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Identification of NaCl and $NaHCO_3$ stress responsive proteins in tomato roots using iTRAQ-based analysis

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ABSTRACT

Soil salinity and alkalinity are common constraints to crop productivity in low rainfall regions of the world. However, the physiological difference of plant response to these two stresses was short of deep investigation. This study has identified a set of differentially expressed proteins of tomato root exploring to NaCl and $NaHCO_3$ stress by iTRAQ (isobaric tags for relative and absolute quantitation) assay. A total of 313 proteins responsive to NaCl and $NaHCO_3$ were observed. Among these proteins, 70 and 114 proteins were up-regulated by salt and alkali stress, respectively. While down-regulated proteins were 80 in salt treatment and 83 in alkali treatment. Only 39 up-regulated proteins and 30 down-regulated proteins were shared by salt and alkali stresses. The majority of the down-regulated proteins accounted for metabolism and energy conversion, and the up-regulated proteins were involved in signaling or transport. Compared with salt stress, alkali stress down-regulated proteins related with the respiratory metabolism, fatty acid oxidative metabolism and nitrogenous metabolism of tomato roots, and up-regulated protein with the reactive oxygen species (ROS) scavenging and ion transport. This study provides a novel insight into tomato roots response to salt and alkali stress at a large translation level.

1. Introduction

Salinity–alkalinity negatively affects crop production in semi-arid and arid regions. Worldwide, 831 million hectares of soils are affected by excessive salinity–alkalinity in the world. Of this, 434 million hectares are sodic soils (alkaline), compared to 397 million hectares of saline soils (FAO). Saline soil mainly due to the accumulation of NaCl, and alkaline soil is mainly due to the accumulation of $NaHCO_3$ and Na_2CO_3 [1]. Therefore, conditions of high percentage of exchangeable sodium and high pH are provided by alkaline soil. The response of higher plant to neutral salt stress has previously been extensively studied, but the adaption mechanism to alkali stress in plants is short of deep investigation. With the increasing recognition of alkaline threat to agricultural production, literatures about higher plant response and adaption to alkaline stress have flourished in recent years [2].

The identification and functional characterization of salt–alkali responsive proteins may provide some attractive candidate genes and valuable information on both defining the tolerance mechanism of plants to salt--alkali stress and improving salt–alkali tolerance of plants by genetic engineering. Large-scale studies intended to identify salt or alkali stress-related genes have been done with the development of transcriptomics. But most of them were reported by measuring changes in gene expression in halophyte [3]. However, it is known that the metabolic reaction of glycophyte is different from halophyte under salt–alkali stress [4]. Furthermore, there is poor or no correlation between changes in mRNA and protein abundance, and only direct protein measurements will reveal real changes that occur at protein levels [5]. As glycophyte, tomato is a worldwide vegetable crop whose tolerance mechanism and proteomics of salt stress have been investigated extensively and deeply [6]. However, until now the research of salt stress mainly emphasizes NaCl as the subject. To our knowledge, there is no report about alkali stress on tomato yet. Root is the main organ for carrying water and mineral nutrients to the rest of the plant. As the primary site of perception and injury for salt and alkali stress, roots provide an ideal target for study of the molecular mechanism underlying plant salt and alkaline stress tolerance and adaptation [7]. In the present study, NaCl and $NaHCO_3$ were respectively used to simulate salt and alkali stress. The objective of comparing proteome differences of tomato roots under salt

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and alkali stress was analyzed by iTRAQ. This work provides a theoretical basis for understanding the different mechanism of salt and alkali stress on tomato roots.

In this study, 1915 proteins were identified in tomato roots, where 150 and 199 proteins were found to respond to NaCl and $NaHCO_3$ stress, respectively. This analysis revealed the common and different pathways between salt and alkali stress in tomato roots, and added a new layer of information regarding tomato plant abiotic stress physiology.

