

Proteome Analysis of Roots in Cucumber Seedlings Under Iso-Osmotic NaCl and Ca(NO₃)₂ Stresses

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Abstract The growth and productivity of cucumber are severely affected by salinity. To understand the complex salt response mechanism, the physiological and biochemical responses of cucumber seedlings to iso-osmotic NaCl and Ca(NO₃)₂ stresses were investigated. In this study, the biomass was significantly decreased under iso-osmotic NaCl and Ca(NO₃)₂ stresses, and the inhibitory effect of Ca(NO₃)₂ stress was less than that of NaCl stress. The soluble protein contents were increased under Ca(NO₃)₂ stress, whereas they were decreased after 6 days of NaCl stress. A sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis revealed that there were 14 differentially expressed protein bands in roots under iso-osmotic NaCl and Ca(NO₃)₂ stresses at 0, 3, 6, and 9 days, and seven protein bands were little expressed under NaCl stress at 6 and 9 days. Based on these results, 2-D gel electrophoresis was used to separate cucumber root proteins in response to iso-osmotic NaCl and Ca(NO₃)₂ stresses at 3 days. A total of 43 protein spots changed under salt stress.

Of these proteins, 33 were successfully identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and liquid chromatography electro-spray ionization tandem mass spectrometry (LC-ESI-MS/MS) and categorized into classes, including those corresponding to antioxidants and defense-related proteins and energy and metabolism. The functions of the significantly differentially expressed root proteins were analyzed, which may facilitate a better understanding of different salt response mechanisms, and we suggest that cucumber seedlings showed a more powerful ability to resist Ca(NO₃)₂ stress.

Keywords Cucumber · NaCl stress · Ca(NO₃)₂ stress · Biomass · SDS-PAGE · 2-DE

Introduction

The secondary salinization caused by the overuse of chemical fertilizers has become a major limiting factor for crop growth and productivity and seriously limits the sustainable development of agricultural production in China (Yu et al. 2005). In greenhouse soils, the main cation and anion are Ca²⁺ and NO₃⁻, respectively, whereas Na⁺ and Cl⁻ are the main ionic forms present in seaside or inland soils (Zhang and Duan 2011).

Proteins are important biomacromolecules and play important roles regulating the metabolism of cells, and stress response proteins play important roles in plant resistance. The soluble protein could be separated using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) to find specific proteins related to salt tolerance. To understand the mechanisms of stress, proteomics has been employed to analyze protein changes in response to environmental changes (Oh et al. 2014). Proteomics is a thorough technique for

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analyzing the molecular mechanisms of stress responses in plants, and it allows for the indirect selection of inherited traits. Donnini et al. (2010) found that 57 differentially expressed proteins responded to Fe deficiency in cucumber roots. Li et al. (2013) found that 38 protein-associated spots changed in cucumber leaves exposed to salt stress.

Cucumber is an important horticultural crop, and it is highly sensitive to salinity. The plant root is the first organ to suffer from salt stress (Steppuhn and Raney 2005). Several previous studies have investigated the differentially expressed proteins in cucumber leaves or roots exposed to NaCl stress (Du et al. 2010; Li et al. 2013). However, there are no reports regarding the effects of iso-osmotic NaCl and $\text{Ca}(\text{NO}_3)_2$ stresses on proteomic changes in cucumber seedlings roots. The aim of this study was to analyze the specific proteins affected in cucumber roots grown under iso-osmotic NaCl and $\text{Ca}(\text{NO}_3)_2$ stresses, understand the response mechanism to different salt stress, and discuss the reasons of the different effects of two salt stresses on plant growth from the perspective of protein level.

Materials and Methods

Plant Material, Treatments, and Growth Conditions

Cucumber (*Cucumis sativus* L., cv. ‘Jinchun No. 2’) seeds were obtained from Tianjin Kernel Cucumber Research Institute, China. Seeds were germinated on filter papers in Petri dishes in the dark for approximately 24 h at 29 ± 1 °C, then the seeds were sown in sand and moved to a plastic greenhouse. After the full development of the second leaves, they were transplanted into plastic containers containing half-strength Hoagland’s nutrient solution (pH 6.5 ± 0.1 , EC 2.0 – 2.2 days m^{-1}). The nutrient solution was aerated using an air pump at an interval of 20 min to keep the dissolved oxygen concentration at 8.0 ± 0.2 mg L^{-1} during the experiment. The concentrations of NaCl and $\text{Ca}(\text{NO}_3)_2$ were selected based on a previous study of Tong (2012). The cucumber seedlings were divided into three groups. After pre-culturing for 3 days with half-strength Hoagland solution, the seedlings were treated using the following:

1. Cont, 0 mM NaCl+0 mM $\text{Ca}(\text{NO}_3)_2$
2. NaCl, 84 mM NaCl
3. $\text{Ca}(\text{NO}_3)_2$, 56 mM $\text{Ca}(\text{NO}_3)_2$

To prevent salt stimulation, each salt treatment was performed in two portions, on average, with the nutrient solution. The iso-osmotic solutions were 84 mM NaCl and 56 mM $\text{Ca}(\text{NO}_3)_2$, with osmotic potentials of -49.2 MPa. After 0, 3, 6, and 9 days of salt treatment, root samples were harvested, immediately frozen in liquid

nitrogen, and stored at -70 °C until processed for protein extraction.

Morphological Analyses

After 3, 6, and 9 days of treatment, the seedlings were sampled for the determination of plant height and root length. After leaves and roots were washed with deionized water and excess water was wiped off, their fresh weights were measured. After determining their fresh weights, leaves and roots were placed in a forced air oven at 105 °C for 15 min, then placed at 70 °C for 72 h to determine the dry weights.

Soluble Protein Content Assay

Root fresh samples (1.5 g) frozen at -70 °C were homogenized in 1.0 mL 30 mM Tris–HCl extraction buffer (pH 8.7, containing 0.7 M sucrose, 1 mM EDTA- Na_2 , 1 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonylfluoride (PMSF), 1 mM ASA, and 1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) on ice. The homogenate was centrifuged at $12,000 \times g$ for 15 min at 4 °C. The resulting supernatant was centrifuged again under the same conditions. The protein in the final supernatant was quantified according to the method of Bradford (1976) using bovine serum albumin as the standard.

SDS-PAGE Gel Electrophoresis

Proteins in roots were prepared according to the methods described by Huang et al. (2006) and were fractionated by 1-D SDS-PAGE using the Laemmli (1970) buffer system with 1-mm-thick 12.5 % separating gels and 1-mm-thick 5 % stacking gels. The concentrations of each protein were measured according to Bradford (1976) and then equivalently sampled. The electrode buffer was Tris–Gly (pH 8.3). The gels were stained with Coomassie Brilliant Blue R-250 (CBB) overnight and then washed until the background color turned transparent. The electrophoresis figures were produced using an Image Scanner. Analysis software (Gel Image System 1D version 4.1.2) was used for the banding strength analysis. Based on the electrophoretic mobility of standard proteins, the putative molecular weights of the unknown proteins were estimated. The standard protein used was a low SDS-PAGE molecular weight marker for proteins (MBI Fermentas).

Protein Extractions

The roots of cucumber seedlings were prepared for protein fractionation after 3 days of treatment. Harvested roots (2 g fresh weight) were finely ground in liquid nitrogen using a mortar and pestle, then the proteins were extracted according to the procedure described by Hurkman and Tanaka (1986).

The ground samples were suspended in 30 mM Tris–HCl (pH 8.7) containing 1 mM EGTA, 1 mM DTT, and 1 mM PMSF and then centrifuged at $15,000\times g$ for 20 min. An aliquot (1 mL) of the resulting supernatant was placed into a tube and precipitated with acetone containing 10 % tricarboxylic acid (TCA) and 0.07 % β -mercaptoethanol. The resulting protein-containing suspension was allowed to precipitate overnight at $-20\text{ }^{\circ}\text{C}$ and was then centrifuged at $20,000\times g$ for 30 min. The pellet was rinsed three times with cold acetone containing 0.07 % β -mercaptoethanol and allowed to stand at $-20\text{ }^{\circ}\text{C}$ for 1 h. Finally, the protein pellet was air dried, aliquoted, and stored at $-70\text{ }^{\circ}\text{C}$ until used in 2-D electrophoresis (2-DE).

2-DE

Isoelectric focusing (IEF) was performed according to the methods of Duncan and Hershey (1984) and O'Farrell (1975). Protein levels were quantified according to the Bradford (1976) method. The immobilized pH gradient (IPG) strips (pH 4–7, 17 cm length; Bio-Rad Laboratories) were rehydrated at room temperature for 12–16 h in 350 μL rehydration solution (8 M w/v urea, 1 M w/v thiourea, 2 % w/v CHAPS, 65 mM DTT, 0.8 % v/v IPG buffer 4–7, and 1 % w/v bromophenol blue) in a rehydration tray. Following rehydration, the IPG strips were run on an Ettan IPGphor 3 (GE Healthcare, USA). The voltage for IEF was set at 100 V for 1 h, 200 V for 1 h, followed by 200 V for 1 h, 500 V for 1 h, 1,000 V for 1 h, 4,000 V for 1 h, a gradient of 10,000 V for 1 h, and 10,000 V rapid focusing, reaching a total of 75,000 V h. The cell temperature was maintained at $20\text{ }^{\circ}\text{C}$ with a maximum current of 50 mA per strip. After running the first dimension, IEF strips were equilibrated for 15 min with 10 mL DTT buffer containing 6 M urea, 30 % (v/v) glycerol, 2 % SDS, 1 % w/v DTT, and 50 mM Tris–HCl (pH 8.8) and then with iodoacetamide buffer with 2.5 % w/v iodoacetamide instead of DTT for 15 min. 2-DE SDS-PAGE was performed using running gels (Hoefer SE600 Ruby Standard Vertical System, GE Healthcare; 12.5 % polyacrylamide) as described by Laemmli (1970). The strips were embedded on the top of the SDS gel and then sealed using 1 % molten agarose solution. Electrophoresis was carried out at 15 mA per gel until the bromophenol blue dye reached the bottom of the gel. After 2-DE, the gels were stained with CBB.

