Comparative Proteomic Analysis of Two *Manilkara* Species Leaves Under NaCl Stress

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Abstract

**Background:** Salinity is a major environmental limiting factor, which affect agricultural production. The two *Manilkara* seedlings (*M. roxburghiana* and *M. zapota*) with high economic importance, could not adapt well to higher soil salinity and little is known about their proteomic mechanisms.

**Objectives:** The mechanisms responsible for the effects of salinity on the two *Manilkara* species leaves were examined by means of proteomic analysis.

**Material and Methods:** The seedlings were cultivated in a greenhouse and treated with NaCl. Leaves of control and the salt-stressed seedlings were sampled for phenol protein extraction. Proteins were separated by two-dimensional gel electrophoresis coupled with mass spectroscopy to study the change of proteins under different NaCl concentration.

**Results:** For *M. roxburghiana* leaves, 21 protein spots exhibited significant abundance variations between the control and the 6‰, 8‰ NaCl treatments, of these 13 proteins were identified. They included L-ascorbate peroxidase, chloroplast carbonic anhydrase, phosphoglycerate kinase, 5 heat-shock proteins (HSPs) which were all down-regulated; For *M. zapota* leaves, 35 protein spots exhibited significant abundance variations, then 24 proteins were identified, including 7 down-regulated HSPs as well as glyceraldehyde-3-phosphate dehydrogenase, Cell division protein, putative mitochondrial NAD-dependent malate dehydrogenase, ATP synthase, Rubisco large subunit-binding protein, Cytochrome c peroxidase.

**Conclusions:** Based on the common identified proteins between the two *M.* species, our results indicated that the identified proteins in the two *Manilkara* species were involved in carbohydrate metabolism, photosynthesis, defense and stress. HSPs exhibited variation strictly related to NaCl stress. The down-regulated HSPs meant the function to repair cells that have suffered damage weaken during stress process. Furthermore, except for HSP70 in *M. zapota* leaves, the HSPs in the two species were all small heat shock proteins (sHSPs) with molecular weights ranging from 15 to 42 kDa.

**Keywords:** 2-DE; MS; *Manilkara roxburghiana*; *Manilkara zapota*

1. Background

Salinity is a major environmental limiting factor which affect agricultural production. The majority of tropical fruit trees are salt sensitive and unable to adapt to soil salinity which result in economic losses and ecological destruction. As a result, there is strong interest in studying the physiological response or mechanisms of salinity tolerance in plants (1, 2). However, we know little about the main mechanisms about their lifecycle of plants under salinity stress (3). Significant progress has been made in understanding the mechanism at the cellular levels when plants are subjected to high salinity (4).

Comparative proteomics research on various plant species such as rice, wheat etc (5, 6) had been conducted to understand the molecular mechanism of plant response to stress. Three salt stress-responsive proteins in rice were identified by 2-DE (two-dimensional gel electrophoresis) and MS (mass spectroscopy) analysis found PvPR1 and PvPR2 specific protein in bean were induced under Cu stress (7). Ping Wang et al firstly...
reported *Gossypium hirsutum* parvulin-type PPlases involved in the salt stress response (8).

The two *Manilkara* species (*M. roxburghiana* and *M. zapota*), which natural habitat was tropical area such as Cuba or Brazil, are excellent tropical fruit trees not only for food but also for enjoy. They were introduced from Brazil to Xiamen overseas Chinese subtropical plant introduction garden (Xiamen city, Fujian province, China) in 1996. Now they grow and reproduce well in Xiamen city (24.26 N, 118.04E). The adaptability, biology characteristics, physiological characteristics and propagation techniques were already investigated in our previous study (9). However, the two 3-year-old *Manilkara* species seedlings showed salt injury on external characteristics under certain NaCl concentration and knowledge of proteomic analysis under NaCl were still limited. Here, differences in expression levels in the proteome of the two *Manilkara* species among different NaCl content treatment were examined.

2. Objectives

The mechanisms responsible for the effects of salinity on the two *Manilkara* species leaves were examined by means of proteomic analysis. This work further facilitates process of the biochemical mechanisms of their tolerance to NaCl stress on the impact of protein spectrum.