2. Materials and methods

2.1. Plants and growth conditions

Tomato seeds (*Solanum lycopersicum* L.) were germinated on moisture filter paper in the dark at 28 °C for 3 d, and germinated seedlings were transferred to the growth chamber filled with vermiculite and grown in greenhouse for 15 d. Then, batches of five seedlings were grown hydroponically in a plastic container filled with 5 L of Hoagland nutrient solution . The treatments were started after 15 d of pre-culture. The experimental design consisted of a control (0 mM NaCl and $NaHCO_3$), 50 mM NaCl treatment and 50 mM $NaHCO_3$ treatment, which were arranged in a randomized. Each treatment contained ten black plastic containers with 50 tomato seedlings, giving a total of 30 containers. The plants were cultivated under natural conditions in a glass greenhouse, and exchange the nutrient solution every day to ensure the steady environment. After 72 h treatment, 50 seedlings' roots in each treatment were taken and mixed abundantly for protein extraction to reducing individual error, the experimental roots were store in liquid nitrogen temporarily.

2.2. Protein extraction, quantification and digestion

Protein extraction was performed according to the method of Lan et al. [8] with some modifications. Roots from different treatments were ground in liquid nitrogen and suspended in 10-fold volume of pre-cooled acetone (−20 °C) containing 10% (v/v) TCA and 0.1% (v/v) 2-mercaptoethanol. Proteins were precipitated at −20 °C for 2 h, then were collected by centrifuging. The protein pellets were washed three times and were dried by lyophilization and immediately extracted using protein extraction buffer. The protein concentration was quantified by Bradford Protein Assay Kit. Take out 100 μg protein for treatment from each sample solution accurately. Digest the protein with Trypsin Gold at 37 °C for 4 h. Add Trypsin Gold with the same ratio once more and digest for 8 h unceasingly.

2.3. iTRAQ labeling

After trypsin digestion, peptide was dried by vacuum centrifugation, which was reconstituted in 0.5 M TEAB and processed according to the manufacturer's protocol for 8-plex iTRAQ (Applied Biosystems). Peptides from digestion were labeled with different iTRAQ tags in the group, respectively (Control treatment samples, NaCl treatment samples and $NaHCO_3$ treatment samples were separately labeled with iTRAQ reagents with molecular masses of 116, 117 and 118 Da). The pooled mixtures of iTRAQ-labeled peptides are fractionated by SCX chromatography.

2.4. Fractionation by strong cationic exchange (SCX)

For SCX chromatography using the Shimadzu LC-20AB HPLC Pump system, the peptide from digestion is reconstituted with 4 ml buffer A (25 mM NaH_2PO_4 in 25% ACN, pH 2.7) and loaded onto a 4.6 × 250 mm ultremex SCX column containing 5 μm particles (Phenomenex). The peptides was eluted at a flow rate of 1 ml/min with a gradient of buffer A (2% ACN, 0.1% FA) for 10 min, 5–35% buffer B (25 mM NaH_2PO_4, 1 M KCl in 25% ACN, pH 2.7) for 11 min, 35–80% buffer B for 1 min. The system was then maintained in 80% buffer B for 3 min before equilibrating with buffer A for 10 min. Elution was monitored by measuring absorbance at 214 nm, and fractions were collected every 1 min. The eluted peptides are pooled as 12 fractions, desalted by Strata XC18 column (Phenomenex) and vacuum-dried. Each fraction was resuspended in mobile phase A and the final concentration of peptide is about 0.25 μg/μl on average.