Image Acquisition and Data Analysis

The CBB-stained 2-D gels were scanned using an Image Scanner III (GE Healthcare). Spot detection, quantification, and matching were performed with Imagemaster™ 2D Platinum version 5.0 (GE Healthcare). At least three gels from each treatment in three independent experiments were used for the analysis. The intensities of spots were quantified based

on their relative volumes, which were determined by the ratio of the volume of a single spot to the whole set of spots. Only spots with significant (at least 1.5-fold quantitative changes) and reproducible changes in three replicates were analyzed by mass spectrometry. Student's *t* test with a significance level of 95 % was used for the statistical analysis of the gels. Only spots showing a statistically significant difference in protein abundance between the treatments were considered to be differentially expressed spots.

Image Analysis and Database Searching

Protein spots were excised from CBB-stained preparative polyacrylamide gels. Proteins were identified using a 5800 matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS) (AB SCIEX, Foster City, CA, USA) operating in a result-dependent acquisition mode. Peptide mass maps were acquired in positive ion reflector mode (2 kV accelerating voltage). Monoisotopic peak masses were automatically determined within the mass range of 800–4,000 Da. The most intense ions were selected as precursors for MS/MS acquisition, excluding common trypsin autolysis peaks and matrix ion signals. In MS/MS-positive ion mode, spectra were averaged, the collision energy was 2 kV, and the default calibration was set. The MS, together with MS/MS spectra, were searched against the National Center for Biotechnology Information (NCBI) Viridiplantae (V.2010.12.10, 184,045 sequences) database using the software GPS Explorer™, version 3.6 (Applied Biosystems). The parameters used for searching were: trypsin cleavage, one missed cleavage allowed; carbamidomethyl (C) set as a fixed modification; oxidation of methionines allowed as variable modification; peptide mass tolerance within 100 ppm; fragment tolerance set to ± 0.4 Da; and minimum ion score confidence interval for MS/MS data set to 95 %. Figure 1 describes a typical workflow of proteomic studies (including 2-D electrophoresis (1-DE) and 2-DE design) on plant tissues.

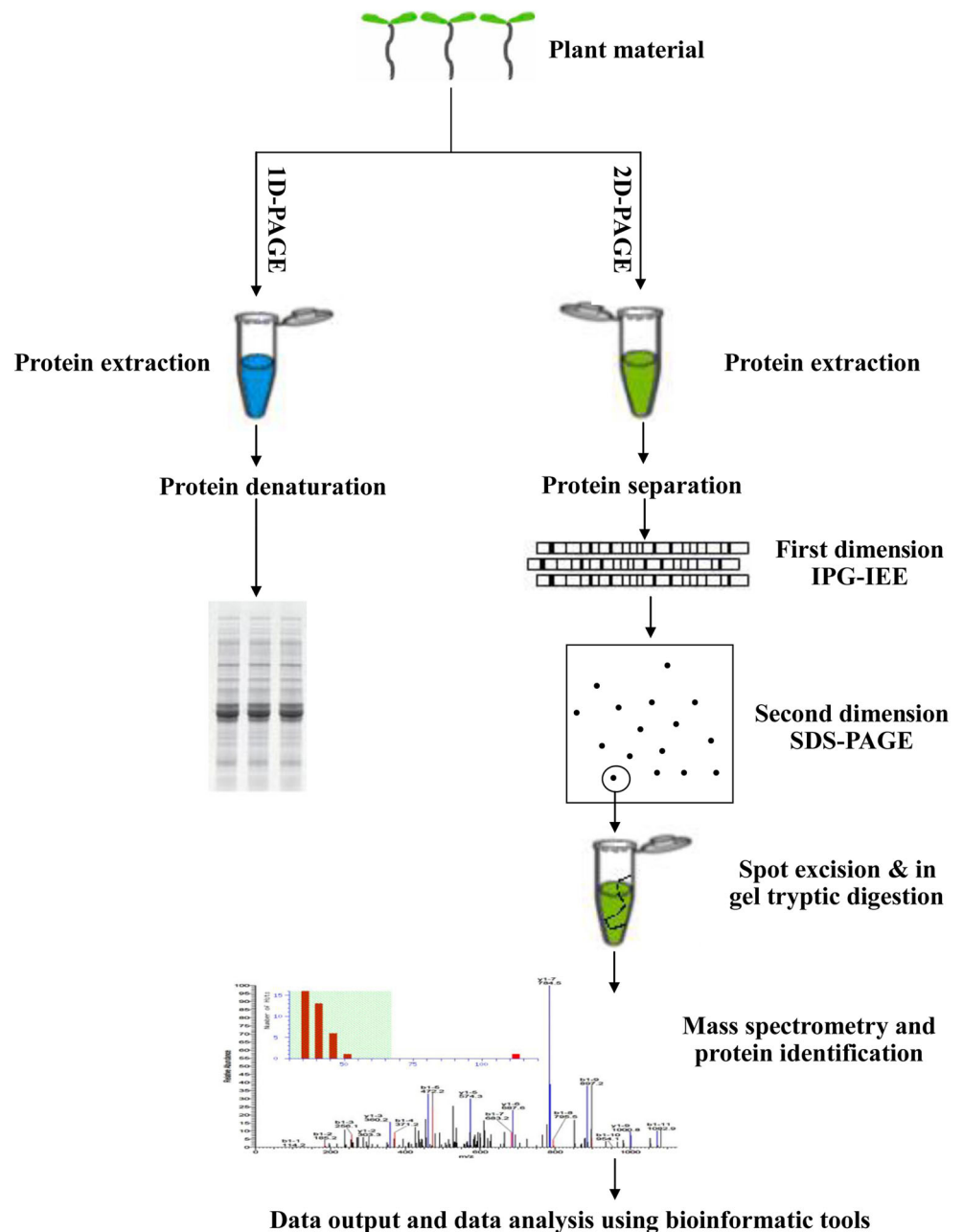
Results

Plant Biomass

The growth of cucumber was significantly influenced by NaCl and $\text{Ca}(\text{NO}_3)_2$ stresses. As shown in Table 1, salt stress damage to the plant was more obviously increased with the extension of salt treatment time. The plant height, root length, shoot fresh weight, root fresh weight, shoot dry weight, and root dry weight were remarkably affected in response to NaCl stress after 9 days with reductions of 67.6, 58.4, 70.0, 76.6, 66.1, and 70.4 %, respectively, while these values in response to $\text{Ca}(\text{NO}_3)_2$ stress after 9 days were reduced by 29.6, 39.1,

Fig. 1 A general workflow of proteomic experiments (including 1-DE and 2-DE design) in roots of cucumber seedlings.

According to the different methods, different proteins are extracted, which are used for 1-DE and 2-DE, respectively. Proteins are subjected to separation via gel (1-DE and 2-DE). As per the requirement, 2-DE protein mixtures convert into peptides. Separated peptides are analyzed through MS and MS/MS for determining protein identity. The detected protein list is then used for data analysis using various bioinformatic tools



42.8, 30.4, 27.0, and 18.3 %, respectively, as compared with the corresponding controls. Those results suggested that NaCl stress damage to the plant was much larger than $\text{Ca}(\text{NO}_3)_2$ stress on plant damage.

Soluble Protein Content in Cucumber Roots

Under NaCl stress, the soluble protein content began to reduce after 6 days of salinity and reached its lowest value of 20.5 % in roots at 9 days (Fig. 2). Under $\text{Ca}(\text{NO}_3)_2$ stress, the soluble protein content was remarkably increased by 34.7, 28.2, and 29.8 % at 3, 6, and 9 days, respectively. During the salting

period, the soluble protein contents under $\text{Ca}(\text{NO}_3)_2$ stress was always significantly higher than under NaCl stress.

