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Research Article

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

Yumei Liu [1,*], Chongling Yan [2], Zhiyu Song [3], Shuang Zhou [3]

[1] Xiamen overseas Chinese subtropical plant introduction garden, Xiamen, Fujian, China
[2] Key Laboratory of the Ministry of Education for Coastal and Wetland Ecosystems, Xiamen University, Xiamen, Fujian, China
[3] Fujian Institute of Subtropical Botany, Xiamen, Fujian, China

[*] **Corresponding author:** Yumei Liu, Xiamen overseas Chinese subtropical plant introduction garden, Xiamen, Fujian 361006, China. Tel: +8613666055422; Fax: +8605922061150; E-mail: xcong@163.com

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 identificated 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 PPIases 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 provice, 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. roxburghian*a 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 (1g) of control and NaCl-treated plants were grounded 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 IPG strips (Immobiline Drystrip™, pH 4–7) and then resolved on a 12.5% slab gel with SDS-PAGEl. 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 immobiline 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 meaned 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.

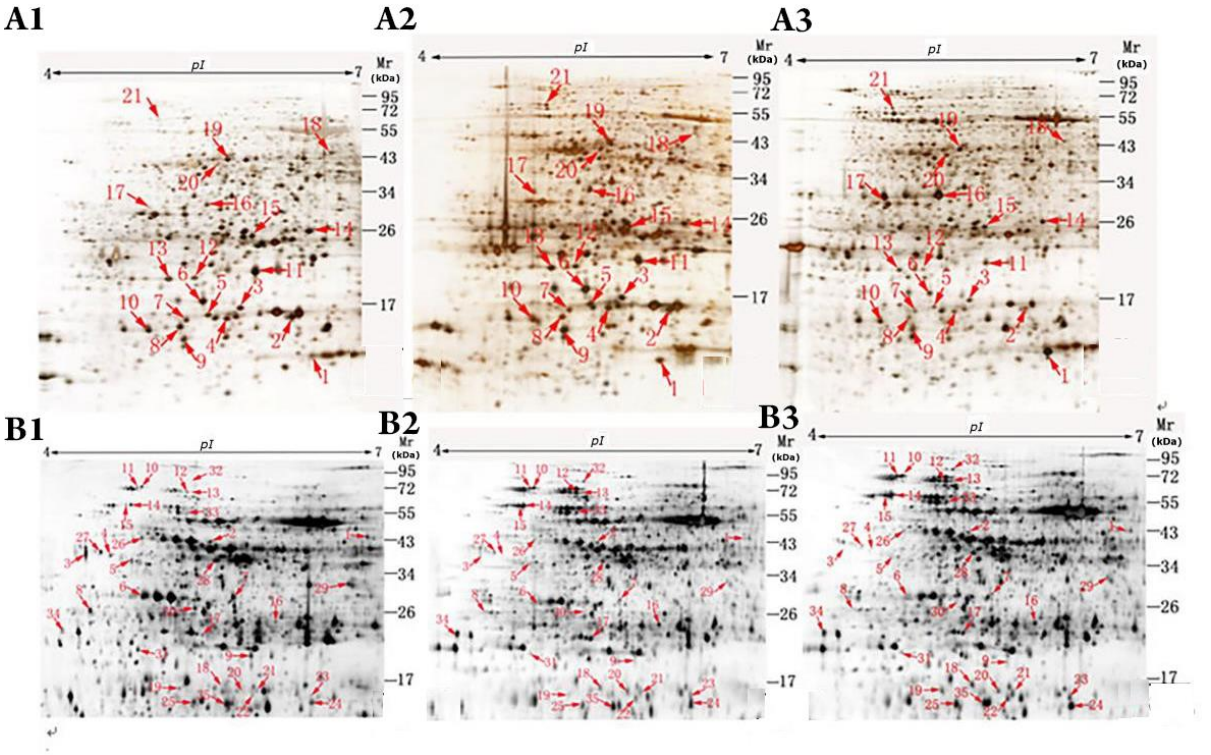

Figure 1. Comparison of 2-DE maps of the two *Manilkara* species leaves under NaCl stress.
A1, A2, A3 were 2-DE maps of *M. roxburghiana* under 0, 0.6%, 0.8% NaCl stress respectively and the B1, B2, B3 were maps of *M. zapota* under 0, 0.6%, 0.8% NaCl stress respectively.

For *M. zapota* leaves, the electrophoretograms were similar in all which meaned the stability of proteins, but each of 3 ones had specificity in detail. 1746 were detected in the map of B1, 1947 in B2, and 1997 in B3. The similarity between the control and the others was 57.62% (B2&B1), 58.83% (B3&B1) in turn. The quantitative analysis of proteins revealed that a total of 35 proteins showed a more than 2 fold differences in expression values in the 5 stage of leaves. Of these, 20 proteins spots (spot 10-25, 32-35) showed a decrease in abundance. The abundance of spot 1-9, 26-31 spots increased. For the two *Manilkara* species, the difference in spot numbers was mainly due to different species.

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 identificated (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). The method was adopted as much for *M. zapota* leaves, 35 protein spots were subjected to identification and as a result a total of 24 were identificated (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

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 identificated 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%), bingding (3 spots, 13%) and unknown (5 spots, 21%).

