SOS1 (salt overly sensitive), HAK, HKT (high-affinity potassium transporter) and NHX (Na^+/H^+ exchanger) [104].

Under saline stress, plants generally activate the cascades of molecular networks involved in stress perception or sensing [65], signal transduction [54], as well as the induction of specific stress-related genes and their metabolites to survive [60]. Although some of the signaling pathways are specific, others may cross talk such as MAPK cascades and biotic signaling [39]. Previous studies on various plant species demonstrated that cross talk included the complex networks of gene regulation [19, 46], which are influenced via plant hormones, such as ethylene (ET), jasmonic acid (JA) and abscisic acid (ABA) [98], and transcription factors (TFs) [91]. Cross talk also results in the expression alternation of other various genes for osmoregulation and cell protection [9].

NADPH oxidase expression (RBOH) leading to an increase in ROS, MAP kinase, as well as gene expression of *ACC* synthase induced the activation of SOS1 antiporter and K^+ channels in *Arabidopsis* under salt stress [22, 102]. Moreover, salinity stress induces the production of ethylene that can be as a downstream signal altering other genes expression in various pathways related to salinity [96]. In general, the knowledge of signaling pathways in long and short terms is considered for understanding the regulation of different genes interconnected at several points under various stresses [14].

The RNA-Seq technique utilizing next-generation sequencing (NGS) is an efficient tool for sequencing cDNA that has been derived from an RNA sample, and hence produces millions of short reads. This technique also analyzes the differentially expressed (DE) genes and biological processes under salinity [9]. Moreover, the RNA-Seq method has considerable potential to generate high-resolution transcriptome

maps sensitive enough to display transcripts even with low levels of expression [40]. The present study utilized RNA-Seq analysis in two genotypes including a salt-tolerant mutant line “M4-73-30” and its wild-type cultivar “Zarjou” at an early time (six hours) after exposure to the salinity stress in order to evaluate transcriptome analysis of salt-related genes, physiological differences and processes. It will improve the understanding of the tolerant mechanisms and effects of the mutation in barley response to salt stress.

Results

Analysis of RNA-Seq datasets

In this research, the results of RNA-seq analysis indicated that a total number of 3184 up-regulated transcripts with differential expression was identified in salt-tolerant mutant “73-M4-30” and its wild-type “Zarjou” genotypes at seedling stage after six hours of exposure to the salt stress (300 mM NaCl), comprised 1586 and 1479 transcripts in the salt-tolerant mutant and the wild-type genotypes, respectively. These DE genes of the gene ontology analysis were assigned to three main categories; molecular function, biological process, and cellular component. The results of gene anthology analysis demonstrated that the greatest number of genes involving in final molecular functions in the salt-tolerant mutant genotype were calcium ion binding, peroxidase activity, transcription factor, and serine-type endopeptidase inhibitor activity. Moreover, the differentially expressed genes related to the final biological process in this genotype included potassium ion transport, cation transmembrane transport, phenylalanine catabolic process, and regulation of transcription DNA-templated. Up-regulated genes detected in parts of the apoplast and endoplasmic reticulum membrane of the salt-tolerant mutant genotype were involved in the cellular component.

On the other hand, the highest transcript abundance of the genes involving in the final molecular functions in the wild-type genotype included electron transporter in the photosynthesis activity and the cytochrome-c oxidase activity. In addition, the differentially expressed genes involved in the biological process in the wild-type genotype comprised a response to ROS, photosynthetic electron transport in photosystem I, glycine metabolic process, as well as regulation of photosynthesis. According to involvement in the cellular component, the differentially upregulated genes were in parts of mitochondria and chloroplast of the wild-type genotype.

All the DE transcripts were mapped to reference standard pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (<http://www.genome.ad.jp/kegg/>) to identify the different pathways that were activated at six hours of the exposure to the salt stress in the two barley genotypes. In this study, 99 pathways were totally identified in the salt-tolerant mutant genotype that 32 of which had the significant DE that were comprised of 461 up-regulated genes. Moreover, metabolic pathways, biosynthesis of secondary metabolites and phenylpropanoid biosynthesis included the highest number of the genes in the salt-tolerant mutant genotype. On the other hand, the results of KEGG analysis detected that 29 pathways out of the 96 identified pathways included 515 differentially expressed genes in the wild-type

genotype. Furthermore, pathways with the highest number of genes involved in the metabolic pathways, the biosynthesis of secondary metabolites, and photosynthesis in the wild-type genotype.