SDS-PAGE Electrophoresis Analysis of Cucumber Root Proteins

SDS-PAGE electrophoresis profiles in cucumber seedling roots showed that each lane was about 26 protein bands, with relative molecular weights ($\times 10^3$) ranging from 10×10^3 to 150×10^3 . There were 14 differentially expressed protein bands in the different sampling times and treatments (Fig. 3). It may be that these 14 protein bands were related

Table 1 Effects of iso-osmotic NaCl and Ca(NO₃)₂ stress on growth of cucumber seedlings

Treatment time (days)	Salt treatment	Plant height (cm)	Root length (cm)	Shoot fresh weight (g/plant)	Root fresh weight (g/plant)	Shoot dry weight (g/plant)	Root dry weight (g/plant)
3	Cont	19.01±1.17 ^a	28.00±0.38 ^a	18.39±1.55 ^a	7.82±0.64 ^a	1.78±0.06 ^a	0.26±0.02 ^a
	NaCl	11.09±0.67 ^c	15.07±0.41 ^c	10.32±1.44 ^c	1.83±0.19 ^c	0.61±0.46 ^b	0.10±0.03 ^c
	Ca(NO ₃) ₂	16.18±0.76 ^b	19.24±0.57 ^b	14.66±0.90 ^b	3.79±0.29 ^b	1.28±0.08 ^a	0.18±0.01 ^b
6	Cont	32.85±1.35 ^a	44.05±2.01 ^a	35.47±1.85 ^a	13.87±0.58 ^a	3.24±0.09 ^a	0.51±0.07 ^a
	NaCl	11.99±0.51 ^c	17.92±0.64 ^c	12.30±0.91 ^c	3.36±0.13 ^c	1.24±0.11 ^c	0.17±0.02 ^c
	Ca(NO ₃) ₂	21.43±1.15 ^b	27.36±0.95 ^b	18.40±1.12 ^b	5.16±0.07 ^b	1.69±0.06 ^b	0.31±0.02 ^b
9	Cont	42.80±4.22 ^a	56.30±11.37 ^a	49.23±11.63 ^a	17.89±5.52 ^a	4.57±0.81 ^a	0.74±0.17 ^a
	NaCl	13.93±2.75 ^c	23.54±7.08 ^c	14.47±3.01 ^c	4.03±2.17 ^c	1.54±0.21 ^c	0.22±0.13 ^c
	Ca(NO ₃) ₂	30.68±2.18 ^b	34.79±8.27 ^b	27.94±4.19 ^b	12.33±2.66 ^b	3.40±0.62 ^b	0.58±0.11 ^b

to the salt stress tolerance of cucumber seedlings. There were seven protein bands with relative molecular weights ($\times 10^3$) of 87.7, 57.0, 49.1, 46.2, 34.3, 22.1, and 17.0 kDa expressed under all conditions. The expression levels of these protein bands were lower under Ca(NO₃)₂ stress than that under NaCl stress or in the controls at 3 days, and the expression levels were stronger under Ca(NO₃)₂ stress than that under NaCl stress at 6 and 9 days. The expression of seven protein bands with relative molecular weights ($\times 10^3$) of 132.2, 78.8, 71.6, 66.2, 61.6, 30.6, and 18.5 kDa almost disappeared under NaCl stress at 6 and 9 days.

According to the changes in protein expression levels under NaCl and Ca(NO₃)₂ stresses, further studies on the different effects of the two salt stresses on the cucumber root's proteome were performed.

2-DE Analysis of Root Proteomics

Triplicate gels were obtained from three independent experiments, and representative gels for Cont, NaCl, and Ca(NO₃)₂ treatments are illustrated in Fig. 4a–c. Using the modified TCA/acetone extraction protocol and CBB staining, the average number of detectable spots reached about 500 on each 2-DE gel. A total of 43 protein spots showed significant changes in a relative volume (>1.5 -fold) and were excised from the gels for MALDI-TOF/TOF MS analyses. A total of 33 proteins were successfully identified. The remaining 10 proteins did not show any significant hits in the database and hence were not considered. The MS and MS/MS data were searched against the cucumber genomics database (cucumber.genomics.org.cn) successfully. The identified spots are presented in Table 2. For most of the identified proteins, there were functional annotations in the databases, whereas one protein, spot 23, had no functional annotations.

Functional Classification of Differentially Expressed Proteins

An overall analysis of the gels revealed that out of the matching spots between the control and NaCl stress treatment, 60.6 % of proteins were significantly increased abundance, and 27.3 % of proteins were significantly decreased abundance (Fig. 4d). Out of the matching spots between the control and Ca(NO₃)₂ stress treatment, 51.5 % of proteins were significantly increased abundance, and 33.3 % of proteins were significantly decreased abundance (Fig. 4e).

A Venn diagram analysis of the quantitative expression patterns of the increased and decreased abundance of proteins from cucumber roots under NaCl and Ca(NO₃)₂ stresses is illustrated in subpanels f and g Fig. 4, respectively. As shown in Fig. 4f, 20 proteins under NaCl stress and 17 proteins under Ca(NO₃)₂ stress were increased abundance. Of these, 10 proteins were decreased abundance under both stresses. As shown in Fig. 4g, the expressions of nine proteins under NaCl stress and 11 proteins under Ca(NO₃)₂ stress were decreased. Of these, the expressions of three proteins were decreased under both stresses.

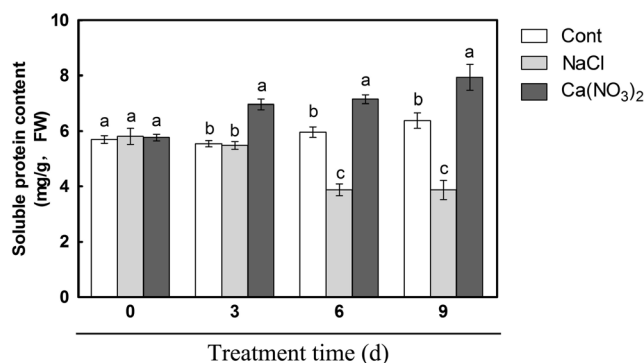
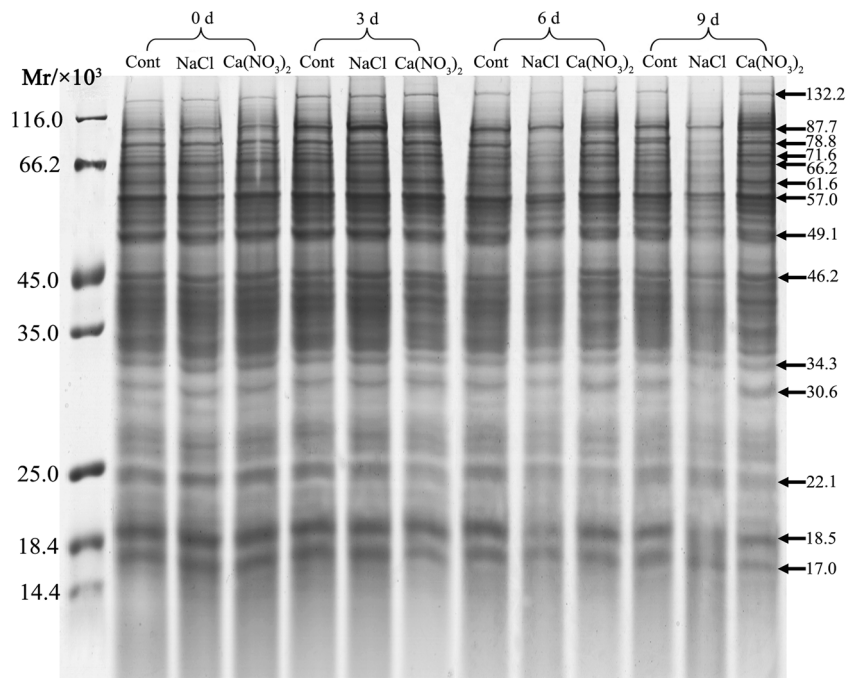


Fig. 2 Effects of iso-osmotic NaCl and Ca(NO₃)₂ stresses on the soluble protein content in roots of cucumber seedlings. Values are the means of three replicate samples. Letters indicate significant differences at $P < 0.05$ according to Duncan's multiple range tests

Fig. 3 Effects of iso-osmotic NaCl and $\text{Ca}(\text{NO}_3)_2$ stresses on soluble protein SDS-PAGE profiles in roots of cucumber seedlings



In this study, the functions of the identified proteins were classified into seven categories, involving antioxidant and defense (11 protein spots), energy and metabolism (nine protein spots), amino acid metabolism (two protein spots), protein synthesis, folding, and degradation (five protein spots), cell wall-related proteins (two protein spots), signal transduction (one protein spot), and other functional proteins (three protein spots) (Fig. 4h).

Discussion

Physiological Responses of Cucumber Seedlings and the Content and SDS-PAGE Electrophoresis Analyses of Cucumber Root Proteins in Response to Iso-Osmotic NaCl and $\text{Ca}(\text{NO}_3)_2$ Stresses

Growth inhibition is a common symptom observed in plants subjected to salt stress. The growth and biomass production of cucumber were considerably suppressed by salt stress, with more suppression under NaCl stress than under $\text{Ca}(\text{NO}_3)_2$ (Table 1).

Salt can decrease the protein content by inhibiting protein synthesis and accelerating protein degradation. It is also possible to increase the content of proteins by inducing new protein expression. Debouba et al. (2006) reported that the greater decrease in soluble protein content was associated with growth decrease. In this work, the soluble protein content was reduced under NaCl stress, while this value was significantly induced under $\text{Ca}(\text{NO}_3)_2$ stress in roots (Fig. 1). Under NaCl stress, there were no significant differences between salt

stress and control at 3 days in roots, which might be an adaptation mechanism of plants to environmental stresses (Yan et al. 2012). SDS-PAGE electrophoresis profiles in cucumber seedling roots showed that there were 14 differentially expressed proteins related to salt stress. Seven protein bands were expressed under all conditions, and the expression intensities of these protein bands had contrasting relationships between 3 and 6 and 9 days under NaCl and $\text{Ca}(\text{NO}_3)_2$ stresses. On the sixth and ninth days, seven protein bands were little expressed under the NaCl stress. It was similar to the change in soluble protein contents in roots. The results showed that the influence of NaCl stress on plant proteins was much larger than that of $\text{Ca}(\text{NO}_3)_2$ stress.