3. Materials and Methods

3.1. Plant Materials and Growth Conditions

*M. roxburghiana* seedlings grew in a greenhouse under a light/dark regime of 14/10 h at 28–30 °C, and relative humidity between 70–80%. The 3-year-old seedlings were then treated respectively with 0 (control), 0.2%, 0.4%, 0.6%, 0.8% NaCl for a period (2009.12.21–2010.06.16). The seedlings which were under 0.6%, 0.8% NaCl stress showed visible injury. The mature leaves were carefully collected and immersed into liquid nitrogen, and stored at -80 °C.

3.2. Protein Sample Preparation

Leaf samples (1 g) of control and NaCl-treated plants were ground in liquid nitrogen and homogenized in an extraction buffer containing 100 mg PVPP. The homogenate was suspended in 4 ml ice-cold phenol extraction buffer (0.7M sucrose; 0.1M KCl; 50mM EDTA, 0.5M Tris–HCl, 1% (w/v) DTT, pH 7.5; complete protease inhibitor cocktail (Roche Applied Science)) and immediately added 4 ml ice-cold Tris buffered phenol and vortexed for 10 min. After centrifugation (30 min, 3354 ×g, 4 °C) the phenolic phase was collected and the sample was re-extracted, then vortexed for 10 min and repeated twice. The supernatant was removed and the pellet was rinsed twice in ice-cold acetone/0.2% DTT. The sample was incubated for 60 min at -20 °C and then air-dried.

Protein concentration was determined by standard Bradford assay using bovine serum albumin as standard (10).

3.3. Two-dimensional Gel Electrophoresis (2-DE) Analysis

Protein (1mg) was subjected to IEF using an IPGphor III system (Gelifescience, Xiamen, China) with 24 cm IEP strips (Immobiline Drystrip™, pH 4–7) and then resolved on a 12.5% slab gel with SDS-PAGE. The gel was overlaid with 0.5% agarose (dissolved in running buffer containing bromophenol blue) and 2-DE was run using an Ettan DALTsix Vertical System (Gelifescience, USA) at 1 W/gel for 30 min, and then at 15 W/gel until the dye front reached the bottom of the gel. IEF was carried out as Wang (11).

3.4. Protein Visualization, MS Analysis and Quantification

After 2-DE, gels were scanned using a PowerLook1100 scanner (UMAX). After scanning, the protein spots were quantified using the vol. %. Those with 2 fold changes (p < 0.05) were considered to be differentially accumulated proteins in relative abundance in NaCl-treated plants compared to control. The significant spots were manually excised from silver stained 2-DE gels and digested with trypsin using a Spot Handling Workstation (100 μg protein per gel was added 12.5 ng Trypsin). Tubes containing the gel pieces were then placed into an air circulation incubator at 37 °C for 12 h. Trypsin digestion was carried out as Wang (11). After gel digestion, 1.4 L peptide solution was mixed with 0.4 L matrix in 30% acetonitrile (CAN) and 0.1% trifluoroacetic acid (TFA) before spotting onto the target plate. MALDI-TOF and tandem TOF/TOF MS were then carried out using an AB SCIEX MALDI-TOF-TOF™ 5800 Analyzer.

3.5. Peptide and Protein Identification by Database Search

Proteins were identified by searching against a comprehensive non-redundant sequence database used for database searching by MASCOT software search engine (12). The identification was mostly considered to be correct at a > 100% confidence interval for the protein score.