In addition, among all these genes with differential expression, 11 important families of transcription factors such as WRKY, ERF, AP₂/EREBP, NAC, Cytochrome P450, CTR/DRE, AP₂/ERF, MAD, MIKC, HSF, and bZIP 91 genes were found in the salt-tolerant mutant genotype. Moreover, three families of transcription factors of TFIIID, HSP, and Cytochrome P450 in the wild-type genotype were identified that were comprised of 24 genes relating to the salt stress response.

The expression pattern of genes involved in Na⁺ homeostasis under the salt stress

Expression pattern analysis of *Rboh* (Fig. 1a, b), *ACC synthase* (Fig. 1c, d), *HAK* (Fig. 1e, f), and *HVP* (Fig. 1g, h) genes in the shoot and the root samples of the salt-tolerant mutant “M4-73-30” and its wild-type “Zarjou” in compared to the control (0) at different time points (3, 6, 12, 24, 48, 72, and 96 hours after exposure to the salt stress) were evaluated by qRT-PCR technique in contrast to *α-tubulin* as a house-keeping gene under 300 mM NaCl (Fig. 1).

The gene expression pattern of *Rboh* was increased significantly and reached to its maximum level until 48 hours of the exposure to the salt stress in the root sample of salt-tolerant mutant genotype compared to the control (0). Moreover, the amount of this considerable increase was approximately more than 250-fold in the root sample of the mutant genotype in contrast to that of the wild-type genotype. Afterward, a decreasing trend was observed until 96 hours. Moreover, the gene expression after a sudden significant increase at six hours (more than 280-fold) had a gradual increase till 72 hours in the shoot sample of the salt-tolerant mutant genotype. On the other hand, the expression level of *Rboh* gene in the shoot sample of the wild-type genotype had the highest level at 24 hours and 96 hours. However, the expression of this gene in the root and shoot samples of the wild-type genotype was not detected as a regular trend in contrast to those of the mutant genotype (Fig. 1a, b).

The gene expression of *ACC synthase* up-regulated until 72 hours and then down-regulated at 96 hours in the root sample of the salt-tolerant mutant genotype. Moreover, the expression pattern of this gene in the shoot of mutant genotype was approximately similar to that of the root of the mutant genotype (except at 24 hours and 48 hours). Meanwhile, the expression pattern of this gene in the root and the shoot samples of the wild-type was similar, so that the highest gene levels were at 24 hours and 96 hours of exposure to the salt stress (Fig. 1c, d).

The gene expression of *HAK* in the root sample of the salt-tolerant mutant genotype considerably increased by six hours in compared to the control (0) which was a 2-fold increase in contrast to that of the wild-type genotype. The increase in this gene expression level was also observed at 24 hours and 48 hours of the exposure of the salt stress. Furthermore, the expression level of this gene in the shoot sample of the salt-tolerant mutant genotype increased at six hours and continued till 12 hours that reached to the greatest level. However, the highest level of this gene expression in the shoot of the wild-

type genotype was observed at 48 hours compared to that of the salt-tolerant mutant genotype (Fig. 1e, f).

The expression level of *HVP* gene in the root and shoot samples of the salt-tolerant mutant genotype generally increased at 3 hours and 6 hours after exposure to salinity conditions, respectively. Meanwhile, the highest amount of this gene expression in the root and shoot samples of the wild-type genotype was observed at 96 hours and 12 hours after the salt stress compared to the control (0) (Fig. 1g, h).

