Physiological and protein responses in leaves of *Nitraria billardieri* seedlings to moderate salt stress

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**ABSTRACT**
Salt stress is a major environmental factor affecting plant growth and geographical distribution. Halophytes are considered valuable resources for investigating plant tolerance mechanisms. The halophyte *Nitraria billardieri* is widely distributed in saline soil in Australia and China. To investigate salt stress-induced changes at the physiological and molecular levels in *N. billardieri*, three-month-old seedlings were subjected to salt stress treatments. The physiological and biochemical analyses showed that *N. billardieri* seedlings could adapt to and strongly tolerate salt stress by accumulating soluble sugars and proline as organic osmolytes and increasing the activities of antioxidative enzymes. Comparative proteomic and metabolic pathway analyses revealed 130 differentially expressed proteins, which displayed various response patterns under salt stress. A protein interaction analysis found that the interaction consisting of amino acid and carbohydrate metabolism coupled with redox homeostasis and protein synthesis may play an essential role in response to salt stress in *N. billardieri* seedlings. Overall, salt stress treatment disrupted a cascade of normal metabolic programming and subsequently caused oxidative and osmotic stress by altering protein fates, signal transduction and redox homeostasis.

**Introduction**
Among the various abiotic stresses, salt is a major source of stress that limits plant growth and development and the geographical distribution of plants (Greenway and Munns 1980; Stepien and Johnson 2009). It was reported that almost 6% of the world’s land is affected by salt, and more than 20% of irrigated land suffers from salt damage (Zhu 2001b; Munns and Tester 2008; Cheng et al. 2015). Therefore, deciphering salt stress response mechanisms is critical for maintaining plant growth and developing breeding strategies that increase salt stress tolerance.

Plant tolerance to salt stress is a complex trait and involves various physiological, biochemical and molecular mechanisms (Zhang and Shi 2013). To survive salt stress, plants have evolved complex defense mechanisms and use various strategies to cope with the stress, one of which is the response and regulation of gene expression. Distinct sets of genes can interact with each other to regulate specific physiological and biochemical processes to enhance adaptation to salt and tolerance of salt through complex regulatory networks (Ji et al. 2013). Previous studies in the model plant Arabidopsis have reported that plants respond to salt stress by initiating several key determinants of salt tolerance and the common salt-responsive signaling pathway (Zhu 2001a). The cloning of Salt Overly Sensitive (SOS) genes and the functional elaboration of the SOS signaling pathway in particular have increased our understanding of plant salt tolerance mechanisms (Ji et al. 2013; Zhang and Shi 2013). Considering the abnormal growth of non-halophytes and normal growth of halophytes in the same saline environment, the salt tolerance mechanisms found in non-halophytes cannot completely represent those for halophytes; non-halophytes and halophytes may possess common and unique salt tolerance mechanisms to cope with salt stress. Therefore, it is also essential to discover the salt tolerance mechanisms and signaling pathways specific to halophytes.

Halophytes could adapt to a saline environment by controlling salt uptake or using compartmentalization to avoid salt damage (Flowers and Colmer 2008). However, there is still considerable variation in salt tolerance and tolerance mechanisms among halophytes (Wang et al. 2002). The halophyte *Nitraria* L. belongs to the family Zygophyllaceae; the genus includes 11 species distributed in Africa, Asia, Australia and Europe. However, every species of *Nitraria* L. has a different salt tolerance (Ni et al. 2012; Chun et al. 2016). *N. billardieri* is widely distributed and grows in saline soil in Australia and China. However, research on the salt tolerance characteristics of *N. billardieri* on physiological and protein level has not been reported.

In this study, a moderate dose of salt stress with a 200 mM NaCl concentration was applied to *N. billardieri* seedlings to elucidate the tolerance mechanisms involved in the response to moderate salt stress. The morphological and physiological traits of *N. billardieri* seedling leaves were investigated, and protein profiles were analysed using the Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) approach. Bioinformatics analysis was conducted to reveal diverse metabolic pathways containing the differentially expressed proteins affected by salt stress.

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Materials and methods

Growth and salt treatment of *N. billardieri* seedlings

*N. billardieri* seedlings were cultivated in plastic pots filled with soil and grown in a greenhouse under 14 h light (600–800 µmol m$^{-2}$ s$^{-1}$) at 28 ± 2 °C and 10 h darkness at 22 ± 2 °C; the relative humidity was maintained at 65–75%. When growing for three months, pots were thinned to two or three plants, and were divided into two groups, one being normally watered, while the other was subjected to 200 mM NaCl treatment for 9 days. The seedling leaves were collected from the stress treatments at day 0, 3, 6, and 9 of salt treatment for physiological and biochemical determination, and the leaves collected at day 9 of salt stress treatment were only used for proteome analysis. At least three independent replicates were used in each treatment for all experiments.