4. Results

4.1. Proteomic Analysis of Proteins in the Two *Manilkara* Species Leaves

Protein spots showing at least a 2-fold difference in abundance between control and treatments were selected and excised manually. The selection of a 2-fold change as an arbitrary threshold allowed us to focus on the most responsive
proteins for subsequent characterization (shown in Fig. 1).
The pH 4-7 immobile pH gradients were used to separate the different proteins under NaCl stress by IEF-SDS-PAGE comparing the control and the treatments under four different NaCl concentrations, and then the proteins in electrophoretogram were detected by the software of Image Master TM 2D Platinum. For *M. roxburghiana* leaves, the electrophoretograms were similar in all which meant the stability of proteins, but each of 3 ones had specificity in detail. 783 spots were detected in the map of A1. 925 spots in A2, and 1158 spots in A3. The similarity between the control and the others was 72.21% (A2 and A1), 65.84% (A3 and A1) in turn. The quantitative analysis of proteins revealed that a total of 21 proteins showed a more than 2 fold differences in expression values in the 3 stage of leaves. Of these, 18 proteins spots (spot 2-15, 18-21) showed a decrease in abundance. The abundance of spot 1, 16, 17 increased.

4.2. Indetification of Differentially Expressed Proteins of the Two Manilkara Species

For *M. roxburghiana* leaves, these 21 protein spots were subjected to identification by MALDI-TOF-TOF/MS and protein sequencing. Some of these proteins had no MS/MS data. Their theoretical MW and pI did not fit well to the experimental ones though they could be identified by PMF data. Their identities need to be further confirmed. Thus, a total of 13 were identified (Tab.1). They were phosphoglycerate kinase correlating carbohydrate metabolism, chloroplast carbonic anhydrase involved in photosynthesis, L-ascorbate peroxidase correlating anti-oxidation, 5 HSPs relating to defense and stress (Spot No.3,5-8) and 5 unknown proteins. They all were down-regulated except for Spot No.1 (predicted protein).

For *M. zapota* leaves, these 35 protein spots were subjected to identification and as a result a total of 24 were identified (Tab.2). They were cell division protein ftsH, ATP synthase, ankyrin-repeat protein relating to binding, 2 peptidyl-prolyl cis-trans isomerase, 3 Rubisco involved in photosynthesis, putative mitochondrial NAD-dependent malate

Figure 1. Comparison of 2-DE maps of the two Manilkara species leaves under NaCl stress. A1, A2, A3 were 2-DE maps of *M. roxburghiana* under 0, 0.6%, 0.8% NaCl stress respectively and the B1, B2, B3 were maps of *M. zapota* under 0, 0.6%, 0.8% NaCl stress respectively.
dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase which related to carbohydrate metabolism and 7 HSPs (Spot No. 10-11, 18, 21, 24-25, 35) which had defense and stress function, Cytochrome C Peroxidase which was antioxidant and 5 unknown proteins. Within the identified proteins, the cell division protein ftsH, 2 Rubisco, ATP synthase CF1 alpha subunit and all HSPs were down-regulated. The low numbers of identified protein and differentially expressed protein were partly caused by experiment skills which should be improved.

4.3. Functional Classification of Relevant Proteins under NaCl Stress

For M. roxburghiana leaves, the successfully identified protein spots were grouped according to the biological process (Fig. 2). The identified proteins fall into 4 functional categories including defense and stress (6 spots, 46%), photosynthesis (1 spot, 8%), carbohydrate metabolism (1 spot, 8%) and unknown (5 spots, 38%). While for M. zapota leaves, the 5 functional categories including defense and stress (10 spots, 42%), photosynthesis (4 spots, 16%), carbohydrate metabolism (2 spots, 8%), binding (3 spots, 13%) and unknown (5 spots, 21%).

4.4. The common of Identified Proteins Between the Two Manilkara Species

The common identified proteins between the two M. species were HSP, peroxidase and chloroplast protein (Tab 3. the unknown or hypothetical proteins were not listed). The common was as followed: Firstly, except for cell division protein ftsH in M. zapota leaves relating to binding, the proteins were involved in carbohydrate metabolism, photosynthesis, defense and stress. Secondly, all the proteins related to defense and stress were HSPs and most of the HSPs were sHSPs (15-42KDa). About the category of defense and stress, there were 5 HSP besides L-ascorbate peroxidase in M. roxburghiana leaves and 7 HSP besides Cytochrome c peroxidase in M. zapota leaves. The percentage of sHSP in HSP were 100% in M. roxburghiana.
leaves while 71% in M. zapota leaves (the others were HSP70). These results indicated that HSPs especially sHSPs exhibited variation strictly related to the M. species under NaCl stress.