The gene expression analysis of *Rboh* (Fig. 2a, b), *ACC synthase* (Fig. 2c, d), *HAK* (Fig. 2e, f), and *HVP* (Fig. 2g, h) in the root and shoot samples of the salt-tolerant mutant genotype when compared to its wild-type genotype at different time points under the salt stress were shown in Fig 2. The interesting parallel of the up-regulation of *Rboh*, *ACC synthase*, *HAK*, and *HVP* genes were identified in the short term (six hours) in the root and shoot samples of the salt-tolerant mutant in contrast with those of the wild-type genotypes. However, the gene expression increase of *Rboh* was detected at 12 h and 24 h as well. Furthermore, the genes expression of *Rboh*, *ACC synthase*, *HAK* and *HVP* in the shoot of a salt-tolerant mutant genotype in comparison to that of its wild-type genotype was parallel at six hours after exposure to NaCl which reached to the maximum level (Fig. 2).

Discussion

Plants utilize many various strategies to tolerate salinity conditions. Plant requires a high cytosolic K^+/Na^+ ratio in the cytoplasm to survive under salt stress. Previous studies in various crop species such as barley, rice and maize indicated that the limiting of Na^+ accumulation or the ability to maintain K^+ in the shoot under salinity has been positively correlated with salt tolerance [16, 89]. Under salinity stress, some tolerance mechanisms are evolved in plants to regulate K^+ and Na^+ leading cellular homeostasis under salinity stress. Restriction of Na^+ uptake, Na^+ exclusion to the soil and compartmentation of Na^+ from cytosol to vacuoles and control of Na^+ loading in xylem are crucial mechanisms for tolerance to salinity [57, 82]. Many of these salt tolerance mechanisms depend on H^+ , K^+ and Na^+ transporters, such as SOS1, HKT, HAK and NHX to maintain the cellular ionic homeostasis for salt tolerance [5].

Salinity stress reduces the soil water potential, thus induces water deficit and rapidly transmits a water deficit signal from root to shoot and finally contributes intracellular turgor reduction [5]. Therefore, salinity causes a reduction in stomatal conductance that is the most significant response that immediately occurs after plant exposure to salt. Due to stomatal closure, salinity stress reduces the rate of transpiration (water loss) limiting a load of toxic ions within the transpiration stream in plant, and photosynthesis (CO_2 uptake), thus inhibits carbon fixation and the accumulation of the ions in the shoot part of the plant affecting plant growth [43, 65, 92]. These events have been found in several plant species [10, 30]. Koyro [45] showed that a decrease in stomatal conductance is an adaptive mechanism to salinity via maintaining salts at subtoxic levels.

Cytosolic calcium is a second messenger in signalling of abiotic stress such as salinity. The initial increase in cytosol Ca^{2+} could be due to the production of ROS (mainly H_2O_2) sourced by the membrane NADPH oxidases (encoded by RBOH) are involved in signaling during stress [34]. The results of this study showed that the gene expression of *Rboh* was significantly up-regulated in the root and shoot samples of salt-tolerant mutant genotype in compared to its wild-type genotype in the short term (six hours) after exposure to salt stress. Moreover, ROS increase the cytosol Ca^{2+} concentration resulting in the regulation of ion homeostasis via the activity of SOS1 antiporter, MAP kinase, SOS2 and CBLs [104]. According to the overexpression of these genes in the salt-tolerant mutant genotype at the same time of six hours, these events were observed in the present and the previous study [101].

Drerup et al, [27] demonstrated that RBOH in Arabidopsis can be activated by CIPK26/CBL1/9. In this study, the parallel up-regulation of these was shown in the salt-tolerant mutant genotype at six hours after exposure to the salinity in comparison to its wild-type genotype. ROS function as important signaling molecules in adaptive and developmental processes of plants to abiotic and biotic stresses that can activate several signal pathways, such as hormonal signaling networks to enhance the salinity tolerance [81]. In addition, ROS activate K^+ ion channels leading to retain cellular homeostasis [26, 28].