Measurement of leaf relative water content

To measure leaf relative water content (LRWC), leaves were collected from randomly selected plants in each treated group, and the fresh weight of the leaves was measured immediately after collection. The fully turgid fresh weight of the leaves was measured after immersing the leaves in deionized water at 22°C for 6 hrs, and the dry weight was measured after drying the leaves at 105°C for 20 hrs. LRWC was estimated as (fresh weight–dry weight) / (turgid weight–dry weight) × 100% (Chen et al. 2012).

Photosynthesis measurement

The net photosynthetic rate (Pn), transpiration rate (Tr), intercellular CO$_2$ (Ci), and stomatal conductance (Gs) were measured during 10:00–11:00 at every collection time using a LI-COR 6400 Photosynthesis System (LI-COR Inc., Lincoln, NE, USA) according to the manufacturer’s instructions. The measurement settings were as follows: leaf chamber [CO$_2$] using a LI-COR CO$_2$ injection system; 1500 µmol m$^{-2}$ s$^{-1}$ photon flux density; and CO$_2$ in the sample chamber supplied at 400 µmol mol$^{-1}$ air by the CO$_2$ mixer.

Determination of relative electrolyte leakage, malondialdehyde content, proline and soluble sugar

Relative electrolyte leakage (REL) was measured with the method reported by Cheng et al. (2015), with minor modification. The leaves from the plants of each treatment were cut into 0.5-cm segments, placed in ultra-pure water and incubated for 2 h at 28 °C. Electrical conductivity (Lt) was measured using a conductometer (DDSJ-308A, China). The leaf segments were then incubated at 100 °C for 30 min and 28 °C for 1 h to measure the electrical conductivity (L0). REL was estimated as Lt/L0 × 100%.

Lipid peroxidation of seedling leaves was measured using the method reported by Dhindsa and Matowe (1981), with minor modification, as follows: leaves were ground in a buffer of trichloroacetic acid (0.1%, v/v) and then centrifuged at 8000 × g for 40 min. The super-natant was mixed with trichloroacetic acid (20%, v/v) and thiobarbituric acid (0.5%, v/v) and then heated at 95 °C for 30 min. After rapidly cooling, it was centrifuged at 8000 × g for 40 min. The supernatant was used to measure MDA content.

The osmolyte contents, such as proline and soluble sugar, can reflect the tolerance status of plants to osmotic stress caused by salt treatment. The proline content was determined as described in Inan et al. (2004) and Kant et al. (2006). The total soluble sugar content in seedling leaves was determined according to the methods reported by Zhang et al. (2010) and Wang et al. (2013).

Activity determination of antioxidative enzymes

The extraction of enzyme solution was conducted according to Ramel et al. (2009), with minor modification. Leaves were ground into powder in liquid nitrogen and suspended in enzyme extraction buffer containing 50 mM phosphate buffer (pH 7.8), 0.1% Triton X-100 (v/v), 0.5% polyvinylpyrrolidone (PVP-40) (w/v), 1 mM EDTA (pH 7.4) and protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 22 °C. The mixture was centrifuged at 8000 × g for 30 min at 4°C. Supernatants were collected for measurement of enzyme activities. The protein concentration of the enzyme extracts was measured using a protein assay kit (Bio-Rad, Hercules, CA, USA).

The enzyme activities of superoxide dismutase (SOD) were determined using the methods reported by Beauchamp and Fridovich (1971) and Yang et al. (2015). Enzyme extracts were added to a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.5), 750 µM NBT, 130 mM methionine, 100 µM EDTA, and 20 µM riboflavin; the reaction mixture was illuminated under a transilluminator and then measured for SOD activities. Enzyme extracts were added to a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.5) to determine the activity of catalase (CAT) with a Spectro UV-Vis Auto UV-2602 (Labomed Inc., CA, USA), as described by Aebi (1984). An assay of peroxidase (POD) enzyme activity was performed using the method reported by Yang et al. (2013).