Most of the Antioxidant and Defense-Related Proteins Were Increased Abundance by Salt Stress

The exposure of plants to salt stress increased the production of reactive oxygen species (ROS), which cause oxidative damage to different cellular components, including membrane lipids, proteins, and nucleic acids (Apel and Hirt 2004; Tanaka et al. 2006; Zhu et al. 2000). Plants can develop antioxidant enzymatic system to scavenge these toxic compounds, and plant salt tolerance has been found to be positively associated with a more efficient antioxidant system.

In the present study, peroxidase 2-like (spot 20), which acts as a biological catalyst to scavenge H_2O_2 (Ikehata et al. 2005), was induced under NaCl stress but not changed under $\text{Ca}(\text{NO}_3)_2$ stress. Superoxide dismutase (SOD) is an essential antioxidative enzyme, which dismutates two superoxide radicals to produce hydrogen peroxide and oxygen. The

Table 2 Root proteins responsive to NaCl and Ca(NO₃)₂ stress identified by MALDI-TOF/TOF MS

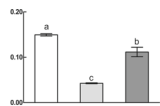
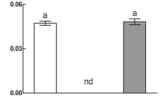
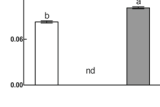
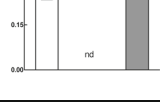
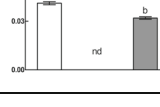
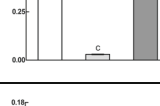
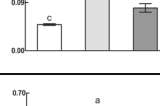
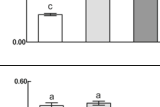
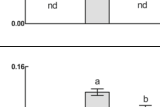
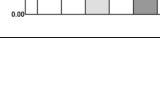
^a Spot No.	Protein name	Groups	Accession No.	^b TpI/EpI	^c TMr/EMr	Score	^d MP	^e Cov (%)	^f Protein expression (%vol) A B C
1	Protein disulfide-isomerase-like [<i>Cucumis sativus</i>]	Protein synthesis, folding, and degradation	gi 449464162	4.88/4.87	57.3/68.0	67	11	30.39	
5	Malate dehydrogenase, cytoplasmic-like [<i>Cucumis sativus</i>]	Energy and metabolism	gi 449458824	5.76/5.50	36.1/24.6	408	10	41.14	
9	Acetyl-CoA acetyltransferase, cytosolic 1-like [<i>Cucumis sativus</i>]	Energy and metabolism	gi 449453612	6.25/6.68	41.9/43.1	297	12	51.60	
10	Fructose-bisphosphate aldolase, cytoplasmic isozyme-like [<i>Cucumis sativus</i>]	Energy and metabolism	gi 449440201	6.49/6.69	38.9/39.9	285	13	45.53	
11	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic-like [<i>Cucumis sativus</i>]	Energy and metabolism	gi 449503692	6.62/6.74	36.5/37.9	96	6	24.41	
13	Peptidyl-prolyl cis-trans isomerase 1-like [<i>Cucumis sativus</i>]	Protein synthesis, folding, and degradation	gi 449527215	8.68/6.87	18.3/17.5	329	11	82.56	
15	Luminal-binding protein 5 [<i>Cucumis melo</i>]	Antioxidant and defense related proteins	gi 659074058	5.10/4.77	73.7/83.8	131	21	40.45	
16	Enolase [<i>Cucumis melo</i>]	Energy and metabolism	gi 659125144	5.40/5.50	47.9/58.3	409	12	40.99	
17	Peroxidase [<i>Cucumis sativus</i>]	Antioxidant and defense related proteins	gi 167533	4.94/4.67	34.8/38.4	445	8	42.24	
18	Actin-7 [<i>Cucumis melo</i>]	Cell wall-related	gi 659080849	5.31/5.39	41.9/50.1	265	5	19.36	
19	Malate dehydrogenase, cytoplasmic [<i>Cucumis melo</i>]	Energy and metabolism	gi 659121409	5.75/5.71	36.1/36.8	445	11	52.55	

Table 2 (continued)

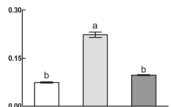
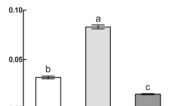
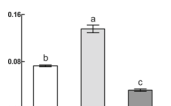
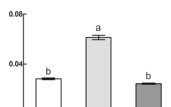
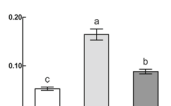
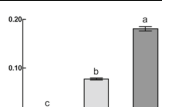
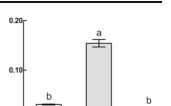
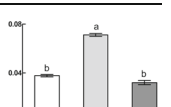
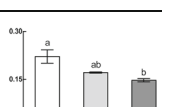
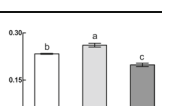
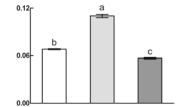
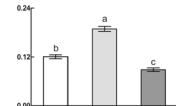
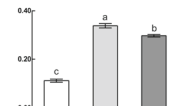
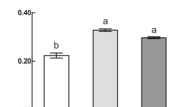
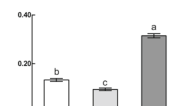
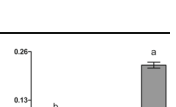
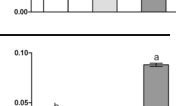
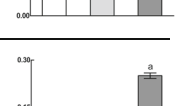
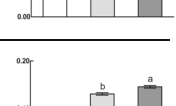
20	Peroxidase 2-like [<i>Cucumis sativus</i>]	Antioxidant and defense related proteins	gi 449467745	5.64/6.37	37.7/38.1	732	13	39.83	
21	Fructose-bisphosphate aldolase, cytoplasmic isozyme-like [<i>Cucumis sativus</i>]	Energy and metabolism	gi 449451108	7.57/6.93	38.7/39.0	835	21	57.54	
23	Uncharacterized protein At2g39795, mitochondrial [<i>Cucumis sativus</i>]	Other function proteins	gi 449441362	4.69/4.46	28.8/27.3	96	6	33.20	
24	Proteasome subunit alpha type-5 [<i>Cucumis melo</i>]	Protein synthesis, folding, and degradation	gi 659118266	4.75/4.74	26.1/26.6	354	7	41.35	
25	Caffeoyl-CoA O-methyltransferase 1-like [<i>Cucumis sativus</i>]	Cell wall-related	gi 449438410	5.29/5.28	28.1/31.7	491	13	71.89	
26	Ascorbate peroxidase [<i>Cucumis sativus</i>]	Antioxidant and defense related proteins	gi 315115497	5.32/5.52	27.5/29.7	227	11	51.81	
27	Ascorbate peroxidase [<i>Cucumis sativus</i>]	Antioxidant and defense related proteins	gi 315115497	5.32/5.67	27.6/28.3	478	9	45.38	
28	Glutathione S-transferase DHAR2-like [<i>Cucumis sativus</i>]	Antioxidant and defense related proteins	gi 449459946	6.18/6.35	23.9/31.0	373	11	66.67	
29	Glutathione S-transferase-like [<i>Cucumis sativus</i>]	Antioxidant and defense related proteins	gi 449498465	5.98/6.31	24.0/26.6	176	8	52.09	
30	Acylpyruvate FAHD1, mitochondrial-like [<i>Cucumis sativus</i>]	Other function proteins	gi 449432876	6.21/6.45	23.6/26.9	86	8	47.73	
31	Flavoprotein WrBA-like [<i>Cucumis sativus</i>]	Antioxidant and defense related proteins	gi 449466103	6.43/6.44	21.7/24.1	515	6	43.84	
32	Flavoprotein WrBA-like [<i>Cucumis sativus</i>]	Antioxidant and defense related proteins	gi 449466103	6.43/6.43	21.7/23.3	444	7	51.23	

Table 2 (continued)

33	Translationally-controlled tumor protein homolog [<i>Cucumis sativus</i>]	Signal transduction	gi 449432858	4.56/4.64	18.8/19.8	253	5	41.67	
34	Glycine cleavage system H protein, mitochondrial-like [<i>Cucumis sativus</i>]	Amino acid metabolism	gi 449455395	4.98/4.57	16.9/15.2	68	5	42.58	
35	MLP-like protein 328 [<i>Cucumis melo</i>]	Antioxidant and defense related proteins	gi 659098664	5.07/5.17	17.4/14.8	295	8	60.27	
36	Superoxide dismutase [Cu-Zn]-like [<i>Cucumis sativus</i>]	Antioxidant and defense related proteins	gi 449485788	5.43/5.76	15.5/15.1	78	4	36.84	
37	NAD-dependent dihydropyrimidine dehydrogenase subunit PreA-like [<i>Cucumis sativus</i>]	Other function proteins	gi 449439483	6.07/5.48	46.5/47.2	207	14	45.76	
38	S-adenosylmethionine synthase 1-like [<i>Cucumis sativus</i>]	Energy and metabolism	gi 449436329	5.50/5.69	43.7/47.0	253	16	55.98	
39	S-adenosylmethionine synthase 2-like [<i>Cucumis melo</i>]	Energy and metabolism	gi 659122902	5.58/5.69	43.6/44.8	290	11	37.91	
40	Cysteine synthase-like [<i>Cucumis sativus</i>]	Amino acid metabolism	gi 449442895	5.92/5.92	34.6/36.0	348	11	41.41	
41	Cysteine proteinase RD19a-like [<i>Cucumis sativus</i>]	Protein synthesis, folding, and degradation	gi 449464688	6.07/5.51	41.4/35.4	404	8	28.65	
43	40S ribosomal protein S12 [<i>Cucumis melo</i>]	Protein synthesis, folding, and degradation	gi 659122493	5.76/5.90	15.2/15.0	142	7	72.15	

