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

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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 beet salt-tolerant. Results Here, leaves and roots were used to identify the differentially abundant protein species 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]. Although the growth of most crops is adversely affected by soil salinity, some cultivars are able to adapt to saline conditions to achieve good harvests.

Sugar beet (Beta vulgaris ssp. vulgaris or B. 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]. Cultivar ‘O68’ is an excellent parent used in traditional crossbreeding with strong salt tolerance. Our previous study showed that under 300 m $mol \cdot L^{-1}$ NaCl treatment, the relative germination rate of this cultivar was more than 70% and the seedling can grow normally [8]. In addition, it has a strong regeneration capability of petiole explants which is ideally suited for use in molecular breeding. Therefore, O68 is a good choice for studying the mechanism of salt-stress response in B. vulgaris.

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 [9–11]. Li et al. analyzed the changes of membrane proteins under salt stress using iTRAQ technology in sugar beet monosomic addition line M14[12]. Yu et al. analyzed the changes in proteome and phosphoproteome of M14 leaves induced by short-term salt stress (30 min and 1 hour)[13]. Wu et al. studied changes in the proteome of beet seedlings treated with 50 mm NaCl for 72 h and 30 and 105 differentially expressed proteins were identified in the shoots and roots, respectively[14]. 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 [15, 16]. In addition, as suggested by Shavrukov [17], 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. As the increase of salt concentration in nature usually occurs gradually, a method of gradual adding NaCl is used for treatment.

In the present study, iTRAQ-based quantitative proteomic analysis was employed to identify differentially abundant protein species (DAPs) in leaves and roots of cultivar beet ‘O68’, respectively. The final concentration of treatment was set as 300 mM, and the treatment time was 24 hours after reaching the final concentration. 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 B. vulgaris.

Results

The purpose of this study was to compare the protein levels changes associated with physiological and biochemical mechanisms involved in salt response between leaves and roots of *B. vulgaris*. To achieve this, we performed iTRAQ analysis of leaves sampled and roots sampled from plants treated with gradually increasing NaCl levels.

Effects of salinity on physiological indexes of B. vulgaris

Acetone extraction is used to determine the content of chlorophyll, a 0.76-fold decrease in chlorophyll content was detected in leaves under salt stress compared to control plants (Figure. 1a). While salt-stress leaves displayed 3.6-fold increased proline content than control (Figure. 1b). Moreover, MDA (malondialdehyde) content was 1.6-fold higher in leaves of stressed plants (Figure. 1c). 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. 1d)

Primary Data Analysis and Protein Identification Information by iTRAQ

To investigate the mechanism of sugar beet against to salt stress, the iTRAQ-based comparative proteome analysis at 36 hour after NaCl treatment of leaf and root was performed. A total of 31,438 and 39,522 MS/MS counts were generated from leaves and roots of *B.vulgaris*, 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. Also 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). Indeed, for individual proteins, the responses to salt stress were distinct between leaves and roots. 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 are provided in Table S1 and Table S2.

Bioinformatics Analysis of DAPS Identified by iTRAQ

The putative functions of salt-stress-responsive DAPs in *B. vulgaris* 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). 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).

To further explore the potential clustering of the DAPs in specific metabolic pathways, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was carried out. 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 [18]. 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.

