iTRAQ-based quantitative proteomic analysis of salt stress in Spica Prunellae

Zixiu Liu1,2,3,4, Lisi Zou1,2,3, Cuihua Chen1,2,3, Hui Zhao1,2,3, Ying Yan1,2,3, Chengcheng Wang1,2,3 & Xunhong Liu1,2,3

Spica Prunellae is an important Chinese herbal medicine. Because of its good curative effect on various diseases, this herb is consumed in large quantities in clinical applications. The metabolites of Spica Prunellae are known to change under salt stress; however, the difference in protein levels of Spica Prunellae between saline and normal conditions is unclear. In this study, we used proteomics techniques to identify differentially expressed proteins in Spica Prunellae under different saline conditions. (iTRAQ) MS/MS was used to detect statistically significant changes in protein between salt stress and normal conditions. Ultimately, we detected 1,937 proteins, 89 of which were detected in two different comparison. Based on GO, STRING and KEGG analyses, 35 significantly differentially expressed proteins were selected for further analysis. The results of functional and signal pathway analyses indicated that the cellular protein and carbohydrate metabolism of Spica Prunellae was weaker, calcium ion transport was higher, photosynthesis was higher, and protein production was faster under saline conditions than under normal conditions. This study provides useful information for studying the causes of differences in secondary metabolites in Spica Prunellae under salt stress and the protein mechanisms related to their quality.

Soil salinity is a major external environmental stressor of plants worldwide because it has a very strong negative effect on plant growth, development and production. Under salt stress, aspects of plant growth, including seed germination, root length, and plant height are significantly inhibited. With the rapid development of industry and agriculture, the soluble salt in the soil has increased in recent years. According to reports by the Food and Agriculture Organization (FAO 2008; http://www.fao.org/ag/agl/agll/spush/), more than 400 million hectares of land worldwide have been exposed to high salinity. Irrigation water, which always contains sodium chloride (NaCl), is the main reason for soil salinization worldwide. Soil salinity also affects photosynthesis, protein synthesis, and energy metabolism, and is likely to cause plant physiological drought, ion toxicity, and physiological metabolic disorders. Plants have developed a series of tolerance mechanisms for growth under harmful conditions. This tolerance depends primarily on a variety of biochemical processes that lead to the production of compatible permeants, antioxidants, transporters and specific proteins that control salt damage. For example, plants have developed strategies to reduce osmotic damage, primarily by reducing water loss, while relying on many permeants, organic solutes, inorganic ions, and water channels to maximize moisture absorption. Wiciarz, M revealed the salt tolerance mechanism in Arabidopsis thaliana and Eutrema salsugineum. StDWF4 overexpression in potato seedlings enables them to resist salt stress by mitigating the negative effects of the stressor. Copper, zinc superoxide dismutase (CuZnSOD) and ascorbate peroxidase (APX) overexpression together enhance the tolerance of sweet potato under salt stress. Overexpressing SOD and APX improves salt stress tolerance. Therefore, studying the physiological processes and molecular genetic mechanisms associated with salt stress resistance will certainly help clarify the complex biological responses of plants to high salinity and contribute to the genetic engineering of stress-resistant plants.

Spica Prunellae (Xia Ku Cao in Chinese), comprises the fruit spikes of the perennial plant Prunella vulgaris L., which is widely distributed in Asia and Europe. As a well-known traditional Chinese medicinal herb, Spica

1College of pharmacy, Nanjing University of Chinese Medicine, Nanjing, China. 2Collaborative Innovation Center of Chinese Medicinal Resources Industrialization, Nanjing, China. 3National and Local Collaborative Engineering Center of Chinese Medicinal Resources Industrialization and Formulae Innovative Medicine, Nanjing, China. 4Department of Pharmacy, No. 454 Hospital of PLA, Nanjing, China. Correspondence and requests for materials should be addressed to X.L. (email: liuxunh1959@sohu.com)
Prunellae is commonly used to treat various cancers. Spica Prunellae can alleviate liver fire, improve vision, and disperse swelling, and is mainly used to treat eye pain, headaches, dizziness, and swollen breasts. Modern clinical practice has demonstrated that the herb possesses anti-inflammatory, antimicrobial, antioxidant, antiviral and immunomodulator properties and can be used to lower blood pressure, alleviate sore throat, reduce fever, treat thyroditis and accelerate wound healing. Spica Prunellae extract reportedly induces programmed apoptosis in human colon carcinoma cell lines. Spica Prunellae induces efflux transporter expression via activation of the Nrf2-mediated signalling pathway in HepG2 cells. In recent years, as the abundance of Spica Prunellae in the wild has declined and the demand for original medicines has risen annually, it has become imperative to carry out work on the artificial cultivation of Spica Prunellae. When Spica Prunellae is cultivated in the field, many abiotic stress factors affect its yield and quality. Among these stressors, salinity is one of the most severe environmental factors limiting the quality of Spica Prunellae. Planting Spica Prunellae in salinized land can effectively alleviate the discrepancy between its current supply and strong market demand. Therefore, it is very important to study the salt tolerance mechanism of Spica Prunellae to increase the yield and quality of this important herb. There are currently no reports on the mechanism of Spica Prunellae response to salt stress.

Proteomics is a frontier method in proteomic research and is widely used in the study of plant resistance to external stress. Proteomics-based technologies have been widely used to identify proteins responsible for salt tolerance in several crops, such as cotton, cucumber, and wheat, promoting advancements in the study of molecular activities under salt stress. iTRAQ technology has been used to identify and quantify 2,165 proteins and 1,815 proteins in developing rice embryos and wheat grains, respectively.

