

Full Length Research Paper

Effects of salinity on sucrose metabolism during tomato fruit development

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Accepted 7 January, 2010

The objective of this study was to investigate the effects of salt stress on content of carbohydrate and activity of sucrose-metabolizing enzyme and gene expression patterns and to provide a new evidence for tolerating salt stress of cultivated tomato. The related enzymes' activities of sucrose metabolism including invertase, sucrose synthase and sucrose phosphate synthase and the expression of acid invertase and sucrose synthase mRNAs were determined. The results indicated that hexoses (fructose and glucose) accumulated to higher levels and the content of sucrose and starch is lower in mature fruit under salt stress treatments; the salinity can maintain or enhance the invertase activities of tomato fruit in a long period of time (20 - 60 days after anthesis) and elevate the expression of acid invertase mRNA. Salinity could also regulate the sucrose synthase activity by controlling its gene expression. But the effects of salinity treatment on sucrose phosphate synthase activities were weak under the condition of salt stress. It showed that salinity could regulate the activity of sucrose-metabolizing enzymes by controlling its gene expression under salt stress.

Key words: *Lycopersicon esculentum*, salinity, sucrose metabolism, gene expression.

INTRODUCTION

Salinity is a major abiotic stress and affects almost every aspect of the physiology and biochemistry of plants, significantly reducing yield. Great effort has been devoted to understanding physiological aspects of response to salinity in plants, as a basis for plant breeders to develop salinity-tolerant genotypes (Cuartero et al., 2006). The yield of tomato crops is decreased to some extent under salinity. However, during the early stage of fruit development salt stress can increase soluble sugar content and sugar acid ratio in mature tomato fruit (Jiang et al., 2007; Shi and Tadashi, 2001; Balibrea et al., 2003).

The growth of different sink organs depends firstly on the use of sucrose, which requires cleavage into hexoses by either invertases or sucrose synthase. In this regard,

sucrose transport and metabolism are strong determinants of crop yield and quality as they affect both growth and composition of harvestable sinks. Sucrose is also accumulated in many plant tissues in response to environmental stress (Balibrea et al., 1997, 2003), for playing a role in osmoregulation and cryoprotection. Under saline conditions, the competition between different physiological processes and sink organs for carbon supplies significantly affects overall plant growth and crop yield. Carbohydrate content and composition are important factors determining tomato fruit quality and flavour. The decrease in tomato fruit weight and therefore in crop yield, under saline conditions has been partially explained in terms of sucrose transport and metabolism (Balibrea et al., 1999, 2000). It has been reported that soluble acid invertase is a major sucrolytic enzyme in cultivated tomato. This activity is considered to be involved in the composition of stored sugars (Klann et al., 1996; Miron and Schaffer, 1991). The sucrose synthase activity plays an important role in the control of sucrose import and fruit growth parameters (D'Aoust et al., 1999). In these studies, the sucrolytic activities have mainly been studied in relation to fruit quality and salinity

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Abbreviations: HLPC, High-performance liquid chromatograph; cDNA, complementary; PCR, polymerase; SS, sucrose synthase; AI, soluble acid invertase; SAI, insoluble acid invertase; NI, neutral invertase; SPS, sucrose phosphate synthase; DAA, days after anthesis.

influences sucrose metabolism in different ways, perhaps depending on the species, the development stage, the duration of the treatment, etc. Thus, sugar may have different and specific functions under salinity stress conditions. Following a search of the literature no information is available about the effect of salinity on the activity in relation to the expression gene of sucrose-metabolizing enzyme in the different parts of developmental fruit. For this reason, the objective of this work is to study whether the sucrose metabolism is modified to long-term salinity during the fruiting period and what strategies these plants develop to regulate sucrose metabolism and the enzyme activity and gene expression in sink organs.

MATERIALS AND METHODS

Plant material and fruit harvesting

The cultivated tomato hybrid Liaoyuanduoli (*Solanum lycopersicum* L.) was grown in plug containing a substrate composed of nutrient soil and vermiculite (2:1, V/V). Half-strength Hoagland's solution was applied from emergence of the first leaf and from six true leaves that emerged. 120 seedlings of uniform size were transferred to eight tanks (2 × 1.5 × 0.1 m) after carefully rinsing the roots with deionized water. The containers were filled with 200 L of full-strength Hoagland's nutrient solution (pH 6.5 ± 0.1), where the plants were to be grown. The planting pattern was of 0.5 m between rows and 0.45 m between plants within rows and plants were trimmed to only one stem, eliminating all axillary buds. The salt treatments were carried out by adding 0 (control), 25, 50 or 75 mM NaCl to the nutrient solution. The nutrient solution was changed every 7 days and the levels adjusted every 3 days.

