

Proteomics and metabolomics analysis of tomato fruit at different maturity stages and under salt treatment

Huimeng Tang¹, Xu Zhang¹, Biao Gong, Yanyan Yan, Qinghua Shi*

State Key Laboratory of Crop Biology, Shandong Collaborative Innovation Center of Fruit & Vegetable Quality and Efficient Production, College of Horticulture Science and Engineering, Shandong Agricultural University, Taian 271018, PR China

ARTICLE INFO

Keywords:

Proteomics
Metabolites
Tomato fruit quality
Salt treatment
Fruit ripening

ABSTRACT

Proteomics and metabolomics were used to study the changes in proteins and metabolites in tomato fruits at different ripening stages and the effect of salt treatment on fruit quality. The results showed 2607 and 153 differentially expressed proteins in ripe fruits compared with mature green fruits and in NaCl-treated ripe fruits compared with control ripe fruits, respectively. KEGG analysis indicated that these proteins were mainly involved in photosynthesis, pentose and glucuronate interconversions in different ripening stages of fruits, and salt-induced proteins were involved in flavonoid biosynthesis and linoleic acid metabolism. A series of metabolites, including carbohydrates and amino acids showed significantly different accumulations between ripe and mature green fruits and between salt-treated and control fruits. Combined analysis explored glycine, L-alanine, D-xylose and sucrose and some proteins involved in multiple metabolic pathways under salt conditions. Their interactions might affect fruit development and fruit quality under salt treatment.

1. Introduction

Tomatoes are popular worldwide because of their unique flavor and abundant nutrients, which are dependent on the fruit color, firmness, sugar content and volatile aromatics. Large numbers of proteins and metabolites are changed in the process of tomato fruit growth and development, which ultimately determine the fruit quality. Fruit ripening is the result of a combined change of structure, physiological and biochemical levels (Meng et al., 2016), and in recent years, some results have reported on aspects of fruit development and maturation and the changes in physiological and biochemical processes under adverse conditions (Carrari & Fernie, 2006; Rodríguezruiz, Mito, & Palma, 2016). With the ripening of tomato fruit, the color transforms from green into red, accompanied by cell wall degradation and the accumulation of volatile aromatics and some primary metabolites (Zhu et al., 2018). In addition, related proteins responsible for essential physiological processes also change with fruit maturation (Rocco et al., 2010), which could regulate the process of fruit quality formation. Furthermore, tomato quality is also influenced by environmental factors, such as salt stress (Zhang, Mao, Shi, & Kou, 2017), temperature (Riga, Anza, & Garbisa, 2010) and drought (Liu et al., 2010).

According to incomplete statistics from the UNESCO and FAO, there are 954.3 million hectares of saline soil in the world, of which 99.13

million hectares are in China. Salt stress has a negative impact on plant growth, resulting in plant nutritional imbalance, oxidative stress, photosynthesis inhibition and respiratory changes. Zhang et al. (2017) reported that the expression profiles of some vital genes participating in carbohydrate and amino acid metabolic pathways are obviously changed under salt stress. Proteins related to energy metabolism, redox reactions and ripening process were affected by salt treatment in tomato fruit (Manaa et al., 2013). Although salt stress resulted in lower productivity, research has indicated that salt treatment could significantly improve fruit quality (Li, Wang, Li, Yao, & Hao, 2013). Keutgen and Pawelzik (2008) found that the total amino acid content in strawberry was increased by salt treatment, and many key genes involved in cell wall metabolism and flavonoid and phenylpropanoid pathways were changed under salt treatment (Galli et al., 2016). In tomato, enzyme activities associated with the sucrose metabolic pathway, such as acid invertase and sucrose synthase activities were enhanced by salt treatment (Lu, Li, & Jing, 2010). However, the mechanism of salt treatment regulation requires further investigation.

Proteomics and metabolomics technologies have become increasingly important in systemically exploring the mechanisms of plant growth and development and the responses to biotic and abiotic stresses. Szymanski et al. (2017) reported that the expression of proteins was differently changed in fruit flesh and skin tissues during

* Corresponding author.

E-mail address: qhshi@sdaa.edu.cn (Q. Shi).