In this study, a currently popular high-throughput proteomics technology (iTRAQ technology) was adopted to reveal the protein activity of Spica Prunellae under salt stress and to study the important protein-mediated stress mechanism of Spica Prunellae under salt stress. The energy metabolism and stress-related metabolism of this herb were revealed. The findings of this study provide important reference information and a theoretical basis for future research on salt tolerance in Spica Prunellae.

Material and Methods

Plant materials and salinity stress. Seedlings of Spica Prunellae were collected from Chuzhou City, Anhui Province. The seedlings were sown in plastic pots filled with garden soil and grown under greenhouse conditions (20 ± 2 °C, 50 ± 5% relative humidity, 16/8 h photoperiod) until mature ears were fully developed. For the salt treatments, soluble NaCl was gradually incorporated into the soil until the salt concentrations reached 0 (CK), 50 (Na50), 100 (Na100) or 150 (Na150) mM. The incorporation of NaCl was repeated 5 times for each group. Plants were watered once with Hoagland nutrient solution for ten days. When the plants were approximately five months old (on February 26), the Spica Prunellae seedlings were treated with Hoagland nutrient solution as a control or Hoagland nutrient solution with 50, 100 or 150 mM NaCl as a salt stress treatment five times in fifty days. Samples were obtained at 10:00 a.m. on June 14 by pooling the fruit spikes from four plants in the same group, and two replicates were analysed per treatment. Later in sample processing, 30 fruit spikes of Spica Prunellae from each group were crushed and mixed in liquid nitrogen, and 5 g of the material from each group was taken as the sample for subsequent proteomic processing.

Protein extraction. Spica Prunellae proteins were extracted using phenol, and few modifications were made. Briefly, the stored Spica Prunellae samples were pulverized in liquid nitrogen and then mixed with 10% polyvinylpolypyrrolidone (PVPP). The precipitate was then dissolved in protein extraction buffer containing sucrose (0.8 M), KCl (100 mM), ethylenediaminetetraacetic acid (EDTA) (50 mM, pH of 8.0), phenylmethanesulfonfyl fluoride (PMSF) (1 mM), Tris-HCl (50 mM, pH of 8.5), and dithiothreitol (DTT) (1%) at 4 °C. Afterwards, a mixture of equal volumes of Tris-buffered phenol (pH of 8.0) and the sample was thoroughly vortexed. Then, the samples were centrifuged at 12,000 rpm for 30 minutes at 4 °C. The supernatant was carefully removed and back extracted with an equal volume of protein extraction buffer. The mixture was vortexed at room temperature for 5 minutes and centrifuged, and this sequence was repeated 6 times. The final recovered phenol phase was poured into a new tube. Finally, the pellets were air-dried. The protein was then determined using the bicinchoninic acid (BCA) method with bovine serum albumin (BSA) as the standard.

In-Solution digestion and iTRAQ labelling. After protein quantification, approximately 200 µg of protein was digested with trypsin overnight at 37 °C in a 1:50 trypsin-to-protein mass ratio. After digestion, peptides were reconstituted in 0.5 M triethylammonium bicarbonate (TEAB), and an 8-plex iTRAQ kit (AB Sciex, Framingham, MA, USA) was implemented following the manufacturer’s protocol. Briefly, one unit of thawed iTRAQ reagent was reconstituted in 70 L of isopropanol. Peptides from the salt treatment and control groups were marked with different iTRAQ tags by incubation for 2 h at room temperature. The peptide mixtures were then combined and dried by vacuum centrifugation. The pooled mixtures of labelled peptides were separated by strong cationic exchange (SCX) chromatography.

hpRP chromatography. High-pH reverse-phase fractionation (hpRP) chromatography was performed using a Dionex UltiMate 3000 high-performance liquid chromatography (HPLC) system with built-in micro collection options for autosampling and ultraviolet (UV) detection. iTRAQ-labelled tryptic peptide was reconstituted in buffer A (20 mM NH₄HCO₃, pH of 10) and loaded onto a Gemini-NX C₁₈ column (3 µm, 2 × 150 mm, 110 A, Phenomenex) with 20 mM NH₄HCO₃ as buffer A and 80% acetonitrile (ACN) + 20% 20 mM NH₄HCO₃ as buffer B. The peptide was eluted at a flow rate of 200 µL/min with a gradient of 0–5% buffer B for 10 minutes and then 5–15% buffer B for 5 minutes. Elution with 15–50% buffer B lasted for 45 minutes, and with 50–90% buffer B lasted for 10 minutes. Twenty-four fractions were collected at 1-minute intervals.
were dissolved in buffer A [0.1% formic acid, 2% ACN] and pelleted at 13,500 rpm for 20 minutes. Nano-LC-MS/MS was performed using a Q Exactive mass spectrometer (Thermo Scientific). The supernatant was identified with the Q Exactive system (Thermal Scientific) after being loaded onto analytical columns. Mobile phase A was formic acid (0.1%) and mobile phase B was 80% ACN with 0.1% formic acid. The flow rate was set at 300 nL/min for the analytical columns, and the peptides were analysed with a 3-step gradient (80% ACN in 0.1% formic acid from 4% to 50% over 45 minutes, from 50% to 90% over 5 minutes and kept at 90% for 5 minutes) for 65 minutes. The primary MS parameters included a scan range of 350 to 1,800 m/z with a resolution of 70,000 and a maximum injection time of 40 ms. The second-grade MS spectra were obtained at a resolution of 17,500 with a maximum injection time of 60 ms, and the 20 top precursors per MS cycle were selected.