Chung et al, [22] showed that the activity of salt stress-induced SOS1 transcript requires RBOH through increasing of Ca^{2+} concentration in the cytosol. Ca^{2+} is sensed by CBL4 (SOS3) protein which after interaction with the serine/threonine protein kinase CIPK24 (SOS2) produces the SOS3/SOS2 complex. This complex activates the plasma membrane Na^+/H^+ antiporter SOS1 for Na^+ efflux from cell to control ion homeostasis [75, 97, 101]. On the other hand, SOS3/SOS2 complex induces the activity of ion transporters such as NHX1 to compartmentalize Na^+ from the cytoplasm to the vacuoles [20]. The driving force for this transporter is provided by two main vacuolar H^+ -pumps; H^+ -ATPase and H^+ -pyrophosphatase (V-PPase) indicating those have essential roles in response to salt conditions [29, 37]. In our previous study, the overexpression of the parallel genes (*SOS1-3*) in SOS pathway, *NHX1* transporter and *HVA* pump were observed in the salt-tolerant mutant genotype in contrast with wild-type genotype at 6 h after exposure to salinity stress [101].

Under salt stress, NADPH oxidase leading to the generation of ROS results in MAP Kinase activity that linked to salt tolerance [99]. MAPK superfamily is as a part of the serine/threonine kinases, is a key player in some of the critical roles in plant signaling networks and is tolerant to various stresses including drought and salinity [88]. Moreover, many studies have indicated that in addition to MAPK pathway activity, salinity stress also induces hormones production and signals, such as ethylene in plants [49]. The plant hormones such as ethylene have a major role in plant development, throughout germination, growth, and response to stress conditions. Previous studies have indicated ROS are essential for transduction of ethylene signal in the regulation of Na^+ and K^+ homeostasis to initiate the tolerance [70]. Moreover, it is shown that salt tolerance was induced by ethylene in Arabidopsis [13, 94]. Ethylene production is as a mediator component in the response to stress conditions in barley. To produce ethylene, S-adenosyl-L-methionine is converted to ACC by ACC synthase (or ACS). Finally, ACC is modified

by ACC oxidase (or ACO), which can influence the expression of another set of genes [24]. Salt and osmotic stress induced the conversion of ACC to ethylene in the halophyte *Allenrolfea occidentalis* [21]. Ethylene binding to the receptor which interacts with Constitutive Response (CTR), initiates a transcriptional cascade and downstream ethylene responses [11]. Li et al, [48] demonstrated that ethylene production and activity of ACO were significantly increased in cucumber seedlings under salinity stress (75 mM NaCl). Moreover, the ethylene-responsive element binding factor (ERF) was vital in cotton under stress conditions [41]. Overexpression of sugarcane and soybean ERFs in tobacco conferred tolerance to high salinity stress (200 mM) [103]. Regarding parallel overexpression of these genes at hormonal pathway networks of ethylene production, these mechanisms were observed in the salt-tolerant mutant genotype after six hours of exposure to the salt stress to enhance the salinity tolerance.

Moreover, potassium (K^+) is an essential factor in osmotic processes for resistance to salinity and drought. K^+ decreases the toxic effects of Na^+ and maintains high K^+ / Na^+ ratio in shoots, especially in leaves, which is important in glycophytes for more tolerance to salinity conditions [33]. On the other hand, the production of ROS depends on K^+ availability. Reduction in K^+ content is associated with increased enzymes activity involved in the detoxification of H_2O_2 . Therefore, the increase in K^+ content reduces ROS using limiting the membrane NADPH oxidase resulting in salinity tolerance [83]. K^+ channels are vital to maintain and support the development and plant growth. Researches categorized six gene families, including three channel families and three transporter families (HAK/KUP/KT, HKT, and CPA) [31, 85].

The main transporter of salt stress tolerance is HKT antiporter [65, 78]. HKT transporters have two Sub-families that sub-family1 transporters are only permeable to Na^+ , while sub-family2 transport both K^+ and Na^+ [23]. HKT1;5 is a Na^+ selective transporter, expressed in the plasma membrane of parenchyma cells surrounding xylem vessels [4, 67]. HKTs are involved in Na^+ long-distance translocation by contributing to Na^+ unloading through the xylem, preventing the large accumulation of Na^+ in leaves [80]. The up-regulation of the HKT1 transporters activity causes a decrease in leaf Na^+ content [25, 73]. Therefore, it increases the ability of control of K^+ / Na^+ homeostasis for salinity tolerance [77]. It is revealed that *OsHKT1;5* reduced the root to shoot delivery of Na^+ and increased salinity tolerance in rice [36, 64, 77]. In the present study, the gene expression of HKT1;5 was up-regulated at 6 h after exposure to salt stress in the salt-tolerant mutant genotype. In general, in parallel of increase in *Rboh* gene expression, the up-regulated genes of *MAPK*, *ACC synthase*, *HAK*, *HVP* and *HKT* were observed at the same time point (6 h) of exposure to salt stress in the salt-tolerant mutant genotype in comparison to the wild type genotype that was shown in Fig 2.