The flowers were tagged at anthesis in the first trusses and salt treatment started at 20 days after anthesis (20 DAA). Each treatment was randomly applied to two blocks of 30 plants. Fruits were harvested every 10 days until the red ripe stage (60 days after anthesis). Fruits at each stage were weighed and cut into small pieces, frozen with liquid nitrogen and stored at -86°C until analysis. Sink strength was expressed as the dry matter accumulation rate which was calculated for each period according to the equation $(DW_{t2}-DW_{t1})/(t2-t1)$. The "t" stands for the number of days after pollination. Sink activity was expressed as the increase in dry weight per unit of dry weight present per unit of time using the equation $(\ln DW_{t2}-\ln DW_{t1})/(t2-t1)$ as described by Wang and Zhang (1993).

Photosynthesis

The photosynthetic activity of the 8th true leaf (no cotyledon) was measured just before sampling by using a Li-Cor LI-6400 XT Portable Photosynthesis System (Li-Cor Inc., Lincoln, NE, USA). These leaves remained healthy over the whole period tested in both salt stresses and control. The ambient CO₂ inside the chamber was 400-450 ppm. Each measurement (15 s) was performed 3 times. Total plant photosynthesis was calculated multiplying the net photosynthetic rate.

Carbohydrate determination

Soluble sugars (fructose, glucose and sucrose) were measured using a Waters 600E high-performance liquid chromatograph

(HPLC). Carbohydrate column and 2410 refractive index monitor were used. The mobile phase was 75% acetonitrile and ultra water (75:25). The mobile rate was 1.0 ml/min and the temperature of the column was set to 35°C. Water Millennium software was used to interpret data. The starch content was measured using perchloric acid hydrolyzed method (Cao et al., 2007). Total soluble sugar content was measured using anthrone method (Cao et al., 2007). Organic acid content was assayed with 0.1 mol/L NaOH titrate (Cao et al., 2007).

Enzyme extraction and assays

Enzyme extracts were prepared essentially as described in Miron and Schaffer, (1991) and Scholes et al. (1996). Fruit material was homogenised in 10 ml of ice-cold homogenising medium (50 mM Hepes-NaOH, pH 7.5) and centrifuged at 12000 g for 20 min at 4°C. The supernatant were dialyzed for approximately 20 h against 5 mM Hepes-NaOH (pH 7.5). The supernatant contained soluble sucrose-metabolizing related enzymes. The insoluble pellet was washed two times in homogenising medium and then incubated, with shaking, for 4 h in ice-cold homogenising medium containing 1 M NaCl. Following centrifugation, the supernatant contained apoplasmic invertase activity.

Invertase activities were measured as described in Qi et al. (2005). The soluble and insoluble acid (E.C 3.2.1.25) and neutral (E.C 3.2.1.26) invertases (6-D-fructofuranoside fructohydrolase) activities were assayed in a final volume of 25 ml, containing 0.2 ml of dialyzed enzymatic extract, 0.8 ml of reaction solution (pH 4.8 or 7.2 0.1 M Na₂HPO₄-0.1 M sodium citrate, 0.1 M sucrose for acid invertase and neutral invertase, respectively). The activities were measured by the quantity of reducing sugars released in the assay media with dinitrosalicylic acid. The reducing sugars were revealed by incubation at 100°C for 5 min and read at 520 nm in a Cary 100UV: VIS spectrophotometer (GBC Scientific Equipment Pty Ltd, Heareus, Germany). Decomposition direction of sucrose synthase (EC 2.4.1.13) was measured as described in Balibrea et al. (2000).

Synthesis direction of sucrose synthase (EC 2.4.1.13) was measured by using 0.4 ml reaction solution (0.05 M fructose, 0.82% UDPG, 0.1 M Tris, 10 mM MgCl₂) adding 0.2 ml enzyme, incubating at 37°C for 30 min followed by 1 min at 100°C, 0.6 ml distilled water and 0.1 ml 2 M NaOH was added, incubated in a boiling water bath for 10 min and cooled in water to room temperature, then adding 3.5 ml 30% HCl and 1ml 0.1% resorcinol. Blank controls were obtained by adding the distilled water to the reaction medium containing resorcinol. The reducing sugars were revealed by incubation at 80°C for 10 min and read at 480 nm in a Cary 100UV: VIS spectrophotometer (GBC Scientific Equipment Pty Ltd, Heareus, Germany). Sucrose-phosphate synthase (EC 2.4.1.14) was assayed by measurement of sucrose produced from fructose 6-phosphate plus UDP-glucose (Vassey and Sharkey, 1989).

