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Full Length Research Paper

Sodium chloride causes variation in organic acids and proteins in tomato root

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Tomato is a salt-tolerant crop. The purposes of this research are to evaluate changes of organic acids and proteins in tomato grown in environment of different NaCl concentrations (0, 0.25 and 0.5%, respectively). The results showed that oxalic acid, malic acid, fumaric acid and proteins in which P69C, Cytochrome P450 proteins, Lucinerich protein, phototropin-1 and retrotransposon gag protein were upregulated in 0.5% NaCl treatment. However, there were some proteins were apparently inhibited in 0.5% NaCl treatment. Tomato roots under high NaCl concentration could be characterized by the cellular activities involved in carbohydrate metabolism, organic acid production, energy metabolism, alleviating redox damage, root phenotypical change etc, which are critical for plant survival under high NaCl concentration. This study may provide an important direction to future research on salt resistance mechanisms in tomato.

Key words: Tomato, salt tolerance, physiology, organic acids, proteins.

INTRODUCTION

There is an area of 9.6×10^8 Km^2 in the world that is salt-affected land and such land is increasing at 10^5 Km^2 annually (Szabolcs, 1989). The salted land is formed mainly by sea salt invasion and its main component is NaCl transported through wind, under long period of dry condition coupled with high level of ground water table (Wang et al., 1989). Tomato (*Lycopersicon esculentum* Mill) in general can tolerate soil that has a conductivity of 4 - 6 mS/cm (Chen, 1993). Katerji et al. (2001) conducted salt-tolerant experiments on eight crops: tomato, bean, corn, rye, potato, sugar-beet, sunflower, broad bean and found that tomato was the most salt-tolerant crop; it could grow normally in an environment with 0.5% NaCl concentration (Kalarmari et al., 2009). Salt tolerance was found better for cherry tomato than for larger, normal one (Sanchis et al., 1991). Plants grown in salty environment may have some salt-tolerance as their own characteristics. When salt tolerant plants grown in high salt environment, their growth may be quickly enhanced, probably because sodium (Na) in the salt is their required element (Brownell et al., 1965). The alternative explanation is that these plants grow rapidly to reduce their salt concentrations (Brownell et al., 1972; Mandak and Pysek, 1990). Roots of plants are the first encounter of stresses in soil. Previous studies indicated that proteins were different between plants suffering heavy metal toxicities and those not exposed to heavy metal (Cambell et al., 1994; Van-Assche and Cijlsters 1990; Talor et al., 1997). Le Van et al. (2004) showed that an acid (pI 3.8) and low molecular weight (23 kD) protein was identified in Al-treated Cayenne root tips following Al stress, but not in no Al-treated one. About the study of salt-tolerant plants, many researchers recognized that salt-tolerant proteins in roots which enhance salt-tolerance of the plants (Singh and Haseguwa, 1987; Degeuhandt et al., 2000; Dhingra and Varghese, 1985). The salt-tolerant proteins and physiological effect of tomato roots in high salt environment have rarely been studied. This research aims to evaluate the changes of organic acids and proteins when tomato is cultivated in hydroponic solution at different NaCl concentration. The results may be referred to improve crops grown

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Abbreviations: LC-MS, Liquid chromatography-mass spectrometry; **2D,** two-dimension; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Table 1. Organic acid concentrations of tomato roots after treatment of different NaCl concentrations.

NaCl concentration	Organic acids (nmol/h.g root FW)				
	Oxalic acid	Citric acid	Malic acid	Succinic acid	Fumaric acid
0%	7.1[b]*	5.1[a]	10.3[c]	3.0[a]	4.2[c]
0.25%	9.2[b]	4.5[a]	22.6[b]	2.5[a]	11.3[b]
0.5%	14.5[a]	5.4[a]	34.9[a]	2.1[a]	21.6[a]

*The same letter in the same column indicates no significant difference at 0.05 level according to Duncan's multiple range test.

in salty land.