Protein extraction, digestion and quantitative proteomic analysis

Leaf proteins were extracted according to a previously described method (Yang et al. 2016). The quantification of each protein extract was performed using a Bradford protein assay kit, with bovine serum albumin as the standard. The abovementioned protein extracts were washed and precipitated with cold acetone. After precipitation and drying, the pellets were reduced using dithiothreitol, alkylated using iodoacetamide and, finally, digested using trypsin buffer (Yang et al. 2014).

The peptides from digested proteins were labeled using iTRAQ Reagents based on the manufacturer’s instructions (Foster City, CA, USA). After labeling, the fractionation of peptide mixtures and analysis of tandem mass spectrometry were performed according to previously described methods (Cheng et al. 2015).

Protein identification and relative quantification

Protein identification was also performed with a method previously described by Cheng et al. (2015). The mass spectrometry data were submitted for sequence query using...
ProteinPilot software (Applied Biosystems, CA, USA) against a local Tangut Nitraria protein/peptide sequence database previously used by Cheng et al. (2015). Protein relative quantification was conducted using ProteinPilot software, with default settings. A protein was identified as having differential expression in response to salt stress treatment if it had a fold change $>1.5$ or $<0.66$ and a $p$-value $<0.05$ (Wan and Liu 2008; Pang et al. 2010; Neilson et al. 2011; Yang et al. 2014).

Bioinformatics analysis of identified proteins

The functions of the identified proteins in the abovementioned proteomic analysis were assigned using the InterPro database and the Pfam database (Apweiler et al. 2001; Finn et al. 2007), and the identified proteins were annotated and categorized based on the methods used for Arabidopsis (Bevan et al. 1998).

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed on proteins with salt-induced differential expression using the GO annotation tool Blast2GO and the data from the National Center for Biotechnology Information (NCBI) and The Arabidopsis Information Resource (TAIR) databases (Conesa and Gotz 2008). The protein-protein interaction network was constructed using the Cytoscape program, based on the protein interaction databases of Search Tool for the Retrieval of Interacting Genes/Proteins (DIP) (Franceschini et al. 2013).

Statistical analysis

For the measurements taken in the abovementioned experiments, all data were subjected to analysis of variance using SPSS software, and the means ($n=5$) were separated with $>95\%$ confidence intervals using Fisher’s least-significant difference test.

Results

Effects of salt stress on leaf phenotypes of N. billardieri seedlings

To characterize the impact of salt stress on the morphology of N. billardieri, three-month-old seedlings were tested in response to salt stress with 100, 200 and 500 mM NaCl. The results demonstrated that salt treatments for one week at three different concentrations had different stress effects on the morphology of N. billardieri seedlings. Specifically, the 100 mM NaCl treatment did not cause any obvious visible changes in morphology, and moderate stress with 200 mM NaCl caused slightly rolling of leaves; however, the 500 mM NaCl treatment caused the relatively severe symptoms of leaf curling (Images not recorded). Hence, the moderate salt stress treatment with a 200 mM NaCl concentration was chosen for further investigation.

Salt stress treatment for 3 days did not cause any visible changes of phenotypes in seedling plants of N. billardieri. With continued stress, a slight loss of turgor in the leaves of seedlings was observed 6 days after salt stress induction, and salt treatment of up to 9 days caused the leaf margins to slightly roll inward (Figure 1). However, Arabidopsis seedlings, a salt-sensitive model plant, displayed an obvious rolling at 3-day treatment of same NaCl concentration, and this phenotype gradually worsened with continuing stress treatment over time (Figure S1). The LRWC of seedling leaves was determined at the beginning of stress treatment and at 3, 6 and 9 days after salt stress induction; the LRWC decreased from 77.5% at day 0 and 77.9% at day 3 to 73.6% and 67.4% at days 6 and 9, respectively (Figure 2(A)). The results showed that N. billardieri had a high salt tolerance, similar to the other species of the genus Nitraria L. (Ni et al. 2012; Chun et al. 2016).

Salt stress increased the REL value of seedling leaves (Figure 1(E)), implying that salt stress treatments might damage the cell membrane. Salt stress tends to cause osmotic imbalance in the cytoplasm of plants (Inan et al. 2004). The accumulation of osmolytes, such as proline and soluble sugar, can enhance tolerance of osmotic stress (Inan et al. 2004; Kant et al. 2006; Dassanayake et al. 2011). Our results revealed that the proline content increased and reached the highest value at 6 days after salt treatment (Figure 2); it is possible that the accumulated proline content protected proteins against denaturation (Kant et al. 2008; Zhang et al. 2010). The soluble sugars also showed a similar continuous increase during the salt treatment period (Figure 2); this result is consistent reported findings that salt stress increased the accumulation of soluble sugars in plants (Inan et al. 2004; Bartels and Sunkar 2005; Gong et al. 2005).