RNA isolation and hybridization

RNA was extracted using the Trizol Regent (Invitrogen Life Technologies, Carlsbad, CA, USA). 15 µg of total RNA were separated by electrophoresis through 1.2% (w/v) formaldehyde-agarose gels and blotted onto a nylon membrane (Hybond-N+; Amersham-Pharmacia Biotech, USA) by capillary transfer. Probes for the detection of acid invertase (AI - GenBank accession number AF465613) and sucrose synthase (SS - GenBank accession number AJ011535) transcripts were PCR-amplified with primers of AI2F (5'-AACTCCGCTCTCGTTACACA-3') and AI2R (5'-TAGGATGGTAGCGACCCTG-3'), SuS2F(5'-AACTTTAGCTGCTCACCGAA-3') and SuS2R(5'-TTGCCCTTATAATGGTGAGCG-

Table 1. Influence of different concentrations of NaCl treatments on tomato fruit fresh weigh and quality.

NaCl (mM)	Soluble sugar (%)	Organic acid (%)	Brix:acid ratio	Starch (mg/g)	Dry weight (g)
0	2.29 ± 0.27	0.71 ± 0.02	3.22	7.75 ± 0.31	18.91 ± 2.17
25	2.80 ± 0.27	0.75 ± 0.03	3.73	5.31 ± 0.26	19.18 ± 1.99
50	3.92 ± 0.21	0.84 ± 0.06	4.61	3.49 ± 0.13	17.86 ± 1.92
75	4.03 ± 0.17	0.88 ± 0.09	4.58	5.43 ± 0.27	16.53 ± 1.72

Values are the mean ±SD of five replicate samples.

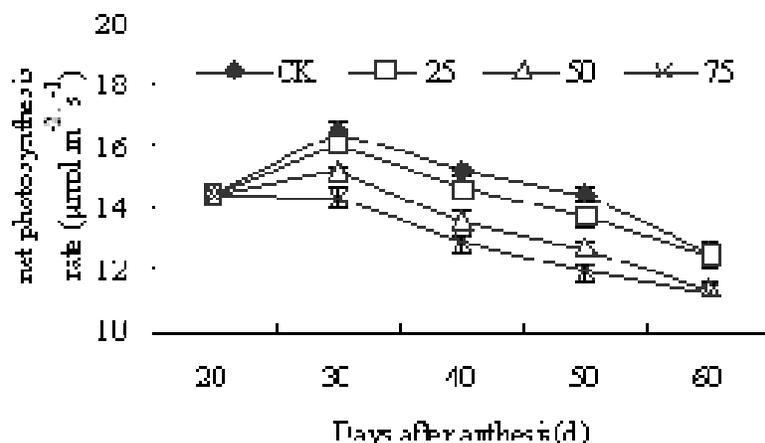


Figure 1. Net photosynthesis rate of the 8th true leaf for NaCl treatments 25, 50 and 75 mM and no NaCl control –CK. . Each point represents the mean ± SD of five measurements in different plants.

3'). The gene-specific probes were labeled according to the manufacturer's instructions with DIG using DIG High Prime DNA Labeling and Detection Starter Kit (Roche Diagnostics GmbH, Mannheim, Germany).

Quantitative real time-PCR analysis

PCR primers were designed with the Primer Express program Version 1.0 from PE Biosystems (USA) to produce a short amplicon (100-200 bp). The primer sequences (*lin5*, *tiv-1*, *sus2* and *sus3*) can be found in Kortstee et al. (2007). RNA was extracted using RNeasy pure kit (Qiagen). cDNA was prepared with the Quantscript cDNA first-strand synthesis kit for Real Time PCR (Qiagen). SYBR® Green PCR Master Mix (Qiagen) was used on an Applied Biosystems 7500 Real Time PCR System. The program of the thermocycler was set at: denaturation for 3 min at 95°C followed by 40 cycles at 95°C for 30 s and annealing at 57°C for 30 s and extension for 1 min at 68°C. Relative quantification of the target RNA expression level and standard deviation was performed using the comparative Ct method according to the 7500 software v2.0.1 (Applied Biosystems).

