iTRAQ protein profile analysis of leaves and roots of sugar beet (*Beta vulgaris*) differing in response to salt stress

Junliang Li
Harbin Institute of Technology
Jie Cui (cuijie2006@163.com)
Harbin Institute of Technology https://orcid.org/0000-0002-3247-082X
Dayou Cheng
Harbin Institute of Technology
Cuihong Dai
Harbin Institute of Technology
Tianjiao Liu
Harbin Institute of Technology
Congyu Wang
Harbin Institute of Technology
Chengfei Luo
Harbin Institute of Technology

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Abstract

Background Salinity is one of the most serious threat to agriculture worldwide. Sugar beet is an important sugar-yielding crop and has a certain tolerance to salt. However, the molecular mechanism of salt tolerance in Beta vulgaris are poorly understood. Proteomics can provide a new perspective and deeper understanding for the research of salt-tolerant sugar beet.

Results Here, leaves and roots were used to identify the differentially abundant protein species (DAPs) between salt-stress and control conditions in beta vulgaris . As a result, 70 and 76 DAPs were identified in leaves and roots, respectively. The functions were determined for the classification of the DAPs, mainly involved in cellular processes, environmental information processing, genetic information processing and metabolism. These processes can work cooperatively to reconstruct the favorable equilibrium of physiological and cellular homeostasis under salt stress. Some candidate DAPs are closely related to salt resistance such as choline monooxygenase, betaine aldehyde dehydrogenase, glutathione S-transferase (GST) and F-type H + -transporting ATPase. The expressional pattern of 10 DAPs encoding genes were consistent with the iTRAQ data.

Conclusions Our results demonstrated that during adaptation of beet to salt stress, leaves and roots have distinct mechanisms of molecular metabolism regulation. This study provided some significative insights into the molecular mechanism underlying the response of higher plant to salt stress, and identified some candidate proteins against salt stress.

Background

Salinity is one of the most severe abiotic threats that affects the growth and development of crops [1, 2]. Soil-salinization is a growing problem for agriculture that may negatively decrease the quality and yield of crops. The common effect of soil salinity on plants comes from the inhibition of growth by Na^+ and Cl accumulation [3]. Unlike other abiotic stresses, salinity causes both osmotic stress and ion toxicity in plants [4]. Plant growth may rapidly impaired by osmotic stress in a first phase and then specific ion toxicity primarily from Na^+ and Cl accumulation may cause membrane disorganization, the generation of reactive oxygen species, metabolic toxicity, inhibition of photosynthesis, and the attenuation of nutrient acquisition in a second phase of salt stress [5, 6]. Some soils are actually hyperhaline for certain crop life, however, some salt-tolerant varieties can adapt to these conditions and achieve a good harvest. Sugar beet (*Beta vulgaris ssp. vulgaris*) is one of the most important sugar-yielding crops in the world. As recently domesticated crop, cultivated beets inherited certain salt-tolerance traits from its wild ancestor *Beta vulgaris ssp. maritima* (*B. maritima* or ‘sea beet’) [7].

Analysis of the proteome responses to stress provides more direct insights into the molecular phenotype, since proteome is a better reflection of organism's actual response to environmental changes than the transcriptome. Isobaric tags for relative and absolute quantitation (iTRAQ) is one of the most reliable labeling techniques available for proteome quantification [8-10]. Li et al. analyzed the changes of

membrane proteins under salt stress using iTRAQ technology in sugar beet monosomic addition line M14 [11]. Yu et al. analyzed the changes in proteome and phosphoproteome of M14 leaves induced by short-term salt stress (30min and 1 hour) [12]. Wu et al. studied changes in the proteome of beet seedlings treated with 50mm NaCl for 72h and 30 and 105 differentially expressed proteins were identified in the shoots and roots, respectively [13]. However, how plants respond to salinity depends on the organ, intensity and duration of the stress, which may lead to various changes at the proteome level [14, 15]. In addition, as suggested by Shavrukov [16], salt treatments can be divided into two types: salt stress (gradual exposure to rising salt levels) or shock (immediate exposure to a high-salt environment). Due to the difference in response to salt-stress between the two approaches[7], the method of salinity application should be carefully considered with respect to the interpretation of results.

In the present study, iTRAQ-based quantitative proteomic analysis was employed to assess proteomic changes and identify differentially abundant protein species (DAPs) in leaves and roots of cultivar beet 'O68' under salt-stress, respectively. NaCl was used in the Hoagland medium to imitate environmental salt stress. A total of 70 and 76 DAPs were identified in leaves and roots, respectively. Then Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups of proteins (COG) and STRING were used to elucidate the function of the DAPs in response to salt stress. These results will provide some insights into the underlying molecular mechanisms of stress responses and improve the understanding of the salt-stress response of sugar beet.

Results

Effects of salinity on physiological indexes of sugar beet

Chlorophyll content was detected with a 0.76-fold decrease in leaves under salt stress compared to control plants (Figure. 1c). In contrast, salt-stress leaves displayed 3.6-fold increased proline content than control (Figure. 1d). Moreover, MDA (malondialdehyde) content was 1.6-fold higher in leaves of stressed plants (Figure. 1e). TTC (triphenyltetrazolium chloride) reduction was used to evaluate root activity. A reducing capacity of 1.5-fold greater was detected under salt stress compared to control plants. (Figure. 1f)

Primary Data Analysis and Protein Identification Information by iTRAQ

To investigate the salt-tolerance mechanism of sugar beet, iTRAQ-based comparative proteome was used to analyze and compare the differentially expressed proteins in roots and leaves of salt-treated sugar beet plants and untreated plants. A total of 31,438 and

39,522 MS/MS counts were generated from leaves and roots of sugar beet, respectively. In leaf, 10121 unique peptides and 3,175 proteins were identified against the UniProt database, 61.6% proteins (1,966) of which had at least two unique peptides. In root, 13248 unique peptides and 3,935 proteins were identified, of which 64.6% proteins (2,541) had at least two unique peptides. The length and number distribution of the peptides are provided in Figure. S1, statistical analyses showed that most peptides have 8-15 amino acids. The peptide number distribution of proteins indicated that 90% of identified proteins contain less than 8 segments (Figure. S2). The mass of the identified proteins suggested that 60 and 70 low molecular weight proteins (Mr < 10 kDa), 300 and 417 high molecular weight proteins (Mr > 100 kDa) were identified using the iTRAQ strategy (Figure S3), respectively. The distribution of protein coverage showed that coverage with less than 10 %, 10 % -30 %, and 30 % 100% accounted for 57.6 %, 33.9 % and 8.4% in leaf (Figure S4a), and 55.6 %, 30.6 % and 13.8 % in root (Figure S4b).

Identification of Differential Abundance Protein Species (DAPS)

Proteins with at least two unique peptides in this study were used to screen DAPS. The fold change >1.2 and p value <0.05 were set as the criteria. In leaf, a total of 70 DAPS including 44 up-accumulated and 26 down-accumulated were identified under salt stress versus control condition (Figure 2a). Correspondingly in root, 76 DAPS were identified, of which, 40 were up-accumulated and 36 were down-accumulated (Figure 2b). A Venn diagram including the total 143 proteins was generated (Figure 2c). For individual proteins, the responses to salt stress were distinct between leaf and root tissue. Only 2 (1.4%) up-regulated proteins were conserved between leaf and root, and one protein (0.7%) had opposite expression patterns between leaves and roots. Detailed information of DAPs were provided in Table S1 and Table S2.

Bioinformatics Analysis of DAPS Identified by iTRAQ

The putative functions of salt-stress-responsive DAPs in sugar beet were investigated using Gene ontology (GO) enrichment, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment and Clusters of Orthologous Groups (COG) analyses (Figure 3). Gene

ontology (GO) enrichment analysis showed that oxidation-reduction process (GO:0055114) was the most significantly enriched term under biological process in both leaves and roots. In addition, transport (GO:0006810), response to gibberellin (GO:0009739), response to cytokinins (GO:0009735), sulfate assimilation (GO:0000103), and cell redox homeostasis (GO:0045454) were enriched specifically in leaves(Figure 3a), while response to hypoxia (GO:0001666), cell wall organization (GO:0071555), peroxisome organization (GO:0007031), toxin catabolic process (GO:0009407), response to anoxia (GO:0034059), sucrose metabolic process (GO:0005985), response to water deprivation (GO:0009414), and defense response to bacterium (GO:0042742) were enriched specifically in roots(Figure 3b). For cellular component, leaves and roots had similar enrichment in cytoplasm (GO:0005737), plasma membrane (GO:0005886), cell wall (GO:0005618), plasmodesma (GO:0009506), extracellular region (GO:0005576), integral component of membrane (GO:0016021), and apoplast (GO:0048046), but thylakoid (GO:0009579) was enriched only in leaves. The most enriched molecular function category in leaves were protein binding (GO:0005515) and RNA binding (GO:0003723), while in roots were metal ion binding (GO:0046872) and glutathione transferase activity (GO:0004364).