**Protein identification and data analysis.** Protein data analysis was performed according to the method used in a previous study on *Pseudostellaria heterophylla* [23]. Briefly, the initial raw files were converted into mgf files via Proteome Discoverer 1.4 (Thermo, American). Then, Protein Pilot 5.0 (AB Sciex, USA) was used to analyse the mgf files. The Paragon algorithm integrated in Protein Pilot 5.0 was used for database searching. The custom database consisted of protein sequences predicted from RNA data.

The parameter settings were as follows: "Done ID" mode with 95% confidence, iTRAQ peptide labelling, and Cys and trypsin digestion by methyl methanethiosulfonate (MMTS) oxidation. To increase confidence levels, proteins with iTRAQ ratios above 20 or below 0.05 were excluded, and only proteins with a reasonable ratio in all channels were considered quantifiable. Further functional analysis was performed for differential protein expression analysis, including whether the proteins were downregulated or upregulated. The change was determined compared to the CK, and p < 0.05 in the t test was used to indicate a significant difference between *Spica Prunellae* cultivated under salt stress and its blank control. The protein showing an average fold change of ≥ 1.3 or ≤ 0.77 in the experiment and with a minimum of two peptide matches were considered significantly differentially expressed. QuickGO software was used for Gene Ontology (GO) analysis of differentially expressed proteins (DEPs); the software searched the databases most commonly used in bioinformatics research to generate biological process, molecular function and cellular composition information for *Spica Prunellae*. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/pathway.html) was used to exploit the knowledge of current biochemical pathways and other types of molecular interactions.

**Results**

**Primary data and protein profiling.** Mascot software was used to analyse all MS/MS spectra. In total, there were 179,744 spectra, among which 54,837 spectra were quantified. Subsequently, 18,745 distinct peptides were found; based on these peptides, we obtained 5,066 primary proteins, and 1,937 of the primary proteins were retained (Fig. 1A, Table S1). The results of protein sequence and mass analysis indicated that approximately 78% of the identified proteins contained at least two peptides (Fig. 1B). More than 49% of the peptide sequences exhibited a sequence coverage of more than 10%, and more than 24% of the proteins exhibited a coverage of more than 20%, indicating high confidence (Fig. 1C). Regarding the protein mass distribution, 93% of the mass values were between 10 and 100 kDa, indicating a good average coverage.

**DEP classification.** Analysis of changes in protein profiles revealed DEPs (p value ≤ 0.05). Sixty-six proteins in the Na50 group increased in expression by 1.3 times or more and 132 decreased by 0.77 times or less compared to the CK group. The expression level of 80 proteins increased and of 98 proteins decreased in the Na100 group compared with the CK group (178 in total). The level of 142 proteins increased and the level of 202 proteins decreased in the Na150 group compared with the CK group (344 in total) (Fig. 2A, Table S2). Therefore, more protein species displayed a change in abundance in response to the 150 mM NaCl treatment than in response to the other treatments. A Venn diagram of these down- and upregulated protein species between the CK and salt treatments is shown in Fig. 2B.C. We identified proteins that were differentially expressed in *Spica Prunellae* among the three treatments with different salt concentrations. The DEPs of sucrose synthase, pyruvate decarboxylase, bifunctional aspartate aminotransferase, glutamate/aspartate-prephenate aminotransferase, pyruvate, orthophosphate dikinase, D-3-phosphoglycerate dehydrogenase and tyrosine aminotransferase were regulated by only a low salt concentration. These proteins are mainly related to the metabolism of carbohydrates and amino acids, which may indicate that the metabolism of carbohydrates and amino acids in *Spica Prunellae* is abnormally active in the early stage of salt stress to prepare for later adaptation to salt stress. However, the DEPs of glutathione S-transferase, peroxin-14, F-type H+ -transporting ATPase subunit alpha, L-ascorbate peroxidase, nicotinamide adenine dinucleotide (NADH), photosystem II P680 reaction center D1 protein, photosystem II CP47 chlorophyll protein, photosystem II CP43 chlorophyll protein, and photosystem II oxygen-evolving enhancer protein 2 were regulated only under high salt stress. Functional annotations showed that these DEPs were mainly related to photosynthesis and redox functions, which suggested that *Spica Prunellae* was more active in terms of photosynthesis and antioxidant function under high salt stress than under the other concentrations.

**GO and KEGG pathway enrichment analysis for DEPs.** GO and KEGG analysis of these DEPs was performed to unveil the global pattern of protein species abundance. For the GO analysis, comparing the Na50 and CK groups, in the biological process category, 18 proteins were related to biosynthetic process, followed by small molecule metabolic process (17), cellular nitrogen compound metabolic process (16), and response to stress.
Figure 1. Initial analysis of date and protein identification. (A) Basic protein mass distribution. (B) Peptide number distribution. (C) Protein mass distribution. (D) Distribution of proteins sequences coverage.

Figure 2. Identification and statistics analysis of DEPs under different salt concentration. (A) Number of up- or downregulated proteins between the CK group and different salt concentrations. (B) Venn diagram analyses for upregulated proteins. (C) Venn diagram analysis for downregulated proteins.
For the cellular component category, 20 proteins were related to intracellular and 20 to protein complex, followed by nucleus (13). In the molecular function category, ion binding was represented by 53 proteins, followed by oxidoreductase activity (28) (Fig. 3A, Table S3). In contrast, in the comparison of the Na100 group with the CK group, in the biological process category, biosynthetic process (with 13 proteins) was the dominant term, followed by small molecule metabolic process (12) and translation process (11). In the cellular component category, a total of 16 and 15 proteins were related to protein complex and intracellular, respectively, while ion binding (45) and oxidoreductase activity (20) were the major functions in the molecular function category (Fig. 3B, Table S4).