¹ These authors equally contributed to the work.

tomato fruit development, and the phenylpropanoid pathway played an important role in fruit ripening, especially in skin tissue. In oriental melon, proteomics analyses found that many proteins were found to be up or down regulated, and most of these proteins participated in sugar and energy metabolism pathways (Guo, Xu, Cui, Chen, & Qi, 2017). Yue et al. (2018) found that a series of proteins in maize responding to rough dwarf disease and some metabolic pathways were changed by employing Tandem Mass Tag (TMT)-labeled proteomics. Lee et al. (2016) identified a total of 48 specific metabolites at different mulberry fruit maturation stages using gas chromatography-mass spectrometry; among those compounds, levels of fructose, glucose and citrate were obviously improved. Baldina et al. (2016) reported that the contents of amino acids, glycoalkaloid compounds and total phenolics in three fruit shape typologies of tomato were significantly different. Yin et al. (2010) revealed changes of amino acids during tomato fruit ripening and found that the content of aspartate clearly increased and that γ -aminobutyric acid levels decreased gradually, while organic acid did not show an obvious change under salt stress. Thus, omics technology is a highly effective tool for systemically clarifying the metabolic pathways and protein expression levels in different development stages or under different surroundings. Despite the fact that some progress has been made in the study of fruit quality, the systemic understanding is still limited; thus, to further investigate the mechanism of fruit quality formation at different ripening stages and under salt treatment, integrated omics can be a powerful tool. In the present experiment, integrated proteomics and metabolomics methods were applied to explore the different protein expression and metabolites profiles in tomato fruits at different stages and in ripened fruits under salt treatment, and the proteomics and metabolomics co-analyses were applied. The results will provide a theoretical basis for understanding the quality formation of tomato fruit in the ripening process and will expand the understanding of fruit quality improvement during tomato development in a salt environment.

2. Materials and methods

2.1. Plant materials and treatments

This experiment was carried out in the glass greenhouse of Shandong Agricultural University. The climate condition of the glass greenhouse is 12 h/12 h, 25–28 °C/15–20 °C (day/night), and the humidity level is 50–70%, with natural lighting. Tomato plants (*Solanum lycopersicon*, cv. Micro-Tom) were cultivated using Hoagland nutrient solution through hydroponic culture. There were 6 pots in the experiment and 6 plants in one pot; every pot was one replicate, and there was a total of 36 tomato plants in all pots. Flowers were marked at the anthesis, and 3–5 flowers with the same flowering period were selected for marking of each tomato plant; and the other inflorescences were removed. The first fruit sample was taken at the mature green fruit stage (G) (Fig. S1), which corresponds to 22 days after flowering, after which 60 mM NaCl (N) nutrient solution was added into 3 pots, with the other 3 pots kept under normal conditions (R). The nutrient solution was changed every 7 days. When the fruits turned mature red (45 days after flowering) (Fig. S1), the pericarp was taken and immediately placed in liquid nitrogen and kept at –80 °C. Each sample consisted of 1 g from six tomato plants for proteomics and 1 g of pericarp from two tomato plants for metabolomics. The repetitions for proteomics and metabolomics analysis were three and eight, respectively.

2.2. Proteomics analysis methods

2.2.1. Protein extraction, trypsin digestion, and TMT labeling

The 1 g frozen samples taken from –80 °C storage were ground in liquid nitrogen into powder and then transferred to a 5 mL centrifuge tube. Four volumes of lysis buffer containing 8 M urea (Sigma-Aldrich, Saint Louis, USA), 10 mM dithiothreitol (Sigma-Aldrich, Saint Louis, USA), 2 mM ethylenediaminetetraacetic acid (Sigma-Aldrich, Saint

Louis, USA) and 1% protease inhibitor cocktail VI (Merck Millipore 539133-1ML, Billerica, USA) was added to each sample, which was then subjected to sonication (ultrasonication in an ice water bath for 5 min, on 3 s, off 5 s, ultrasonic intensity 30%), and was then centrifuged at 20,000g (4 °C) for 10 min. After that, the supernatant was aspirated and added to 2.7 mL of 20% trichloroacetic acid for 2 h at 4 °C, followed by centrifugation at 12,000 g (4 °C) for 3 min, after which the supernatant was discarded and the sediment was washed three times with precooled acetone. Finally, the precipitate was redissolved in 10 mL of 8 M urea and tested using a BCA kit (Beyotime, Shanghai, China) to determine the protein concentration.

For digestion, dithiothreitol was added to the protein solution to a final concentration of 5 mM and reduced at 56 °C for 30 min, followed by the addition of iodoacetamide (Sigma-Aldrich, Saint Louis, USA) to a final concentration of 11 mM, after which the sample was placed at room temperature in the dark for 15 min. Finally, the concentration of urea in the sample was diluted to < 2 M. Trypsin (Promega, Madison, USA) was added at a 1:50 trypsin-to-protein mass ratio for the first digestion overnight at 4 °C and a 1:100 trypsin-to-protein mass ratio for a second 4 h digestion.