^a Spot numbers are given in Fig. 1^b TpI and EpI are the theoretical isoelectric point and experimental isoelectric point, respectively^c TMr and EMr are the theoretical molecular mass and experimental molecular mass, respectively^d The total number of identified peptides^e Percentage of sequence coverage by matched peptides^f The relative levels of protein expression. A: Control; B: NaCl; C: Ca(NO₃)₂

overexpression of different types of SODs is known to confer protection against oxidative stress (Bowler et al. 1992; Mckersie et al. 1996). In this study, we observed an increased abundance of SOD [Cu-Zn]-like (spot 36) under both NaCl and $\text{Ca}(\text{NO}_3)_2$ stresses. Under NaCl stress, two ascorbate peroxidases (APXs, spots 26 and 27) and two glutathione *S*-transferases (GST, spots 28 and 29) were all significantly increased abundance, whereas under $\text{Ca}(\text{NO}_3)_2$ stress, only one APX (spot 26) and one GST (spot 29) were significantly increased abundance. An increase in luminal-binding protein 5 (BiP 5,

spot 15) was observed under both stresses. Recently, the ER-bound HSP70 luminal-binding protein (BiP) was found to be a main molecular chaperone regulating the biogenesis and degradation of membrane-bound immune receptors (Kim et al. 2014). Soybean BiP can enhance the capacity of drought resistance and delay the leaf senescence induced by drought (Irsigler et al. 2007; Valente et al. 2009). The expression of flavoprotein WrbA-like (spot 32) was increased abundance under NaCl stress, whereas these proteins (spots 31 and 32) were decreased abundance under $\text{Ca}(\text{NO}_3)_2$ stress. The

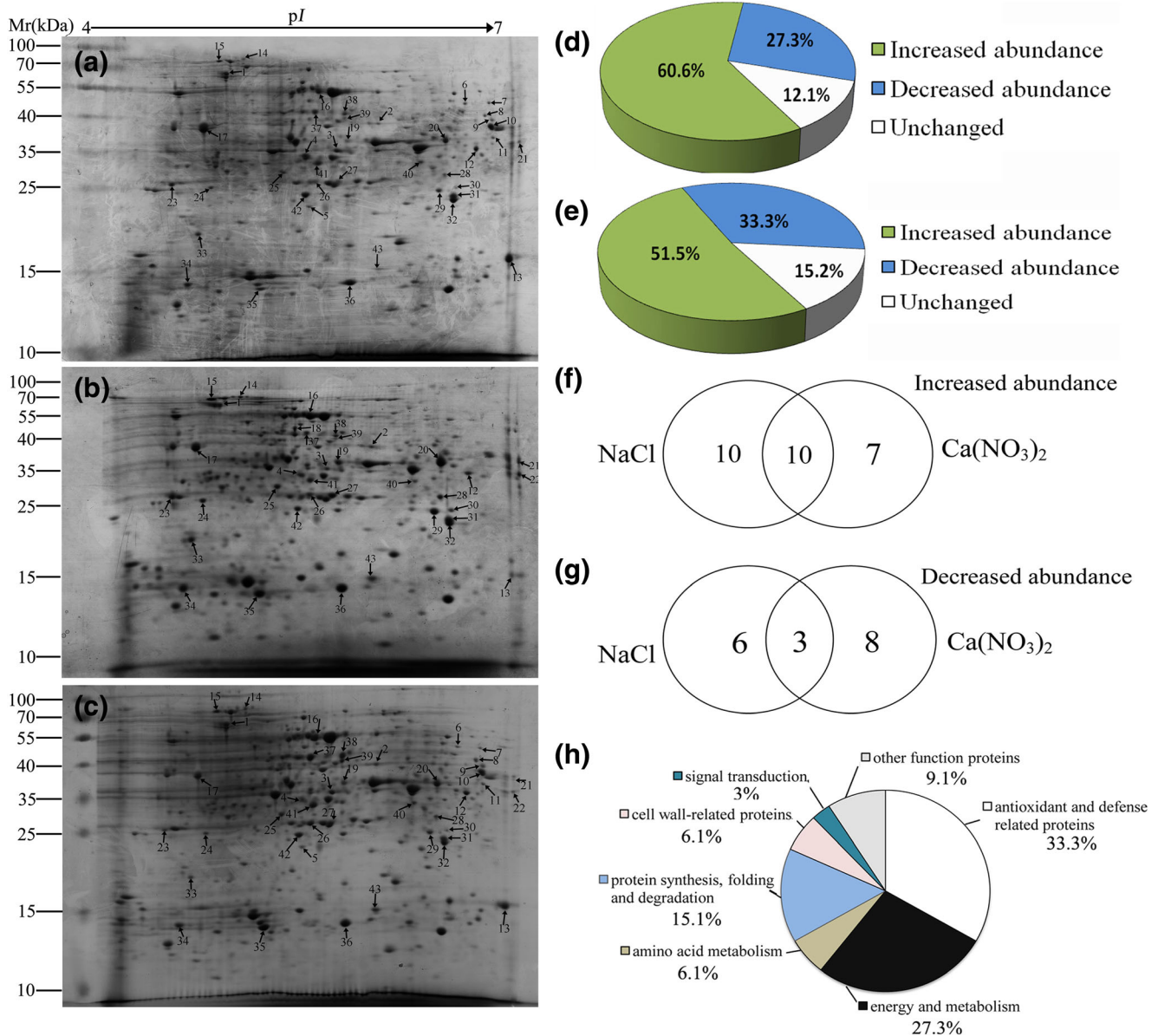


Fig. 4 2-DE protein profile, diagrammatic representation, and Venn diagrams of cucumber root protein expression patterns under salt stress. A 2-DE protein profile of cucumber roots in normal (a), 84 mM NaCl (b), and 56 mM $\text{Ca}(\text{NO}_3)_2$ (c) treatments. Total proteins were extracted and separated by IEF/SDS-PAGE and then stained with Coomassie Brilliant Blue R-250. An equal amount (800 mg) of total proteins was loaded on each gel strip. The numbers of the 43 differentially expressed proteins are

marked and annotated according to the numbering in Table 1. A diagrammatic representation of cucumber root protein expression patterns under NaCl (d) and $\text{Ca}(\text{NO}_3)_2$ (e) stresses. Venn diagram analysis of increased abundance (f) and decreased abundance (g) root proteins under iso-osmotic NaCl and $\text{Ca}(\text{NO}_3)_2$ stresses. Functional classification of proteins identified by the MS analysis (h)

flavoprotein WrbA, originally described as a tryptophan (*W*) repressor-binding protein in *Escherichia coli*, has recently been shown to exhibit the enzymatic activity of a NADH:quinone oxidoreductase. This finding points towards a possible role in stress response and in the maintenance of a supply of reduced quinone (Andrade et al. 2007). In the present study, an allergen, MLP-like protein 328 (spot 35), exhibited increased abundance under both stresses. Interestingly, there were 11 proteins participating in antioxidant and defense responses to salt stress. Six of them (spots 15, 26, 28, 29, 35, and 36) were increased abundance under both NaCl and $\text{Ca}(\text{NO}_3)_2$ stresses, and the expression levels of five proteins (spots 15, 28, 29, 35, and 36) were higher under NaCl than that under $\text{Ca}(\text{NO}_3)_2$. Three of them (spots 20, 27, and 32) were increased abundance only in NaCl stress. Therefore, increased abundance of these proteins in roots under salt stress suggests that defense mechanisms on antioxidant and detoxification were activated by salt stress, and the influence of NaCl stress was much larger than that of $\text{Ca}(\text{NO}_3)_2$ stress, which seems showed that cucumber seedlings were more tolerant to NaCl stress than $\text{Ca}(\text{NO}_3)_2$ stress, probably because it was short-term response to NaCl stress, and the long-term responds to those two salt stresses required further studies.

Energy- and Metabolism-Related Proteins

There were nine identified proteins as participating in the energy and metabolism responses to salt stress, including two malate dehydrogenases cytoplasmic-like (spots 5 and 19), one acetyl-CoA acetyltransferase cytosolic 1-like (spot 9), two fructose-bisphosphate aldolase cytoplasmic isozyme-like (spots 10 and 21), one glyceraldehyde-3-phosphate dehydrogenase cytosolic (GAPDH, spot 11), one enolase (spot 16), and two *S*-adenosylmethionine synthases (SAMs, spots 38 and 39) proteins.

Malate dehydrogenases (MDH) is a key enzyme in the TCA cycles of prokaryotes and eukaryotes. It catalyzes a reversible reaction that forms malate and NAD from oxaloacetate and NADH (Ding and Ma 2004). In this study, one MDH (spot 19) was increased abundance in cucumber roots under both stresses, and the MDH expression abundance was more prominent under NaCl stress than that under $\text{Ca}(\text{NO}_3)_2$ stress. The other MDH (spot 5) expression was unchanged under $\text{Ca}(\text{NO}_3)_2$ stress but not observed in roots under NaCl stress. Therefore, the specificity in response to different stimuli might be a reason for the different forms of MDH displaying different expression levels in cucumber seedlings roots after being subjected to salt stress.