Furthermore, ethylene causes the preservation of cellular K^+ via an increase in transcript level of *AtHAK* transporter in Arabidopsis under salt stress [104]. Under salt stress, HAK transporter plays a key role in K^+ uptake through the root, and K^+ long-distance transport through loading and unloading in the vascular tissue [79, 87]. Shin and Schachtman (2004) revealed that *HAK5* expression gene in Arabidopsis root depended on RBOH activity and ROS. Moreover, *HAK5* activity in Arabidopsis roots is regulated by CIPK23, CBL1, CBL8, CBL9, and CBL10 proteins [76, 97]. In the current research, an increase in gene expression of

HAK in the root and shoot samples of the salt-tolerant mutant genotype in addition to six hours, was considerably observed at 24 hours and 48 hours in the root sample of that after exposure to salt stress in contrast to its wild-type genotype.

TPK1/KCO1 channel, another important family of K⁺ channel have been localized in the vacuolar membrane. This channel contains the binding sites for Ca²⁺ and 14-3-3 proteins involved in the K⁺ transport from vacuole to cytosol to contribute a favorable Na⁺/K⁺ ratio and ion homeostasis [85, 97]. In the present study, the transcript level of TPK1/KCO1 gene and 14-3-3 protein was up-regulated after 6 h of exposure to salinity in the salt-tolerant mutant genotype in comparison with its wild type genotype.

Salt stress causes the ROS production inducing some enzymatic antioxidants to remove them for salinity tolerance [6]. In this research, the higher expression of peroxidase was observed to remove H₂O₂ content in the salt-tolerant mutant genotype in comparison to its wild-type genotype after 6 h of exposure to salinity. Under salt stress, APX is one of the most significant antioxidant enzymes in plant cells and plays an essential role in the control of ROS levels regulated by redox signals and H₂O₂ [84]. Many researchers reported an increase in APX activity in response to abiotic stresses such as salinity, drought, chilling, and metal toxicity [32, 55]. Moreover, APX has a much higher affinity for H₂O₂ than CAT, making efficient scavengers of H₂O₂ with high concentration under stress conditions [95]. Therefore, due to more accumulation of H₂O₂ in the wild-type genotype, the genes expression of enzymatic antioxidants, such as CAT, POX, and APX for ROS scavengers were significantly up-regulated in contrast to salt-tolerant mutant genotype in the short term of six hours after exposure to the salt stress. The results of this study were similar to the results of Kiani et al, [44]. Furthermore, because of stomatal closure, the rate of evaporation via transpiration stream and H₂O₂ production via photosystems (I and II) were reduced in plants, which is an important mechanism of salt tolerance [7, 65].

Many researchers indicated that salt stress induces MAPK pathway, also activation of transcription factors, such as WRKY in Arabidopsis [50]. Transcription factors are the most important regulators that control a wide range of gene expressions in different signaling pathways through binding to the specific cis-acting element in the promoters of genes [18]. Among all transcription factors, bZIP, WRKY, MYB, CTR/DRE, AP2, NAC, C2H2 zinc finger gene, and DREB families observing high expression levels, comprise a large number of stress-responsive members [42, 59]. In this research, the results of mRNA-seq analysis revealed that the expression levels of important transcription factors were increased in the salt-tolerant mutant genotype in comparison to its wild-type genotype at six hours after exposure to the salinity stress that was included WRKY, ERF, bZIP, AP₂/ERF, NAC, AP₂/EREBP, Cytochrome P450, CTR/DRE, MICK, MAD, and HSF. Many researchers demonstrated that transcription factors, such as WRKY and NAC have main roles in ROS signaling pathways in response to stresses resulting in salt tolerance in Arabidopsis [15, 51]. Nakashima et al, [66] revealed that the upregulation of a NAC in both rice and wheat plays a vital role in salt tolerance. Moreover, Song et al, [86] demonstrated that NAC overexpression induced by ROS (H₂O₂) in rice under salinity stress, which may regulate the synthesis and

accumulation of components, such as proline, sugar, and LEA proteins that play the important roles in tolerance to stress.