RESULTS

Fruit weight and quality

At the end of the salinization period, 50 and 75 mM NaCl reduced the single fruit dry weight by 5.6 and 13%,

respectively (as compared with control plants), while 25 mM NaCl increased the single fruit dry weight by 1.4% in mature fruit (Table 1). The content of soluble sugar and titratable acids increased along with the increase of NaCl concentration, the treatment of 50 mM NaCl had the highest ratio of brix- acid in mature fruit. It is showed that treatment with a certain concentration of salt will improve the quality of the fruit flavor and nutritional value.

Photosynthesis

Net photosynthesis reaches a maximum at 30 DAA, followed by a gradual decline; this pattern was also observed in saline treated plants (Figure 1). However, the saline conditions resulted in a lower photosynthesis rate relative to the concentration of NaCl, that is, 25 mM NaCl had a smaller reduction in photosynthesis rate than 75 mM NaCl (Figure 1). The largest difference in net photosynthesis rate was observed between the control and treated plants at 50 DAA, which decreased significantly up to 17.7% under 75 mM NaCl stress condition compared with control; it was between 7.5% (30 DAA) and 12% (50 DAA) lower under 50 mM NaCl stress in the different stages of fruit developments (Figure 1). Compared with control conditions, the net photosynthesis rate was more reduced by 75 mM NaCl stress

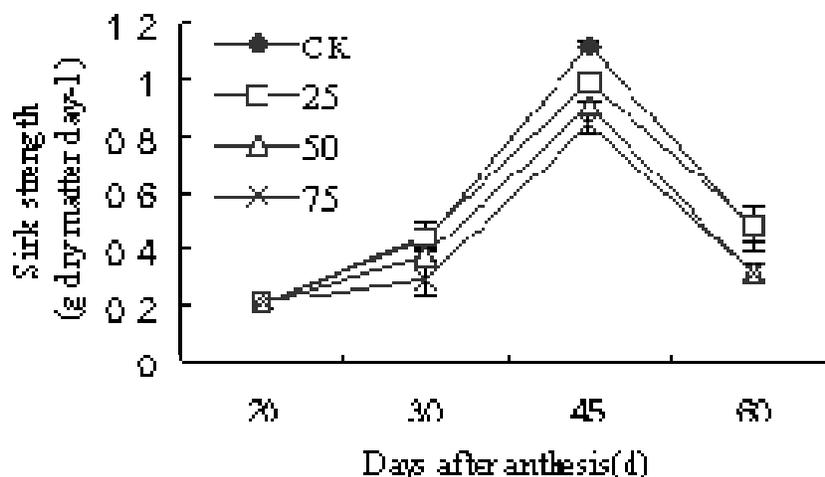


Figure 2. Influence of NaCl treatments 25, 50 and 75 mM and no NaCl control-CK on sink strength of tomato fruit. Values are the mean \pm SD of five replicate samples.

than 50 mM NaCl stress and in a similar extent under 25 mM NaCl stress condition.

Sink strength

The sink strength, a measure of dry fruit weight gain, may be considered as an import rate. In control fruits, the fastest growing period occurred between 30 and 45 DAA (Figure 2). During this critical period, salinity reduced the sink strength by 11% (25 mM NaCl) and by more than 25% (75 mM NaCl). At the end of the growing period (60 DAA), there was no difference in this parameter between 25 mM NaCl stress and control fruits and 50 and 75 mM NaCl stress.

Hexose, sucrose and starch contents

As the fruit developed, the hexose (fructose and glucose) was the predominant sugars and gradually increased with the highest level presenting at ripe stage (Figure 3). Hexoses accumulated to higher levels in mature fruit under salt stress treatments. 75 mM NaCl stress provoked the highest hexose concentrations increased proportionally with increasing NaCl stress.

In the early stage of development, starch accumulated between 20 and 45 DAA but then declined sharply during the ripening stage, 45 – 60 DAA. The content of sucrose slightly decreased in tomato mature fruits under salt stresses than under control. Different concentrations of NaCl treatments produced similar impact on the concentration of sucrose and starch (Figure 3).

Activities of sucrose-metabolizing related enzymes

In order to determine whether changes in carbohydrate

content could be due to an increase in enzymes mobilizing sucrose or hexose, we measured sucrolytic activities and sucrose biosynthetic activities at the start of ripening and at the fully ripe stage under salt stress.

The results suggest that under non-saline conditions, there were differences in the activities of enzymes measured in the mesocarp and radial pericarp in comparison with the placental tissue. The placental tissue had consistently increased the activities of several enzymes in comparison with the mesocarp and radial pericarp. This is presumably because the different tissues serve different roles.