Majority protein IDs	Flod Change (ST/CK)	pval	qPCR 2-ΔΔCt	pval	trends	
					iTRAQ	qPCR
LEAF						
tr|A0A0J8B583|A0A0J8B583_BETVU	0.81	0.000	0.51	0.017	-	-
tr|A0A0J8CVF6|A0A0J8CVF6_BETVU	0.82	0.002	0.31	0.001	-	-
tr|A0A0J8B8Y7|A0A0J8B8Y7_BETVU	0.79	0.000	0.96	0.388	-	=
tr|Q4H1G6|Q4H1G6_BETVU	1.48	0.016	15.84	0.000	+	+
tr|Q4H1G0|Q4H1G0_BETVU	1.45	0.000	10.47	0.001	+	+
tr|A0A0J8B9V6|A0A0J8B9V6_BETVU	1.22	0.000	3.02	0.001	+	+
ROOT						
tr|A0A0J8B2J0|A0A0J8B2J0_BETVU	0.81	0.000	0.97	0.522	-	=
tr|A0A0J8BHH5|A0A0J8BHH5_BETVU	0.80	0.001	0.22	0.000	-	-
tr|A0A0J8CRX9|A0A0J8CRX9_BETVU	0.79	0.004	0.32	0.002	-	-
tr|A0A0J8BAU3|A0A0J8BAU3_BETVU	1.21	0.003	3.20	0.004	+	+
tr|Q4H1G6|Q4H1G6_BETVU	1.21	0.005	9.62	0.001	+	+
tr|Q84RC0|Q84RC0_BETVU	1.50	0.017	7.07	0.001	+	+

*-: down-regulated, =: no significantly change, +: up-regulated

Discussion

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 3 DAPs were differentially expressed in both roots and leaves, while the remaining 140 DAPs were differentially expressed only in leaves or roots. The functional divergence of the DAPs 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 B. vulgaris. 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. However, many footprints revealing the divergent responses to salt stress between leaf and root. 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. High salinity will destroy chloroplasts and affect photosynthesis. The experiment showed that chlorophyll content in leaves decreased 0.76-flod under high salt stress in *B. vulgaris* (Figure 1a). Changes must be implemented in response to this reduction. 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, protein-protein interaction analysis showed that the psbQ-like protein 1 was interacted with another up-regulated protein PPI (A0A0K9RJJ3 |peptidyl-prolyl cis-trans isomerase fkbp16-4) (Figure 4a). The same up-regulation has been shown in other studies [19] 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[20]. Furthermore, DNA repair RAD52-like protein (A0A0K9RXT2) and DNA-damage-repair/toleration protein DRT100-like (A0A0K9S3X5) were also up-regulated proteins in chloroplastic, these DAPs may help protect chloroplast DNA from high salt and enhance salt tolerance in plant [21, 22].

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. Betaine is an important osmotic regulator, which is produced from choline through two-step oxidation in plants [23-25]. 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. In addition, SEX4 (A0A0J8B9Z0 |STARCH-EXCESS 4, also known as Dual specificity protein phosphatase 4, DSP4) acts as a bridge between light-induced redox changes and protein phosphorylation in the regulation of starch accumulation [26], 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 was LS (A0A0J8E4J4 |6,7-dimethyl-8-ribityllumazine synthase), which catalyzes 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 [27]. 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 (A0A0K9Q9I3 |Thiamine thiazole synthase) 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[28]. 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 [29, 30].

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 [31-33]. 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 [34]. 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 *B. vulgaris*.

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^+) [35]. 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 [36-38]. 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[39, 40]. 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[41], 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[42]. 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 [43, 44]. 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 strategies 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 [45-47]. 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[48]. 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 [49, 50]. 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 [51, 52]. 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[53]. 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[54]. 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 [55]. 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[56]. 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 [57]. 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-regulated of these DAPs illuminates 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[58]. Choline /ethanolamine kinase (CEK: A0A0K9RLT3) catalyzes the initial reaction step of choline metabolism that produces phosphoethanolamine [59]. Phosphoethanolamine N-methyltransferase (PEAMTs: Q4H1G5) is a rate-limiting enzyme that catalyzes the phosphoethanolamine to produce choline [60]. 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 concern 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[61]. Many studies have demonstrated that GR-RBP plays a remarkable role in response to stress [62, 63]. 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 surmised 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) [64]. 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 B. vulgaris, 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 *B. vulgaris*.