The RNA-seq, as high-throughput sequencing of cDNA is the most important powerful application for analysis during these decades. Recently, a genome sequence and transcript profiling of barley has been reported for the stress-responsive genes [9, 93]. In this research, sequencing of the cDNA samples of the salt-tolerant mutant and its wild-type genotypes in the early time point (six hours) after exposure to the salt stress (300 mM NaCl treatment) yielded about 20 million reads for each genotype. Moreover, a total number of differential expression transcripts included 7116, 1586, and 1479 DE transcripts of which with significant overexpression were obtained in the salt-tolerant mutant and its wild-type genotypes, respectively.

Many types of research demonstrated that the tolerant plants displayed a decreased respiratory rate, whereas sensitive plants displayed an increased respiratory rate. The certain halophytes decrease respiration under salt stress, due to deploying their carbon reserves in the shoot for reproducing and maintaining tissue tolerance [12, 38]. Moreover, Jacoby et al, [40] suggested that the tolerant varieties to salt stress allocate less of its fixed carbon into respiration, and more into growth. According to the up-regulation of some genes related to respiration pathway including glycolysis, Krebs cycle, citric acid cycle, and the electron transport chain in mitochondria in the wild type genotype, RNA-seq analysis data was detected that the respiratory rate was higher in this genotype when compared to the salt-tolerant mutant genotype. Therefore, instead of maintaining the plant tissues, the stored energy and carbon was consumed in the wild-type genotype at an early time point under salinity stress.

Methods

Plant Materials and salt stress conditions

Two barley (*Hordeum vulgare* L.) genotypes, a salt-tolerant mutant line “M4-73-30” and its wild-type cultivar “Zarjou” were used for the present study. They were supplied by the Seed and Plant Improvement Institute, Karaj, Iran [44, 101]. A salt-tolerant mutant genotype was produced by the gamma irradiation approach and evaluated for salt tolerance in the farm trials. It had been introduced as a salt-tolerant cultivar “Roodasht” by the Seed and Plant Improvement Institute, Karaj, Iran [56]. Previous studies [44, 56, 101] revealed that a salt-tolerant mutant had a higher ability to tolerate salt stress than its wild-type genotype by physiological response related to salinity tolerance.

Uniform seeds of both genotypes were surface-sterilized using 5% sodium hypochlorite for five minutes and were rinsed with sterile water for 10 minutes. Sterilized seeds of each of the genotypes were germinated on moistened germination paper in 15 cm diameter glass Petri dishes in an incubator at a temperature of 22 ± 1 °C under dark conditions for four days. After germination, all the seedlings were transferred into a half-Hoagland nutrient solution at pH 6.0 [35]. The plants were grown in controlled conditions at 25/22°C day/night, 16 hours for a light duration, and 70% humidity in a greenhouse at Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran. Afterward, the seedling

with the two-leaf stage was stressed by adding NaCl at a concentration of 300 mM to the nutrient solution including CaCl₂ to maintain Na⁺/Ca²⁺ ratio of 10:1 on a molar basis. The root and the shoot samples were harvested separately in three replications (including six plants for each replication) after three, six, 12, 24, 48, 72, and 96 hours exposure to the salt stress. The plant seedlings that were grown only under normal Hoagland nutrient solution were used as the control (0). All samples were immediately frozen in liquid N₂ and stored at -80°C for further use.