Using KEGG, all DAPs in both leaves and roots were divided into four main categories: “Cellular Processes”, “Environmental Information Processing”, “Genetic Information Processing”, and “Metabolism”. Specifically, 70 DAPS in leaves were further divided into 12 subclass and mapped to 43 pathways (Figure 3c), while 76 DAPs in roots were further divided into 17 subclass and mapped to 45 pathways (Figure 3d). The analysis results showed that a pathway related to membrane transport (ko02010) was specially enriched in leaves, while compared with leaves, there were more metabolism-related pathways enriched in roots.

A total of 70 and 76 DAPS in leaves and roots were classified into 14 and 13 categories of Clusters of Orthologous Groups of proteins (COG), respectively, among which Function unknown Carbohydrate transport and metabolism, and Posttranslational modification, protein turnover, chaperones represented the largest group in both leaves and roots. The difference is that the leaves contain DAPs classified into Defense mechanisms and

Cytoskeleton (Figure 3e), while the roots contain DAPs classified into to Transcription (Figure 3f).

STRING protein interaction database was used to analysis the protein-protein interaction (PPI) for DAPs in leaves and roots, respectively [17]. Ultimately, 31 and 26 interacting proteins were identified in the leaves and roots, respectively (Figure 4). Among these DAGs, eight KEGG pathway were significant enrichment in leaves including: Biosynthesis of amino acids (bvg01230), Metabolic pathways (bvg01100), Cysteine and methionine metabolism (bvg00270), Biosynthesis of secondary metabolites (bvg01110), Carbon metabolism (bvg01200), Glycerophospholipid metabolism (bvg00564), Sulfur metabolism (bvg00920) and Photosynthesis (bvg00195); while six KEGG pathway were significant enrichment in roots including: Glycolysis / Gluconeogenesis (bvg00010), Metabolic pathways (bvg01100), Purine metabolism (bvg00230), Biosynthesis of secondary metabolites (bvg01110), RNA polymerase (bvg03020) and Pyrimidine metabolism (bvg00240).

Transcriptional Analyses of the Corresponding Genes Encoding DAPS

To know the correlation between the abundance of DAPs and the transcript level of their corresponding genes, twelve DAPs (six from leaves and six from roots with one intersection) were selected for qRT-PCR analyses. The results showed that ten of the twelve selected DAPs keep the same express trend between transcript and protein level, the other two DAPs showed no significantly changed at transcript level (Table 1). This discrepancy may be due to temporal differences between the transcriptional level and the protein level of the salt stress response.

Table1. Comparison of expression pattern at the mRNA and protein level of DAPS.

[Please see the supplementary files section to view the table.]

Discussion

Salt stress may have distinct effects on different organs of plants. As shown in the iTRAQ data, we identified 1966 and 2541 proteins in leaves and roots that possessed more than two unique peptides. Furthermore, 70 and 76 DAPs were identified in leaves and roots, respectively. Among these DAPs, only three were differentially expressed in both roots and leaves, while the remaining 140 DAPs were differentially expressed only in leaves or roots. Functional divergence of the proteins from leaves and

roots suggested that leaf and root may have distinct responses to salt stress and may make different contributions to stress-resistance in sugar beet. This result may not represent the entire landscape of protein patterns in leaf and root under stress or control condition owing to the technical limitations of iTRAQ. The possible biological significance of some key DAPS and their relevant metabolic pathways in salt stress adaptation are discussed below.

Analysis of the DAPs response to salt stress in Chloroplast

Chloroplasts, which only exist in leaves, are the most sensitive organelles to salt stress in plants [18]. High salinity will destroy chloroplasts and affect photosynthesis [19]. The experiment showed that chlorophyll content in leaves decreased 0.76-flod under high salt stress in sugar beet (Figure 1a). Unsurprisingly, 22 of the identified DAPs in leaves were related to chloroplasts including 14 up-regulated DAPs and 8 down-regulated DAPs. Three proteins psbQ-like protein 1 (A0A0K9RS47) and Plastocyanin (A0A0J8B4F7) and NAD(P)H quinone oxidoreductase subunit U (A0A0K9R1T8) from the photosynthetic electron transport chain were up-regulated under salt-stress, which may contribute to the maintenance of photosynthesis intensity. In addition, the STRING network analysis showed that the psbQ-like protein 1 was interacted with another up-regulated protein peptidyl-prolyl cis-trans isomerase fkbp16-4 (PPI) (A0A0K9RJJ) (Figure 4a). Other studies have shown the same up-regulation [20] which imply that plants may respond to salt stress by accumulating PPI to accelerate protein synthesis. Another up-regulated protein A0A0K9QN40 is thioredoxin Y1 that has the ability to regulate the activity of photosynthetic enzymes[21]. Furthermore, DNA repair RAD52-like protein (A0A0K9RXT2) and DNA-damage-repair/toleration protein DRT100-like (A0A0K9S3X5) were also up-regulated proteins in chloroplast, these DAPs may help protect chloroplast DNA from damage under salt stress and enhance salinity tolerance in plant [22, 23].

Glycine betaine is considered to be the best osmotic regulator, which is not only involved in the osmotic regulation of cells, but also plays an important role in stabilizing the structure and functions of biological macromolecules under osmotic conditions, such as protecting the major enzymes and terminal oxidases of TCA (tricarboxylic acid) cycle and stabilizing the peripheral peptides of the light system under salt stress [24-26]. Betaine is an important osmotic regulator, which is produced from choline through two-step oxidation in plants [27-29]. The synthesis of betaine is catalyzed by two enzymes, choline monooxygenase (Q4H1G6) and betaine aldehyde dehydrogenase (Q4H1G7), which are significantly up-regulated under salt-stress [28]. In addition, SEX4 (STARCH-EXCESS 4, also known as Dual specificity protein phosphatase 4, DSP4) (A0A0J8B9Z0) acts as a bridge between light-induced redox changes and protein phosphorylation in the regulation of starch accumulation [30]. The accumulation of SEX4 in this study may suggest that SEX4 may promote the decomposition of transitory starch into soluble sugar to regulate the osmotic pressure in plant cells under salt stress.

Another up-regulated protein LS (6,7-dimethyl-8-ribityllumazine synthase) (A0A0J8E4J4) has been shown to catalyze the penultimate step in the synthesis of riboflavin. In addition to catalyzing riboflavin synthesis and regulating intracellular REDOX reactions, it has been reported that LS plays a role in the JA

signaling pathway and participates in plant defense reactions [31]. We observed that ABC transporter B family member 26 (A0A0K9QZ15) was up-regulated under salt stress which may play specific transport role in salt stress response. THI1 (Thiamine thiazole synthase) (A0A0K9Q9I3) was down-regulated in the present study, it has been demonstrated to take part in both guard cell abscisic acid (ABA) signaling and the drought response in *Arabidopsis* [32]. The abundance of enolase 1 (A0A0J8CFG6) in plastids was down-regulated under salt stress. Previous studies have also documented that the isoenzyme expression of this protein is down-regulated under salinity [33, 34].

Analysis of the DAPs resistant to salt-stress

Osmotic imbalance, ion injury and reactive oxygen species (ROS) coupled with salt stress, which threaten the normal growth and development of plants. Besides betaine, soluble sugar and proline are also essential osmotic regulators. A 3.6-fold increase of proline was detected in leaves (Figure 1b), unfortunately, we did not find differentially accumulated of Proline metabolism-related enzymes (like *P5CS*) in both leaves and roots. However, we found 2 differentially accumulated sucrose synthase (Q6SJP5 and V7C8M2) in roots. As a widely existing glycosyltransferase in plants, sucrose synthase (SuSy) is a kernel enzyme in sucrose metabolism which can catalyze the decomposition and synthesis of sucrose. The accumulation of SuSy under abiotic stress has been found in many plants, especially in roots [35-37]. It has been reported that SuSy is not only involved in osmotic regulation of plants, but also functions at a branch point to allocation sucrose between cell wall biosynthesis and glycolysis [38]. Thus, choline monooxygenase and betaine aldehyde dehydrogenase may play important roles in osmotic regulation of leaves under salt stress, while SuSy may be pivotal factors in the osmotic regulation of roots in sugar beet.