Materials And Methods

Plant Materials and Treatments

The cultivar sugar beet O68 were from our own laboratory (Heilongjiang, China). Let the seeds soaked in water for 10h, 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, seedlings were selected, 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). 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, then 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 [65], respectively. 0.5g Fresh roots were collected for the determination of root activity by TTC reduction method base on Gao[65]. 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 [66]. 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 [67, 68]. For protein identification, the beet (*B. vulgaris*) protein database of UniProt (https://www.uniprot.org/uniprot/?query=beta%20vulgaris&fil=reviewed%3Ano&sort=score) 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 (http://geneontology.org/) and KEGG (http://www.genome.jp/kegg/) databases [69, 70]. 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 (http://www.ncbi.nlm.nih.gov/KOG/) 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 (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi). 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 ($p < 0.05$).

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

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 for publication

Not applicable.

Availability of data and materials

The materials used during the current study will be freely available upon request to corresponding author: cuijie@hit.edu.cn

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

Conceptualization, Junliang Li and Jie Cui; Data curation, Junliang Li; Formal analysis, Junliang Li; Funding acquisition, Jie Cui and Dayou Cheng; Methodology, Tianjiao Liu and Congyu Wang; Project administration, Jie Cui, Cuihong Dai and Chengfei Luo; Resources, Jie Cui and Dayou Cheng; Validation, Junliang Li and Tianjiao Liu; Writing – original draft, Junliang Li; Writing – review & editing, Junliang Li.