The trypsin-digested peptide was desalted by Strata X C 18 (Phenomenex, Torrance, US). After enzymatic hydrolysis, Strata X C18 (Phenomenex) was used to remove salt. The simplified steps were as follows: the sample was acidified to pH 2–3 with TFA, the salt column was activated once with methanol, the sample was balanced twice with 0.1%TFA, the sample was added, the sample was carried out again with flow, 0.1%TFA was removed salt three times, and 80% ACN was eluted. After these steps, the sample was vacuum freeze-dried. Peptide was dissolved in 100 μ L of 0.5 M tetraethylammonium bromide (Sigma-Aldrich, Saint Louis, USA), and the peptides were marked according to the brief introduction for the TMT kit (ThermoFisher Scientific, Waltham, USA). Following the addition of 41 μ L of acetonitrile to dissolve the TMT reagent, the sample was incubated for 2 h at room temperature. Finally, the labeled peptides were pooled, desalted and dried by vacuum centrifugation.

2.2.2. HPLC fractionation

A one-milliliter sample of peptides was fractionated into fractions by high pH reverse-phase HPLC. The columns used were Agilent 300 Extend C 18 (5 μ m particles, 4.6 mm ID, 250 mm length, Agilent, Santa Clara, USA). First, peptides were separated with a gradient of 8% to 32% acetonitrile (pH 9.0) over 60 min into 60 fractions. Second, the peptides were combined into 18 fractions and then dried by vacuum centrifugation prior to the next operation.

2.2.3. LC-MS/MS analysis

The tryptic peptides were dissolved in 0.1% formic acid (Solvent A, consisting of 0.1% formic acid and 2% acetonitrile) and detached through the EASY-nLC 1000 UPLC system (ThermoFisher Scientific, Waltham, USA). The separated peptides were entered into an NSI source and then followed by MS/MS in Q ExactiveTM Plus (ThermoFisher Scientific, Waltham, USA) coupled online to the UPLC. The ion source was set at 2.0 kV. The m/z scan range was 350–1800 for the full scan, and intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were then selected for MS/MS using an NCE setting as of 28, and the fragments were detected in the Orbitrap at a resolution of 17,500. To improve the efficiency of mass spectrometry, the data-dependent procedure alternated between one MS scan followed by 20 MS/MS scans with 30 s dynamic exclusion. Automatic gain control was set at 1E4, and the fixed first mass was set at 100 m/z .

2.2.4. Database search

The MAXQUANT search engine (v.1.5.2.8) and the *Solanum lycopersicum* database (includes 33,952 sequences) were used to obtain the MS/MS results. The search parameters were as follows: the cleavage enzyme was trypsin, allowing up to 2 missing cleavages, the mass

tolerance of precursor ions in the first set of search was 20 ppm, and that of the main search was 5 ppm, and the mass tolerance of the fragment ions was set to 0.02 Da. The quantitative method was set to TMT-10 plex, and the FDRs of protein identification and PSM identification were both adjusted to < 1%. The full data for protein identification were submitted to PRIDE.

2.2.5. Gene Ontology analysis

Gene Ontology (GO) annotation of the proteome was obtained from the UniProt-GOA database (www.ebi.ac.uk/GOA/). First, identified protein IDs were changed to UniProt IDs and were then mapped to GO IDs by protein ID, and the related information was obtained from the UniProt-GOA database according to GO ID. If some identified proteins were not annotated in the UniProt-GOA database, InterProScan software (<http://www.ebi.ac.uk/interpro/>) was used to annotate the protein's GO function based on a protein sequence alignment method. These proteins were then classified according to biological process, cellular component and molecular function.

2.2.6. Subcellular localization

Wolfpsort (v. PSORT/PSORT II, Japan) was used to predict subcellular localization for proteins. The plant sequence was first selected in the software, and the identified protein sequence was then input to predict its location.

2.2.7. Enrichment of pathway analysis

The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was applied to enrich the pathway analysis (Yue et al., 2018). A two-tailed Fisher's exact test was used to test the differentially expressed proteins against all identified proteins. A p-value less than 0.05 was regarded as a significant change in the enrichment pathways.