2.5. LC–ESI-MS/MS analysis based on Triple TOF 5600 system

A splitless nanoAcquity system (Waters) was coupled to the Triple TOF for analytical separation. The system uses micro fluidic traps and nanofluidic columns packed with Symmetry C18 (5 μm, 180 μm × 20 mm) for online trapping, desalting, and nanofluidic columns packed with BEH130 C18 (1.7 μm, 100 μm × 100 mm) for analytical separations. Solvents were composed of water/acetonitrile/formic acid (A: 98/2/0.1%; B: 2/98/0.1%). A 2.25 μg (9 μl) portion of sample was loaded, and trapping and desalting were carried out at 2 μl/min for 15 min with 99% mobile phase A. At a flow rate of 300 nl/min, separation was maintained 5% B for 1 min, then 5–35% B for 40 min, 35–80% B for 5 min and maintained for 5 min. Data was acquired using an ion spray voltage of 2.5 kV, curtain gas of 30 PSI, nebulizer gas of 15 PSI, and an interface heater temperature of 150 °C.

2.6. Database search and quantification

The 2.3.02 version of the Mascot software (Matrix Science) was used to simultaneously identify and quantify proteins. Only unique peptides used for protein quantification can be chosen. Searches were made against the *S. lycopersicum* protein database (ftp://ftpmips.helmholtz-muenchen.de/plants/tomato/tomato_genome/ITAG_annotation/ITAG2.3_release/ITAG2.3_proteins.fasta). The search parameters were as follows: trypsin/P was chosen as the enzyme with two missed cleavages allowed; fixed modifications of carbamidomethylation at Cys, variable modifications of oxidation at Met and iTRAQ 8-plex at Tyr; peptide tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.02 Da. Peptide charge was set Mr +2 ~ +5, and monoisotopic mass was chosen. iTRAQ 8-plex was chosen for quantification during the search simultaneously.

The search results were passed through additional filters before exporting the data. For protein identification, the filters were set as follows: significance threshold $P < 0.05$ (with 95% confidence) and ion score or expected cutoff less than 0.05 (with 95% confidence). For protein quantitation, the filters were set as follows: "weighted" was chosen for protein ratio type (http://mascot-pc/mascot/help/quant_config_help.html); minimum precursor charge was set to 1 and minimum peptides was set to 2; only unique peptides were used to quantify proteins. Summed intensities were set as normalization, and outliers were removed automatically. The peptide threshold was set as above for homology.

2.7. Statistical analysis

In brief, the mean and SD from the log2 ratios of the 1915 quantified proteins overlapping in both biological repeats was calculated. Next, 95% confidence (Z score = 1.96) was used to select those proteins whose distribution was removed from the main distribution. The cutoff value for the down-regulated proteins was 0.83-fold and for the up-regulated proteins was 1.2-fold.

3. Result and discussion

3.1. Effects of salt and alkali stress on expression changes of the tomato root proteome

Integrated root cell was considered to investigate the responsive mechanism of tomato root to salt and alkali stress in this study. Considering the composite qualification, we used 50 mM NaCl and $NaHCO_3$ to simulate the salt and alkali stress for 3 d. To combine our large-scale tomato root proteome analysis with quantitative information on proteins modulated under salt and alkali stress, we used isobaric tags for relative and absolute quantification (iTRAQ) [9]. Proteins were extracted from roots of control, salt stress and alkali stress plants with the experimental scheme in Supplemental Fig. S1.

Analysis of the tomato proteome database revealed that 313 of the 1915 identified proteins were significantly regulated under salt or alkali stress compare with control treatment. Of these 150 proteins in control treatment vs. NaCl treatment (116 vs. 117) with 70 proteins were up-regulated and 80 proteins were down-regulated; 199 proteins in control treatment vs. $NaHCO_3$ treatment (116 vs. 118) with 114 proteins were up-regulated and 85 proteins were down-regulated (Fig. 1A). To examine the proteins of tomato roots changes during the different stresses, the numbers of proteins up- or down-regulated by salt and alkali stresses were compared in Fig. 1A. It was detected that the responsive proteins to salt and alkali were greatly different. In tomato roots, 39 (26.9%) of the up-regulated proteins and 30 (22.6%) of the down-regulated proteins, respectively, were regulated in common biological process by both of salt and alkali stresses. So it showed that salt and alkali stresses on tomato roots have some commonness but more characteristic including exchangeable sodium, pH and alkaline reactions in a complex network involving multiple physiological and metabolic pathways.