Furthermore, in the comparison of the Na150 and CK groups, in the biological process category, small molecule metabolic process was detected for 36 proteins, followed by translation (29), transport (28), and biosynthetic process (27). In the cellular component category, the majority of proteins (33) were associated with intracellular, followed by proteins (30), and cytoplasm (17). Finally, in the molecular function category, 90 proteins were related to ion binding, and 45 were related to oxidoreductase activity (Fig. 3C, Table S5).

As shown in Table 1, the 125 DEPs were further investigated using the KEGG database, and in Na50, they were found to be enriched in Carbon metabolism (with 16 proteins), Biosynthesis of amino acids (9), Ribosome (9), Oxidative phosphorylation (8), Photosynthesis (7) and Glycolysis/Gluconeogenesis (7). The top 10 enriched KEGG pathways in the Na100 group are shown in Table 2; Ribosome (with 7 proteins) was the most enriched,
followed by Photosynthesis and Carbon metabolism (7), and Biosynthesis of amino acids (6). In the comparison of the Na150 and CK groups (shown in Table 3), Carbon metabolism (with 21 proteins) was the most enriched pathway, followed by Ribosome (20), Biosynthesis of amino acids (14), RNA transport (13) and Photosynthesis (13).

**Discussion**

The ultimate effect of salt stress on plants is protein change. Soil salinity is one of the many serious challenges that plants currently face. Genomics-based technologies, including transcriptomic and proteomic technologies, have been widely used for proteomic analysis in many plant studies. This study represents the first time that iTRAQ has been used to provide a comprehensive explanation and accurate measurement of protein expression at different salt concentrations in the cultivated Chinese traditional herb *Spica Prunellae*. Protein is the ultimate driver of plant life activities. Although the expression level of proteins across the genome is generally stable under normal conditions, it always changes in response to a change in environmental conditions, such as salt stress. Different external environmental factors may induce changes in genome expression, which ultimately results in the differential expression of proteins and leads to the accumulation of differences in secondary metabolic processes. Therefore, proteomics research can help investigate the accumulation of the active constituents of *Spica Prunellae* and the mechanisms that affect its quality. For this research, iTRAQ was used to study the protein changes and related molecular mechanisms in *Spica Prunellae* under salt stress. Based on the results of the GO, KEGG and STRING analyses, 35-significant DEPs were divided into several categories; these DEPs and categories are discussed below (Table 4).

| KEGG Pathway | Pathway Name | Number of proteins |
|--------------|--------------|-------------------|
| ko03010      | Ribosome     | 7                 |
| ko01200      | Carbon metabolism | 7             |
| ko00195      | Photosynthesis | 7               |
| ko01230      | Biosynthesis of amino acids | 6          |
| ko00010      | Glycolysis/Gluconeogenesis | 5        |
| ko00190      | Oxidative phosphorylation | 4        |
| ko00620      | Pyruvate metabolism | 4        |
| ko04141      | Protein processing in endoplasmic reticulum | 4        |
| ko04015      | Rap1 signaling pathway | 3        |
| ko00940      | Phenylpropanoid biosynthesis | 3        |

Table 2. Top ten KEGG pathways for the identified protein species (CK vs Na100).

| KEGG Pathway | Pathway Name | Number of proteins |
|--------------|--------------|-------------------|
| ko01200      | Carbon metabolism | 21            |
| ko03010      | Ribosome     | 20                |
| ko01230      | Biosynthesis of amino acids | 14        |
| ko03013      | RNA transport | 13               |
| ko00195      | Photosynthesis | 13               |
| ko00190      | Oxidative phosphorylation | 10      |
| ko00010      | Glycolysis/Gluconeogenesis | 10   |
| ko04141      | Protein processing in endoplasmic reticulum | 9       |
| ko00710      | Carbon fixation in photosynthetic organisms | 8       |
| ko00620      | Pyruvate metabolism | 7        |

Table 3. Top ten KEGG pathways for the identified protein species (CK vs Na150).

Salt stress induces changes in transcription. Nucleic acid-binding, OB-fold-like protein was down-regulated in replication, recombination and repair progress. These three functions are very important in RNA transcription. Histone H2A 6 and Histone H2A 10 were downregulated in DNA binding and transcription process; their role is to stabilize the DNA structure and directly increase the transcription of proteins. These proteins participate in the promotion of DNA unwinding, replication and transcription and chromatin structure stability under salinity. This finding is consistent with the findings of previous reports on rice and wheat.

Salt stress induces signal transduction change. Calcium-transporting ATPase and Calmodulin 5 were upregulated while Calcium-binding EF-hand family protein, Calmodulin 7, Centrin2 and Nucleoside diphosphate kinase 1 were downregulated in Signal transduction mechanisms. In plants, the calcium signalling...
pathway plays a crucial role in initiating complex responses to stress conditions. In our proteomic analysis, several key calcium-signalling components were found to have an altered abundance. It is well established that these DEPs act as important Ca\(^{2+}\) signalling sensors in this signalling process\(^{29}\). Furthermore, several other protein species related to the calcium signalling pathway, including 14-3-3-like proteins, annexin, calreticulin, phospholipase C (PLC) and phospholipase D (PLD), accumulated under salt stress. For example, 14-3-3 proteins perform a key step in calcium mediated signal transduction by binding to phosphorylated target proteins\(^{30}\). Our results suggested that two 14-3-3-like protein species were significantly upregulated under salt stress especially in the Na\(^{100}\) treatment, which was consistent with the results of a previous study on salt-stressed sugar beet\(^{31}\). Generally, the abundance of the majority of these signalling-related protein species