MATERIALS AND METHODS

Cultivation of tomato seedlings

Healthy tomato seedlings are selected and cultivated in 1L hydroponic solution based on Konishi's modified ingredient (Konishi et al., 1985). Its components in mM include 1.10 $(NH_4)SO_4$, 0.35 $Ca(NO_3)_2$, 1.0 Na_2HPO_4, 0.51 K_2SO_4, 0.35 $CaCl_2$, 1.00 $MgSO_4$ and that in μM include 6.3 Fe-EDTA, 9.3 H_3BO_3, 18.0 $MnSO_4$, 1.5 $ZnSO_4$, 0.4 $CuSO_4$, and 0.5 Na_2MoO_4. The seedlings were moved to growth chamber (temperature and relative humidity were 27°C and 65% respectively, for day time and 23°C and 85% respectively, for the night) which was aerated for 10 min at an interval of 2 h. The hydroponic solution was replaced at 2-day intervals, after a week the seedlings were moved to hydroponic solutions containing 0, 0.25 and 0.5% NaCl solutions separately. Each treatment contained 30 seedlings, and the experiments were carried out in triplicates, all under the same cultivation conditions. Two weeks later all tomato roots were collected and stored at liquid nitrogen temperature.

Extractions of organic acids and proteins from tomato roots

According to Delhaize et al. (1993), each stored root sample was ground with 1 mL 0.6N $HClO_4$, then centrifuged for 5 min at 15000 g. The supernatant at 0.9 mL was mixed with 80 μL of K_2CO_3 (69 g/100 mL) and then centrifuged again for 5 min at 15000 g. Deionized water at 0.85 mL was added into the supernatant for analyses of organic acids. The roots obtained from different treatments were collected and put in Eppendorf tube for extraction of proteins with extractant (2% Triton, 50 Tris, pH 7.4). Then trichloroacetic acid was added to precipitate for protein analysis.

Analysis of organic acids

According to the method of Jin et al. (2008). Briefly, a high performance liquid chromatography (HPLC) (Shimatz, LC10A) was used with 50 mM $HClO_4$ (Merck, 70 - 72%) as a mobile phase at 1 μL/min flow rate. Two chromatographic columns (Shodex RSpark KC811, Japan) were set at 45°C and sampling volume was 5 μL. A refractive index detector (RID) was used to detect and measure oxalic acid, citric acid, malic acid, succinic acid and fumaric acid quantitatively.

Protein analysis and identification

Two-dimensional electrophoresis

Protein sample at 400 μg was dissolved in a buffer solution (8 M urea, 2% CHAPS, 0.5% IPG buffer and 7 mM DTT) and then put in 13 cm (the medium format 2-DE) IPG gel strip (pH 4 - 7, linear distribution) for 1D electrophoresis with IPGphorⅡ system (GE Healthcare, Piscataway, N J) set at 60,000 - 75,000 voltage-hours. SDS buffer solution was used to equilibrate the completed electrophoresis gel strip. Then 12.5% SDS-PAGE was used to proceed for two-dimension (2D) electrophoresis for difference in molecular weight (Gorg et al., 1988).

Protein gel staining and image analysis

2D electrophoresis gel film was stained with SYPRO Ruby gel stain kit. The stained gel film was excited at 488 nm and emitted at 610 nm wave length for photo image using a charge coupled device (CCD) photographic system (Perkin Elmer, MA). Molecular weight was calculated based on the photo image using phoretix 2D Elite software program.

MS sample preparation (Walker, 2002)

Manually cut out gel strip of 1 mm width, protein spot was treated with MS-grade Trypsin Gold at 37°C for 8 h to disintegrate protein, followed by 10 μL Milli-Q ultra-pure water and 20 μL 0.1% TFA, concentrated by vacuum concentrator, then dissolved in 1μL of 5% CAN with 0.5% TFA.

Identification of proteins with ESI-MS/MS

Mass spectrometer (MicroMass Q-TOFZ, UK) was used for protein measurement and MASCOT search engine (www.matrixscience.com) was applied for data analysis and comparison with the data bank. Search conditions and data bank were as following : NCBInr, Taxonomy, green plant, enzyme, trypsin, fixed modification, carbamidomethylation, variable modification, methionine oxidation, peptide tolerance, 0.3 Da, MS/MS tolerance, 0.3 Da, allowance of one missed cleavage.