5. Discussion

5.1. Photosynthesis Related Proteins

The protein spots involved in photosynthesis was 8% (chloroplast carbonic anhydride) for M. roxburghiana and 16% for M. zapota (chloroplast ribosomal protein S1, 3 Rubisco). Among these photosynthesis related proteins, chloroplast ribosomal protein and 1 Rubisco were up-regulated, the others were down-regulated. Chloroplast carbonic anhydride was reported to be associated with a Calvin cycle enzyme complex in Nicotiana tabacum (13). Studying on how wheat chloroplasts proteins respond to salt stress could be identified as marker proteins (14). The chlorophyll synthesis in the two Manilkara species was obviously restricted under 0.6%, 0.8% NaCl stress (10). Chloroplast carbonic anhydrase evidently correlated with the drop of photosynthesis for M. roxburghiana. Manaal et al. (14) studied two contrasting tomato genotypes seedlings cultivated under 0, 100 and 200 mM NaCl stress and found that some proteins related to the degree of genotype tolerance. The up-regulation of Rubisco activases and Rubisco large subunit was correlated with an increase in abundance level of proteins which are involved in energy metabolism (Malate dehydrogenase, Glucose-6-phosphate dehydrogenase, pyruvate dehydrogenase), especially in salt-tolerant genotype.

In the result that silicon nutrition and mycorrhizal inoculations improved growth, nutrient status, K+/Na+ ratio and yield of Cicer arietinum L. genotypes under salinity stress also showed that the Rubisco activity increased (15). As Miranda et al. (16) report, the improved tolerance to salinity stress in Sorghum bicolor plants was strongly correlated with the higher instantaneous carboxylation efficiency of Rubisco. As for M. zapota, it was Rubisco in induced proteins may correlate with the drop of photosynthesis.

Table 2. Identification of differentially expressed proteins of M. zapota leaves under NaCl stress by MALDI-TOF-TOF /MS

| Spot No. | Protein name | Regulated circumstances | Species | Accession No. NCBI | Protein MW | Protein PI | Score |
|----------|--------------|------------------------|---------|--------------------|------------|------------|-------|
| 1        | Glyceraldehyde-3-phosphate dehydrogenase | up-regulated | Vicia sativa | gi|296784038 | 40824 | 8.56 | 96 |
| 2        | Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic | up-regulated | Cucumis sativus | gi|266893 | 45909 | 7.57 | 152 |
| 3        | Putative ankyrin-repeat protein | up-regulated | Vitis aestivalis | gi|17625031 | 38089 | 4.53 | 159 |
| 4        | Peptidyl-prolyl cis-trans isomerase CYP38 | up-regulated | Arabidopsis thaliana | gi|186509663 | 39344 | 6.08 | 162 |
| 5        | Hypothetical protein Osl_20703 | up-regulated | Oryza sativa Japonica Group | gi|222635252 | 42622 | 6.20 | 207 |
| 7        | Cytochrome c peroxidase, mitochondrial precursor, putative | up-regulated | Ricinus communis | gi|255558656 | 40989 | 7.70 | 94 |
| 9        | Hypothetical protein SELMODRAFT_407197 | up-regulated | Selaginella moellendorfii | gi|302765154 | 22539 | 9.71 | 76 |
| 10       | Chloroplast heat shock protein 70-1 | down-regulated | Arabidopsis thaliana | gi|15233779 | 76575 | 5.07 | 245 |
| 11       | Heat shock protein 70 | down-regulated | Arabidopsis thaliana | gi|6746592 | 77230 | 5.13 | 249 |
| 12       | Unknown | down-regulated | Picea sitchensis | gi|148910696 | 71665 | 5.07 | 222 |
| 13       | Cell division protein ftSH1, putative | down-regulated | Ricinus communis | gi|255558698 | 75504 | 6.43 | 271 |
| 14       | Rubisco large subunit-binding protein subunit alpha, chloroplastic CPN-60 alpha | down-regulated | Brassica napus | gi|135130 | 57714 | 4.84 | 166 |
| 15       | Rubisco large subunit-binding protein subunit alpha, chloroplastic CPN-60 alpha | down-regulated | Brassica napus | gi|135130 | 57714 | 4.84 | 90 |
| 16       | Unknown protein product | down-regulated | Vitis vinifera | gi|296090101 | 21562 | 7.00 | 169 |
| 17       | Class I heat shock protein | down-regulated | Kandcha candel | gi|12401095 | 15250 | 5.58 | 121 |
| 18       | 17.7kDa heat shock protein | down-regulated | Helianthus annuus | gi|1235898 | 17662 | 6.19 | 89 |
| 19       | HSP19 class II | down-regulated | Citrus x paradise | gi|305755570 | 19111 | 8.01 | 120 |
| 20       | HSP19 class II | down-regulated | Citrus x paradise | gi|305755570 | 19111 | 8.01 | 136 |
| 21       | Chloroplast ribosomal protein S1 | up-regulated | Cucumis sativus | gi|117662841 | 10410 | 6.40 | 118 |
| 22       | Peptidyl-prolyl cis-trans isomerase, putative | up-regulated | Ricinus communis | gi|222635252 | 42622 | 6.20 | 207 |
| 23       | Putative mitochondrial NAD-dependent malate dehydrogenase | up-regulated | Solanum tuberosum | gi|21388550 | 36429 | 8.48 | 230 |
| 24       | Unknown | down-regulated | Populus trichocarpa | gi|118488171 | 92819 | 5.36 | 195 |
| 25       | ATP synthase CF1 alpha subunit | down-regulated | Hydrocotyle sp. SRD-2010 | gi|340034097 | 55938 | 5.35 | 298 |
| 26       | HSP19 class II | down-regulated | Citrus x paradise | gi|30575570 | 19111 | 8.01 | 97 |
5.2. Defense and Stress Related Proteins