Liu et al. (2014) reported that there is consistency between salt-tolerant ability and expression of glycolysis-related proteins and the activation in glycolysis protects rice seedlings from salt stress impacts by supplying the energy needed to repair salt-induced damage. Fructose-bisphosphate aldolase

(FBP aldolase) is an essential enzyme involved in glycolysis. It catalyzes the reversible cleavage reaction of fructose-1,6-bisphosphate (F-1,6-BP) into D-glyceraldehydes-3-phosphate, which produces dihydroxyacetone phosphate and ATP (Matsumoto and Ogawa 2008). According to Konishi et al. (2005), FBP aldolase is involved in gibberellins A (GA)-stimulated rice (*Oryza sativa* L.) root growth through the activation of the glycolytic pathway. In our study, one of two FBP aldolase cytoplasmic isozyme-like proteins (spot 10) was not detected under NaCl stress but increased abundance under $\text{Ca}(\text{NO}_3)_2$ stress, whereas the other FBP aldolase cytoplasmic isozyme-like protein (spot 21) was increased abundance under NaCl stress but decreased abundance under $\text{Ca}(\text{NO}_3)_2$ stress. Enolase is also an essential glycolytic enzyme that catalyzes the dehydration of 2-phosphoglycerate to phosphoenolpyruvate and plays an important role in providing plants with carbohydrates and energy during various abiotic and biotic stresses. Enolase is expressed in many plant species in response to various environmental stresses, such as salt stress (Liu et al. 2014; Manaa et al. 2011), cold (Lee et al. 2009), and drought (Sengupta et al. 2011). In the present study, enolase (spot 16) appeared to be increased abundance under both NaCl and $\text{Ca}(\text{NO}_3)_2$ stresses, which was more prominent under NaCl stress than that under $\text{Ca}(\text{NO}_3)_2$ stress. It was reported that expression abundance of GAPDH was decreased in tomato roots under aluminum potassium sulfate (Zhou et al. 2009) and soybean roots under salt stress (Sobhanian et al. 2010). In our study, GAPDH expression abundance (spot 11) was also decreased in cucumber roots under $\text{Ca}(\text{NO}_3)_2$ stress but was not detected under NaCl stress. The data indicate that glycolysis plays important functions in plant response to salt stress.

In salt-treated cucumber roots, SAM (spots 38 and 39) was identified. SAM is related to ethylene-mediated inhibition of root growth, and/or its stimulation could be involved in the alteration of cell wall and polymer structures in roots (Fukuda et al. 2007). In addition, SAM is an important enzyme involved in methyl cycling, which plays a key role against oxidative stress (Kunstmann and Osiewacz 2008). Witzel et al. (2009) reported that SAM expression was higher in the salt-tolerant than in the salt-sensitive barley cultivar. In this study, the expression of SAMs was increased abundance only under $\text{Ca}(\text{NO}_3)_2$ stress (spots 38 and 39). This result showed that compared with the NaCl stress, cucumber seedlings had stronger adaptability and tolerance to $\text{Ca}(\text{NO}_3)_2$ stress, and it might be one of the reasons that lead to the growth and development of the root system of plant under $\text{Ca}(\text{NO}_3)_2$ stress which was better than that under NaCl stress.

Amino Acid Metabolism

Cysteine synthase (CS) catalyzes the biosynthesis of cysteine in plants and is a key enzyme in the sulfur assimilation

responsible for the production of antioxidants and metal chelators, such as GSH, metallothionein (MT), and phytochelatin (Yang et al. 2007). Noji et al. (2001) showed that overexpression of cysteine synthase in tobacco helped protect against sulfur-containing environmental pollutants. In salt-treated cucumber roots, cysteine synthase-like (spot 40) was decreased abundance under NaCl stress but increased abundance under $\text{Ca}(\text{NO}_3)_2$ stress. The glycine cleavage system (GCS) catalyzes a cycle of glycine catabolism reactions producing methyl-tetrahydrofolate, NADH, and CO_2 , and it can function in the opposite direction (Douce et al. 2001). In eukaryotes, the cycle is typically localized in the mitochondrion. In this study, the glycine cleavage system's mitochondrial-like H protein (spot 34) was increased abundance under NaCl stress but decreased abundance under $\text{Ca}(\text{NO}_3)_2$ stress. According to Ashraf and Harris (2004), amino acids are accumulated in higher plants under salinity and besides act as an indicator for salt tolerance. In this experiment, high accumulation of enzyme protein involved in the biosynthesis of amino acids and decreased abundance of enzyme protein involved in the catabolism of amino acids will probably play an important role to intensify a resistance to $\text{Ca}(\text{NO}_3)_2$ stress of cucumber seedlings.

Protein Synthesis, Folding, and Degradation

Protein synthesis and degradation, which depends on proteolytic enzymes, are essential parts of the plant's responses to abiotic stresses (El-Maarouf et al. 1999; Ingram and Bartels 1996). Ribosomes are highly conserved large ribonucleoprotein particles, which consist of a large 60S subunit and a small 40S subunit, that perform protein synthesis. The 40S ribosomal protein S12 is an important component of the 40S subunit of ribosomes. Decreased abundance of the 40S ribosomal protein S12 was reported in cucumber under salt stress (Li et al. 2013). But, in this study, the 40S ribosomal protein S12 (spot 43) was increased abundance under both stresses, which was more pronounced under $\text{Ca}(\text{NO}_3)_2$ stress than that under NaCl stress. This result indicated that the resistance of cucumber to the inhibitory effect of salt on protein biosynthesis under $\text{Ca}(\text{NO}_3)_2$ stress was better than that under NaCl stress.

Proteolysis in plants is a complex process involving many enzymes in many pathways in various cellular compartments. Cysteine proteinases are an important group of enzymes in this process (Grudkowska and Zagdańska 2004). Cysteine proteinases accumulate when tissues are exposed to different abiotic stresses (Callis 1995; Koizumi et al. 1993). Proteasomes are multi-protein complexes, widely known to participate in protein degradation by ubiquitin-dependent and ubiquitin-independent proteolysis. The 20S proteasome is a barrel-shaped structure formed by four heptameric rings: the two outer rings are made of alpha-type subunits and the two inner rings are made of beta-type subunits. The beta-

subunits are responsible for proteolysis, while the main function of the alpha-rings is to regulate the access of substrates to the proteolytic chamber (Mittenberg et al. 2008; Tanaka 2009). In our study, the cysteine proteinase RD19a-like (spot 41) was overexpressed only under $\text{Ca}(\text{NO}_3)_2$ stress, and the proteasome subunit alpha-type 5 (PSMA5, spot 24) was overexpressed only under NaCl stress. Those results indicated that increased abundance of cysteine proteinase RD19a-like and decreased abundance of PSMA5 involved in the protein degradation will probably play an important role to intensify a resistance to $\text{Ca}(\text{NO}_3)_2$ stress of cucumber seedlings.

Protein disulfide isomerase (PDI) is an enzyme in the endoplasmic reticulum of eukaryotes that catalyzes the formation and breakage of disulfide bonds between cysteine residues within proteins as they fold (Gruber et al. 2006; Wilkinson and Gilbert 2004). This allows proteins to quickly find the correct arrangement of disulfide bonds in their fully folded state, and therefore, the enzyme acts to catalyze protein folding. Peptidyl-prolyl *cis-trans* isomerase (PPIase) catalyzes the *cis-trans* isomerization of proline imidic peptide bonds in oligopeptides and has been shown to accelerate the refolding of several proteins in vitro. It is thought to be essential for protein folding during protein synthesis in the cell. Thus, Takahashi et al. (1989) proposed that the peptidyl-prolyl *cis-trans* isomerizing activity of PPIase may be involved in events, such as those occurring early in T cell activation, that are suppressed by cyclosporin A. In our study, similar behaviors for these two proteins were observed, and PDI-like (spot 1) and PPIase 1-like (spot 13) were decreased abundance under both stresses, which was less pronounced under $\text{Ca}(\text{NO}_3)_2$ stress than that under NaCl stress. Hence, those results suggested that the influence of $\text{Ca}(\text{NO}_3)_2$ stress on those proteins was less severe than NaCl stress, and cucumber seedlings were more tolerant to $\text{Ca}(\text{NO}_3)_2$ stress than NaCl stress.

Cell Wall-Related Proteins

Two cell wall-related proteins putatively involved in cell wall reinforcement were identified, actin-7 (spot 18) and caffeoyl-CoA O-methyltransferase 1-like (spot 25), and these proteins were more highly expressed under NaCl stress. Actin-7 is a subunit of the cellular microfilament, and its major functions are to impart mechanical strength to the cell, assist in cell wall synthesis, and direct cell expansion, apart from being involved in cytokinesis, mitosis, and intracellular localization of organelles and vesicles (Niini et al. 1996). In this study, although actin-7 (spot 18) was not detected under control condition and $\text{Ca}(\text{NO}_3)_2$ stress, it was induced under NaCl stress. This result suggests that the expression of actin-7 under NaCl stress represents a stress response in the cucumber seedlings. Caffeoyl-CoA O-methyltransferase plays a vital role in lignin biosynthesis of the cell wall (Martz et al. 1998). Veeranagamallaiah et al. (2008) demonstrated that caffeoyl-CoA O-

methyltransferase was present under control conditions and increased under the NaCl stress treatment in foxtail millet seedlings. In this study, caffeoyl-CoA *O*-methyltransferase 1-like (spot 25) was increased abundance under both stresses, which was more pronounced under NaCl stress than that under $\text{Ca}(\text{NO}_3)_2$ stress. Budak et al. (2013) reported that some cell wall-related enzymes exhibited highest levels in the modern wheat variety Kızıltan than in the drought-tolerant wild genotype. Hence, we assumed that cucumber seedlings were more tolerant to $\text{Ca}(\text{NO}_3)_2$ stress than NaCl stress.