The damage of NaCl to plants is mainly caused by ion toxicity of sodium ions and chloride ions, as well as the production of ROS induced by stress. The 1.6-flod increase of MDA in leaves reflected the oxidative damage caused by stress (Figure 1c). In plant, excess ingestion of Na^+ can affect the absorption of mineral nutrients, such as calcium (Ca^{2+}), magnesium (Mg^{2+}) and potassium (K^+) [39]. However, as a salt-tolerant plant, beet can use Na^+ replaces of K^+ for many functions like osmotic regulation, stomatal regulation and, long-distance transport of anions and so on [40-42]. High levels of chlorine ions in the environment can inhibit the uptake of NO_3^-, correspondingly, a significantly decrease in the content of high-affinity nitrate transporter (A0A0J8B2J0) under salt stress was observed in roots. In addition, the increase of ROS in plants under salt stress will affect physiological and biochemical processes such as photosynthesis, metabolism and signal transduction. Subjected to salt stress also lead to oxidative burst in plants. However, plants have their own detoxification system which can be divided into three stages: in the first stage, exogenous toxins or cytotoxins are oxidized, reduced or hydrolyzed by enzymes such as cytochrome P450 monooxygenase; then, a coupling reaction of processed products and sugar (or GSH) are catalyzed by enzymes, such as coupling with GSH catalyzed by GST; in the third stage, these conjugates are recognized by ATP coupling transporters in vacuoles or plasma membranes and eventually transported to vacuoles or expelled from the cell[43, 44]. Proteomic studies of roots have shown

that two CYP family members (A0A0K9RP46 and A0A0K9RFX0), 3 GST family members (A0A0J8B2G1; A0A0J8CMV9; A0A0J8BAU3) and a F-type H^+-transporting ATPase (A0A369ANY6) were differentially expressed. The detection results of root activity showed a 1.5-fold increase of TTC reduction capacity under salt stress (Figure 1d), which is likely to depend on the accumulation of GSTs. These proteins may play an important role in detoxification against salt stress in roots.

Another adaptation of sugar beets to high salt stress results from compartmentalization[45], excessive salt is selectively distributed to different tissues or organs. Generally, higher salt content is found in petioles and older leaves while lower salt content is found in new leaves, which is conducive to ensuring the function of these functional leaves[46]. Unlike in roots, there was no accumulation of CYP and GST but differential expression of two peroxidase family members (A0A0K9R0G7 and A0A0J8B8Y7) were found in leaves. In addition to the protective enzymes, flavonoids also play an important role in scavenging effect to ROS as non-enzymatic reaction [47, 48]. Chalcone synthase (CHS) and flavanone-3-hydroxylase (F3H) are key enzymes in flavonoid metabolism. An accumulation of CHS (A0A0K9R791) and F3H (A0A0J8CVF6) were detected under salt-stress in leaves. This may be due to the sampling of functional leaves (the third-pair euphylla), and further studies are needed to determine the differences between new leaves and old leaves. These proteins may play an important role in detoxification against salt stress in leave.

Furthermore, non-symbiotic hemoglobin (NsHb) is also an important strategy that plants have evolved to resist stress, which can reduce the damage caused by oxidative stress. Overexpression of NsHb can improve the activity of antioxidant enzyme system in plants [49-51]. Two Non-symbiotic hemoglobin protein (V5QQP3 and V5QR23) and one Non-symbiotic hemoglobin protein (V5QQV5) were increased expression in roots and leaves, respectively. V5QR23, in particular, was upregulated more than two-fold, this intensely induced by salt stress implies it may play an underestimated role in resistant to salt-stress.

Analysis of the DAPs associated with Apoplast and Cell wall

Based on the GO analysis results, a large number of DAPs were associated with apoplast and cell wall in both roots and leaves. However, there were significant differences in response to salt stress between root and leaf cell wall DAPs. The apoplast is the first plant compartment encountering environmental signals[52]. Studies have shown that apoplast protein is not only involved in the response of various environmental signals, but also in the perception and transduction of signals in collaboration with the plasma membrane [53, 54]. The cell wall is the outermost barrier of plant cell, which first senses the stress signal and transmits them into cell to regulate the activity of cell [55, 56]. It is a reticulate structure composed of polysaccharides, enzymes and structural proteins, while changes in composition affect ductility, mechanical support and defense functions of cell wall. In response to stress, cell wall proteins play an important role in cell wall structure, metabolism and signal transduction. It is very interesting that all the DAPs related to apoplast and cell wall were up-regulated in leaves. Specifically, β-galactosidase (A0A0J8B708), β-D-xylosidase 5 (A0A0K9QCY3), endo-1,3;1,4-β-D-glucanase (A0A0J8B9V6), xyloglucan endotransglucosylase/hydrolase protein 24-like (A0A0K9QWM7) were significant accumulation under

salt stress in leaves. In higher plants, β-galactosidase is the only enzyme that can inner cleaves β-1,4-galactose to further cleavage of galactose residues from cell wall polysaccharides[57]. Xylan is the main polysaccharide structure in plant cell wall, β-D-xylosidase is a kind of O-glycosyl hydrolases that can hydrolyze Glycosyl bonds between xylans[58]. Endo-1,3(4)-β-D-glucanase has A specific digestive effect on cellulose microfibers and plays an important role in regulating plant cell wall structure [59]. Xyloglucan endotransglucosylase/hydrolases (XTHs) is a family of xyloglucan modifying enzymes that play an essential role in the construction and restructuring of xyloglucan cross-links[60]. In general, the up-regulation of these genes led us to speculate that beet leaves respond to salt stress by maintaining the ductility of cell walls. Leaf cells may increase in volume to compensate for the loss of photosynthetic intensity due to chlorophyll damage, thus ensuring energy supplies.

Diametrically opposed, α-xylosidase 1 (A0A0K9RU87), xyloglucan endotransglucosylase/ hydrolase (A0A0J8CRX9 and A0A0K9QMQ7), β-galactosidase 5 (A0A0K9R1V6), Expansin-like A2 (A0A0K9QJR1), and proline-rich protein 3 (A0A0J8FJ16) were down-regulated under salt stress in root. Expansin is a kind of cell wall relaxation protein, and it has been shown that its accumulation is an important biochemical mechanism for the salt tolerance reaction of wheat varieties [61]. PRP protein (proline-rich protein) is structural protein of plant cell wall that plays an important role in cell wall construction and defense. Overall, the down-regulation of these DAPs suggested that the beet roots resist salt stress by inhibiting cell wall relaxation.

Analysis of DAPs related to Metabolism

The DAPs involved in carbohydrate and energy metabolism are indispensable. Here, NADH-ubiquinone reductase complex 1 MLRQ subunit (Q1H8M8) and Cytochrome c oxidase subunit 5C (A0A0K9Q6K8) participated in energy metabolism were up-accumulated in leaves under salt stress. Both of the two proteins are components of electron transport chain, which are involved in cellular respiration. More widely, 2 EMP components 6-phosphofructokinase (A0A0K9R9W4) and glyceraldehyde-3-phosphate dehydrogenase (A0A0K9R3B7); succinyl-CoA ligase, β subunit (S3HB42) belonging to TCA; ADH (A0A0K9QY28), PDC1 (A0A0K9QG18) and PDC2 (A0A0K9QMI0); ATP synthase α subunits (A0A369AIG8) and ATP synthase β subunits (S3HC31) were all up-accumulated in roots. PDC (Pyruvate decarboxylase) and ADH (Alcohol dehydrogenase) can convert pyruvate metabolism pathway from the synthesis of lactic acid into the synthesis of ethanol. Ethanol is much less toxic to plants than lactic acid or the intermediate acetaldehyde, and spreads easily. These results indicated that sugar beet is able to adapt salt stress by improving energy metabolism.

Phosphatidylcholine (PC) is a crucial metabolite of plant growth and development, and also the main lipid component of plant cell membrane. Besides its structural role, PC is the source of signaling molecules, Serine decarboxylase (SDC: Q4H1G0) can catalyzes the conversion of serine into ethanolamine, which is the first step in PC biosynthesis[62]. Choline /ethanolamine kinase (CEK: A0A0K9RLT3) catalyzes the initial reaction step of choline metabolism that produces phosphoethanolamine [63]. Phosphoethanolamine N-methyltransferase (PEAMTs: Q4H1G5) is a rate-

limiting enzyme that catalyzes the phosphoethanolamine to produce choline [64]. The up-accumulated of SDC, CEK and PEAMTs in leaves may not only be involved in the synthesis of cell membranes, but also in the synthesis of betaine and phosphatidic acid (PA) in response to salt stress. Unsurprisingly, the abundance of SDC (Q4H1G0) and GPI ethanolamine phosphate transferase 1 isoform X2 (A0A0J8B1W3) were reduced in roots. Besides, dirigent protein (A0A0K9QD33) involved in yielding lignans was down-regulated in roots after salt stress. These results support our analysis on cell wall related DAPs, which postulates that leaf cells strive to increase volume while root cells maintain it under salt stress.

A similar trend, the abundance of DAPs involved in protein Folding, and degradation were increased in leaves including Tubulin-folding cofactor D (A0A0K9RSC6), Aspartic proteinase nepenthesin-1 (A0A0K9RL53) and Ubiquitin carboxyl-terminal hydrolase 12-like (A0A0K9RR35); while prefoldin subunit 4 isoform X1 (A0A0K9RGH7), DnaJ protein homolog ANJ1(A0A0K9REY5) and Basic 7S globulin 2-like (A0A0K9QE67) were reduced in roots.