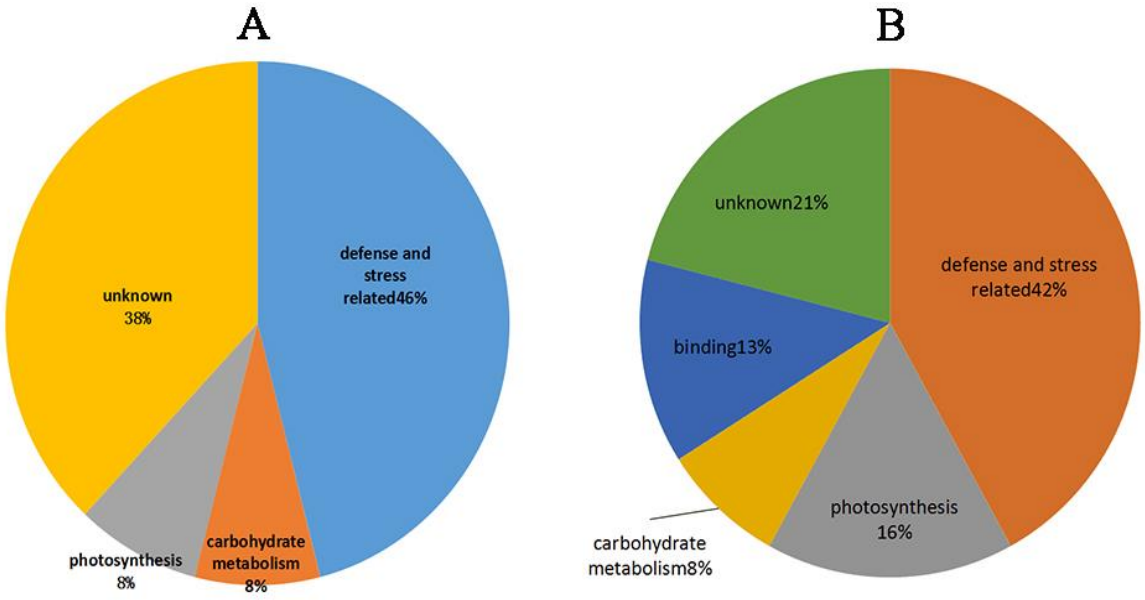

Figure 2. Functional categories and percentage in each functional category of identified protein spots differentially expressed in the two *Manilkara* species leaves under NaCl stress. A : *M. roxburghiana*; B : *M. zapota*

Table 1. Identification of differentially expressed proteins of *M. roxburghiana* leaves under NaCl stress by MALDI-TOF-TOF /MS

Spot No.	Protein name	Species	Accession No. NCBI	Protein MW	Protein PI	Score
1	Predicted protein	*Populus trichocarpa*	gi|224132044	18321	5.31	101
3	HSP 18.2	*Arabidopsis thaliana*	gi|15238509	18223	6.77	79
4	Predicted protein	*Populus trichocarpa*	gi|224132044	18321	5.31	100
5	HSP 22.5	*Ricinus communis*	gi|255558872	22520.6	8.76	209
6	HSP 15.9 (type 2 Cytosolic class I)	*Rhododendron rubropilosum*	gi|283482280	15931.2	5.22	147
7	HSP19 (class II)	*Citrus x paradisi*	gi|30575570	19111	8.01	138
8	HSP17.5 (Cytosolic class II)	*Rosa hybrid cultivar*	gi|140083605	17571.2	5.95	182
9	Predicted protein	*Populus trichocarpa*	gi|224132044	18321	5.31	100
13	L-ascorbate peroxidase	*Bruguiera gymnorhiza*	gi|289467897	27391	5.65	168
14	Chloroplast carbonic anhydrase	*Pachysandra terminalis*	gi|112292669	35635	6.46	133
15	Hypothetical protein	*Vitis vinifera*	gi|225452452	36920.8	8.06	173
18	Unnamed protein product	*Pisum sativum*	gi|20733	48079.8	7.7	195
19	Phosphoglycerate kinase	*Ricinus communis*	gi|255544584	50114	8.74	823

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 anhydrase) 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 anhydrase 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*. Manaa *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 indentificated 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|37625031	38089	4.53	159
4	Peptidyl-prolyl cis-trans isomerase CYP38	up-regulated	*Arabidopsis thaliana*	gi|186509663	39344	6.08	162
5	Hypothetical protein OsJ_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 moellendorffii*	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 ftsH, 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|1351030	57714	4.84	166
15	Rubisco large subunit-binding protein subunit alpha, chloroplastic CPN-60 alpha)	down-regulated	*Brassica napus*	gi|1351030	57714	4.84	90
17	Unnamed protein product	down-regulated	*Vitis vinifera*	gi|296090101	21562	7.00	169
18	Class I heat shock protein	down-regulated	*Kandelia candel*	gi|32401095	15250	5.58	121
21	17.7 kDa heat shock protein	down-regulated	*Helianthus annuus*	gi|1235898	17662	6.19	89
24	HSP19 class II	down-regulated	*Citrus x paradise*	gi|30575570	19111	8.01	120
25	HSP19 class II	down-regulated	*Citrus x paradise*	gi|30575570	19111	8.01	136
26	Chloroplast ribosomal protein S1	up-regulated	*Cucumis sativus*	gi|117662841	10410	6.43	118
27	Peptidyl-prolyl cis-trans isomerase, putative	up-regulated	*Ricinus communis*	gi|255552604	51547	4.97	84
29	Putative mitochondrial NAD-dependent malate dehydrogenase	up-regulated	*Solanum tuberosum*	gi|21388550	36429	8.48	230
32	Unknown	down-regulated	*Populus trichocarpa*	gi|118488171	92819	5.36	195
33	ATP synthase CF1 alpha subunit	down-regulated	*Hydrocotyle sp. SRD-2010*	gi|340034097	55938	5.35	298
35	HSP19 class II	down-regulated	*Citrus x paradise*	gi|30575570	19111	8.01	97

Table 3. The contrast of identified proteins between the two *Manilkara* species

M. roxburghiana			***M. zapota***		
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 kinasehydrate 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

5.2. 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 *Tamaris 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 a 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 CO_2 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 identificated 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.

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 identificated 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 seeding resistant to NaCl.

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