Signal Transduction

Translationally controlled tumor protein (TCTP) binds calcium with a high affinity but does not belong to any known family of calcium-binding proteins (Sanchez et al. 1997). Moreover, TCTP is associated with salt stress signaling via calcium-binding (Gong et al. 2001). Fatehi et al. (2012) found that during salt stress, TCTP was increased abundance in salt-resistant barley, but Witzel et al. (2010) found that TCTP was decreased abundance in both tolerant and sensitive barley genotypes. Interestingly, in our experiments, TCTP (spot 33) was increased abundance under NaCl stress but decreased abundance under $\text{Ca}(\text{NO}_3)_2$ stress. Cucumber seedlings contained higher amounts of calcium under $\text{Ca}(\text{NO}_3)_2$ stress than that under NaCl stress (Tong 2012); thus, we presumed that more calcium combined with TCTP leads to the decrease of TCTP under $\text{Ca}(\text{NO}_3)_2$ stress, and it might play a role in the enhancement of tolerance to $\text{Ca}(\text{NO}_3)_2$ stress.

Other Function Proteins

In our investigation, some other proteins were identified, including NAD-dependent dihydropyrimidine dehydrogenase subunit PreA (spot 37), acylpyruvase FAHD1, mitochondrial-like (spot 30), and one unknown protein (spot 23), which has no functional annotations.

NAD-dependent dihydropyrimidine dehydrogenase subunit PreA is involved in pyrimidine base degradation and catalyzes the reduction of uracil to 5,6-dihydrouracil (DHU) using NADH as a specific cosubstrate. It also catalyzes the reverse reaction and the reduction of thymine to 5,6-dihydrothymine (Mihara et al. 2008). This protein at spot 37 was decreased abundance under NaCl stress but increased abundance under $\text{Ca}(\text{NO}_3)_2$ stress. This protein may contribute to the $\text{Ca}(\text{NO}_3)_2$ stress tolerance of cucumber seedlings. Interestingly, protein spots 30 and 23 exhibited a higher level in $\text{Ca}(\text{NO}_3)_2$ stress. According to this result, it can be suggested that those proteins might have a negative regulatory role on salt stress.

Conclusion

Salt stress significantly inhibited the growth of cucumber seedlings, of which the restraining levels were different that may partly due to the different sensitivity and tolerance of cucumber seedlings to different stress. We observed that the damage of NaCl stress on biomass and the content and expression of soluble protein was more severe than $\text{Ca}(\text{NO}_3)_2$ stress. It suggested that cucumber seedlings showed a more powerful ability to resist $\text{Ca}(\text{NO}_3)_2$ stress. And cucumber seedlings more tolerant to $\text{Ca}(\text{NO}_3)_2$ stress would contribute to the expression of proteins involved in SAM biosynthesis, amino acid metabolism, protein synthesis, folding, degradation, cell wall, and signal transduction. These findings further our understanding of the response mechanism to different salt stresses in cucumber seedlings.

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Supplemental Table1 The different proteins identified by MALDI-TOF/TOF MS.

Spot NO.	Protein Name	Accession No.	Identified peptides	Identified by
1	Protein disulfide-isomerase -like	gi 449464162	LVEYNGDR GETSVSGPVVR ELATQFEIR LSGDEFNNFK FNVEADQIAPWVK SKEDIINFIETNR LRTDYDFGHTLDAK LFKPFDEQFVDFK NVLLEFYSPWCGHCK FDATANDIAVGDFEVQGYPTLYFR FIESSSIPTVTEFNNDPSNHVYLSK	MASCOT
5	Malate dehydrogenase, cytoplasmic-like	gi 449458824	NISCLTR ALGQISER LNVQVSEVK EFAPSIPER MELVDAAFPLLK SSALSAASAACDHIR KLSSALSAASAACDHIR DGEWSIVQGLSIDEFSR ELVADDAWLNGEFISTVQQR NVIIWGNHSSTQYPDVNHATVK	MASCOT

9	Acetyl-CoA acetyltransferase, cytosolic 1-like	gi 449453612	KLRPSFK DVCIVGVAR LGSLAIEAALKR APAAAAADESINPR EAQDDYAVQSFER VNVHGGAVSLGHPLGCSGAR DSNAFEWEIVPVEVSGGR GKPSTVVSSDEGLGKFDPK GYADAAHEPEFFTTAPALAVPK VDPALVQEVIFGNVLSANLGQAPAR LLGISPDKVN HGGAVSLGHPLGCSGAR AKVDPALVQEVIFGNVLSANLGQAPAR	MASCOT
10	Fructose-bisphospha te aldolase, cytoplasmic isozyme-like	gi 449440201	YYEAGAR CAEVTER EGGVLPQIK TAAGKPFVDVLK KPWSLSFSFGR VAPEVIAEYTIR LASINVENVESNR GILAADESTGTIGKR LASINVENVESNRR GDAELSEGAAESLHVK YQDELIANATYIGTPGK IGPTEPSQLSINENAYGLAR GTVELAGTNGETTTQGLDGLAQR	MASCOT
11	Glyceraldehyde-3-phos phate dehydrogenase, cytosolic-like	gi 449503692	IGINGFGR AVTVFGFR VPTVDVSVDLTVR VPTVDVSVDLTVR GGRAASFNIIPSSTGAAK GILGYTEDDVSSDFVGDSR	MASCOT

13	Peptidyl-prolyl cis-trans isomerase 1-like	gi 449527215	TDWLDGK FADENFIK FADENFIKK IIMELYADVTPR VFFDMTIGGTPAGR TSKPVVIADCGQLS TAENFRALCTGEK HVVFGQVVEGMNVVK PNPKVFFDMTIGGTPAGR HTGPGILSMANAGPGTNGSQFFICTAK VIPNFMCGGDFTAGNGTGGESIYGAK	MASCOT
15	Luminal-binding protein 5	gi 659074058	ETAEAFGLK FDLTGIPPAPR GRLSQEEIDR MKETAEAFGLK LIPRNTVIPTK FEELNNDLFR IMEYFIKLIK VQQLLKDYFEGK NQIDEIVLVGGSTR ITPSWVAFTDSER ARFEELNNDLFR NGHVEIANDQGNR VFSPEEISAMILTK IINEPTAAAIAYGLDK DAVVTVPAYFNDAQR LIGEAAKNQAAVNPER IDARNSLETYIYNMK IKDAVTVPAYFNDAQR VEIESLFDGTDSEPLTR LKEVEAVCNPIITAVYQR SQVFTTYQDQQTTSIQVFEGER	MASCOT

16	Enolase	gi 659125144	YNQLLR AGWGVMAHR RAGWGVMAHR AAVPSGASTGIYEALRL IEEELGSAAVYAGVNFR GNPTVEVDIVLSDGTLAR LAMQEFMILPIGASSFK SGETEDTFIADLSVGLATGQIK YGQDATNVGDEGGFAPNIQENK AGYTSQVVIGMDVAASEFYGSDK IEEELGSAAVYAGVNFRKPVAPY TAIKAGYTSQVVIGMDVAASEFYGSDK	MASCOT
17	Peroxidase	gi 167533	CMFFSGR NYYTNLQNNQGLLR DAVTLASGQGWTVQLGR LPSPFESLSNIQGIFR DRLSPFESLSNIQGIFR DVGLNDNTDLVALSGAHTFGR TAVENVCPGVVSCADILALGSR SDQVLFSTPGASTIATVNSLASSESFAFADAFAQSMIR	MASCOT
18	Actin-7	gi 659080849	AVFPSIVGRPR IWHHTFYNELR LDLAGRDLTDALMK NYELPDGQVITIGAER DLYGNIVLSGGSTMFPGIADR	MASCOT

19	Malate dehydrogenase, cytoplasmic	gi 659121409	NISCLTR ALGQISER MELVDAAFLLK LSSALSAASAACDHIR VLVVANPANTNALILK DGEWSIVQGLSIDEFSR VLVTGAAGQIGYALVPMIAR ELVADDAWLNGEFISTVQQR NVIIWGNHSSTQYPDVNHATVK GVVATTDVVEACTGVNIAIMVGGFPR GVLLGPNQPVILHMLDIPPAAESLNGVK	MASCOT
20	Peroxidase 2-like	gi 449467745	DSRIANK KAIESDIR DSVDVQGGPSWR ANFDPVTPDVFDK SFLEGVCSAGADTR ECPGIVSCADILAQASK TGADSNLASPFETLDQLK NVGLNTMDLVSLGAHTFGR QSMINMGNIKPLTGGQGEIR QSMINMGNIKPLTGGQGEIRR ANFDPVTPDVFDKNYYTNLQVGK GLLQSDQELISTPGADTIVIVNSFAER ECPGIVSCADILAQASKDSVDVQGGPSWR	MASCOT