Analysis of DAPs involved in transcription and translation processes

Unlike animals, plants have to adapt to environmental changes continuously, they need to adjust their growth and processes of life timely in response to such alterations. Transcription and translation play an irreplaceable role in this adaptation process. A down-regulated accumulation of six DAPs associated with RNA and /or Protein binding were observed in leaves (Figure 4a). Of interest is Glycine-rich RNA-binding protein 2 (GR-RBP2) (A0A0J8FG78), which can affect the expression of genes encoded by mitochondrial genome and thus regulate respiration[65]. Many studies have demonstrated that GR-RBP plays a remarkable role in response to stress [66, 67]. Similarly, a number of DAPs involved in transcription and translation are down-regulated in roots like DNA-directed RNA polymerases II, IV and V subunit 3 (A0A0K9RPV4), DEAD-box ATP-dependent RNA helicase 7 (A0A0J8BDH3), H/ACA ribonucleoprotein complex subunit 2-like protein (A0A0J8BPQ1), and so on. But unlike leaves, the accumulation of 50S ribosomal protein L14 (A0A023ZRD6) and 50S ribosomal protein L22 (A0A369ACK0) during salt stress were higher compared with the control. More remarkable, these two ribosomes are located in mitochondria. Thus, we hypothesize that the global intensity of transcription and translation in beet were decreased during salt stress, while root cells enhanced the synthesis of mitochondrial-related proteins on local level. Such specific regulation may help ensure the proper functioning of mitochondria to obtain sufficient energy against stress.

Analysis of DAPs in regard to Plant hormones

Plant hormones are active substances induced by specific environmental signals and have obvious physiological effects at very low concentrations. Gibberellin regulated protein (A0A0K9RKJ8) was observed to accumulate in the leaves. 1-aminocyclopropane-1-carboxylate oxidase (ACO) is a key enzyme in the ethylene biosynthesis pathway. Long chain acyl-CoA synthetase (LACS) has been reported to have catalytic ability to activate biosynthetic precursors of jasmonic acid (JA) [68]. ACO1 (A0A0J8B2W2), LACS 4-like (A0A0K9RTE2) and auxin-binding protein ABP19a (Q84RC0) were up-accumulated while abscisic

acid receptor PYL4 (A0A0J8BHH5) was down-regulated in roots under salt stress. Besides, a significant accumulation of carboxylesterase 1(A0A0J8CQ53), which can demethylate inactive methyl salicylate (MeSA) and methyl jasmonate (MeJA) into active salicylate acid and jasmonic acid, was observed in both leaves and roots under salt stress. These results help us to analyze the potential regulatory functions of diverse hormones in different organs under salt stress.

Conclusion

ITRAQ was used to reveal the divergent responses of leaves and roots to salt stress in sugar beet, and 70 and 76 DAPs were identified in leaves and roots, respectively. Based on functional analysis and previous studies, we concluded that roots and leaves exhibited different coping strategies under salt stress. In this study, leaves showed a relatively robust metabolism at global level, particularly in ensuring the photosynthesis, in an effort to gain more energy for the body to cope with environmental pressures. And the homeostasis of leaf cells may be attributed mainly to the accumulation of betaine, and ROS scavenging by the coordination of enzymatic and non-enzymatic system. While roots exhibited a relatively dispirited metabolism at global level. The accumulation of GST family members in root may conducive to the survival of root under salt stress. In the future, studies on the function of DAPs found in this study will be helpful to explore the mechanisms of beet resistance to salt stress. In summary, our proteomics analysis not only increased our understanding of molecular mechanisms response to salt stress in different organs of beet, but also provided the theoretical foundation for the improvement of salt-tolerant in sugar beet.

Methods

Plant Materials and Treatments

Cultivar ‘O68’ is an excellent parent used in traditional crossbreeding with strong salt tolerance. Our previous study showed that under 300 m mol·L^{-1} NaCl treatment, the relative germination rate of this cultivar was more than 70% and the seedling can grow normally [69]. In addition, it has a strong regeneration capability of petiole explants which is ideally suited for use in molecular breeding. Therefore, cultivar ‘O68’ is a good choice for studying the mechanism of salt-stress response in sugar beet. The seeds of O68 used in this study were from our own laboratory (Heilongjiang, China). The seeds were soaked in water for ten hours, then sterilized in 0.1% (v/v) $HgCl_2$ for 10 min, washed repeatedly with distilled water, and germinated on wet filter paper in germination box at 26 °C for 2 days. After germination, budding seeds were transferred to plastic pots (45 cm × 20 cm × 14 cm, 12 plants per pot) filled with quarter-strength Hoagland solution. The germinating seeds were cultivated under 16/8 light photoperiod at 24 °C (day)/18 °C (night) in a phytotron (Friocell 707, Germany). As the increase of salt concentration in nature usually occurs gradually, a method of gradual adding NaCl is used for treatment. For proteomics research, in order to make the salt treatment more close to the salt stress under natural conditions, four-week-old plants (three-pairs-euphylla) were treated with half-strength Hoagland medium supplemented with increasing concentrations of NaCl (increase 50mM /12 h), until the final

concentration of 300 mM were reached, after which the treatments were continued for 24 hours. NaCl-free nutrient solution was used as a control. Leaves representing the third pair of euphylla and roots were collected and immediately frozen in liquid nitrogen, then stored at -80 °C until further use.

Physiologic Indexes Detection

A total of 0.1g, 0.5g and 1g fresh leaves from the third pair of euphylla were used to detect chlorophyll content (acetone extraction), proline content (Ninhydrin colorimetry method), and malondialdehyde content (Thiobarbital acid method) following Gao [70], respectively. 0.5g Fresh roots were collected for the determination of root activity by TTC reduction method base on Gao[70]. These parameters were analyzed by a UV-2100PC ultraviolet-visible spectrophotometer (UNICO. LTD), and each treatment was repeated three times.

Protein Extraction, Protein Digestion and iTRAQ Labeling

ITRAQ analysis was carried out in LC Sciences (Hangzhou, China). The leaf or root tissue from every 10 plants was pooled as one biological replicate, and three biological replicates were conducted for iTRAQ-based comparative proteomics analysis. The total proteins of the leaves and roots from each sample were extracted, according to a previous report [71]. The protein concentration was determined by the BioDrop μLite microdetector (BioDrop, UK), and protein quality was measured with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For digestion, 100 μg protein of each sample was first reduced with 20mM dithiothreitol (DTT) at 37 °C for 60 min and then alkylated with 40 mM (final concentration) iodoacetamide at room temperature for 30 min in darkness. The protein pool of each sample was digested with Sequencing Grade Modified Trypsin with the ratio of protein: trypsin=100: 1 mass ratio at 37 °C 12h and 100:1 for a second digestion for 4 h. After trypsin digestion, peptide was desalted by Waters sep-Pak C18 column (Waters Inc., US) and vacuum-dried. Peptide was reconstituted in 40 μl 100mM TEAB and processed according to the manufacturer's protocol for 8-plex iTRAQ kit (AB sciex Inc., US). iTRAQ reagents 114, 115, and 116 were used to label the peptides from CK replicates (leaf and root, respectively), iTRAQ reagents 117, 118 and 121 were used to label the peptides from salt-stress replicates (leaf and root, respectively). The labeled peptide mixtures were desalted by Waters sep-Pak C18 column and vacuum-dried.

Nano-LC-ESI-MS/MS Analysis

The labeled peptides powder was dissolved with 52 μl (10 mM ammonium formate, pH 10) and then fractionated into fractions by Waters E2695 liquid chromatography system using BEH C18 chromatographic column (5 μm, 4.6*250 mm, Waters Inc). According to the chromatographic peak-type, the peptides were combined into 17 fractions in each leaf samples and 18 fractions in each root samples, then freeze-dried. Each fraction was redissolved with 5 μl 0.1% formic acid (FA) and performed by NanoLC-MS/MS system using EASY-nLC 1000 coupled to Q Exactive (Thermo Scientific, US) mass spectrometer. The eluent was sprayed via ESI source at the 2.0 kV electrospray voltage and then analyzed by tandem mass spectrometry (MS/MS) in Q Exactive. The MS scan spectra rang of 350 to 1800 m/z

were acquired in the Orbitrap with a resolution of 70,000. The dd-MS^2 scan spectra was automatic selection and dd-MS^2 resolution of 17,500.

Protein Identification and Quantification

A MaxQuant (version 1.5.5.1) was used for iTRAQ protein identification and quantification [72, 73]. For protein identification, the *B. vulgaris* protein database of UniProt was used with the criterion of a false discovery rate (FDR) < 0.01. The parameters of library searching were as following: fixed modifications include Carbamidomethyl on cysteine residues, iTRAQ 8 plex (N-term) and iTRAQ 8 plex (K); oxidative modification on methionine was set as a variable modification. The peptide mass tolerance was set as ±10 ppm and the fragment mass tolerance was 0.2 Da.

Bioinformatics Analysis

The biological and functional properties of proteins were analyzed with GO and KEGG databases [74, 75]. Hypergeometric test was used to find significantly enriched GO terms and KEGG pathway of DAPs. If the p-value was <0.05, the GO term or KEEG pathway were regarded as a significant enrichment of DAPs. Clusters of Orthologous Groups of Proteins System was also employed for the functional classification of the DAPs.