Changes in photosynthetic parameters in response to salt stress in Nitraria billardieri seedlings

To evaluate the influence of salt stress on photosynthetic capacity, we investigated the changes in photosynthetic parameters of N. billardieri seedling leaves during the period of

![Figure 1](image-url). Morpological changes, water content and membrane permeability in leaves of Nitraria billardieri seedlings after treatment with 200 mM NaCl for 0 (A), 3 (B), 6 (C), and 9 days (D); E depicts water content and membrane permeability. Data represent the mean ± SD. Different letters indicate significant differences ($p<0.05$) based on Tukey’s test between salt treatments and control or between different salt treatment times.
salt treatment. Salt stress treatment significantly decreased the Pn parameter (Figure 3(A)). The influence of salt stress on this trait dropped steadily during the progression of salt stress treatment, as Pn decreased from the initial value of 2.97 µmol CO$_2$ m$^{-2}$s$^{-1}$ to 2.71, 2.16, and 1.67 µmol CO$_2$ m$^{-2}$s$^{-1}$ at day 3, 6, and 9 of treatment, respectively (Figure 3(A)). The Pn in the stress induction treatment was 27.5% and 43.6% lower at 6 d and 9 d than that in normally grown controls, respectively. A similar pattern of change occurred in the Gs parameter in response to salt stress treatment (Figure 3(A)). The Gs decreased by 24.3% and 46.8% at 6 d and 9 d of stress induction, respectively. However, the Gs value slightly decreased throughout the salt stress treatment. The Ci decreased by 6.8% and 12.1% at 6 d and 9 d of salt stress treatment, respectively (Figure 3(B)). In addition, salt stress also reduced the Tr accompanied by stress application (Figure 3(B)). The abovementioned negative effects mainly occurred at day 6 and 9, but 3 days after salt stress induction, there was no significant influence on the photosynthetic parameters.

**Effect of salt stress on membrane status and antioxidative enzyme activity**

Salt stress treatment slightly increased the MDA content in seedling leaves. Three days after salt induction, no change in MDA content occurred; however, the MDA content rose by 1.15% and 1.34% at 6 d and 9 d of stress treatment, respectively. The increase in MDA content showed that continuous salt stress treatment caused membrane lipid peroxidation and membrane damage (Figure 4(A)).

The activities of several key ROS scavenging enzymes, SOD, CAT and POD, were determined to investigate their roles in the antioxidative defence system in leaves of *N. billardieri* during salt stress treatments. It was found that the activities of antioxidative enzymes were enhanced in response to salt stress in the examined leaves of *N. billardieri* seedlings (Figure 4(A and B)). The SOD activity did not significantly change after salt treatment for 3 days and then increased slightly by up to 1.22- and 1.43-fold at 6 and 9 days after salt induction, respectively (Figure 4(B)). The CAT activities displayed a gradual decrease after salt treatment for 3, 6 and 9 days of 1.12-, 1.45-, and 1.63-fold, respectively (Figure 4(B)). Similarly, the POD activities displayed a progressively increasing trend (Figure 4(B)). Hence, *N. billardieri* seedlings under salt stress may enhance their ROS-scavenging enzyme activities to eliminate oxidative stress caused by excessive ROS production due to salt stress.

### N. billardieri leaf proteome patterns in response to salt stress treatments

To understand the salt stress response mechanism of *N. billardieri* at the protein level, comparative proteomic analysis was performed to investigate the changes in protein profiles in the leaves of *N. billardieri* seedlings at 9 days after salt stress induction. Queries of the *Nitraria* protein/peptide sequence database and plant protein database produced 885 proteins, resulting in the identification of 839 proteins. A total of 130 proteins were found to have significant expression changes of >1.50-fold (*p* ≤ 0.05); among them, 76 proteins were up-regulated and 54 proteins were down-regulated in salt-treated leaves compared to control ones (Table S1). Here, several isoforms of some differentially expressed proteins or different types of protein subunits were identified, such as two members of the 3,4-dihydroxy-2-butanoate kinases, two members of the ATP-dependent Clp protease proteolytic subunit-related chloroplastic complex, and three members of the heat shock 90 family. The isoforms displayed the same trends of down-regulation or up-regulation or the opposite trends in response to salt stress. These various response patterns may be caused by protein modification, localization, or functional roles in plants under salt stress treatments. The increase in protein expression may reflect the specific adaptation or sensitivity of seedlings to environments with salt stress, and the...
decrease may represent the stress-induced damage or disorder of metabolic processes caused by salt treatments.