RESULTS

Due to experiments for crop cultivating in hydroponics have much predominance, such as rapidity and high reproducibility (Campbell and Carter 1990; Horst et al., 1997). Table 1 shows that oxalic acid, malie acid and fumaric acid gradually increase their concentrations as NaCl concentration in the hydroponic solution increases. When NaCl concentration in the hydroponic solution is 0.5% in comparison to no NaCl in the solution, oxalic acid in the roots is three times higher. For malic acid, it is also

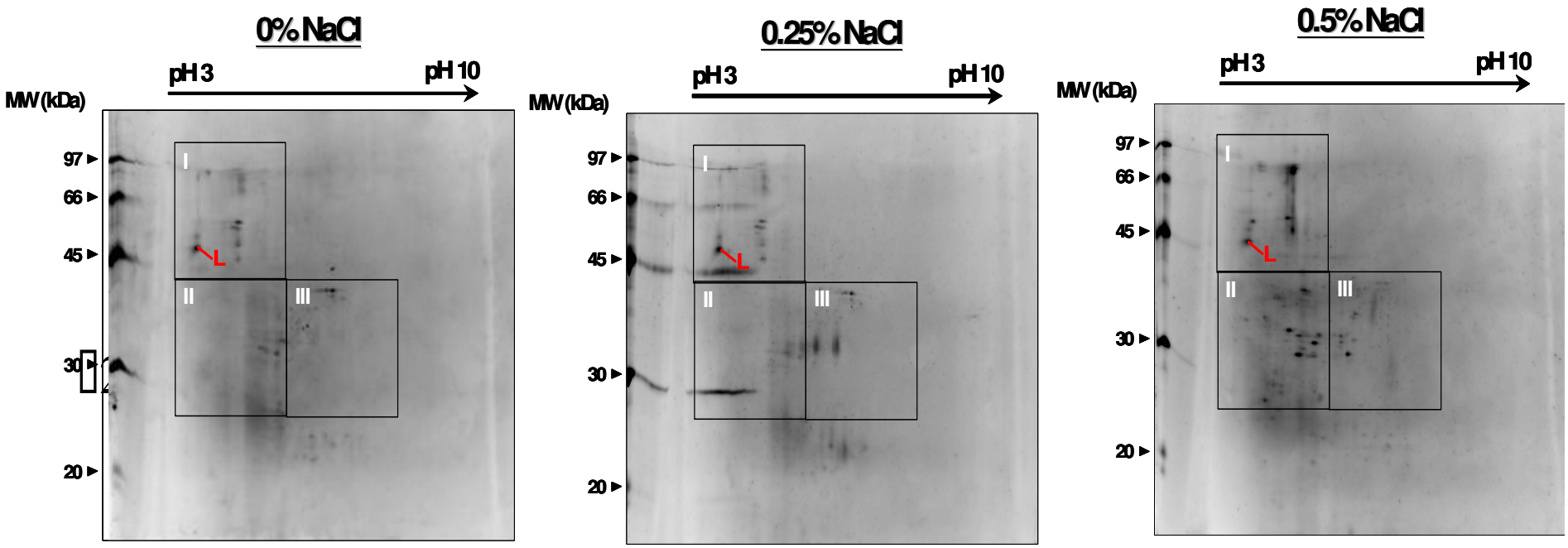

Figure 1. 2D SDS-PAGE plate for proteins of tomato roots after treatment of different NaCl concentrations.

three times higher; for fumaric acid, it is even 5 - 6 times higher. For citric acid and succinic acid, their difference is small no matter the hydroponic solution is treated with or without NaCl. Thus oxalic acid, malic acid and fumaric acid in roots may be related to the salt tolerance of tomato.

Having treated with three different NaCl concentrations, the figures of SDS-PAGE are similar, and 81 spots of protein can be separated (Figure 1). After comparing with the corresponding spots of no Al treatment in abundance, eight apparently differentially expressed spots were selected and analyzed by LC-MS-MS. Among these proteins, five spots appeared significantly in the samples of 0.5% NaCl treatment, but not clear in the no NaCl treatment (Figure 1), suggesting that these proteins were induced under high NaCl concentration, or their concentration was too low to be detected under no NaCl conditions. Three protein spots were decreased in abundance under treatments of 0.5% NaCl. Spots 7 and 8 were significantly decreased and almost disappeared with Al toxicity (Figures 1 and 2). Selected parts of the spots are highlighted in Figure 2 to show the comparisons of high-NaCl-responsive protein spots in tomato root apices between the treatments of 0, 0.25 and 0.5% NaCl treatments.