Hierarchical cluster analysis was used to identify proteins with certain patterns of changes under different stresses (Supplemental Table S1). The differentially regulated proteins were clustered according to similarities in change profiles across all conditions. Dendrogram and colored image were produced as cluster analysis of different samples using cluster 3.0. Dark boxes indicate no change in expression pattern compared to control treatment. Each row of colored boxes was representative of a single protein and each treatment is represented using a single column, the left (116 vs. 117) and the right (116 vs. 118). Five distinguishable clusters are separated by black bars (Fig. 1B). Cluster I includes proteins down-regulating in 116 vs. 117 but little up-regulating in 116 vs. 118. Cluster II, which accounted for the largest portion of total differentially regulation proteins, contained proteins showing up-regulation in both 116 vs. 117 and 116 vs. 118. Cluster III included proteins showing down-regulation in both 116 vs. 117 and 116 vs. 118. Cluster IV comprises proteins with expression levels up-regulation in 116 vs. 117, but little down-regulation in 116 vs. 118. Cluster V contained proteins showing high down-regulation in 116 vs. 117 and 116 vs. 118. According to the hierarchical cluster analysis, most proteins were changed in same direction in cluster II, III, and V, which showed that both salt and alkali stresses had similar effect on the metabolic physiology of tomato root, such as ion unbalance, osmotic stress and dehydration. As the character of alkali treatment, high pH stress could induce those discrepant proteins in cluster I and IV. Similar result could be consulted by Jin et al. [10].

3.2. Protein identification from tomato roots by iTRAQ

Out of the 4964 significantly unique peptides, we assigned at least one GO term to 1915 protein based on *Solanum lycopersicum*