HSPs play important roles in protecting plants against environmental stresses (17). They were generally divided into five conserved groups (HSPs, HSP60, HSP70, HSP90, HSP100) according to molecular mass (18). sHSPs are the most ubiquitous HSP subgroup with molecular weights ranging from 15 to 42 kDa (19), which play an important role in growth, defense and stress resistance (20). Under NaCl stress, all nine ThsHSPs genes were up-regulated at least one stress time-point in both roots and leaves of Tamarix hispida (21). DcHsp17.7 performs molecular chaperone activity in salt-stressed transgenic E. coli, and is involved in tolerance to salinity stresses (22). Overexpression of alfalfa mitochondrial HSP23 in prokaryotic and eukaryotic model systems confers enhanced tolerance to salinity stress (23). The two species shared in common to the highly conservative nonspecific HSPs kept down-regulating under the environmental stress. The degradation of HSPs showed that the defense function weakened with the increment of salinity. These results indicated that small HSPs (sHSPs) exhibited variation strictly related to NaCl stress. The peptidyl-prolyl cis-trans isomerase (PPIase) is important for response to high concentrations of NaCl (24) and played important roles in a variety of stress responsiveness. The purified recombinant G. hirsutum peptidyl-prolyl isomerase (GhPPI) could accelerate the initial velocity of the cis-trans conversion of peptidyl-prolyl bonds of tetrapeptide in a GhPPI concentration-dependent manner. Recombinant GhPPI also suppressed protein aggregation under denaturing conditions (8).

5.3. Carbon Metabolism Related Proteins and Other Proteins

Phosphoglycerate kinase (PGK) is involved in carbon fixation, following Rubisco as the next enzymatic step in the Calvin Cycle.

The expression of PGK under NaCl stress is different for different plants. Expression of major photosynthetic and salt-resistance genes in invasive reed lineages grown under elevated CO₂ and temperature showed that at 20% salinity, most genes were higher expressed in the future than in the ambient climatic conditions. However, the expression of PGK was not negatively affected by the salt treatment (25).

The analysis of salt-responsive proteins has indicated that changes in time-dependent expression of specific proteins occurs following salinization. Of the proteins identified, expression analysis identified only PGK altered specifically within 24 h (26).