The identities of eight distinctive spots were determined using MASCOT search engine according to the similarity of sequences with previously characterized proteins in the NCBInr database. From Table 2, distinctive spots selected from high NaCl (0.5%) treatment include 10 proteins (spots 1 to 5). Nine proteins were obviously inhibited (spots 6 to 8). Among the distinctive proteins, there is one unknown protein, two predicted proteins which are obtained from correlation with *populus trichocarpa* and *Micromonas* sp. Rcc299, respectively. There is one hypothetical protein as correlated from Sorghum bicolos. There are six proteins identified: p69C protein, cytochrome P450, ATP synthase beta subunit, Leucine-rich repeat, phototropine-1 and retrotransposon gag protein. The obviously inhibited proteins derived from 0.5% NaCl treatment relative to no or low NaCl treatment includes one unknown protein, one predicted protein based on correlation with Ostrococcus CCE 9901. Five hypothetical proteins

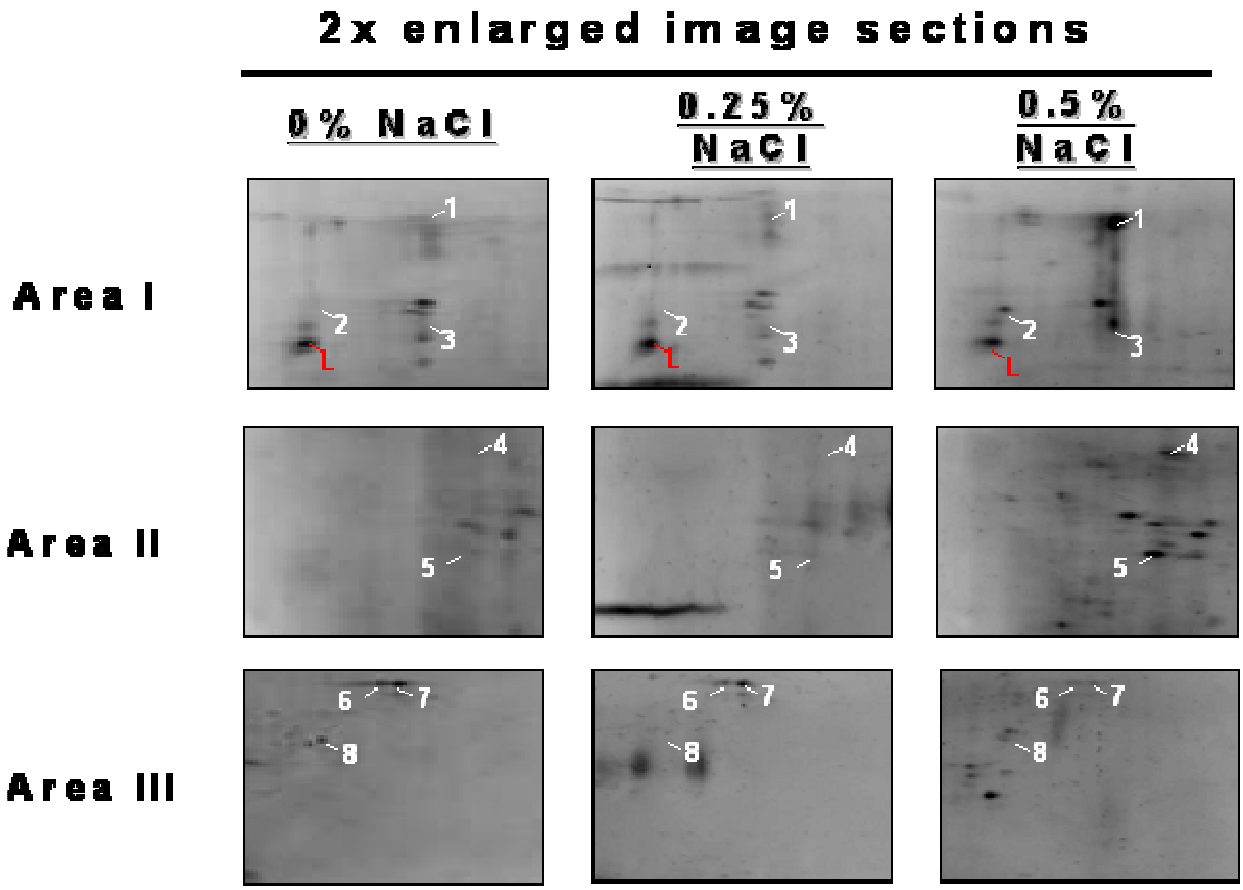

Figure 2. Protein distribution in different areas of the SDS-PAGE plate after treatment of different NaCl concentrations.

are based on correlations with *Vitis vinifera* , OsI_38149, *Oryza sativa* Indica Group, *Oryza sativa* Japonica Group and *Zea mays*. Two proteins identified are TNF receptor-associated factor (ISS) and pcl-like protein OSI-38149.