Functional categorization of differentially expressed proteins

To further identify and annotate proteins with salt-induced differential expression, the NCBI NR protein database was searched for homologous sequences by querying with the Basic Local Alignment Search Tool (BLAST) program (Altschul et al. 1990). The protein sequences that were identical to their homologs by up to 50% were confirmed to have similar functions. A total of 130 differentially expressed proteins were found to have known or putative functions in the Pfam and InterPro databases.

To understand the roles of the differentially expressed proteins associated with salt stress in seedling leaves, we sorted these proteins into 16 functional categories based on their annotated or identified functions (Figure 5) including amino acid metabolism, carbohydrate metabolism, cell cycle and cytoskeleton, cell rescue/defense, energy metabolism, lipid metabolism, membrane and transport, photosynthesis, protein degradation, protein folding and assembly, protein synthesis, redox homeostasis, and others and unknown. Among these categories, signal transduction (15%), carbohydrate metabolism (12%), protein synthesis (10%), protein degradation (8%), and membrane and transport (7%) account for the majority of the differentially expressed proteins (Figure 5(A)). In the up-regulated proteins, the most-represented categories comprised signal transduction, protein synthesis, and carbohydrate metabolism, followed by redox homeostasis and protein degradation (Figure 5(B)). However, the three over-represented categories in the down-regulated proteins were signal transduction, carbohydrate metabolism, and protein degradation (Figure 5(C)). These results imply that proteins involved in signal transduction, carbohydrate metabolism, protein metabolism and redox homeostasis play important roles in the seedling leaves of N. billardieri during response to salt stress.

Gene ontology and KEGG pathway analyses of salt-responsive proteins

To obtain deeper insights into the biological functions in which the proteins with salt-induced differential expression were involved, GO and KEGG pathway analyses was performed, and the results were visualized with Blast2GO (Conesa and Gotz 2008).

For the proteins with salt-induced differential expression, the most highly enriched categories of biological processes (BPs) were cellular nitrogen compound biosynthetic process, organonitrogen compound biosynthetic process, oxaacid metabolic process and cellular protein metabolic process, demonstrating that these biological processes were of functional importance for salt stress responses in the seedling leaves of N. billardieri. The second most highly enriched GO-BP categories were macromolecule biosynthetic process, cellular macromolecule biosynthetic process, phosphate-
containing compound metabolic process, gene expression, cellular amide metabolic process and peptide metabolic process (Figure 6). For the categories of molecular functions (MFs), metal ion binding, ribonucleoside binding, purine nucleotide binding, purine ribonucleotide binding, purine ribonucleoside triphosphate binding and purine nucleoside binding contributed the largest portion of the proteins (Figure 6). For the Cellular Component ontology, the top four categories were mitochondrion, plastid, cytosol and chloroplast part (Figure 6).

In the KEGG pathway analysis of salt stress-responsive proteins, the over-represented categories were biosynthesis of antibiotics, glutathione metabolism, pyruvate metabolism, amino sugar and nucleotide sugar metabolism, oxidative phosphorylation, and TCA cycle (Table S2). Among these categories, the most important pathway was biosynthesis of antibiotics, which contained 11 identified enzymes including hydroxymethyltransferase (EC:2.1.2.1), succinyltransferase (EC:2.3.1.61), carboxykinase (EC:4.1.1.49), kinase (EC:2.7.1.40), dehydrogenase (EC:1.1.1.35), dehydrogenase (EC:1.3.5.1), acetyltransferase (EC:2.3.1.12), dehydrogenase (NADP+-dependent, decarboxylating) (EC:1.1.1.44), reductoisomerase (EC:1.1.1.86), phosphatase (EC:3.1.3.25), and synthase (ferredoxin) (EC:1.17.7.1). These enzymes were involved in mediating the transfer of secondary metabolites during cellular metabolism.

The second most important pathways were those that involve carbohydrate metabolism, including citrate cycle (TCA cycle), glycolysis / gluconeogenesis, and starch and sucrose metabolism. Four enzymes belonged to the TCA cycle, which are key enzymes for the TCA cycle in plants; they are succinyltransferase (EC:2.3.1.61), carboxykinase (ATP) (EC:4.1.1.49), dehydrogenase (EC:1.3.5.1), and acetyltransferase (EC:2.3.1.12). Three enzymes belonged to glycolysis / gluconeogenesis, including carboxykinase (ATP) (EC:4.1.1.49), kinase (EC:2.7.1.40), and acetyltransferase (EC:2.3.1.12). Two enzymes, gentiobiase (EC:3.2.1.21) and adenyllyltransferase (EC:2.7.7.27), belonged to starch and sucrose metabolism.