RNA Extraction and qRT-PCR

Total RNA was extracted from leaves and roots by MiniBEST Plant RNA Extraction Kit (TaKaRa, Japan). Approximately 2 μg of total RNA was reverse-transcribed using High-Capacity cDNA Reverse Transcription Kits in a 20 μl of reaction volume (ThermoFisher Scientific, US). The reactions were incubated for 10 min at 25 °C, followed by 37 °C for 120 min, and finally the reactions were terminated at 85 °C for 5 min. All the primers were listed in Table S3. *PP2A*+ *UBQ5* and *PP2A*+*25S* were used as endogenous control in leaves and roots, respectively. For qRT-PCR, the gene-specific primers were designed using Primer-BLAST online [76]. The qRT-PCR reactions were performed using iTaq Universal SYBR® Green Supermix (BIO-RAD, Hercules, CA) on the CFX Real-time PCR system (BIO-RAD, CA). To avoid non-specific amplification, melting curve was carried out for each PCR product. The expression level of the miRNAs in different samples were calculated by comparative $2^{-\triangle\triangle CT}$ method.

Data Treatment and Statistical Analysis

For the data of the physiological parameters and qPCR analysis, the mean and SD were calculated from three repeats of each treatment, and the differences were analyzed by Duncan's multiple range test ($p < 0.05$) and an independent-samples t-test ($p < 0.05$).

Abbreviations

B. vulgaris: *Beta vulgaris ssp. Vulgaris*; **iTRAQ:** isobaric tag for relative and absolute quantification; **DAPs:** differential abundance protein species; **GO:** Gene Ontology; **KEGG:** Kyoto Encyclopedia of Genes and Genomes; **COG:** Clusters of Orthologous Groups of proteins; **MDA:** malondialdehyde; **TTC:** triphenyltetrazolium chloride; **FDR:** false discovery rate; **qRT-PCR:** quantitative RT-PCR.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publish

Not applicable.

Availability of data and materials

The materials used during the current study will be freely available upon request to corresponding author: cuijie2006@163.com. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the iProX partner repository [77] with the dataset identifier PXD017954.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

Conceptualization, JL Li and J Cui; Data curation, JL Li; Formal analysis, JL Li; Funding acquisition, J Cui and DY Cheng; Methodology, TJ Liu and CY Wang; Project administration, J Cui, CH Dai and CF Luo; Resources, J Cui and DY Cheng; Validation, JL Li and TJ Liu; Writing – original draft, JL Li; Writing – review & editing, JL Li. All authors have read and approved the manuscript.

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References

1. Rengasamy P: **Soil processes affecting crop production in salt-affected soils**. *Functional Plant Biology* 2010, **37**(7):613-620.
2. Munns R, Gilliham M: **Salinity tolerance of crops - what is the cost?** *New Phytologist* 2015, **208**(3):668-673.
3. Tester M, Davenport R: **Na+ Tolerance and Na+ Transport in Higher Plants**. *Annals of Botany* 2003, **91**(5):503-527.
4. Munns R, Tester M: **Mechanisms of Salinity Tolerance**. *Annual Review of Plant Biology* 2008, **59**(1):651-681.
5. Munns R: **Physiological processes limiting plant growth in saline soils: some dogmas and hypotheses**. *Plant Cell & Environ* 1993, **16**(1):15-24.
6. Garthwaite AJ, Bothmer Rv, Colmer TD: **Salt tolerance in wild *Hordeum* species is associated with restricted entry of Na^+ and Cl^- into the shoots**. *J Exp Bot* 2005, **56**(419):2365-2378.
7. Monika S, Marcin G, Katarzyna K, Janusz N, Jacek K, Krzysztof K, Katarzyna W, Waldemar T, Andrzej T, Jarosław T: **Salt stress vs. salt shock - the case of sugar beet and its halophytic ancestor**. *BMC Plant Biology* 2019, **19**:57.
8. Long R, Gao Y, Sun H, Zhang T, Li X, Li M, Sun Y, Kang J, Wang Z, Ding W *et al*: **Quantitative proteomic analysis using iTRAQ to identify salt-responsive proteins during the germination stage of two *Medicago* species**. *Scientific Reports* 2018, **8**(1):9553.
9. Wiese S, Reidegeld KA, Meyer HE, Warscheid B: **Protein labeling by iTRAQ: a new tool for quantitative mass spectrometry in proteome research**. *Proteomics* 2007, **7**(3):340-350.
10. Liu J-Y, Men J-l, Chang M-c, Feng C-p, Yuan L-G: **iTRAQ-based quantitative proteome revealed metabolic changes of *Flammulina velutipes* mycelia in response to cold stress**. *J Proteomics* 2017, **156**:75-84.
11. Li H, Pan Y, Zhang Y, Wu C, Ma C, Yu B, Zhu N, Koh J, Chen S: **Salt stress response of membrane proteome of sugar beet monosomic addition line M14**. *J Proteomics* 2015, **127**:18-33.
12. Yu B, Li J, Koh J, Dufresne C, Yang N, Qi S, Zhang Y, Ma C, Duong BV, Chen S *et al*: **Quantitative proteomics and phosphoproteomics of sugar beet monosomic addition line M14 in response to salt stress**. *Journal of Proteomics* 2016, **143**:286-297.
13. Wu G-Q, Wang J-L, Feng R-J, Li S-J, Wang C-M: **iTRAQ-Based Comparative Proteomic Analysis Provides Insights into Molecular Mechanisms of Salt Tolerance in Sugar Beet (*Beta vulgaris* L.)**. *Int J Mol Sci* 2018, **19**:3866.
14. Ji W, Cong R, Li S, Li R, Qin Z, Li Y, Zhou X, Chen S, Li J: **Comparative Proteomic Analysis of Soybean Leaves and Roots by iTRAQ Provides Insights into Response Mechanisms to Short-Term Salt Stress**. *Frontiers in Plant Science* 2016, **7**:573.
15. Wang Y, Cong Y, Wang Y, Guo Z, Yue J, Xing Z, Gao X, Chai X: **Identification of Early Salinity Stress-Responsive Proteins in *Dunaliella salina* by isobaric tags for relative and absolute quantitation**

(iTRAQ)-Based Quantitative Proteomic Analysis. *Int J Mol Sci* 2019, **20**:599.

16. Shavrukov Y: **Salt stress or salt shock: which genes are we studying?** *Journal of Experimental Botany* 2013, **64**(1):119-127.
17. Mering Cv, Jensen LJ, Snel B, Hooper SD, Krupp M, Foglierini M, Jouffre N, Huynen MA, Bork P: **STRING: known and predicted protein-protein associations, integrated and transferred across organisms**. *Nucleic Acids Research* 2005, **33**:D433-D437.
18. Suo J, Zhao Q, David L, Chen S, Dai S: **Salinity Response in Chloroplasts: Insights from Gene Characterization**. *International Journal Of Molecular Sciences* 2017, **18**(5):1011.
19. Yamane K, Oi T, Enomoto S, Nakao T, Arai S, Miyake H, Taniguchi M: **Three-dimensional ultrastructure of chloroplast pockets formed under salinity stress**. *Plant Cell And Environment* 2018, **41**(3):563-575.
20. Yoon DH, Lee SS, Park HJ, Lyu JI, Chong WS, Liu JR, Kim B-G, Ahn JC, Cho HS: **Overexpression of *OsCYP19-4* increases tolerance to cold stress and enhances grain yield in rice (*Oryza sativa*)**. *Journal of Experimental Botany* 2016, **67**(1):69-82.
21. Pasternak C, Haberzettl K, Klug G: **Thioredoxin is involved in oxygen-regulated formation of the photosynthetic apparatus of Rhodobacter sphaeroides**. *J Bacteriol* 1999, **181**(1):100-106.
22. Samach A, Melamed-Bessudo C, Avivi-Ragolski N, Pietrokovski S, Levya AA: **Identification of plant *RAD52* homologs and characterization of the *Arabidopsis thaliana RAD52*-like genes**. *Plant Cell* 2011, **23**(12):4266-4279.
23. Fujimori N, Suzuki N, Nakajima Y, Suzuki S: **Plant DNA-damage repair/toleration 100 protein repairs UV-B-induced DNA damage**. *DNA Repair* 2014, **21**:171-176.
24. Martino CD, Delfine S, Pizzuto R, Loreto F, Fuggi A: **Free amino acids and glycine betaine in leaf osmoregulation of spinach responding to increasing salt stress**. *New Phytologist* 2003, **158**(3):455-463.
25. Holmström KO, Somersalo S, Mandal A, Palva TE, Welin B: **Improved tolerance to salinity and low temperature in transgenic tobacco producing glycine betaine**. *Journal Of Experimental Botany* 2000, **51**(343):177-185.
26. Riou N, Rudulier DL: **Osmoregulation in *AzuspirilZum bradense*: glycine betaine transport enhances growth and nitrogen fixation under salt stress**. *Journal Of General Microbiology* 1990, **136**:1455-1461.
27. McCue KF, Hanson AD: **Drought and salt tolerance: towards understanding and application**. *Trends in Biotechnology* 1990, **8**(12):358-362.
28. Joseph S, Murphy D, Bhave M: **Glycine betaine biosynthesis in saltbushes (*Atriplex spp.*) under salinity stress**. *Biologia* 2013, **68**(5):879-895.
29. Mitsuya S, Kozaki K, Takabe T: **Tissue Localization of the Glycine Betaine Biosynthetic Enzymes in Barley Leaves**. *Plant Production Science* 2013, **16**(2):117-122.
30. Sokolov LN, Dominguez-Solis JR, Allary A-L, Buchanan BB, Luan S: **A redox-regulated chloroplast protein phosphatase binds to starch diurnally and functions in its accumulation**. *PNAS* 2006,

103(25):9732-9737.

