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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.