Figure 6. Gene Ontology analysis of salt-responsive proteins in the leaves of N. billardieri seedlings.

Another important set of identified pathways was that involved in photosynthesis. This set included seven enzymes: dehydrogenase (EC:1.1.1.35), dehydrogenase (EC:1.3.5.1), phosphatase dikinase (EC:2.7.9.1), carboxykinase (ATP) (EC:4.1.1.49), phosphatase dikinase (EC:2.7.9.1), reductase (EC:1.18.1.2), and IX monomethyl ester (oxidative) cyclase (EC:1.14.13.81). These enzymes are considered to play important roles in carbon fixation and the process of photosynthesis.

Salt stress treatment also influenced glutathione metabolism. Four enzymes were identified, including peroxidase (EC:1.11.1.9), dehydrogenase (NADP+-dependent, decarboxylating) (EC:1.1.1.44), glutamyl transpeptidase (EC:2.3.2.2), and thioredoxin peroxidase (EC:1.11.1.15); these enzymes functioned in maintaining cellular redox homeostasis. Additionally, 11 enzymes involved in amino acid metabolism were identified. They included purine metabolism, lysine degradation, methane metabolism, valine, leucine and isoleucine biosynthesis, valine, leucine and isoleucine degradation, tyrosine metabolism, glycine, serine metabolism and threonine metabolism (Table S2). The accumulation or degradation of these amino acids might play an important role in plants during response to salt stress.

Protein–protein interaction analysis

Proteins in plant cells tend to interact with each other as a network for adaptation to environmental stress. To investigate how N. billardieri seedlings transfer salt stress messages through protein interactions, salt-responsive proteins were used to construct the protein interaction network. Some of the proteins identified as being significantly regulated by salt were predicted to constitute a network by interacting with each other (Figure 7). The abbreviation and name description of the proteins in the networks are shown in Table S3. A set of protein interactions was detected including TIM9 – AT1G26880 – AT5G12110 – AT1G48830 – SAD2 – EIF2 – SHM3 – SHM4 – THFS – AT2G3658 – GLT1 – AT3G24170 – NTRC – ACLB-2 – LPD1 – ACC2 –
Figure 7. Protein–protein interaction network analysis of salt-responsive proteins.

Figure 8. Schematic presentation of salt stress-responsive proteins involved in protein metabolism.
AT1G68300 – GR – HSP60 – HA3. These proteins constituting interaction networks were mainly involved in protein synthesis, protein folding and assembly, protein degradation, membrane and transport, signal transduction, redox homeostasis, secondary metabolism, cell rescue/defence and energy metabolism. Moreover, the interaction consisting of amino acid and carbohydrate metabolism coupled with redox homeostasis, protein synthesis, and protein folding and assembly occupied an important position and might have had an essential function in N. billardieri seedling response to salt stress. In addition, protein hubs and key connectors, such as SHM3, SHM4, GLT1, NTRC and ACLB-2, occupied central positions of the networks; these proteins may play important functions in response to salt stress by facilitating communication within protein interaction networks. Hence, protein connectivity in networks can provide a deep understanding of the importance of these proteins in the response of N. billardieri to salt stress conditions.

Discussion

Morphological alteration might be associated with physiological and biochemical responses in N. billardieri seedlings adapting to salt stress