31. Xiao S, Dai L, Liu F, Wang Z, Peng W, Xi D: **COS1: an Arabidopsis *coronatine insensitive1* suppressor essential for regulation of jasmonate-mediated plant defense and senescence.** *The Plant cell* 2004, **16**(5):1132-1142.
32. Li C-L, Wang M, Wu X-M, Chen D-H, Lv H-J, Shen J-L, Qiao Z, Zhang W: **THI1, a Thiamine Thiazole Synthase, Interacts with Ca^{2+}-Dependent Protein Kinase CPK33 and Modulates the S-Type Anion Channels and Stomatal Closure in Arabidopsis**. *Plant Physiology* 2016, **170**:1090-1104.
33. Yan S, Tang Z, Su W, Sun W: **Proteomic analysis of salt stress-responsive proteins in rice root**. *Proteomics* 2005, **5**(1):235-244.
34. Jiang Y, Yang B, Harris NS, Deyholos MK: **Comparative proteomic analysis of NaCl stress-responsive proteins in *Arabidopsis* roots**. *J Exp Bot* 2007, **58**(13):3591-3607.
35. Sasaki H, Ichimura K, Imada S, Yamaki S: **Sucrose synthase and sucrose phosphate synthase, but not acid invertase, are regulated by cold acclimation and deacclimation in cabbage seedlings**. *Journal of Plant Physiology* 2001, **158**(7):847-852.
36. Sharif I, Aleem S, Farooq J, Rizwan M, Younas A, Sarwar G, Chohan SM: **Salinity stress in cotton: effects, mechanism of tolerance and its management strategies**. *Physiology And Molecular Biology Of Plants* 2019, **25**(4):807-820.
37. Yan L, Dongfeng J, Robert T, Jine C, Tianbao L, Jing H, Jie L, Yan Z, Cankui Z, Zhiqiang L: **Physiological and Proteomic Responses of Mulberry Trees (*Morus alba.* L.) to Combined Salt and Drought Stress**. *Int J Mol Sci* 2019, **20**(10):2486.
38. Albrecht G, Mustroph A: **Localization of sucrose synthase in wheat roots: increased in situ activity of sucrose synthase correlates with cell wall thickening by cellulose deposition under hypoxia**. *Planta* 2003, **217**(2):252-260.
39. Parida KA, Das AB: **Salt tolerance and salinity effects on plants: a review**. *Ecotoxicology and Environmental Safety* 2005, **60**(3):324-349.
40. Subbarao GV, Wheeler RM, Stutte GW, Levine LH: **How far can sodium substitute for potassium in red beet?** *Journal Of Plant Nutrition* 1999, **22**(11):1745-1761.
41. Subbarao GV, Wheeler RM, Stutte GW, Levine LH: **Low potassium enhances sodium uptake in red-beet under moderate saline conditions**. *Journal of Plant Nutrition* 2000, **23**(10):1449-1470.
42. Pi Z, Stevanato P, Sun F, Yang Y, Sun X, Zhao H, Geng G, Yu L: **Proteomic changes induced by potassium deficiency and potassium substitution by sodium in sugar beet**. *Journal of Plant Research* 2016, **129**(3):527-538.
43. Sono M, Roach MP, Coulter ED, Dawson JH: **Heme-containing oxygenases**. *Chemical Reviews* 1996, **96**(7):2841-2887.
44. Rea PA, Li ZS, Lu YP, Drozdowicz YM, Martinoia E: **From vacuolar GS-X pumps to multispecific ABC transporters**. *Annual Review Of Plant Physiology And Plant Molecular Biology* 1998, **49**:727-760.

45. Shahzad M, Witzel K, Zörb C, Mühling KH: **Growth-Related Changes in Subcellular Ion Patterns in Maize Leaves (*Zea mays* L.) under Salt Stress**. *Journal Of Agronomy And Crop Science* 2012, **198**(1):46-56.

46. Plaut Z, Heuer B: **Adjustment, growth, photosynthesis and transpiration of sugar beet plants exposed to saline conditions**. *Field Crops Research* 1985, **10**(1):1-13.

47. Ithal N, Reddy AR: **Rice flavonoid pathway genes, *OsDfr* and *OsAns*, are induced by dehydration, high salt and ABA, and contain stress responsive promoter elements that interact with the transcription activator, OsC1-MYB**. *Plant Science* 2004, **166**(6):1505-1513.

48. Oh M-M, Trick HN, Rajashekar CB: **Secondary metabolism and antioxidants are involved in environmental adaptation and stress tolerance in lettuce**. *Journal Of Plant Physiology* 2009, **166**(2):180-191.

49. Zhao L, Gu R, Gao P, Wang G: **A nonsymbiotic hemoglobin gene from maize, *ZmHb*, is involved in response to submergence, high-salt and osmotic stresses**. *Plant Cell, Tissue and Organ Culture* 2008, **95**(2):227-237.

50. Cantrel C, Vazquez T, Puyaubert J, Reze N, Lesch M, Kaiser WM, Dutilleul C, Guillas I, Zachowski A, Baudouin E: **Nitric oxide participates in cold-responsive phosphosphingolipid formation and gene expression in *Arabidopsis thaliana***. *New Phytologist* 2011, **189**(2):415-427.

51. Garrocho-Villegas V, Gopalasubramaniam SK, Arredondo-Peter R: **Plant hemoglobins: What we know six decades after their discovery**. *Gene* 2007, **398**(1-2):78-85.

52. Hoson T: **Apoplast as the site of response to environmental signals**. *Journal Of Plant Research* 1998, **111**(1101):167-177.

53. Gao DJ, Knight MR, Trewavas AJ, Sattelmacher B, Plieth C: **Self-reporting arabidopsis expressing pH and Ca2+ indicators unveil ion dynamics in the cytoplasm and in the apoplast under abiotic stress**. *Plant Physiology* 2004, **134**(3):898-908.

54. Horst WJ: **THE ROLE OF THE APOPLAST IN ALUMINUM TOXICITY AND RESISTANCE OF HIGHER-PLANTS - A REVIEW**. *Zeitschrift Fur Pflanzenernahrung Und Bodenkunde* 1995, **158**(5):419-428.

55. Zhu J, Alvarez S, Marsh EL, LeNoble ME, Cho I-J, Sivaguru M, Chen S, Nguyen HT, Wu Y, Schachtman DP *et al*: **Cell wall Proteome in the maize primary root elongation zone. II. Region-specific changes in water soluble and lightly ionically bound proteins under water deficit**. *Plant Physiology* 2007, **145**(4):1533-1548.

56. Kong F-J, Oyanagi A, Komatsu S: **Cell wall proteome of wheat roots under flooding stress using gel-based and LC MS/MS-based proteomics approaches**. *Biochimica Et Biophysica Acta-Proteins And Proteomics* 2010, **1804**(1):124-136.

57. Moctezuma E, Smith DL, Gross KC: **Antisense suppression of a beta-galactosidase gene (*TBG6*) in tomato increases fruit cracking**. *Journal Of Experimental Botany* 2003, **54**(390):2025-2033.