21	Fructose-bisphosphate aldolase,cytoplasmic isozyme-like	gi 449451108	VLAACYK	MASCOT
			YYEAGAR	
			AQAALLVR	
			CADVTER	
			CADVTER	
			ALQQSTLK	
			AWAGKDENIPK	
			TAAGKPFVDVLK	
			KPWSLSFSFGR	
			LSSISVENVETNR	
			GILAADESTGTIGKR	
			GKKPWSLSFSFGR	
			ANSEATLGTYKGDAK	
			LSSISVENVETNRR	
			VAPEVIAEHTVLALQR	
			YADELIANAAYIGTPGK	
			ISEGAAESLHVDNYKY	
			GKYADELIANAAYIGTPGK	
IGPNEPSQLSINENANGLAR				
GTVELAGTNGETTTQGLDGLAQR				
VDKGTVELAGTNGETTTQGLDGLAQR				
23	Uncharacterized protein At2g39795, mitochondrial	gi 449441362	SSSDDSLLR	MASCOT
			EYLTWLTK	
			GIKPSTTNFLHEYMINK	
			TGPSLEFSCSAYPDEISIDSLIVK	
			KTGPSLEFSCSAYPDEISIDSLIVK	
			NPEHSDDQIAYEGPDFHDLDENLQK	
24	Proteasome subunit alpha type-5	gi 659118266	GVNTFSPEGR	MASCOT
			LFQVEYAIEAIK	
			VSPTYHLYTPAEVEAVISR	
			GPSLFYTDPSGTFWQCNAK	
			VSPTYHLYTPAEVEAVISRL	
			FSYGEPMTVESTTQALCDLALR	
			FGEGDEESMSRPFVALLIAGHDEK	

25	Caffeoyl-CoA O-methyltransferase 1-like	gi 449438410	HQEVGHK	MASCOT
			ENYELGLPVIEK	
			EGPALPVLDDMVADKK	
			KNHGSYDFIFVDADK	
			IEICMLPVGDGITLCR	
			IEICMLPVGDGITLCRR	
			SLLQSDALYQYILETSVYPR	
			HPWNIMTTSADEGQFLNMLLK	
			ALAADPRIEICMLPVGDGITLCR	
			VGGVIGYDNTLWNGSVVAPPDAPMR	
			NHGSYDFIFVDADKDNYINYHK	
			VGGVIGYDNTLWNGSVVAPPDAPMRK	
			TMEIGVYTGYSLLATALALPEDGTILAMDINR	
26	Ascorbate peroxidase	gi 315115497	TGGPFGTMR	MASCOT
			NCAPLMLR	
			EDKPEPPPEGR	
			SYFTELLTGEK	
			ALLSDPVFRPLVEK	
			GFIAEKNCAPLMLR	
			SELAHGANNGLDI AVR	
			FKSELAHGANNGLDI AVR	
			YAADEDAFFADYAEAHQK	
			SYFTELLTGEKEGLQLASDK	
27	Ascorbate peroxidase	gi 315115497	DVFYTMGLSDQDIVALSGGHTLGR	MASCOT
			TGGPFGTMR	
			NCAPLMLR	
			EDKPEPPPEGR	
			SYFTELLTGEK	
			ALLSDPVFRPLVEK	
			SELAHGANNGLDI AVR	
			FKSELAHGANNGLDI AVR	
			YAADEDAFFADYAEAHQK	
			DVFYTMGLSDQDIVALSGGHTLGR	

28	Glutathione S-transferase DHAR2-like	gi 449459946	AHGPYVAGEK VSPEGKVPVVK LYHVDVALGHFK ATPEHVIAGWEPK DHSEQNLLEELK AAVGAPDEIGDCPFSQR LHLINLSDKPSWFLK DLACLISYKELLFAR YPEPSLVTPPQFSSVGSK KYPEPSLVTPPQFSSVGSK FDDEWVPDSDVIVETLEK	MASCOT
29	Glutathione S-transferase-like	gi 449498465	VLAALYEK LHEGEHKR VHGIPTSTATGR DLHFELVNVK SAVVLTWIEVESHHFDPVAMK YLGGESFSLADLHHLPVLGYLLATQSK EPFISLNPFGQVPAFQDGDNLNFESR REPFISLNPFGQVPAFQDGDNLNFESR	MASCOT
30	Acylpyruvase FAHD1, mitochondrial-like	gi 449432876	SAGLPWTVAK GSTKDMIFK GQDTFTPISSVLSK ASIPDPDDLELWLK SLDHEVELAVVISQR VTAGITNIIDVHFNVENR DVSEASAMDYVGGYALALDMTAR ARDVSEASAMDYVGGYALALDMTAR	MASCOT
31	Flavoprotein WrbA-like	gi 449466103	FGMMAAQFK AFLDATGGLWR GGSPYGAGTFAGDGSR KPTTLELEQAFHQGK VYIVYYSMYGHVEK SDVPIITPSELSEADGFVFGFPTR	MASCOT

32	Flavoprotein WrbA-like	gi 449466103	FGMMAAQFK AFLDATGGLWR GGSPYGAGTFAGDGSR KPTTLELEQAFHQGK LWQVPETLTEDVLGK VYIVYYSMYGHVEK SDVPIITPSELSEADGFVFGFPTR	MASCOT
33	Translationally-controlled tumor protein homolog	gi 449432858	VVDIVDTFR EGATDPTFLYIAPGLK MLVYQDLVSGDELLSDSFYK DLQFFVGESMADDSAMVFAYYK VKDLQFFVGESMADDSAMVFAYYK	MASCOT
34	Glycine cleavage system H protein, mitochondrial-like	gi 449455395	VEVSDSGELK LMDSEQYSK YATSEWVK FCEEEDSKH ATVGITDHAQGHLDVVVELPEVGTSVK	MASCOT
35	MLP-like protein 328	gi 659098664	DIEAHLK SFQGTYK YYEIFK YYEIFKDK YLDLMHTITK FVSELEINAPAEK GPEHSLAVLTLQFEK VAHIPNISPTIFQNVEVHEGDWDTHGHGSIK	MASCOT
36	Superoxide dismutase [Cu-Zn]-like	gi 449485788	QHGAPEDENR GGHELSSSTGNAGAR GGHELSSSTGNAGARVACGIIGLQG ANFTITDCQIPLCGHESIIGR	MASCOT
37	NAD-dependent dihydropyrimidine dehydrogenase subunit PreA-like	gi 449439483	EAIEQRK AAWEELIDR AFDEGWGAVIAK LHNFSSIEDFR	MASCOT

			RAFDEGWGAVIAK ILIASIMEEYNK MASFNLTQFR TK TKSPITNLALNPSR GASLPYFTTHDLVR SGCEGIAAINTIMSVMGIDLK TLRPEPCVEGYSTPGGYSSK VEQTGVDAFEINFSCPHGM PER RMGAAVGQDCALLEEVCGWINAK GQIIGWENIELISDRPLEIMLK	
38	S-adenosylmethionine synthase 1-like	gi 449436329	SIVASGLAR TAAYGHFGR TIFHLNPSGR NGTCPWL RPDGK FVIGGPHGDAGLTGR YLDEKTIFHLNPSGR IIIDTYGGWGAHGGGAFSGK ENFDFRPGMITINLDL K TQVTVEYYNDNGAMVPVR METFLFTSESVNEGHPDK KIIIDTYGGWGAHGGGAFSGK ENFDFRPGMITINLDLKR VLVNIEQQSPDIAQGVHGHFTK TAAYGHFGRDDPDFTWETIKPLK RCIVQVSYAIGVPEPLSVFVD TYK RPEEIGAGDQGHMFGYATDETPELMPLSHVLATK	MASCOT
39	S-adenosylmethionine synthase 2-like	gi 659122902	SIVASGLAR IVRDCR TIFHLNPSGR NGTCPWL RPDGK FVIGGPHGDAGLTGR IIIDTYGGWGAHGGGAFSGK ENFDFRPGMITINLDL K TQVTVEYYNDNGAMVPVR	MASCOT

			ENFDFRPGMITINLDLKR VLVNIEQQSPDIAQGVHGHFTK RCIVQVSYAIGVPEPLSVFVDYK	
40	Cysteine synthase-like	gi 449442895	VDDTCVAR YLKEQNPSIK YLSTVLFESVK LIVAVFPSFGER AFGAELVLTDPAR YLSTVLFESVKR IHYETTGPFIWK LIICMPASMSLER DVTELIGNTPLVYLNK TPNSYILQQFENPANPK LYGVEPVESAILSGGKPGPHK	MASCOT
41	Cysteine proteinase RD19a-like	gi 449464688	DKPYWIIK GVSCPYICK EQDYPYTGTDR SYASKEEHDHR NSWGANWGENGYK APILPTDGLPTDFDWR NGPLAVAINAVFMQTIK HLDHGVLLVGYGSDGYAPIR	MASCOT
43	40S ribosomal protein S12	gi 659122493	SLAHGGLVR KSLAHGGLVR TLGEWSGLCK DFGEDHEALHIVQK ALCAEHNCLMTVPSAK HAAQLCVLGEDCNQPDYVK SADEVAVVAEAPAPALGEPMDIETALQIVLR	MASCOT