| Accession no. | Gene name                  | Protein name                                                                 | CK vs Na50a | CK vs Na100b | CK vs Na150c |
|---------------|----------------------------|-------------------------------------------------------------------------------|-------------|-------------|-------------|
| Pr_PRUVU_94782_1 | AT1G12310                  | Calcium-binding EF-hand family protein; Potential calcium sensor              | 0.617       | 0.686       | 0.432       |
| Pr_PRUVU_93904_1 | CAM7                       | Calmodulin 7                                                                 | 0.512       | 0.709       | 0.380       |
| Pr_PRUVU_92776_2 | CEN1                       | Centrin2                                                                      | 0.694       | 0.747       | 0.484       |
| Pr_PRUVU_46794_1 | CAM5                       | Calmodulin 5                                                                 | 16.955      | 4.920       | 6.370       |
| Pr_PRUVU_63581_1 | NDPK1                      | Nucleoside diphosphate kinase 1                                              | 0.688       | 0.730       | 0.714       |
| Pr_PRUVU_104895_1 | ECA3                      | Calcium-transporting ATPase                                                   | 2.020       | 2.809       | 1.558       |
| Pr_PRUVU_93518_2 | ALDH2B4                    | aldehyde dehydrogenase 2B4                                                   | 1.489       | 1.352       | 2.536       |
| Pr_PRUVU_114134_1 | AT2G07698                  | ATP synthase subunit alpha                                                   | 1.616       | 1.690       | 2.145       |
| Pr_PRUVU_8875_2  | ATPA                       | ATP synthase subunit alpha                                                   | 0.531       | 1.387       | 2.595       |
| Pr_PRUVU_73033_1 | CYTC-2                     | Cytochrome c-2                                                               | 0.346       | 0.506       | 0.172       |
| Pr_PRUVU_92544_1 | AT2G34480                  | 60S ribosomal protein L18a-2                                                 | 1.589       | 1.673       | 1.799       |
| Pr_PRUVU_31752_1 | SKP1                       | 5 phase kinase-associated protein 1                                          | 0.530       | 0.740       | 0.418       |
| Pr_PRUVU_104895_1 | ECA3                      | Calcium-transporting ATPase                                                   | 2.020       | 2.809       | 1.558       |
| Pr_PRUVU_93904_1 | CAM7                       | Calmodulin 7                                                                 | 0.512       | 0.709       | 0.380       |
| Pr_PRUVU_92776_2 | CEN1                       | Centrin2                                                                      | 0.694       | 0.747       | 0.484       |
| Pr_PRUVU_46794_1 | CAM5                       | Calmodulin 5                                                                 | 16.955      | 4.920       | 6.370       |
| Pr_PRUVU_63581_1 | NDPK1                      | Nucleoside diphosphate kinase 1                                              | 0.688       | 0.730       | 0.714       |
| Pr_PRUVU_104895_1 | ECA3                      | Calcium-transporting ATPase                                                   | 2.020       | 2.809       | 1.558       |

Table 4. List of significant differential expressions of protein species identified in *Spica Prunellae* related to salt stress response. The abundance ratio of proteins in *Spica Prunellae* under 50 mM NaCl compared to the CK group. The abundance ratio of proteins in *Spica Prunellae* under 100 mM NaCl compared to the CK group. The abundance ratio of proteins in *Spica Prunellae* under 150 mM NaCl compared to the CK group.
was strikingly increased by salt stress, implying that the calcium signalling pathway plays an essential and positive role in the *Spica Prunellae* response to high salinity.

**Salt stress induces protein metabolism change.** S phase kinase-associated protein 1 was downregulated in ubiquitin-dependent protein catabolic process. Proteasome beta subunit C1 was downregulated in Posttranslational modification, protein turnover, and chaperones, which are included in protein metabolism. There were four upregulated and three downregulated proteins in *Spica Prunellae* that mainly participated in Translation, ribosomal structure and biogenesis in response to salt stress. 60 S ribosomal protein L18a-2, 40 S ribosomal protein S3-2, 60 S ribosomal protein L6-2, and 40 S ribosomal protein S16A were upregulated while 60 S ribosomal protein, 60 S ribosomal protein L22-2, and Nucleic acid-binding, OB-fold-like protein were downregulated in response to salt stress in *Spica Prunellae*. All these proteins participate in protein metabolism. Protein modification, as well as the balance between synthesis and degradation, is a main regulatory pathway that is coordinated to acquire a uniform cellular response to environmental stimulation33. Our proteomic analysis showed that 40 ribosomal protein species mainly belonging to two types (40 S and 60 S) exhibited marked overexpression in response to the salt treatments. Moreover, S phase kinase-associated protein 1 was downregulated in ubiquitin-dependent protein catabolic process. Proteasome beta subunit C1 was downregulated in Posttranslational modification, protein turnover, and chaperones, which are included in protein metabolism33. Most of these ribosomal protein species were elevated in abundance, potentially suggesting that plants cope with salt stress by accelerating protein synthesis to maintain the balance between the synthesis and degradation of proteins. Nonetheless, an obvious trend was observed in which most of these ribosomal protein species decreased in abundance from the low to high NaCl treatment. A reasonable explanation for this decrease may be that the activity of ribosomes is impaired with an elevated intensity of salt stress. Generally, the differential regulation of distinct translation components suggests that protein biosynthesis might be managed by a complex regulatory mechanism to cope with salt stress in *Spica Prunellae*.