DISCUSSION

Organic acids have been confirmed as one of the mechanisms for crop roots to tolerate stress (Larsen, 1998). Large amount of organic acids in root in response to a stress is related to the behavior of some special proteins (Ryan et al. 1995). Our experiments indicate that oxalic acid, malic acid and fumaric acid in tomato roots increases significantly as the NaCl concentration increase (Table 1). Le Van and Masuda (2004) conducted a study on Al-resistance of different pineapple cultivars. They found that Al-resistant Cayenne containing more malic acid and succinic acid than Al-sensitive Soft Touch in their root apices have higher Al concentrations. The root apices of Cayenne contained more 23 kD protein than that of Soft Touch, leading to a conclusion that these materials must be related to Al-resistance of these pineapple cultivars. The role of proteins involved in reactive oxygen species (ROS) detoxification during salinity stress and identified potential candidates for increasing salt tolerance in barley were studied. Witzel et al. (2009) conducted experiments on salt tolerance of barley and found that two proteins involved in the glutathione-based detoxification of ROS were more abundant in the tolerant genotype, while proteins involved in iron uptake were expressed at a higher level in the sensitive one. Tornero et al. (1996) showed that P69C protein (spot 2) might enhance the resistance against the disease of plant roots. Our experiments confirmed that P69C protein in tomato roots may have salt-tolerant capability. Banco et al. (2002) suggested that formation of brassinosteroids in tomato played an important role in the process of elongation of its roots. Under high salt concentration, cytochrome P450 (in spot 2) was contained in large quantity, it may promote synthesis of brassinosteroids and so enhance salt tolerance of tomato as well as its root growth. For our experiments, cytochrome P450 in tomato roots was apparently higher for 0.5% NaCl treatment than for 0.25% or no NaCl treatment, which was similar to that of Bancos et al. (2002). Zhou et al. (2009) studied Al-resistant proteins in tomato and found that the ATP synthase in vacuole of root cells would

Table 2. Identifications of proteins from tomato roots after treatment of different NaCl concentrations.

Spot	Accession Number	Protein description	MOWSE score	Theoretical M_r / p*I*	Highly expressed in
1	gi|116787836	Unknown [*Picea sitchensis*].	50	43.5/8.97	0.5% NaCl
2	gi|3183979	P69C protein [*Solanum lycopersicum*].	52	80.8/8.50	0.5% NaCl
	gi|255553639	Cytochrome P450, putative [*Ricinus communis*].	46	58.7/8.94	
3	gi|224105443	Predicted protein [*Populus trichocarpa*].	53	12.1/8.84	0.5% NaCl
	gi|255085016	Predicted protein [*Micromonas* sp. RCC299].	53	54.7/5.61	
	gi|7706850	ATP synthase beta subunit [*Androcymbium ciliolatum*].	49	50.3/5.09	
4	gi|124359469	Leucine-rich repeat, plant specific [*Medicago truncatula*].	48	46.7/5.01	0.5% NaCl
5	gi|151176133	Phototropin-1 [*Solanum lycopersicum*].	55	115.7/8.35	0.5% NaCl
	gi|242080053	Hypothetical protein SORBIDRAFT_07g028173 [*Sorghum bicolor*].	49	35.4/9.75	
	gi|89179399	Retrotransposon gag protein [*Asparagus officinalis*].	49	106.0/9.44	
6	gi|147804859	Hypothetical protein (*Vitis vinifera*).	63	168.6/9.00	0% NaCl
	gi|145346954	Predicted protein [*Ostreococcus lucimarinus* CCE9901].	51	117.6/4.78	
	gi|255640268	Unknown [*Glycine max*].	42	39.9/4.79	
7	gi|116057152	TNF receptor-associated factor (ISS) [*Ostreococcus tauri*].	47	162.7/6.53	0% NaCl
	gi|147806268	Hypothetical protein (*Vitis vinifera*).	46	54.7/5.88	
	gi|218186751	Hypothetical protein OsI_38149 [*Oryza sativa* Indica Group].	42	401.8/5.00	
8	gi|53792104	pr1-like protein [*Oryza sativa* Japonica Group].	58	37.2/10.27	0% NaCl
	gi|147801039	Hypothetical protein (*Vitis vinifera*).	54	51.8/5.22	
	gi|226509154	Hypothetical protein LOC100279626 (*Zea mays*).	48	67.8/6.35	