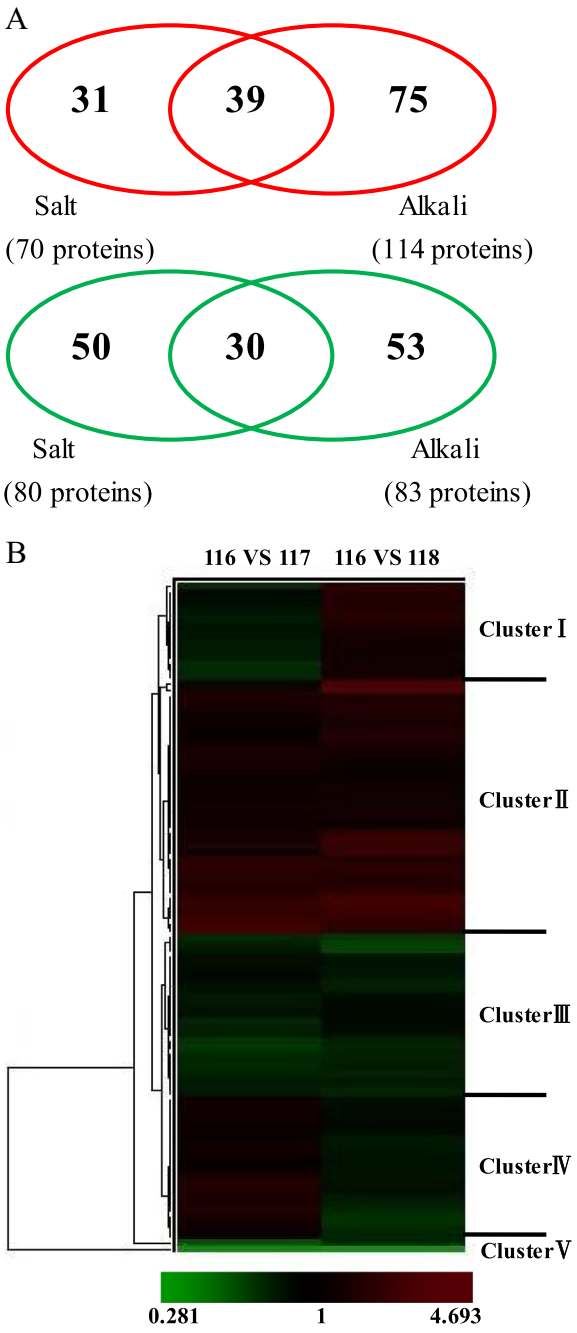

Fig. 1. (A) Salt and alkali stress-induced proteins in tomato roots: up-regulated genes in red; down-regulated genes in green. (B) Hierarchical display of data from differential expression of protein under NaCl and $NaHCO_3$ stresses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

protein database (http://mips.helmholtz-muenchen.de/plant/tomato/index.jsp). The distribution of functional categories was showed in Fig. 2. As seen, 25 GO overrepresented ($P < 0.01$, FDR < 0.05) categories were affected by the salt and alkali stress treatment, including metabolic process, cellular process, response to stimulus, localization and so on. In addition, the majority of the most abundant proteins in tomato roots were related to eleven molecular functions involved in binding, catalytic activity, structural molecule activity, transporter activity, antioxidant activity, enzyme regulator activity, molecular transducer activity, electron carrier activity, nucleic acid binding transcription factor activity, protein binding transcription factor activity and metallochaperone

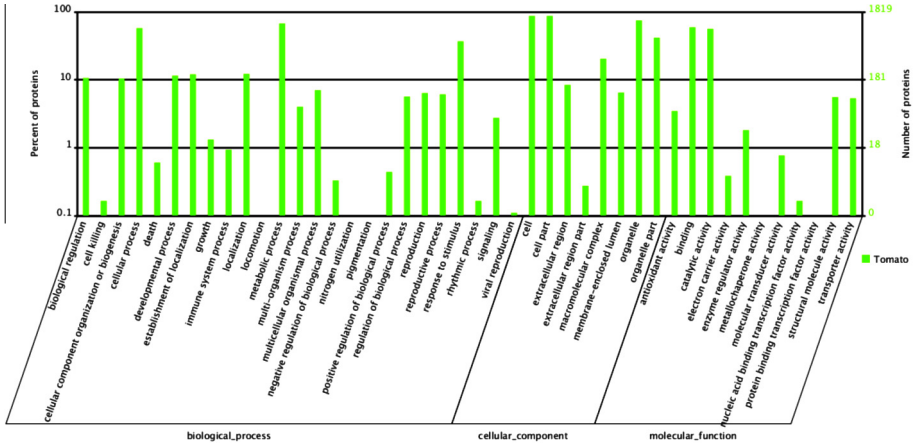

Fig. 2. The biological process, cellular component and molecular function of tomato root in iTRAQ analysis.

activity (Fig. 2). A comparison with a comprehensive proteomics study of tomato roots revealed 80 proteins that were uniquely identified in previous study [11].

3.3. Extension of the biological process model related with salt and alkali stresses revealed by iTRAQ

To obtain an overview of the regulated proteins, we generated a master table (Supplemental Table S1) that summarizes all changed proteins. The master table was useful in relating proteins to biochemical and physiological functions that are modulated by salt and alkali stresses. In order to decipher functional networks, we summarized some of related regulated-proteins in the Supplemental Table S2, which associate with Fig. 3. As the main parts response to salt and alkali stresses, signaling pathways, ROS scavenging pathways, metabolism and energy conversion, transport protein regulating and were summarized in Fig. 3. All of the five parts compose a whole adaptive/injured mechanism of tomato root to salt and alkali stresses.