The phosphoglycerate kinase (PGK) for M. roxburghiana was down-regulated, while the glyceraldehyde-3-phosphate dehydrogenase and NAD-dependent malate dehydrogenase for M. zapota were up-regulated. Compared to M. roxburghiana, M. zapota had more identified proteins relating to binding. Such as cell division protein ftsH, ATP synthase and ankyrin-repeat protein. Among those, cell division protein ftsH and ATP synthase were down-regulated, while ankyrin-repeat protein was up-regulated, under NaCl stress. The unknown proteins were regretfully comparatively large percent perhaps for the test technology.

| Protein name | Spot No. | Regulated circumstances | Protein name | Spot No. | Regulated circumstances |
|--------------|----------|-------------------------|--------------|----------|-------------------------|
| HSP 18.2     | 3        | down-regulated          | Glyceraldehyde-3-phosphate enase carbohydrate metabolism | 1        | up-regulated            |
| HSP 22.5     | 5        | down-regulated          | Rubisco      | 2        | up-regulated            |
| HSP 15.9     | 6        | down-regulated          | Ankyrin-repeat protein binding | 3        | up-regulated            |
| HSP19        | 7        | down-regulated          | Peptidyl-prolyl cis-trans isomerase CYP38 | 4        | up-regulated            |
| HSP17.5      | 8        | down-regulated          | Cytochrome c peroxidase | 7        | up-regulated            |
| L-ascorbate peroxidase | 13      | down-regulated | HSP 70 | 10      | down-regulated            |
| Chloroplast carbonic anhydrase | 14      | down-regulated | HSP 70 | 11      | down-regulated            |
| Phosphoglycerate kinase hydrate metabolism | 19      | down-regulated | Cell division protein ftsH binding | 13      | down-regulated            |
| -            | -        | -                       | Rubisco      | 14      | down-regulated            |
| -            | -        | -                       | Rubisco      | 15      | down-regulated            |
| -            | -        | -                       | HSP15.3      | 18      | down-regulated            |
| -            | -        | -                       | HSP17.7      | 21      | down-regulated            |
| -            | -        | -                       | HSP19        | 24      | down-regulated            |
| -            | -        | -                       | HSP19        | 25      | down-regulated            |
| -            | -        | -                       | Chloroplast ribosomal protein S1 | 26      | up-regulated            |
| -            | -        | -                       | Peptidyl-prolyl cis-trans isomerase | 27      | up-regulated            |
| -            | -        | -                       | NAD-dependent malate dehydrogenase carbohydrate metabolism | 29      | up-regulated            |
| -            | -        | -                       | ATP synthase CF1 alpha subunit | 33      | down-regulated            |
| -            | -        | -                       | HSP19        | 35      | down-regulated            |
5.4. Correlation between Molecular Characteristics Including Protein Expression and Apparent Characteristics

In our previous study, while the seedlings of two Manilkara species were under 0.6%, 0.8% NaCl stress, the leaves showed harm. The chlorophyll synthesis was obviously restricted. The contents of proline or soluble protein were higher than those of the control. In this paper, the HSPs were down-regulated in leaves under 0.6%, 0.8% NaCl stress. This result proved the correlation between the apparent characteristics and physiological change.

6. Conclusions

The mechanisms responsible for the effects of salinity on the two Manilkara species leaves were examined by means of proteomic analysis. In this study, we successfully identified proteins in the two M. species leaves that might be related to NaCl resistance. 2-DE coupled MS were applied to identify differentially expressed proteins resistant to NaCl. The identified proteins in the two M. species were involved in carbohydrate metabolism, photosynthesis, defense and stress. All the proteins related to defense and stress were HSPs and most of the HSPs were sHSPs. The sHSPs down-regulated during stress process may be responsible for two M. species relative to NaCl stress. These findings suggested that the identified proteins are providing important information for plant breeders to develop the seedling resistant to NaCl.

Acknowledgement

This work was supported by Xiamen Research Fund (project number 3502Z20092023, 3502Z20182009), State Key Laboratory of Marine Environmental. Plant Introduction & Quarantine and Plant Product Key Laboratory of Xiamen City.

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