High-salt environments often restrict the growth and development of plants, especially non-halophytes (Ji et al. 2013; Zhang and Shi 2013). Many plants tend to cope with salt stress by changing their osmotic adjustment, growth alteration, injury avoidance and ion homeostasis (Zhu 2001b). The remarkable salt stress tolerance of halophytic Nitraria L. can be largely associated with basic physiological and biochemical mechanisms. Our results demonstrated that continuous salt treatment inhibited the growth of N. billardieri seedlings, which decreased the LRWC and turgor of leaves in a time-dependent manner (Figure 2). Based on the observation of morphological responses to salt stress, continuous salt stress resulted in a gradual increase in growth inhibition of seedling leaves, with the effect being much more pronounced at day 9 after salt stress induction. This result was somewhat similar to that of the morphology of different species of Nitraria L. including N. sibirica, N. tangutorum and N. roborowskii under salt stress (Ni et al. 2012; Cheng et al. 2015). Photosynthetic parameters were affected by salt stress in the leaves of N. billardieri seedlings; significant decreases in the photosynthesis parameters Pn, Gs and Tr at day 9 of salt stress treatment may imply that the seedlings were subjected to stressful conditions (Ni et al. 2012). It can also be proposed that the decreases in the photosynthesis parameters were caused by stomatal closure and subsequent water transpiration, which could even destroy chloroplast organization, with a more pronounced occurrence at a later period of salt stress (Cheng et al. 2015). Moreover, in the proteomic analysis, five identified salt-responsive proteins were associated with photosynthesis, including phosphoenolpyruvate carboxykinase [ATP]-like protein, psbP domain-containing chloroplastic complex, Ycf4-photosystem I assembly complex, magnesium-protoporphyrin IX monomethyl ester [oxidative] cyclase, and phosphate chloroplastic-like protein. The first two proteins exhibited decreasing trends, and the next three proteins showed increased abundances in response to salt stress. These results imply that salt stress influences photosynthesis in seedling leaves by regulating protein expression associated with light reactions and carbon fixation.

Salt stress induced the up-regulation of proteins involved in redox homeostasis

When salt stress-induced growth inhibition occurs, the seedlings might suffer from oxidative stress due to an over-accumulation of ROS, which subsequently activates the ROS scavenging system (Stepien and Johnson 2009). Plants respond to salt stress by the rapid accumulation of reactive oxygen species (ROS), which can disturb the balance of redox status and result in oxidative damage (Zhang et al. 2012). Therefore, the plant can regulate its biochemical and metabolic state to maintain an appropriate balance between ROS and the antioxidative defense system to avoid salt stress-induced damage. For avoiding ROS-caused damage to membrane and protein structures, the ROS scavenging system, including SOD, CAT and POD, is activated to scavenge ROS in leaves of N. billardieri seedlings under salt stress conditions (Figure 4). In the proteomic analysis, 14 differentially expressed proteins known to function in antioxidant reactions were mainly sorted into two categories: redox homeostasis and cell rescue/defense. Of the 7 proteins classified as redox homeostasis-related proteins, 6 (thioredoxin reductase NTRC, monothiol glutaredoxin-S10, peroxisomal (S)-2-hydroxy-acid oxidase, peroxiredoxin-mitochondrial, ferredoxin-NADP root chloroplastic and peroxidase 17) were up-regulated, and one (phospholipid hydroperoxide glutathione peroxidase chloroplastic) was down-regulated in N. billardieri under salt stress. These proteins are involved in the peroxiredoxin/thioredoxin (PrxR/Trx) pathway (including thioredoxin reductase NTRC and peroxiredoxin-mitochondrial), the glutathione peroxidase pathway (including phospholipid hydroperoxide glutathione peroxidase 17) and the glutathione cycle (including monothiol glutaredoxin-S10). Hence, the up-regulation of proteins involved in redox homeostasis implies that the ROS scavenging system may be activated in the leaves of N. billardieri seedlings to alleviate salt-induced oxidative stress and to enhance salt tolerance. Phospholipid hydroperoxide glutathione peroxidase, a key enzyme for degrading H2O2 in the glutathione cycle, displayed a decreasing trend in response to salt stress; this finding is similar to the result observed in leaves of Suaeda aegyptiaca under a 150 mM NaCl concentration (Askari et al. 2006). Mitochondrial peroxiredoxin plays an important role in the redox pathway (Vieira dos Santos and Rey 2006), it was up-regulated in the leaves of N. billardieri seedlings under salt stress, which was consistent with the pattern found in the roots of Cucumis sativus in response to a 50 mM NaCl treatment (Du et al. 2010). Thus, the up-regulated expression of these proteins implies that the ROS scavenging system was activated by salt stress treatment in N. billardieri seedlings in order to alleviate oxidative damage.