The changes in invertase (soluble and insoluble acid invertase and neutral invertase) activity and decomposition direction of sucrose synthase during the development of three different tissues of tomato fruit are shown in Figure 4. The sucrolytic activities (except insoluble acid invertase) are generally increased toward the end of fruit development with ripening. The acid invertase and decomposition direction of sucrose synthase were the main activity responsible for sucrose cleavage in both control and treatment fruits. The sucrolytic activities of fruits were generally higher under salt stress than under control. At 60 DAA, AI and SS activities had a twice increase in mesocarp under 75 mM salinity; the soluble acid invertase activity of fruit was generally higher in the placental tissue than the mesocarp and radial pericarp. SAI and NI activities slightly increased under salt stress than under control conditions between 45 - 60 DAA in the different tissues of tomato fruits. All concentrations of NaCl examined can improve the sucrolytic activities.

The activity of synthesis direction of sucrose synthase (SS) showed low activity at later stages of fruit development (Figure 5). At 45 and 60 DAA, SS activity stayed approximately the same in the different tissues, which also stayed approximately the same under salt stress and control conditions in tomato fruits. Sucrose phosphate

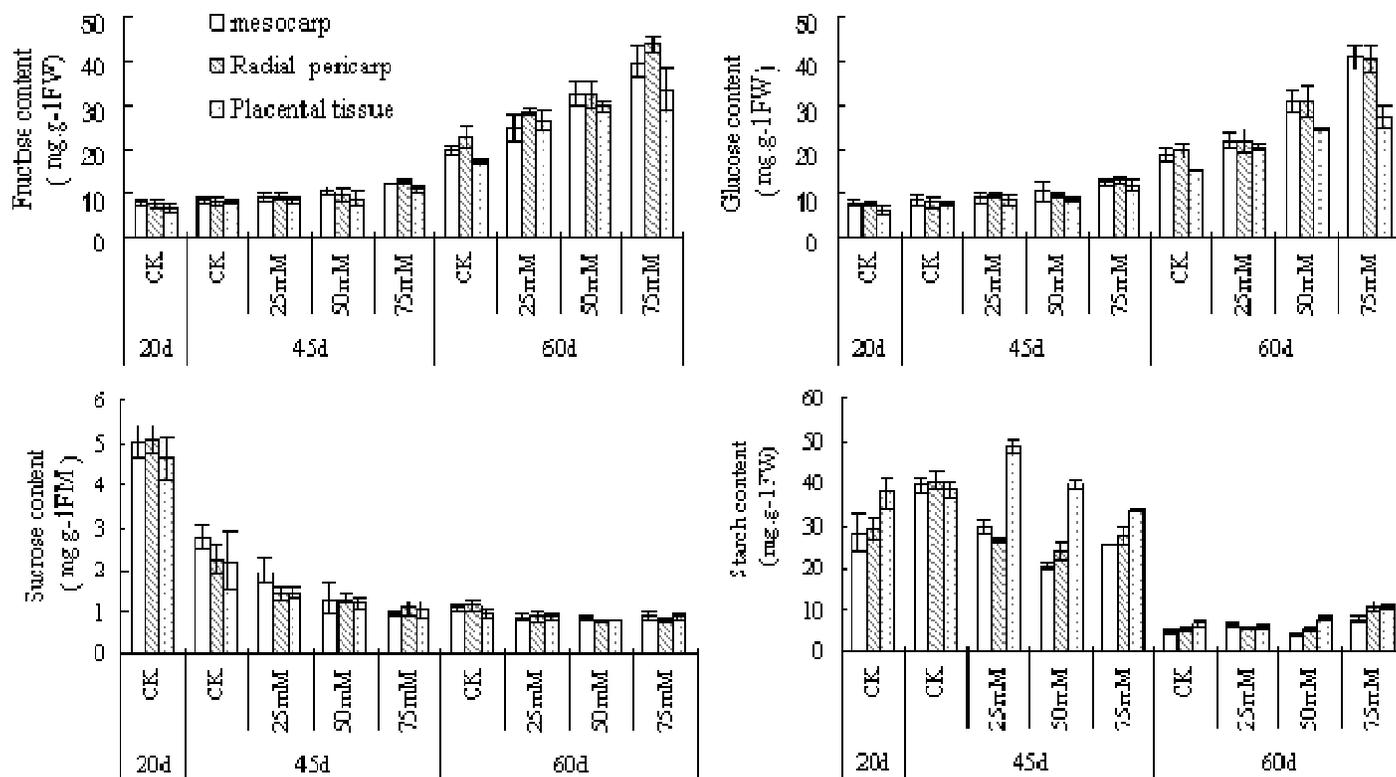


Figure 3. Influence of NaCl Stress on tomato fruit carbohydrate content. Glucose, fructose, sucrose and starch were determined in the same samples. Values are the mean \pm SD of three replicate sample.