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Figures

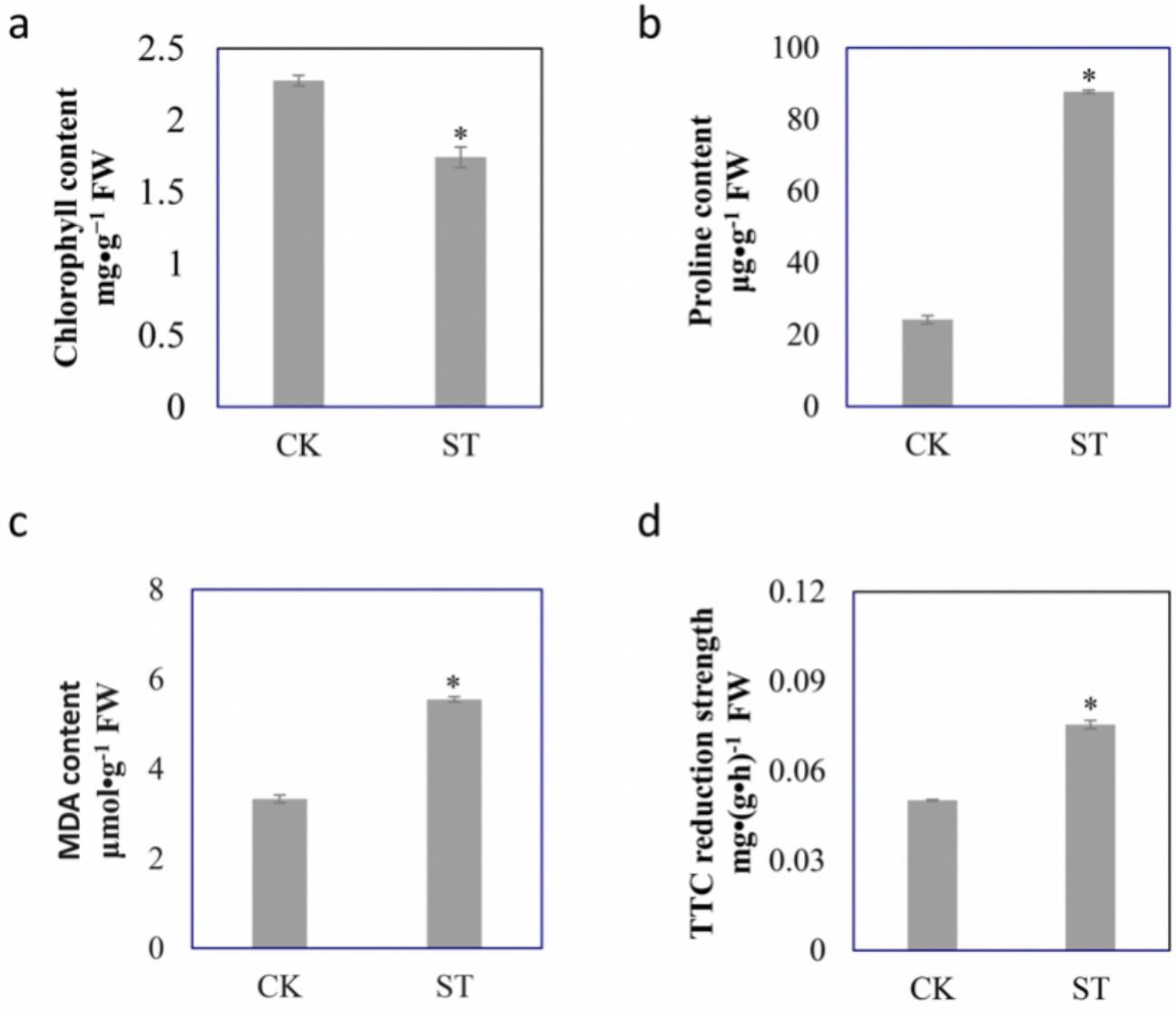

Figure 1

Physiological index determined for B. vulgaris subjected to salt stress. Chlorophyll (a), Malondialdehyde (b), Proline (c), and Root activity (d). 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). (*$p < 0.05$).

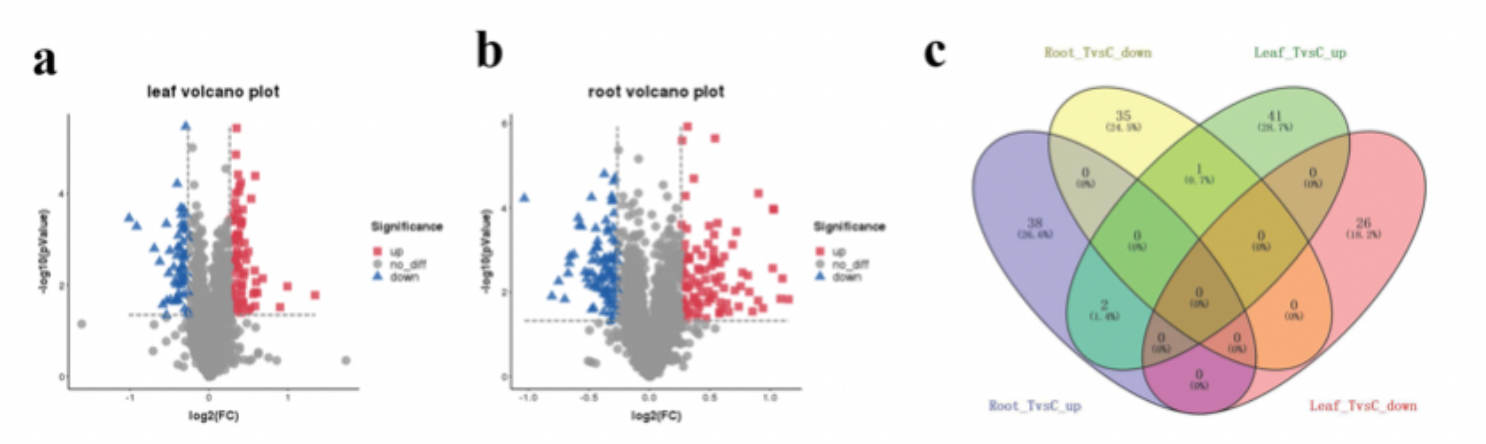

Figure 2

Distribution of salt-stress-responsive differentially abundant proteins (DAPs). Expression pattern of DAPs in leaves samples (a) and roots samples (b) of B. vulgaris. (c). Overlapping of DAPs between leaves and roots samples.