3.3.1. Signaling pathways

The best-characterized roles of ABA were in adaptive responses to abiotic stresses, such as high salt and dehydration stress. This point was best illustrated in abundant researches by plant mutants that cannot produce ABA [12]. ABA biosynthetic strategy involves the one-step hydrolysis of Glc-conjugated ABA (ABA-GE) by a β-glucosidase (βG) homolog, which localizes to the endoplasmic reticulum and increases ABA levels upon dehydration stress through a mechanism called polymerization-mediated activation [13]. In our study, both salt and alkali stresses could up-regulate βG that was accordant with ABA levels. Another role of ABA has to do with induction of genes that encode dehydration tolerance proteins in nearly all cells. Ca^{2+} sensors such as Calmodulin and Calnexin was often induced by various abiotic stresses [14]. They were both up-regulated in alkali stress and down-regulated in salt stress, which was another signal pathway caused by a rapid increase in Ca^{2+} resulting from an oxidative burst in alkali stress in our study [15]. Salt- or alkali-stressed tomato roots up-regulated IMP-β, ES, LEA, HSP, AQU, ASR4, UBC, PR, CPN, WD40, all of which was involved ABA and Ca^{2+} signaling to enhance the cellular dehydration tolerance (Fig. 3).

3.3.2. ROS scavenging pathways

Excessive accumulation of ROS under abiotic stress can cause oxidative damage, like the peroxidation of membrane lipids, the inhibition of protein synthesis, the inactivation of enzyme [16]. Strikingly, compare with control treatment, the contents of H_2O_2, $O_2^{\cdot-}$ and TBARS of tomato roots under salt and alkali stresses were both raised, Which in alkali treatment were significantly higher than salt treatment (Fig. S2). The observed increases in H_2O_2 and $O_2^{\cdot-}$ under salt and alkali stresses probably account for the lipid peroxidation which was indicated by excess accumulation of TBARS. The similar results have been observed by our previous studies [17]. To avoid oxidative damage, plants have evolved efficient antioxidant systems which can protect them from deleterious effects of ROS [18]. SOD, APX, CAT, POD, GPX, GR, GST, PPO and NQO were all up-regulated at the protein level under salt or alkali stresses (Fig. 3 and Supplemental Fig. S3). A constitutively high antioxidant capacity under stress conditions with plant resistance was reported to that particular stress. Consequently, the mechanisms that reduce ROS and increase antioxidant enzyme system in plants have important roles in imparting tolerance in plants under environmental stress conditions [19].

3.3.3. Metabolism and energy conversion

On exposure to osmotic stress as a result of NaCl or $NaHCO_3$, plants accumulate a range of metabolically benign solutes, collectively known as compatible solutes or osmolytes. Many of these compatible solutes are N-containing compounds, such as amino acids and amides or betaines, hence the nitrogen metabolism is of central importance not only in growth but also stressful conditions. In our research, all nitrogen metabolism enzyme including NR, NiR, GS, GOGAT were down-regulation, but only GDH was up-regulation (Fig. 3 and Supplemental Fig. S3). NR was a key enzyme in the overall process of nitrate assimilation by plants. Preceding reports revealed that the salt stress caused an inhibition in NR and NiR enzyme activities [20]. It was suggested that GDH activity could be a measure of utilizing accumulated ammonia under salt and alkali stress when GS and GOGAT systemic was less efficient. Increased activity of GDH and down-regulation of GS and GOGAT in response to salt and alkali stress, which were identified in our study, were also reported [21].

In our study, ACCase was down-regulated, LOX and PKT were up-regulation under $NaHCO_3$ stress, which showed the result of significantly degradation of lipid in root cell (Fig. 3). This result was in accord with TBARS variation trend (Supplemental Fig. S2), which indicated that membrane system was injured more sharply under alkaline conditions compared with salt stress.