**Salt stress induces carbohydrate and energy transport and metabolic change.** Of the proteins that participated in the progression of carbohydrate transport and metabolism related to salt stress, pyruvate kinase, Cinnamyl alcohol dehydrogenase 2, Glyceraldehyde-3-phosphate dehydrogenase and Glyceraldehyde-3-phosphate dehydrogenase B were upregulated under salt stress. Six main DEPs participated in energy production and conversion process; of these DEPs, Aldehyde dehydrogenase 2B4, ATP synthase subunit alpha, ATP synthase subunit alpha, Aldehyde dehydrogenase 2B4, and Pyruvate dehydrogenase complex E1 alpha subunit were upregulated, while Cytochrome c-2 was downregulated. In plants, the glycolysis and tricarboxylic acid (TCA) cycle are principal features of carbohydrate and energy metabolism that not only meet the energy demand but also give rise to many essential cofactors and substrates for other metabolisms34. Of these essential cofactors and substrates, the presence of pyruvate kinases suggested the action of glycolysis, which is required by plants for basic metabolism34. The abundance of most of these identified glycolytic enzymes was notably upregulated in our proteomic study, possibly indicating that glycolytic activity was induced under salt stress in *Spica Prunellae*. As a downstream reaction for glycolysis products in the mitochondria of aerobic organisms, the TCA cycle is responsible for the oxidation of respiratory substrates to drive ATP synthesis assisted by various enzymes. In the current proteomic study, the abundance of several key enzymes involved in the TCA cycle changed. Similar to the results for glycolysis, these results also indicated that the TCA cycle was active under salt stress, which is in contrast to the results observed for salt-stressed cotton17. This discrepancy suggests that a different salt stress mechanism occurs in *Spica Prunellae*. NADPH is involved in the defensive reaction, which can improve resistance to biotic and abiotic stress, such as viral invasion, salt stress, chilling stress, and oxidative stress36. Other studies have suggested that a high level of catalase (CAT) activity is necessary for plants to resist oxidative stress; at the same time, CAT is an important scavenger of superoxides37. CAT is an important antioxidant enzyme found widely in plants that can enhance defence capabilities35, and plays an important role in plant resistance to drought, salinity and heavy metals36,37. In conclusion, the number and expression of the upregulated proteins were higher than those of the downregulated proteins, so we surmise that the ability to respond to stress and the catabolism of oxidoreductases in salt-stressed *Spica Prunellae* were slightly stronger than those in the blank group.

**Salt stress induces stress and a change in defences.** Adenylate kinase and Dehydroascorbate reductase 2 were downregulated in stress and defense process under salt stress. H$_2$O$_2$ in roots can be eliminated by the ascorbate-glutathione (AsA–GSH) cycle. In this process, APX reduces H$_2$O$_2$ to H$_2$O using AsA, which undergoes a redox reaction catalysed by monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR)31. The activities and levels of these enzymes exhibited high diversity in roots under salt treatment. Proteomic studies indicate that the expression patterns of Dehydroascorbate reductase 2 vary among plants and salt treatments. Various APX isoforms in NaCl-treated roots of Arabidopsis and rice also showed differential changes between 6 h and 48 h or among different genotypes36. These findings reveal that long-term salt treatment affects the AsA–GSH cycle by decreasing the activity of Dehydroascorbate reductase 2 in certain plants. This decrease leads to an increase in the H$_2$O$_2$ content and damage to plants.

**Salt stress induces photosynthesis protein change.** Photosystem II D2 protein and Chlorophyll a-b binding protein CP29.2 were upregulated in Photosynthesis under intermediate and high salt stress but downregulated under low salt stress, while COP9 signalosome subunit 6 A was downregulated, which suggested that salt-tolerant plants increased salt stress by enhancing photosynthesis. This finding is consistent with the previous result. At high salt concentrations, plants enhance energy accumulation by enhancing photosynthesis38. Moreover, photosynthesis is a good strategy for resistance to reactive oxygen species (ROS) in plants, and the functional activity of chlorophyll is dependent on a low ROS content. Our results showed that under high salt...
stress, Chlorophyll a-b binding protein was upregulated to reduce ROS production and enhance photosynthesis to increase material accumulation, thus leading to salt stress resistance, which was consistent with the tolerance mechanism observed in corn43,44. Normally, salt stress increases the amount of oxidizing substances in plants. Plants with higher levels of antioxidant ability, either constitutive or induced, reportedly possess greater resistance to different types of environmental stress conditions23.

The regulatory network related to salt stress response. The mechanism of salt response in plants is a very complex process in which a variety of genes and response components are involved. In this study, to reveal the molecular mechanism of salt stress response in Spica Prunellae, a schematic network model was proposed based on the abundant protein information obtained in conjunction with an association analysis of transcriptomic data (Fig. 4). Stress can cause high concentrations of ROS in plant cells. If these substances are not removed by the antioxidant protection system in time, they will lead to oxidative damage and damage transfer. APX, as well as other peroxidases, such as CAT and SOD, has the ability to scavenge ROS. Salt stress is a kind of environmental adversity. Usually, in adverse environments, oxidative substances such as ROS will accumulate in plants, light-harvesting efficiency will be affected, protein synthesis will slow down, and signal transduction will slow down due to changes in osmotic pressure45. In the case of salt stress, plants usually initiate a series of defence mechanisms to transmit signals and activate a series of response mechanisms to increase tolerance. In our study, under salt stress, the accumulation of calcium ions in Spica Prunellae increased, and the response of plants to salt stress was activated. At the same time, the signalling molecules of calcium ions were upregulated to help transport calcium ions, which ultimately led to signal transmission and affected the expression of related genes. The gene transcription increased, accompanied by an increase in protein expression and the enhancement of genomic reorganization and repair ability.

Other consequences of salt stress include carbohydrate and protein metabolic disorders, which are all related to the homeostasis and dynamics of cells. Under salt stress, glycolysis and the TCA cycle are activated, resulting in increased energy metabolism. At the same time, protein metabolism is inhibited, more proteins are resistant to salt stress, and photosynthesis is enhanced, leading to more material accumulation, and resistance. Oxidative capacity is enhanced, which indicates that under salt stress, Spica Prunellae initiates a series of defence mechanisms to resist salt stress and increase tolerance.