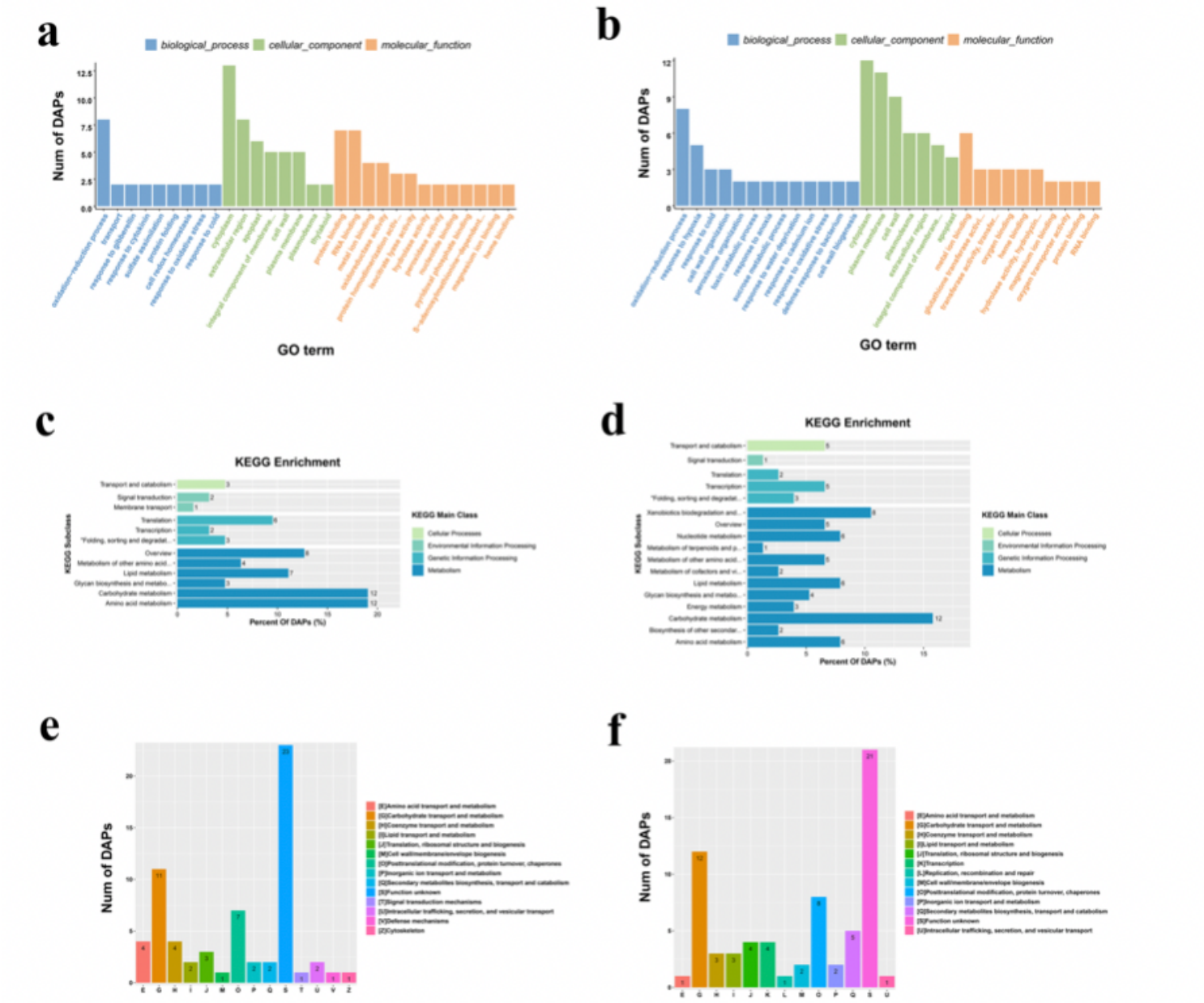

Figure 3

Bioinformatics Analysis of DAPS. Gene ontology (GO) enrichment of DAPs in leaves samples (a) and roots samples (b) of B. vulgaris. 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).