The role of TCA cycle in tomato root had been defined well, and in our experiment there were 4 proteins (DLD, ICDH, MDH, PEPCK) associated with aerobic respiration being up-regulated by salt or alkali stresses. As an essential enzyme catalyzed pyruvic acid to

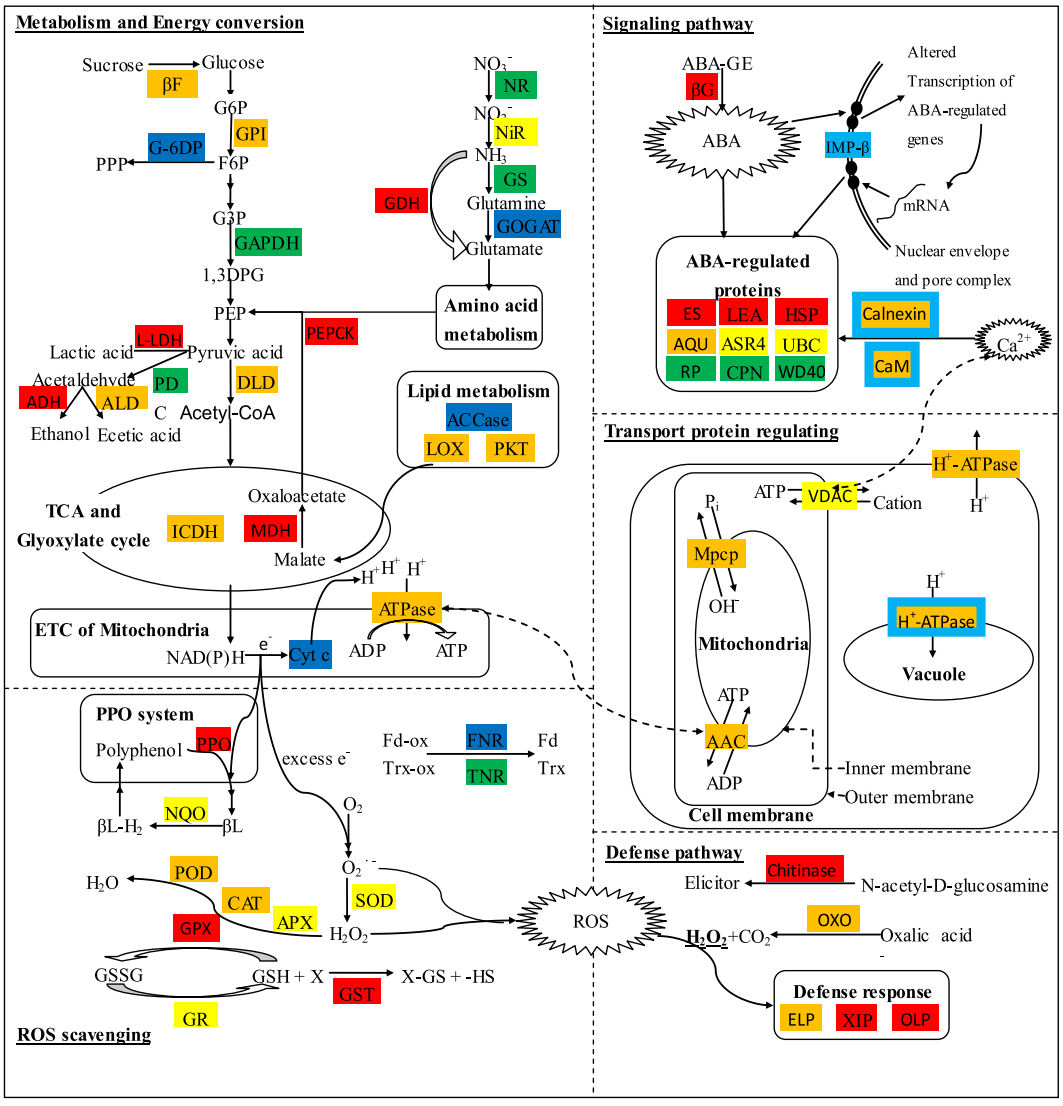

Fig. 3. Summary of some of the biological pathways affected under salt and alkali stresses in tomato root. Red boxes indicate proteins up-regulated in both salt and alkali stresses, orange boxes indicate proteins only up-regulated in alkali stress, yellow boxes indicate proteins only up-regulated in salt stress, and green boxes represent proteins down-regulated in both salt and alkali stresses, oxford blue boxes represent proteins only down-regulated in alkali stress, light blue boxes represent proteins only down-regulated in salt stress. The Dual Color boxes represent proteins that were shown to have mixed expression patterns (both up- and down-regulation). βG, glucan endo-1 3-beta-glucosidase; Calnexin, calcium-binding protein Calnexin; CaM, Calmodulin; IMP-β, importin subunit beta; ES, embryo-specific 3; LEA, late embryogenesis abundant protein; HSP, heat shock protein; AQU, aquaporin; ASR, ABA/WDS induced protein 4; UBC, ubiquitin-conjugating enzyme family protein-like; RP, ribosomal protein; CPN, chaperonin; WD40, WD-40 repeat protein; PPO, polyphenol oxidase; NQO, NAD(P)H-quinone oxidoreductase; TNR, thioredoxin; FNR, ferredoxin; SOD, Cu/Zn-superoxide dismutase; APX, L-ascorbate peroxidase; CAT, catalase; POD, peroxisomal membrane protein; GPX, glutathione peroxidase; GR, glutathione reductase; GST, glutathione S-transferase; βF, acid beta-fructofuranosidase; GPI, glucose-6-phosphate isomerase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase G-6DP, glucose-6-dehydrogenase; L-LDH, L-lactate dehydrogenase; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; DLD, dihydrolipoyl dehydrogenase; ICDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; Cyt c, cytochrome c oxidase subunit VIb; ATPase, F1-ATP synthase delta subunit; NR, nitrate reductase; GS, glutamine synthetase; GOGAT, glutamate