RNA isolation

The total RNAs were extracted separately from the root and the shoot samples by the Biozol method (BioFlax, Japan) [44]. The RNA quality was checked by 1% agarose gel electrophoresis and 30 µg was used for RNA-Seq. Afterward, the RNA samples were shipped to Beijing Genomics Institute (BGI), Hong Kong, China for deep sequencing and generation of datasets. The Agilent 2100 Bioanalyzer System and RNA 6000 Nano were used to generate information on the RNA concentration, the ribosomal ratios and the RNA integrity number (RIN) [61].

Next-generation RNA sequencing

The transcriptome analysis was evaluated using RNA-seq method and sequencing system of Illumina Hi SeqTM 4000 platform for cDNA library formation between the salt-tolerant mutant and its wild-type genotypes after six hours of exposure to 300 mM NaCl. Filtered reads were aligned with the barley genome as the reference after downloading the *Hordeum vulgare* transcript sequences from the NCBI database (<http://www.ncbi.nlm.nih.gov/unigene>). Expected read counts were input into differential expression analysis by Cuffdiff and EdgeR software (version 3.0.0, R version 2.1.5). Moreover, the analysis of gene expression based on log ratio RPKM data to provide a representation of the absolute abundance of transcripts. DE transcripts were annotated and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed using blast-2-GO software (version 2.3.5, <http://www.blast2go.com/>) to identify the different pathway activated under the salt stress.

Moreover, the gene ontology (GO) list of DE genes was analyzed using available AgriGO (version 2.0). Therefore, the GO analysis was described in three categories: biological process, molecular function, and cellular component for the upregulated DE transcripts under the salt stress.

Validation of RNA-seq data by real-time PCR

The validation of the RNA-Seq data for four genes selected was performed using real-time PCR with the *TEF* and *α-tubulin* as a house-keeping gene. Primers were designed with Primer 3 software (www.embnet.sk/cgi-bin/primer3_www.cgi) based on 3' untranslated region (3'-UTR). The primer names and the sequences used for primer designing are given in Table 1. For qRT-PCR, the quantity of mRNA corresponding to each gene was measured by SYBR Green. The PCR mixture consisted of 9.9µl 2X SYBR Bio Pars (SBB, Iran) PCR Master Mix, 0.5µl DMSO, 0.5µl of each gene-specific forward and reverse primers (10 pmol), 0.2µl *Taq* enzyme, and 3µl of the diluted cDNA in a final volume 17µl with double distilled

water. Forty PCR cycles for each gene product included denaturation at 95°C for five min and 10s at 95°C, annealing at 58-60°C for 10s and extension at 72°C for 20 s and five min at 80°C for one cycle. The specificity of the amplicons was checked by melting curve analysis (10s at 55°C) after 81 cycles. α -tubulin as a reference gene by the comparative Ct ($2^{-\Delta\Delta C_t}$) method was used to calculate the relative expression (Livak and Schmittgen, 2001). Expression pattern analysis of *Rboh*, *ACC synthase*, *HAK* and *HVP* genes were detected by qRT-PCR in contrast to *α -tubulin*, a reference gene to normalize data, in the root and the shoot samples under the salt stress. PCR for each sample was performed in three technical and biological replications. Relative expression levels were computed using the following formula which presented by Pfaffl and Hegeleit [71]. The ratio analysis between the amount of the target gene and the housekeeping reference genes was performed by the REST software [72].

Conclusions

It can be concluded that the salt-tolerant mutant genotype utilized the ionic transporters and the channels to control cellular homeostasis promoting the tolerance to the salt stress via retaining of cell homeostasis in contrast to its wild-type genotype. Some important ionic transporters and channels involved in complex pathways of salt-tolerant mutant genotype in the short term of six hours after the exposure to 300 mM NaCl in compared to the wild-type genotype was shown at Fig 3. Therefore, the salt-tolerant mutant genotype used some tolerant mechanisms via a higher expression levels of some important salt-responsive genes to regulate cell homeostasis in contrast to its wild-type genotype. The results totally indicated that the salt-tolerant mutant genotype had a better response at both the osmosis and the ionic phases under the salt stress, causing less damage in the salt-tolerant mutant genotype in comparison to its wild-type genotype. Moreover, the comparisons demonstrated that RNA-Seq is an efficient method for the analysis of genes with differential expression involved in response to salinity and identification of biological processes that are very important in plants breeding. It can be also utilized to improve salt stress tolerance in other important cereal crops using classical methods such as backcross breeding and/or new methods such as gene transformation.