58. Davies G, Henrissat B: **STRUCTURES AND MECHANISMS OF GLYCOSYL HYDROLASES**. *Structure* 1995, **3**(9):853-859.

59. Zhou H-L, He S-J, Cao Y-R, Chen T, Du B-X, Chu C-C, Zhang J-S, Chen S-Y: **OsGLU1, a putative membrane-bound endo-1,4-beta-D-glucanase from rice, affects plant internode elongation**. *Plant Molecular Biology* 2006, **60**(1):137-151.
60. Fu M-M, Liu C, Wu F: **Genome-Wide Identification, Characterization and Expression Analysis of Xyloglucan Endotransglucosylase/Hydrolase Genes Family in Barley (*Hordeum vulgare*)**. *Molecules* 2019, **24**:1935.
61. Zhao M-r, Li F, Han Y-y, Wang W: **Expansins are involved in cell growth mediated by abscisic acid and indole-3-acetic acid under drought stress in wheat**. *Plant Cell Reports* 2012, **31**(4):671-685.
62. Liu Y-c, Gunawan F, Yunus IS, Nakamura Y: **Arabidopsis Serine Decarboxylase 1 (SDC1) in Phospholipid and Amino Acid Metabolism**. *Frontiers In Plant Science* 2018, **9**:972.
63. Lin Y-C, Kanehara K, Nakamura Y: **Arabidopsis CHOLINE/ETHANOLAMINE KINASE 1 (CEK1) is a primary choline kinase localized at the endoplasmic reticulum (ER) and involved in ER stress tolerance**. *New Phytologist* 2019, **223**(4):1904-1917.
64. Chen W, Taylor MC, Barrow RA, Croyal M, Maslea J: **Loss of Phosphoethanolamine *N*-Methyltransferases Abolishes Phosphatidylcholine Synthesis and Is Lethal**. *Plant Physiology* 2019, **179**(1):124-142.
65. Vermel M, Guermann B, Delage L, Grienenberger J-M, chal-Drouard LM, Gualberto† JM: **A family of RRM-type RNA-binding proteins specific to plant mitochondria**. *Proceedings Of the National Academy Of Sciences Of the United States Of America* 2002, **99**(9):5866-5871.
66. Kim JY, Park SJ, Jang B, Jung C-H, Ahn SJ, Goh C-H, Cho K, Han O, Kang H: **Functional characterization of a glycine-rich RNA-binding protein 2 in *Arabidopsis thaliana* under abiotic stress conditions**. *Plant Journal* 2007, **50**(3):439-451.
67. Kim JS, Jung HJ, Lee HJ, Kim KA, Goh C-H, Woo Y, Oh SH, Han YS, Kang H: **Glycine-rich RNA-binding protein7 affects abiotic stress responses by regulating stomata opening and closing in Arabidopsis thaliana**. *Plant Journal* 2008, **55**(3):455-466.
68. Schneider K, Kienow L, Schmelzer E, Colby T, Bartsch M, Miersch O, Wasternack C, Kombrink E, Stuible H-P: **A new type of peroxisomal acyl-coenzyme A synthetase from *Arabidopsis thaliana* has the catalytic capacity to activate biosynthetic precursors of jasmonic acid**. *Journal Of Biological Chemistry* 2005, **280**(14):13962-13972.
69. Shi S, Cui J, Lu Z, Cheng D, Luo C: **Screening of tolerance to NaCl in sugar beet germplasms**. *CHINA BEET & SUGAR* 2008, **4**:7-9.
70. Gao J: **Plant physiology experiment guide**: Higher Education Press; 2006.
71. Zhang G, Zhang J, Wen X, Zhao C, Zhang H, Li X, Yin S: **Comparative iTRAQ-Based Quantitative Proteomic Analysis of *Pelteobagrus vachelli* Liver under Acute Hypoxia: Implications in Metabolic Responses**. *Proteomics* 2017, **17**.
72. Cox J, Mann M: **MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification**. *Nat Biotechnol* 2008, **26**(12):1367-1372.

73. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M: **Andromeda: a peptide search engine integrated into the MaxQuant environment**. *J Proteome Res* 2011, **10**(4):1794-1805.
74. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT *et al*: **Gene ontology: tool for the unification of biology. The Gene Ontology Consortium**. *Nature genetics* 2000, **25**(1):25-29.
75. Kanehisa M, Araki M, Goto S, Hattori M, Hirakawa M, Itoh M, Katayama T, Kawashima S, Okuda S, Tokimatsu T *et al*: **KEGG for linking genomes to life and the environment**. *Nucleic Acids Research* 2007, **36**(suppl_1):D480-D484.
76. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL: **Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction**. *Bmc Bioinformatics* 2012, **13**:134.
77. Ma J, Chen T, Wu S, Yang C, Bai M, Shu K, Li K, Zhang G, Jin Z, He F *et al*: **iProX: an integrated proteome resource**. *Nucleic Acids Research* 2019, **47**(D1):D1211-D1217.

Additional Files

Figure S1. Distribution of peptide length in leaf (a) and root (b) of *B. vulgaris*.

Figure S2. Distribution of peptide number in leaf (a) and root (b) of *B. vulgaris*.

Figure S3. Distribution of protein mass in leaf (a) and root (b) of *B. vulgaris*.

Figure S4. Distribution of protein coverage in leaf (a) and root (b) of *B. vulgaris*.

Table S1 Detailed information of DPAs in leaves

Table S2 Detailed information of DPAs in roots

Table S3 List of primers used for qRT-PCR experiments

Figures

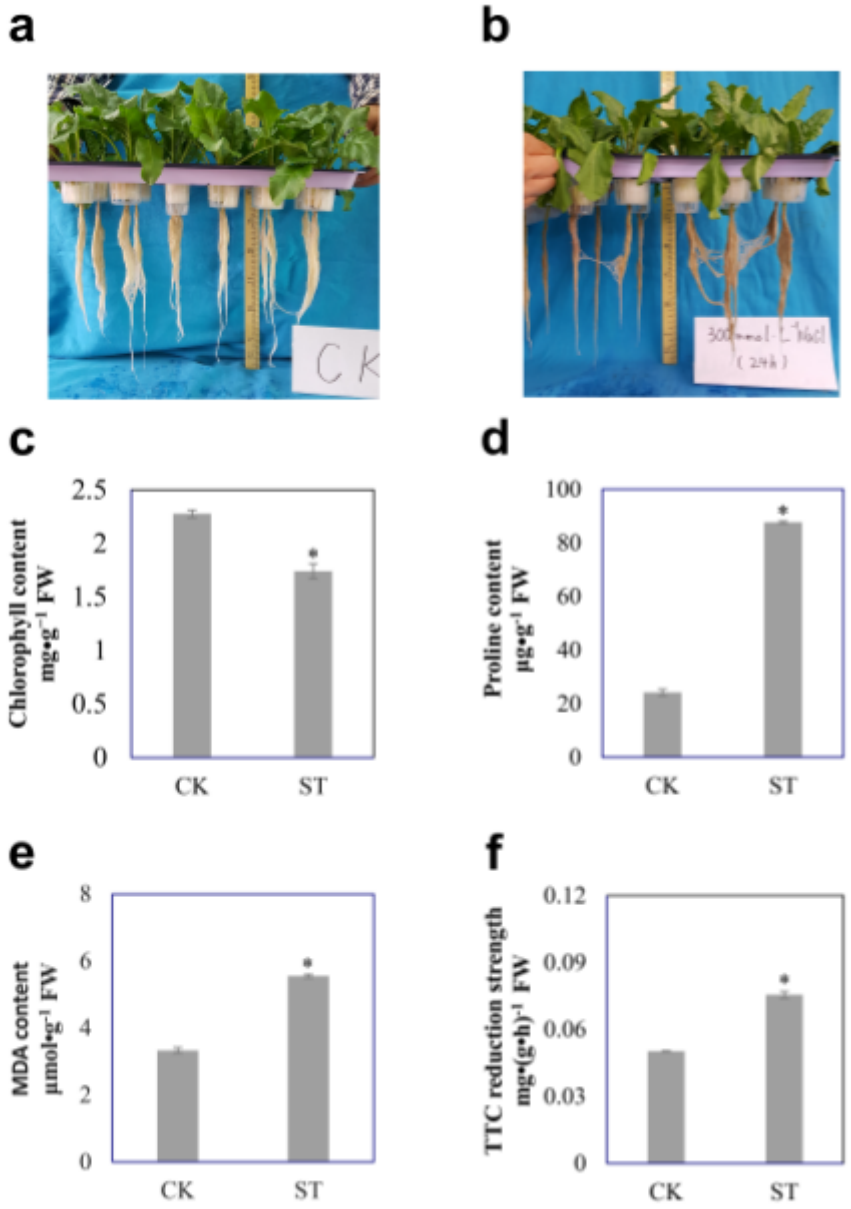

Figure 1

Physiological index determined for sugar beet subjected to salt stress. (a) Control plants; (b) Stressed plants treated with 300 mM NaCl salt; Chlorophyll (c); Malondialdehyde (d); Proline (e); and Root activity (f). The mean and SD were calculated from three repeats of each group, the bars indicate the standard. The asterisk indicates the significant difference among control (CK) and salt-stress (ST). (*p < 0.05).