synthase; GDH, glutamate dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase; ACCase, acetyl-CoA carboxylase; LOX, lipoxygenase; PKT, 3-ketoacyl CoA thiolase; H^+-ATPase, vacuolar H^+-ATPase; VDAC, mitochondrial porin (Voltage-dependent anion channel) outer membrane protein; Mpcp, mitochondrial phosphate carrier protein; AAC, ADP/ATP carrier protein; OXO, oxalate oxidase; ELP, expansin-like protein; XIP, xylanase inhibitor; OLP, osmotin-like protein.

acetyl-CoA, DLD was up-regulation to accelerate TCA cycle. ICDH, except being concerned with TCA cycle, had been proposed to have diverse functions, such as nitrogen metabolism, ammonium assimilation, and degradation of lipids and fatty acids [22].

3.3.4. Transport protein regulating

Five proteins related to transport were identified, distributing in the membrane of mitochondria, vacuole and cell (Fig. 3). The AAC and Mpcp were the most abundant proteins in the mitochondrial carrier, catalyzing the exchange of ADP/ATP and Pi/OH^- separately across the inner mitochondrial membrane [23]. H^+-ATPase catalyzed the translocation of protons across the membranes and played key roles in re-establishment of ion homeostasis under salinity–alkali stress [24]. According to their function, tomato could amend the pH of rhizosphere and accumulate H^+ in vacuolar through up-regulate H^+-ATPase in alkali environment.

3.3.5. Defense pathways

PR proteins were a diverse group of proteins, including Chitinase, OXO, ELP, XIP and OLP that were all up-regulation in our study (Fig. 3). Notably, these PR proteins are induced by not only biotic stress but also abiotic stimuli. OLP accumulating in salt-adapted tobacco cells was found to constitute as much as 12% of the total cellular proteins. Since then, the induction of osmotin-like

protein, or mRNA, has been found in various plant species in response to pathogen infection, such as viroids, viruses, bacteria and fungi, or environmental stresses including high salinity and low temperature [25].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.005.

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