Abbreviations

ACC: Aminocyclopropane-1-carboxylate; ACO: ACC oxidase; APX: Ascorbate peroxidase; CAT: Catalase; CIPK: CBL-interacting protein kinase; DE: Differential expression; ERF: Ethylene-responsive element binding factor; GO: Gene ontology; H₂O₂: Hydrogen peroxide; HAK: High Affinity for K⁺; HKT: High Affinity Potassium Transporter; HVA: H⁺-ATPase; KEGG: Kyoto encyclopedia of genes and genomes; QRT-PCR: Quantitative real-time PCR; MAPK: Mitogen-activated protein kinases; NGS: Next-generation sequencing; NHX1: Na⁺/H⁺ exchanger; POX: peroxidases; RBOH: Respiratory burst oxidase homolog; RIN: RNA integrity number; ROS: Reactive oxygen species; RPKM: Reads per kilobase of transcript per million mapped reads; SOD: Superoxide dismutase; SOS: Salt overly sensitive; TF: Transcription factor; TPK: Tandem-pore K⁺; V-PPase: Vacuolar H⁺-pyrophosphatase.

Declarations

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Tables

Table 1. The name and the sequence of specific primers used for the real-time PCR amplification

Primer name		Sequence	Tm	GC%	Product size (bp)
<i>RBOH</i>	Forward	5'-GCGGGTCTACTTCTACTGGT-3'	55	55	181
	Reverse	5'-CTGCAGCATCACCACCATG-3'	57.8	57.9	
<i>ACC Synthase</i>	Forward	5'-TTGTGCAGATGATGTTCTGGG-3'	58.5	50	158
	Reverse	5'-GCACCGCATGTACTCGATC-3'	58.7	57	
<i>HVP1</i>	Forward	5'-CGTCGCTCAACATCCTCATC-3'	58.8	55	183
	Reverse	5'-GGTTGACCTAAGCCTCCACT-3'	59	55	
<i>HAK</i>	Forward	5'-TGCTCAAAGTCGGGATCACA-3'	59.3	50	199
	Reverse	5'-AAAGAACACCCCTCCCTACC-3'	58.6	55	
<i>α-tubulin</i>	Forward	5'-AGTGTCTGTCCACCCACTC-3'	60.1	60	1629
	Reverse	5'- ATTCAGAGCACCGTCAAACC-3'	57.5	50	

Figures

Figure 1

The expression pattern of genes in the salt-tolerant mutant its wild-type in contrast to control (0). ** indicates significant difference at $P \leq 0.01$.

Figure 2

The expression pattern of genes in the salt-tolerant mutant in contrast to its wild-type as the control.

Figure 3

The schematic diagram of some transporters and upregulated genes in the salt-tolerant mutant genotype. It represents genes involved in salinity-stress signaling pathways and tolerant mechanisms in the salt-tolerant mutant genotype in response to 300 mM salt stress in the salt-tolerant mutant genotype at six hours in comparison to the wild-type genotype. After entering Na⁺ to cell cytoplasm, the activity of RBOH NADPH oxidase was increased to promote ROS formation and cytosolic Ca²⁺ content [47]. Ca²⁺ activated SOS3 and then SOS2 leading SOS1-mediated Na⁺ extrusion [52]. On the other hand, NHX1 was activated by SOS3/SOS2 complex via HVA and HVP pumps providing the driving force to sequester Na⁺ from cytosol to vacuole [17]. Moreover, TPK1/KCO1 is an important vacuolar permeable channel for K⁺ extrusion from vacuole to cytoplasm contributing to homeostasis regulation. salt stress encourages the activity of protein kinases in MAP kinase pathway to produce ethylene [3]. Ethylene can promote K⁺ retention through K⁺ channels, such as HAK and HKT to regulate Na⁺ and K⁺ homeostasis [104].