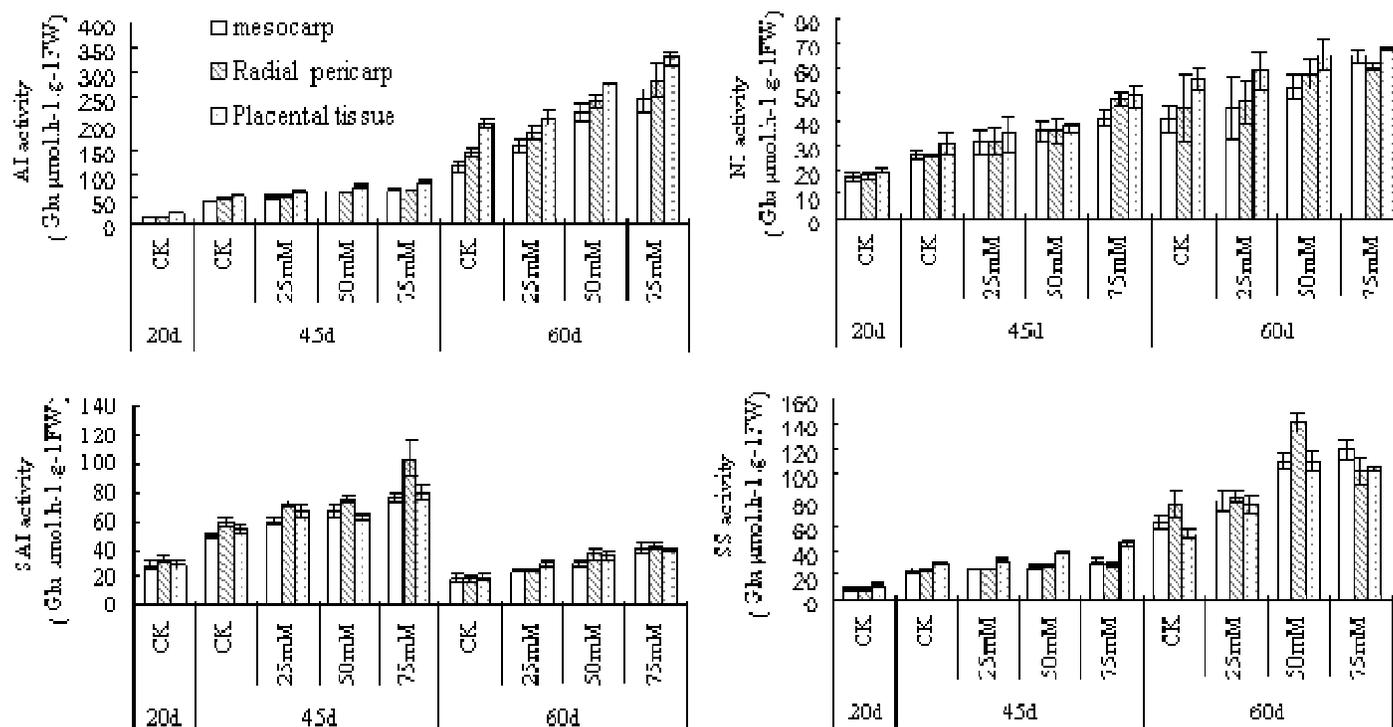


Figure 4. Effect of NaCl Stress on sucrolytic activities in tomato fruit. The enzymes were determined in crude extracts in the same samples. Values are the mean \pm SD of three replicate samples.