Conclusions
To study the changes in global proteins under salt stress, we used NaCl as a source of salt stress to perform comparative proteomic analysis of Spica Prunellae seedlings. In salt-treated Spica Prunellae, 89 protein spots in the Venn diagram were found to be regulated by all three salt concentrations. Of these proteins, 35 were successfully chosen and identified. These identified proteins are involved in different metabolic pathways and processes, including cell rescue/defence, redox homeostasis, signal transduction, photosynthesis, carbohydrate metabolism, nucleotide metabolism, amino acid and nitrogen metabolism, protein biosynthesis, protein folding and assembly, proteolytic proteins, cellular processes, cell wall modifying proteins and unclassified. Some identified proteins may be potential candidates for increasing the salt tolerance of Spica Prunellae. Based on the proteomics data, the mechanism of salt stress response involving salt-responsive proteins was further proposed. Such protein analysis will allow us to further understand and describe possible strategies for managing the cellular activities occurring in the ears of salt-treated Spica Prunellae seedlings.

Figure 4. A schematic genetic regulatory network model of salt stress response in Spica Prunellae.
References

1. Liang, W., Ma, X., Wan, P. & Liu, L. Plant salt-tolerance mechanism: A review. Biochem Biophys Res Commun 495, 286–291 (2018).
2. Abd El-Mageed, T. A., Semida, W. M., Taha, R. S. & Rady, M. M. Effect of summer-fall deficit irrigation on morpho-physiological, anatomical responses, fruit yield and water use efficiency of cucumber under salt affected soil. Scientia Horticulturae 237, 148–155 (2018).
3. Katerji, N., van Hooorn, J. W., Hamdy, A. & Mastrorilli, M. Salinity effect on crop development and yield, analysis of salt tolerance according to several classification methods. Agricultural Water Management 62, 37–66 (2003).
4. Kalaji, H. M., Govindjee, Bosa, K., Kościelniak, J. & Zuó-Gołaszewska, K. Effects of salt stress on photosystem II efficiency and CO2 assimilation of two Syrian barley landraces. Environmental and Experimental Botany 73, 64–72 (2011).
5. Wiciarz, M., Niewiadomska, E. & Kruk, J. Effects of salt stress on low molecular antioxidants and redox state of plastoquinone and P700 in Arabidopsis thaliana (glycophyte) and Eutrema salsugineum (halophyte). Photosynthetic 56, 811–818 (2018).
6. Yu, Y. et al. Melatonin-Stimulated Triacylglycerol Breakdown and Energy Turnover under Salinity Stress Contributes to the Maintenance of Plasma Membrane H(+)–ATPase Activity and K(+)-Na(+) Homeostasis in Sweet Potato. Front Plant Sci 9, 256 (2018).
7. Zhou, X. et al. Functional analysis of SiDWF4 gene in response to salt stress in potato. Plant Physiol Biochem 125, 63–73 (2018).
8. Yan, H. et al. Overexpression of CuZnSOD and APX enhance salt stress tolerance in sweet potato. Plant Physiol Biochem 109, 20–27 (2016).
9. Shafti, A. et al. Transgenic Potato Plants Overexpressing SOD and APX Exhibit Enhanced Lignification and Starch Biosynthesis with Improved Salt Stress Tolerance. Plant Molecular Biology Reporter 35, 504–518 (2017).
10. Yang, Y. et al. Spica Prunellae extract suppresses the growth of human colon carcinoma cells by targeting multiple oncogenes via activating miR-34a. Oncol Rep 38, 1895–1901 (2017).
11. Sun, H. X., Qin, F. & Pan, Y. J. In vitro and in vivo immunosuppressive activity of Spica Prunellae ethanol extract on the immune responses in mice. J Ethnopharmacol 101, 31–36 (2005).
12. Mao, X., Wang, G., Zhang, W. & Li, S. A study on inhibitory effect of Spica prunellae extract on T lymphoma cell EL-4 tumour. Afr J Tradit Complement Altern Med 10, 318–324 (2013).
13. Lin, W. et al. Anti-angiogenic effect of Spica prunellae extract in vivo and in vitro. African Journal of Pharmacy and Pharmacology 5, 2647–2654 (2011).
14. Lin, W. et al. Spica prunellae promotes cancer cell apoptosis, inhibits cell proliferation and tumor angiogenesis in a mouse model of colorectal cancer via suppression of stat3 pathway. BMC Complement Altern Med 13, 144 (2013).
15. Zheng, L. Spica Prunellae extract promotes mitochondrion-dependent apoptosis in human colon carcinoma cell line. African Journal of Pharmacy and Pharmacology 5, 327–335 (2011).
16. Wu, J. et al. Spica prunellae and its marker compound rosmarinic acid induced the expression of efflux transporters through a activation of Nrf2-mediated signaling pathway in HepG2 cells. J Ethnopharmacol 193, 1–11 (2016).
17. Li, W. et al. Identification of early salt stress responsive proteins in seedling roots of upland cotton (Gossypium hirsutum L.) employing (TRAQ-based proteomic technique. Front Plant Sci 6, 732 (2015).
18. Fan, H., Xu, Y., Du, C. & Wu, X. Phloem sap proteome studied by iTRAQ provides integrated insight into salinity response mechanisms in cucumber plants. J Proteomics 125, 54–67 (2015).