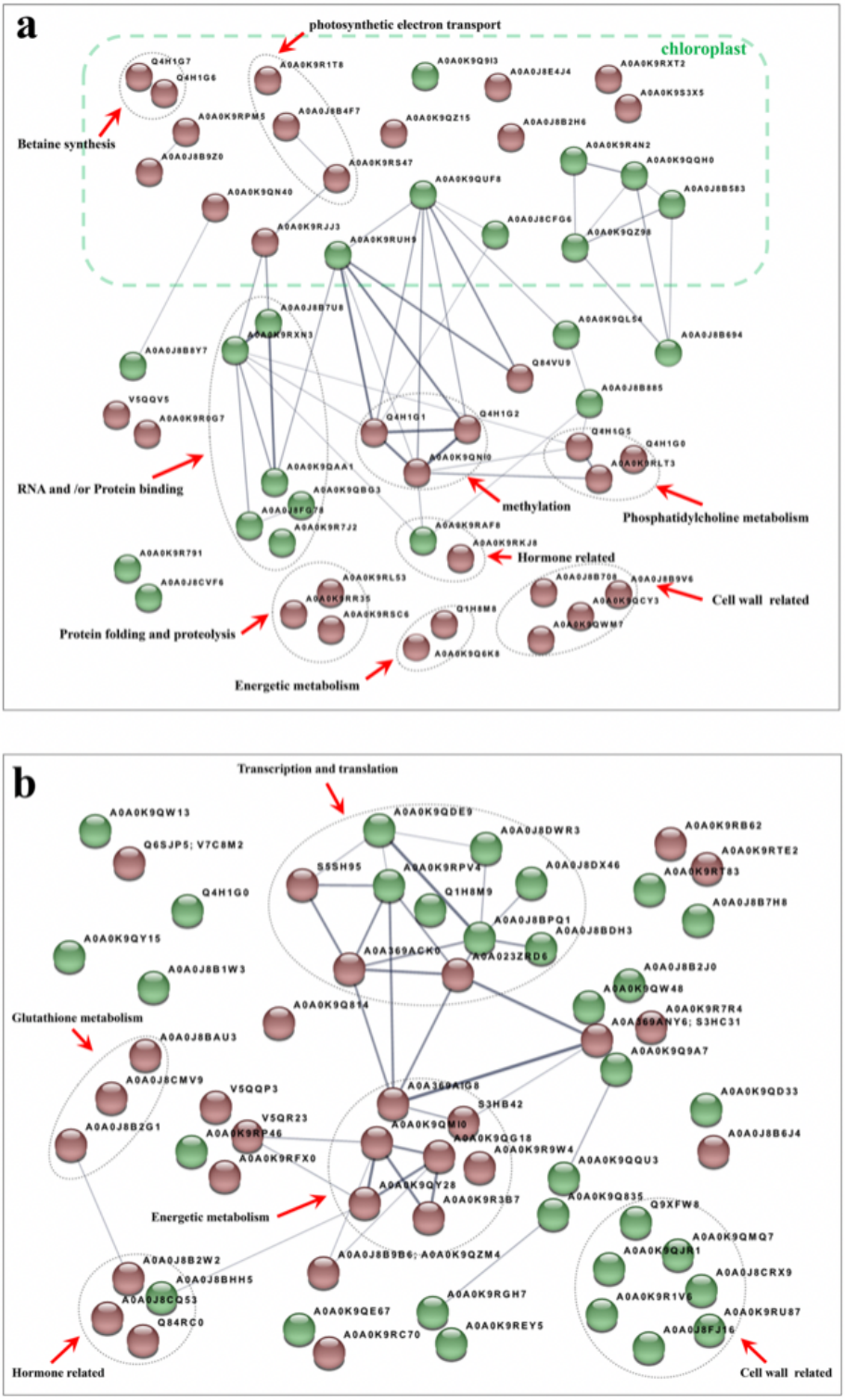

Figure 4

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.

- FigureS1S4.pdf
- TableS2DetailedinformationofDPAsinroots.xlsx
- TableS3ListofprimersusedforqRTPCRexperiments.xlsx
- TableS1DetailedinformationofDPAsinleaves.xlsx