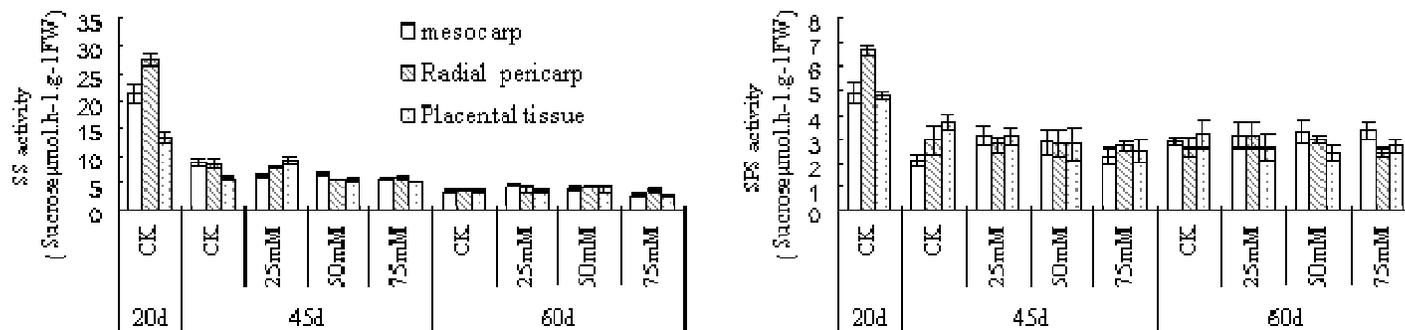


Figure 5. Effect of NaCl Stress on activities of sucrose biosynthetic in tomato fruit. The enzymes were determined in crude extracts in the same samples. Values are the mean \pm SD of three replicate samples.

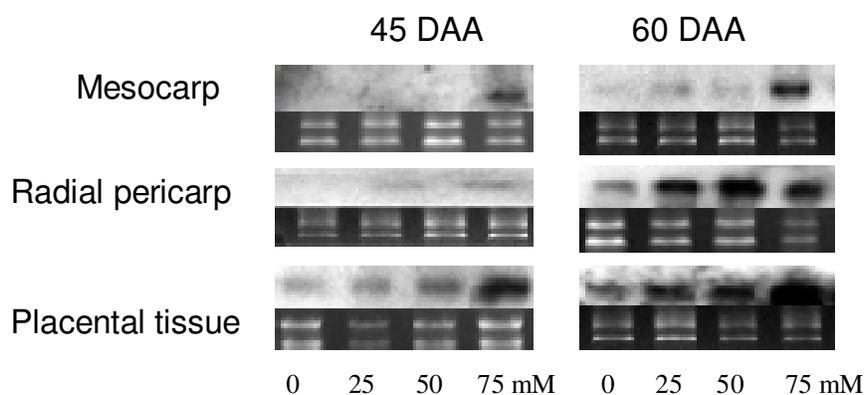


Figure 6. Northern blot analysis of total RNA from mesocarp, radial pericarp and placental tissue at 45 and 60 DAA using partial fragment of AI as probe.

synthase had a similar pattern to the SS. The SPS activity stayed approximately the same between 45-60 DAA under salt stress and control conditions in the different tissues of tomato fruits.

Gene Expression of Sucrose-metabolizing related enzymes

The temporal and spatial expression patterns of acid invertase during tomato fruit development were investigated (Figure 6). In Northern blot analysis, transcriptions of AI were abundant in the placental tissue at 45 and 60 DAA. They were also increased towards the ripening of mesocarp and radial pericarp. After treatment with different concentrations of salt stress, acid invertase gene expression is more complex. There have no detection of gene expression in the mesocarp at 45 DAA and a lower expression at 60 DAA by 25 and 50 mM NaCl. AI mRNA level were increased with the addition of NaCl concentration for all tissue samples. The highest expression activity of acid invertase was found by the 75 mM NaCl in the placental tissue at 60 DAA.

To determine if the differences in enzyme activity in different tomato tissues under different conditions were controlled at the transcriptional level, we measured the expression levels of several genes. The expression of the invertase genes *tiv-1*, *lin5*, *lin6*, *lin7*, *lin8*; the sucrose synthase genes *Sus2* and *Sus3* were studied by means of quantitative real-time PCR. The expression level of each gene was normalized to the expression of actin in the same cDNA sample and the results are shown in Table 2. Expression of *lin6*, *7* and *8* could not be detected in developing fruits. *Tiv-1* expression in tomatoes fruits increased during development, salinity increased the expression levels of this gene at 45 and 60 DAA, except for placental tissue at 45 DAA. Expression of *lin5* in different tissues of tomato fruit decreased with ripening, the expression was further reduced due to salt. The highest expression activity of acid invertase was found in the placental tissue. Expression of *Sus2* was higher in fruits at 45 DAA compared to ripe stage and salinity can decrease the expression levels of this gene. The highest expression activity of *Sus2* was found in the mesocarp. The other sucrose synthase gene, *Sus3*, was expressed at higher levels in fruits at 60 DAA than at 45

Table 2. Relative transcript levels in the mesocarp (M), radial pericarp (R) and placental tissue (P) under 50 mM NaCl treatment (T) and control condition (C) using Real time-PCR analysis of gene expression. Values for expression levels are normalized to actin.