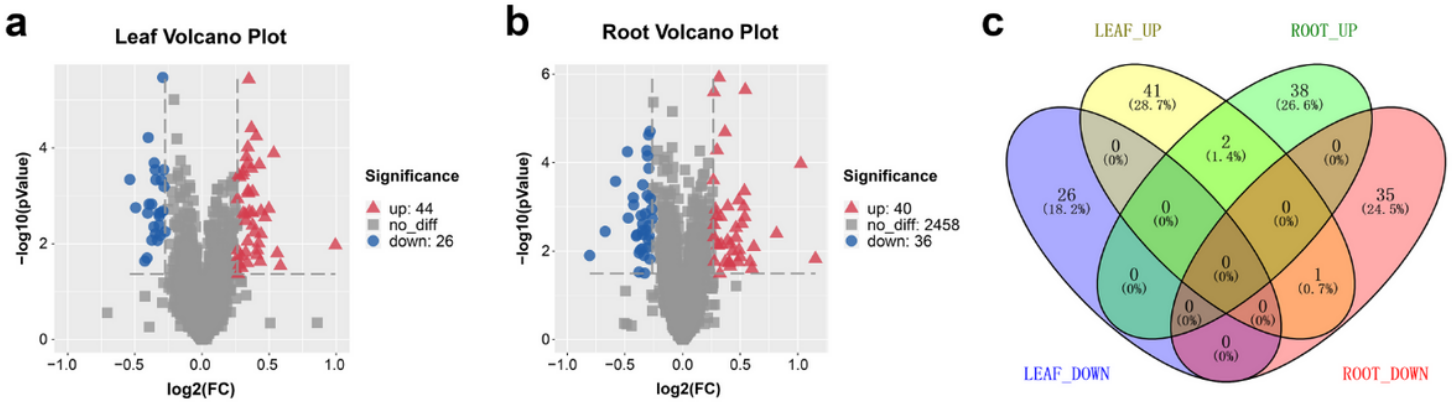

Figure 2

Distribution of salt-stress-responsive differentially abundant proteins (DAPs). Expression pattern of DAPs in leaf group (a) and root group (b) of sugar beet under 300 mM NaCl; Each point represents the difference in expression between the two groups plotted against the level of statistical significance. Proteins represented by a filled red (up) and blue (down) are those with expression that differs at a statistically significant level. (c) Overlapping of DAPs between leaves and roots samples. Each group contains three biological replicates.

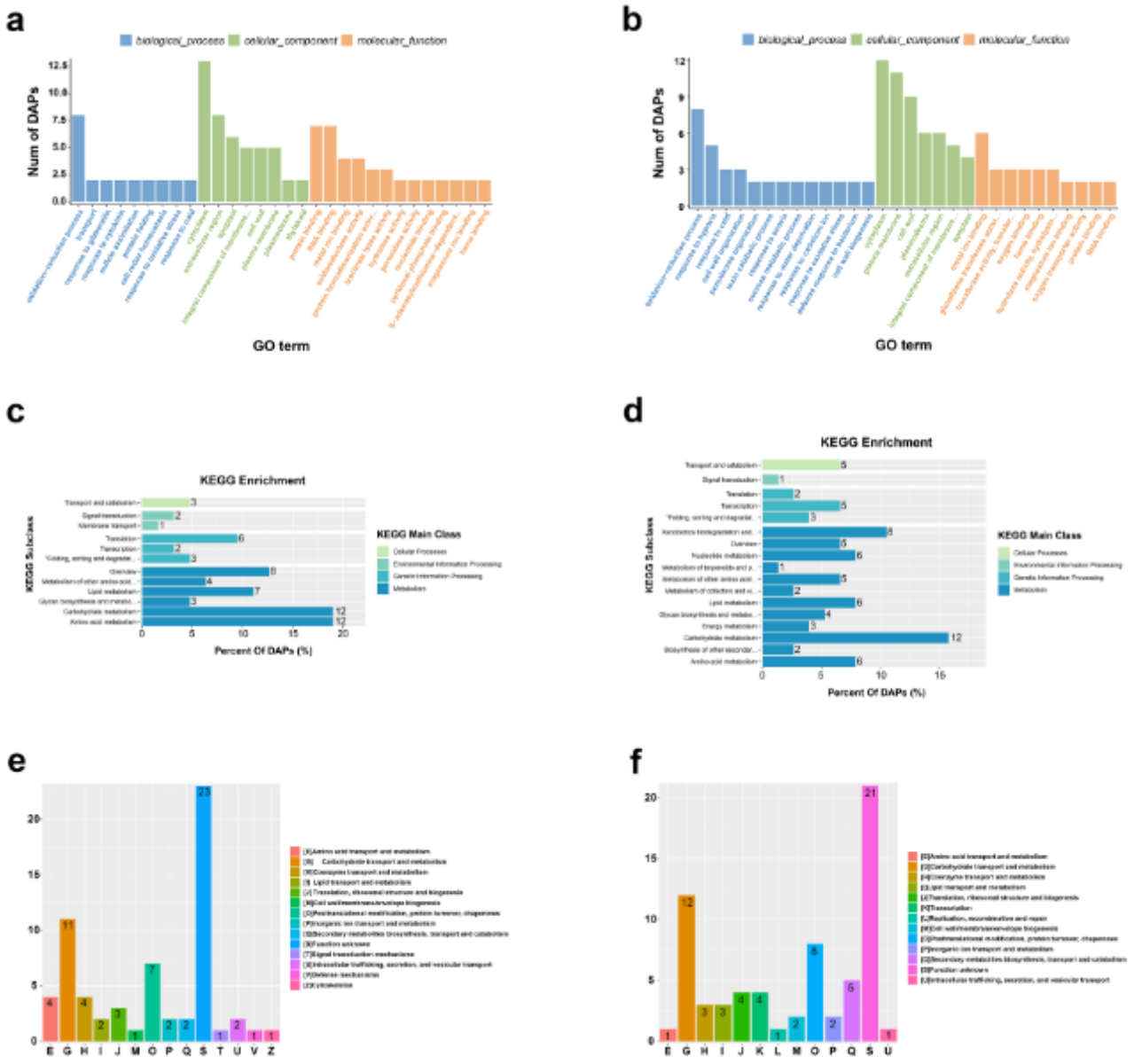

Figure 3

Bioinformatics Analysis of DAPS. Gene ontology (GO) enrichment of DAPs in leaves samples (a) and roots samples (b) of sugar beet. KEGG enrichment of DAPs in leaves samples (c) and roots samples (d). COG Functional Category of DAPs in leaves samples (e) and roots samples (f).

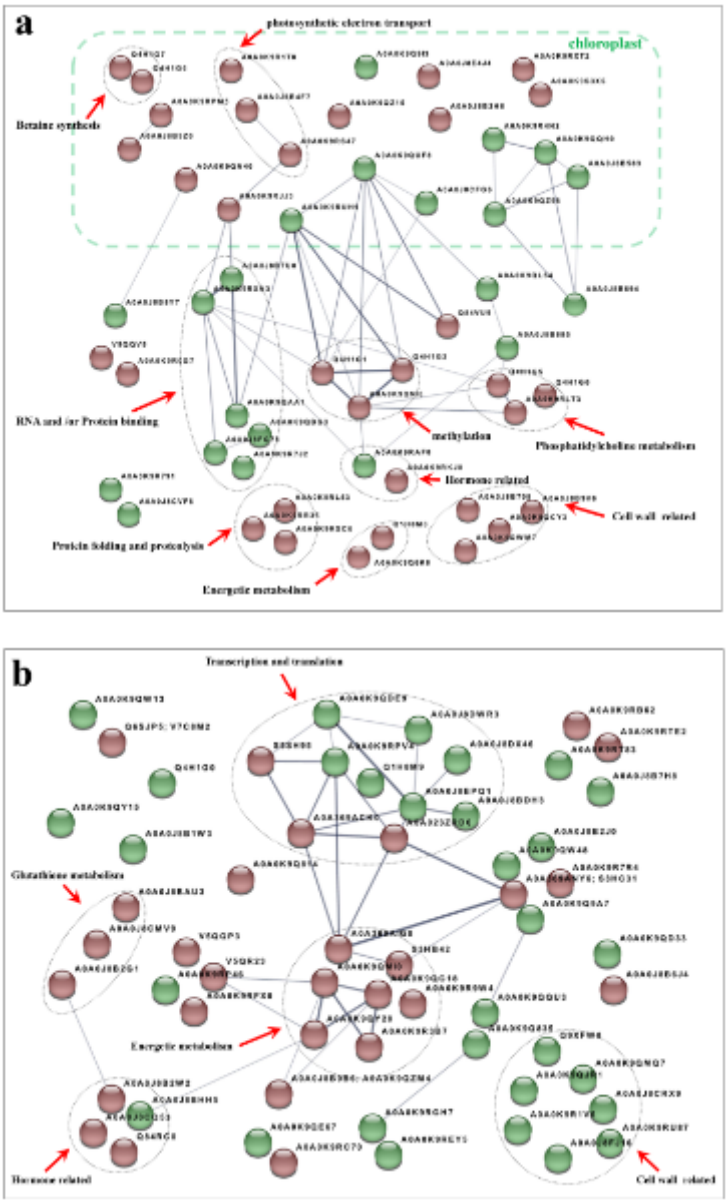

Figure 4

Protein–protein interaction (PPI) analysis of salt stress response DAPs in leaves (a) and roots (b). The circles represent proteins with red representing up-regulated and green representing down-regulated. The thickness of line indicates the strength of data support.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1DetailedinformationofDPAsinleaves.xlsx
- TableS3ListofprimersusedforqRTPCRexperiments.xlsx
- Table1.docx

- FigureS1S4.pdf
- TableS2DetailedinformationofDPAsinroots.xlsx