Salt stress induced the osmotic stress-related metabolic changes

Salt stress can also directly result in osmotic stress (Kant et al. 2006), and increased osmotic stress triggers the accumulation of compatible metabolites, such as sugars and amino acids (Inan et al. 2004; Bartels and Sunkar 2005). Proline and soluble sugars are important osmolytes that might play a key
role in maintaining osmotic homeostasis to decrease damage caused by salt stress (Inan et al. 2004; Yang et al. 2018). The observation of proline accumulation to a relatively high level in leaves is consistent with the finding that the halophyte *Thellungiella* can also increase proline content to prevent water loss during salt stress treatments (Inan et al. 2004; Ghars et al. 2008, 2012). Soluble sugars are also important osmolytes that accumulate in plants to protect molecules, membrane structures or cells during salt stress (Bartels and Sunkar 2005; Wingler et al. 2006). High levels of soluble sugars were found to accumulate in the leaves of three Nitraria L. species including *N. sibirica*, *N. tangutorum* and *N. roborowskii* when they were exposed to salt stress (Ni et al. 2012). Our results showed that salt-treated *N. billardieri* plants accumulated a relatively high level of soluble sugars in leaves treated with 200 mM NaCl (Figure 2), and the proteomic analysis demonstrated that 16 proteins involved in carbohydrate metabolism were regulated in salt-stressed leaves (Table S2).

**Salt stress induced the differential regulation of protein metabolism**

It is well known that salt stress induces changes in many proteins that are involved in various cellular pathways that cooperate or coordinate to respond to and tolerate salt stress. Salt stress was found to have significant effects on protein fates associated with protein synthesis, folding and assembly, and degradation.

Protein synthesis plays an important role in plant response to salt stress. It was found that salt stress induced the change in expression of many components belonging to the protein synthesis machinery (Jiang et al. 2007; Pang et al. 2010; Sobhanian et al. 2010). Our study also identified 13 differentially expressed proteins involved in protein synthesis, including three 40S ribosomal subunits (40S ribosomal S7, 40S ribosomal S6-like and 40S ribosomal S27-2), four 60S ribosomal subunits (60S ribosomal L34, 60S acidic ribosomal P3-like, 60S ribosomal L13-1, and 60S ribosomal L18-2), two translation initiation factors (eukaryotic translation initiation factor 3 subunit C-like and eukaryotic translation initiation factor), two aspartate-tRNA ligases (aspartate-tRNA ligase cytoplasmic and aspartate-tRNA chloroplastic mitochondrial), elongation factor 1-beta 1 and peptide chain release factor (Figure 8). Among these, nine proteins were up-regulated and four proteins were down-regulated under salt stress. Of these regulated proteins, the abundances of three 60S ribosomal subunits and of 40S ribosomal S27-2 decreased under salt stress; this observation was consistent with another study that found that protein synthesis was partially repressed by salt stress (Tuteja 2007).

Correct protein folding and assembly are crucial for maintaining normal cellular functions during salt stress treatments. Heat shock proteins (HSPs), isomerases and chaperones contribute to sub-cellular localization and protein structure stabilization (Vierling 1991). Seven proteins belonged to this functional category, including chaperone chloroplastic isoform X1, heat shock 90-chloroplastic, chaperonin CPN60-mitochondrial, heat shock 90-mitochondrial, and disulphide isomerase-like 2–3, were identified in the leaves of *N. billardieri* seedlings under salt stress conditions. Among these, two proteins localized in the chloroplast (chaperone chloroplastic isoform X1 and heat shock 90-chloroplastic), and two isomerases named disulphide isomerase-like 2–3 and peptidyl-prolyl cis-trans isomerase FKBP16 were up-regulated. Interestingly, three proteins (CPN60 and two heat shock 90 proteins), predicted to be localized in the mitochondrion, were down-regulated in response to salt stress treatments (Figure 8). The aforementioned salt-responsive proteins displaying various alterations might function to maintain normal protein folding and assembly or to repair the damaged proteins caused by stress (Figure 8). Protein degradation also plays an important role in regulating cellular processes; plants can selectively degrade some proteins using proteasome pathways, hydrolase or protease systems. Eleven salt stress-induced proteins, mainly belonging to proteasome components and various proteases, were identified as responsible to salt stress. Taken together, these various expression patterns in protein fates imply that the protein self-regulation system is crucial for salt stress accommodation in plants.

**Conclusion**

Proteomic analysis in combination with physiological and biochemical results has provided novel insights towards understanding the response strategy of *N. billardieri* seedlings to salt stress. Many proteins from various metabolic pathways such as protein metabolism, antioxidative defence system and carbohydrate metabolism exhibited common and differential response patterns in the *N. billardieri* plants that may allow this species to adapt to saline environments. Proteomic analysis indicated that the reprogramming of metabolic processes that contribute to salt tolerance is a complex system that is required to maintain metabolic balance and to alleviate oxidative and osmotic injury. The metabolites and proteins detected here may provide potential targets for improving plant salt tolerance through marker-assisted selection or genome editing.

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