DAA	Gene	MC	MT	RC	RT	PC	PT
45	Lin5	1.000 ± 0.20	0.174 ± 0.06	0.805 ± 0.07	0.104 ± 0.05	1.852 ± 0.78	1.514 ± 0.69
	Tiv-1	1.000 ± 0.12	2.108 ± 0.24	0.655 ± 0.06	0.719 ± 0.04	4.435 ± 0.54	3.442 ± 0.34
	Sus2	1.000 ± 0.25	0.675 ± 0.22	0.843 ± 0.17	0.523 ± 0.06	0.782 ± 0.14	0.516 ± 0.06
	Sus3	1.000 ± 0.16	0.573 ± 0.05	1.326 ± 0.06	0.064 ± 0.07	1.581 ± 0.17	1.412 ± 0.16
60	Lin5	0.262 ± 0.02	0.107 ± 0.01	0.225 ± 0.28	0.102 ± 0.02	0.575 ± 0.02	0.139 ± 0.05
	Tiv-1	29.79 ± 7.41	54.18 ± 5.52	27.85 ± 6.85	45.93 ± 6.27	30.52 ± 8.39	119.8 ± 10.9
	Sus2	0.377 ± 0.23	0.162 ± 0.04	0.126 ± 0.04	0.119 ± 0.02	0.024 ± 0.01	0.015 ± 0.01
	Sus3	1.474 ± 0.61	1.897 ± 0.08	1.700 ± 0.20	3.073 ± 0.45	2.152 ± 0.08	7.288 ± 0.37

Values are the mean ± SD of three replicate samples.

DAA and the expression of *Sus3* increased under salt stress. The expression activity of *Sus3* was higher in the placental tissue than in mesocarp and radial pericarp.

DISCUSSION

Variations in enzyme activities between tomato tissues suggest that enzymes may play specific roles dependent on tissue type. The placental tissue acts as a conduit for nutrients going to the developing seeds, whereas the mesocarp and radial pericarp protect the seeds within the fruit. It is interesting to note that the invertase activity increased dramatically in the final stages of fruit development, especially in the placental tissue. This coincides with a reduction in the sucrose content (Konno et al., 1993). Fructose levels are relatively high in tomato fruit, as was also measured in this study. It has been shown that under *in vitro* conditions SuSy is inhibited by high concentrations of fructose and suggested that this also may be the case *in vivo* (Schaffer and Peterikov, 1997).

The growth capacity of tomato plants under saline conditions have been related to the increase in sink activity of young leaves and roots by the induction of vacuolar acid invertase and sucrose synthase activities (Balibrea et al., 2000). In tomato, most of the invertase activity is attributable to soluble acid invertase, the other alkaline and insoluble invertase isoforms being either absent or present in negligible amounts (Husain et al., 2001). The major function of the high and constant invertase activity in red tomato fruit is to maintain the cellular hexose concentrations. This study found that acid invertase and decomposition direction of sucrose synthase activity is increased by NaCl treatment, while the synthesis direction of sucrose synthase and SPS activity has no significant difference when compared with control in the later development stage of fruit. Increased invertase activity due to salt stress may have resulted in the improved hexose contents of tomato fruit. Gene expression of *tiv-1* and *sus3* changed significantly from 45 to 60 DAA. The enzyme activity did not correlate with the gene expres-

sion in a 1:1 ratio; this may be due to other regulatory factors effecting both transcription and translation. This is presumably because these genes had modification and regulation in the post-transcription. It has been reported that hexose participate in the apple fruit acid-invertase inhibitory post-translational regulation (Wang and Zhang, 2002). This showed that post-translational modification plays an important role in regulation of the sucrose-metabolizing related enzymes activity.

Although only two representative time points have been considered in this study of tomato fruit development, the results show that increase in fruit quality and production by salinity in tomato fruits is related to the sucrolytic activities. But, the identification of different isoforms, location and regulatory mechanisms by endogenous factors such as hormones or sugars could be important in order to determine the role of these enzymes in maintaining sink capacity under salinity. They should be also considered in the scope of the tomato sucrose metabolism in salinity.

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