increase in high salt concentration. Chen et al. (2009) showed that the amount of ATP synthase in root apex would decrease when rice was grown in high salt environment for 2 h, it made the integrity of mitochondria decreasing. In our experiments, tomato roots were not damaged when grown in 0.5% NaCl solution and its ATP synthase beta subunit (spot 3) increased more than that with 0.25% or no NaCl treatment. In the proceeding of ATP formation, ATP synthase beta subunit produced one of the important proteins in tricarboxylic acid cycle (TCA) cycle through respiration. Thus large amount of ATP synthase beta subunit formation might enhance TCA cycle in which citric acid and succinic acid were quickly converted to malic acid, fumaric acid and oxalic acid, resulting in large accumulation of these 3 organic acids. These were similar to the studies conducted by Li et al. (2008) on changes of root proteins of tomato in iron-deficient environment. They showed that the isocitrate dehydrogenase (NADP1), aconitase, succinyl CoA ligase and succinate dehydrogenase for the TCA and ascorbic cycle were unregulated expression and hence the mitochondria TCA cycle was enhanced in response to the stress of iron deficiency. Leucine rich repeat was related to Arabidopsis, perennial ryegrass (*Lolium perenne* L) and bean (*phareolus vulgarize* L) in their disease resistance (Dracatos et al., 2009; Hubert et al., 2009; Farina et al., 2009). Beatty et al. (2009) suggested that Leucine-rich repeat might improve the efficiency of nitrogen use in rice. Thus Leucine-rich repeat in tomato under high salt concentration might promote the assimilation of nitrogen and resulting in no inhibition of tomato growth. The phototropin-1 is associated with photochloroplast relocation, stomatal opening and leaf-flattening responses of plant (Aihare et al., 2008). The phototropin-1 photoreceptor production was thought to be related to plasticity and drought tolerance of Arabidopsis thaliana (Hemm et al., 2004; Galen et al., 2007). Tomato might transport its phototropin-1 photoreceptor production from leaves to protect its roots. Retrotransposon gag protein was related to the resistance of stress for wheat, sweet potato and rice (Snsari et al., 2007; Yamashita Tahara, 2006; Ricolabanas and Mantinez-Izquierdo, 2007; Mathew et al., 2009). We confirmed that retrotransposon gag protein plays a role in salt tolerance of tomato. Finally, stress-resistance of plant roots was related to prl-like protein which was reported by Sautaruceria et al. (2001).

The significantly increasing proteins (from spots 1 to 5) at the treatment of 0.5% NaCl were distributed in the area I (Figure1). The isoelectric points (*pI*) of these proteins were distributed at pH 2 - 4, so the proteins in this area belong to the acid proteins. They may contain much more carboxylic acid, for example P69C protein. Siezen et al. (1997) showed that it contains mainly serine and the main functional group of serine was carboxylic acid. When these proteins are in the environment of pH > 7, the carboxlic acid will be ionized to carboxylic anionic and

then chelate Na^+; hence, that could be the reason for the salt resistance of tomato root. The pH of this experiment declined in the treatments of 0, 0.25 and 0.5% NaCl at the termination of the experiment (data not shown), and this could be as a result of the replacement of H^+ by Na^+ that made the solution acidified.

The tumour necrosis factor (TNF) receptor-associated factor (ISS) and prl-like protein in tomato roots were inhibited when treated with 0.5% NaCl than the treatments of 0 or 0.25% NaCl. These will be worthy of further evaluations.

Conclusion

The accumulation of three organic acids (malic acid, fumaric acid and oxalic acid) in tomato roots may be controlled by some special proteins when tomato was grown in hydroponic solution with 0.5% NaCl. In high NaCl concentration (0.5% NaCl), ten proteins were significantly increasing, in which six proteins have been identified, while four proteins were not yet clearly identified. They may be related to the salt tolerance in tomato root. Among the nine inhibited proteins, only two have been identified, while the rest were unidentifiable. This research has, therefore, confirmed that contents of some organic acids and proteins in tomato roots are different between the environment of high salt concentration and no salt or low salt concentration. This result may be referred to in order to improve the study's crops cultivation in salty land.

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