19. Jiang, Q. et al. iTRAQ-based quantitative proteomic analysis of wheat roots in response to salt stress. Proteomics 17, 1–13 (2017).
20. Wang, B., Hajano, J. U., Ren, Y., Lu, C. & Wang, X. iTRAQ-based quantitative proteomics analysis of rice leaves infected by Rice stripe virus reveals several proteins involved in symptom formation. Virul 12, 1–21 (2015).
21. Jiang, Z. et al. Development of an Efficient Protein Extraction Method Compatible with LC-MS/MS for Proteome Mapping in Two Australian Seagrasses Zostera muelleri and Posidonia australis. Front Plant Sci 8, 1–14 (2017).
22. Hua, Y. et al. iTRAQ-based quantitative proteomic analysis of cultivated Pseudostellaria heterophylla and its wild-type. J Proteomics 139, 13–25 (2016).
23. Aghaee, K., Ehsanpour, A. A. & Komatsu, S. Potato responds to salt stress by increased activity of antioxidant enzymes. J Integr Plant Biol 51, 1095–1103 (2009).
24. Chen, T. et al. iTRAQ-Based Quantitative Proteomic Analysis of Cotton Roots and Leaves Reveals Pathways Associated with Salt Stress. PLoS One 11, e0144877 (2016).
25. Chu, P. et al. iTRAQ-based quantitative proteomics of Brassica napus leaves reveals pathways associated with chlorophyll deficiency. J Proteomics 113, 244–259 (2015).
26. Toone, W. M., Rudd, K. E. & Friesen, J. D. deaD, a new Escherichia coli gene encoding a presumed ATP-dependent RNA helicase, is associated with rice plasma membrane. J Protein Biochem Biophys Res Commun 307, 2144–2154 (2007).
27. Hua, Y. et al. iTRAQ-based Quantitative Proteomic Analysis of Cotton Roots and Leaves Reveals Pathways Associated with Salt Tolerance. J Proteomics 85, 504–518 (2017).
28. P700 in Arabidopsis thaliana (glycophyte) and Eutrema salsugineum (halophyte). Trends Cell Biol 17, 3291–3292 (1991).
29. Wang, M. et al. Proteomic analysis on a high salt tolerance introgression strain of Triticum aestivum/Thinopyrum ponticum. J Proteomics 125, 20–27 (2018).
30. Grant, M. P., Cavanaugh, A. & Breitwieser, G. E. 14-3-3 Proteins Buffet Intracellular Calcium Sensing Receptors to Constrain Signaling. PLoS One 10, 1–19 (2015).
31. Yang, L., Ma, C., Wang, L., Chen, S. & Li, H. Salt stress induced proteome and transcriptome changes in sugar beet monosomal addition line M14. J Plant Physiol 169, 839–850 (2012).
32. Hinkson, J. V. & Elias, J. E. The dynamic state of protein turnover: It's about time. Trends Cell Biol 21, 293–303 (2011).
33. Thornton, S., Anand, N., Purcell, D. & Lee, J. Not just for housekeeping: protein initiation and elongation factors in cell growth and tumorigenesis. J Mol Med (Berl) 81, 536–548 (2003).
34. Sweetlove, L. J., Beard, K. F. M., Núñez-Nesi, A., Fernie, A. R. & Ratcliffe, R. G. Not just a circle: flux modes in the plant TCA cycle. Trends in Plant Science 15, 462–470 (2010).
35. Plaxton, W. C. & Podestá, E. F. The Functional Organization and Control of Plant Respiration. Critical Reviews in Plant Sciences 25, 159–198 (2006).
36. Holscher, C. et al. Defects in Peroxisomal 6-Phosphogluconate Dehydrogenase Isoform PGD2 Prevent Gametophytic Interaction in Arabidopsis thaliana. Plant Physiol 171, 192–205 (2016).
37. Cabane, M., Afi, D. & Hawkins, S. Lignins and Abiotic Stresses. 61, 219–262 (2012).
38. Anderson, L. A., Johnson, A. K., Simms, M. D. & Willingham, T. R. Comparative analysis of catalasas: spectral evidence against heme-bound water for the solution enzymes. FERS Letters 370, 97–100 (1995).
39. Bailey, C. et al. Catalase activity and expression in developing sunflower seeds as related to drying. J Exp Bot 55, 475–483 (2004).
40. Dixit, V., Pandey, V. & Shyam, R. Differential antioxidative responses to cadmium in roots and leaves of pea (Pisum sativum L. cv. Azad). Journal of Experimental Botany 52, 1101–1109 (2001).
41. Miller, G., Suzuki, N., Cifci-Yilmaz, S. & Mittler, R. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ 33, 453–467 (2010).
42. Jiang, Y., Yang, B., Harris, N. S. & Deyholos, M. K. Comparative proteomic analysis of NaCl stress-responsive proteins in Arabidopsis roots. J Exp Bot 58, 3591–3607 (2007).
43. Ma, H. et al. Comparative proteomic analysis of seedling leaves of different salt tolerant soybean genotypes. J Proteomics 75, 1529–1546 (2012).
44. Stepien, P. & Klobus, G. Antioxidant defense in the leaves of C3 and C4 plants under salinity stress. Physiologia Plantarum 125, 31–40 (2005).
45. Deinlein, U. et al. Plant salt-tolerance mechanisms. Trends Plant Sci 19, 371–379 (2014).

Acknowledgements
This work was supported by a grant of the Priority Academic Program Development of Jiangsu Higher Education Institutions of China (No. yxxk-2014).

Author Contributions
X.L. designed the experiments, helped to write the manuscript and critically revised it. L.Z., C.C., H.Z., Y.Y. and C.W. conducted the experiment and performed data analysis. Z.L. provided research idea, supervised the experiments, performed numerical analyses, and wrote the article.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-46043-9.

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019