2.3. Metabolomics analysis methods

2.3.1. Sample preparation

One-gram samples of frozen fruits were taken from -80°C storage and ground into powder in liquid nitrogen. Each sample was precisely weighed, and 15 mg was placed in a microcentrifuge tube, to which 25 mg of prechilled zirconium oxide beads, 10 μl of internal standard and 50 μl of 50% prechilled methanol were added for automated homogenization (BB24, Next Advance, Inc., Averil Park, NY, USA). After centrifugation at 14,000 g and 4°C for 20 min (Microfuge 20R, Beckman coulter Inc., Indianapolis, IN, USA), the supernatant was carefully transferred to an autosampler vial (Agilent Technologies, Foster City, CA, USA), and after adding 200 μl precooled methanol/chloroform (v/v, 3:1) for extraction metabolites, the sample was centrifuged at 14,000 g (4°C) for 20 min. The supernatant (200 μl) was carefully removed to a 1.5 mL automatic injection glass bottle (Agilent Technologies, Foster City, CA, USA) and set in a vacuum centrifuge concentrator (Labconco, Kansas City, MO, USA) for 5 min to remove chloroform, after which the sample was transferred to a low-temperature freeze dryer (Labconco, Kansas City, MO, USA) to completely lyophilize the sample. After the completely dried samples were restored to room temperature, they were filled with high-purity nitrogen (Parker Balston, Lancaster, NY, USA) to protect the samples. The samples were stored by automatic capping and placed on an automatic derivatization platform for silylation and derivation. The brief process was as follows: 50 μl of 20 mg/ml methoxyamine pyridine solution was added to the dried sample, which was then incubated for 2 h at 30°C , followed by the addition of 50 μl MSTFA (containing FAFEs) for 1 h at 37.5°C . After that, the derivatized samples were placed in a time-of-flight mass spectrometry (GC-TOF/MS) system (LECO Corp., St. Joseph, MI, USA) for analysis. GC-TOF/MS system (Pegasus HT, Leco., Joseph, Mo, USA) with an Agilent 7890B gas chromatograph and a Gerstel multipurpose sample MPS2 with dual heads (Gerstel, Muehlheim, Germany). A Rix5-ms capillary column (30 m $\times$ 250 μm i.d., 0.25- μm film thickness;

Restek corporation, Bellefonte, PA, USA) was used for separation. Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The temperatures of the injection and transfer interfaces were both set to 270°C . The source temperature was 220°C . The measurements were made using electron impact ionization (70 eV) in the full scan mode (m/z 50–500).

2.3.2. Metabolite annotation and data analysis

Data generated by GC-TOFMS were loaded into XploreMET software (Metabo-Profile, Shanghai, China) for processing through Chromma TOF software (V. 4.51.6.0, Leco, CA, USA), and were compared to the JiaLib™ database to identify metabolites using a strict matching algorithm incorporated in XploreMET software (Metabo-Profile, Shanghai, China) that uses both retention times and fragmentation patterns in the mass spectrum.

2.3.3. Univariate statistics and metabolic pathway enrichment analysis

The differential metabolites can also be identified using univariate statistical analysis (student T-test). A calculated fold change of 1.5 or a p-value of 0.05 was chosen for statistical significance. XploreMET software uses a proprietary hypergeometric algorithm, which can enrich the information of metabolites and look for key metabolic networks. A p-value < 0.05 was regarded as significantly different, and the importance of the metabolic pathway was used to evaluate the impact-value.

2.4. Heatmap analysis

The R-package was used to draw the heatmap (Gong et al., 2019). The log2-fold changes of R/G and N/R ratios and fold changes were used for heatmap analysis of proteins and metabolites, respectively.

2.5. Determination and analysis of fruit hardness

The hardness of tomato fruits was measured by a durometer (GY-1). Excel 2013 was used for data processing and DPS software was used for difference significance analysis ($p < 0.05$).

3. Results

3.1. Protein profiles of tomato fruit at different maturation stages and protein changes of under salt treatment

To study the protein changes in tomato fruit during the ripening process and under salt treatment, we combined a series of advanced technologies, including TMT labeling, HPLC fractionation technologies and mass spectrometry-based quantitative proteomics technology to quantitatively research the protein composition of tomato fruit tissue. With over 1.3-fold-change and low 1/1.3-fold-change as the up- and down-regulated standards, 2607 differentially expressed proteins were identified between mature red stage and mature green stage (R/G), and 153 differentially expressed proteins were identified between control mature fruits and NaCl-treated mature fruits (N/R), respectively. There were 104 identical differentially expressed proteins in R/G and N/R (Fig. S2). Among these significantly different expressed proteins, 1158 were up-regulated and 1409 were down-regulated in mature red fruits compared with mature green fruits. Furthermore, 59 were up-regulated and 94 were down-regulated in ripening fruits under salt treatment compared to normal maturity fruits (Table S1).

3.2. Functional classification of differentially expressed proteins

We applied Gene Ontology to quantitatively analyze the proteins, which can describe the biological functions of proteins from three aspects: biological process, cellular component and molecular function (Fig. 1). Based on the results, most of the identified proteins focused on

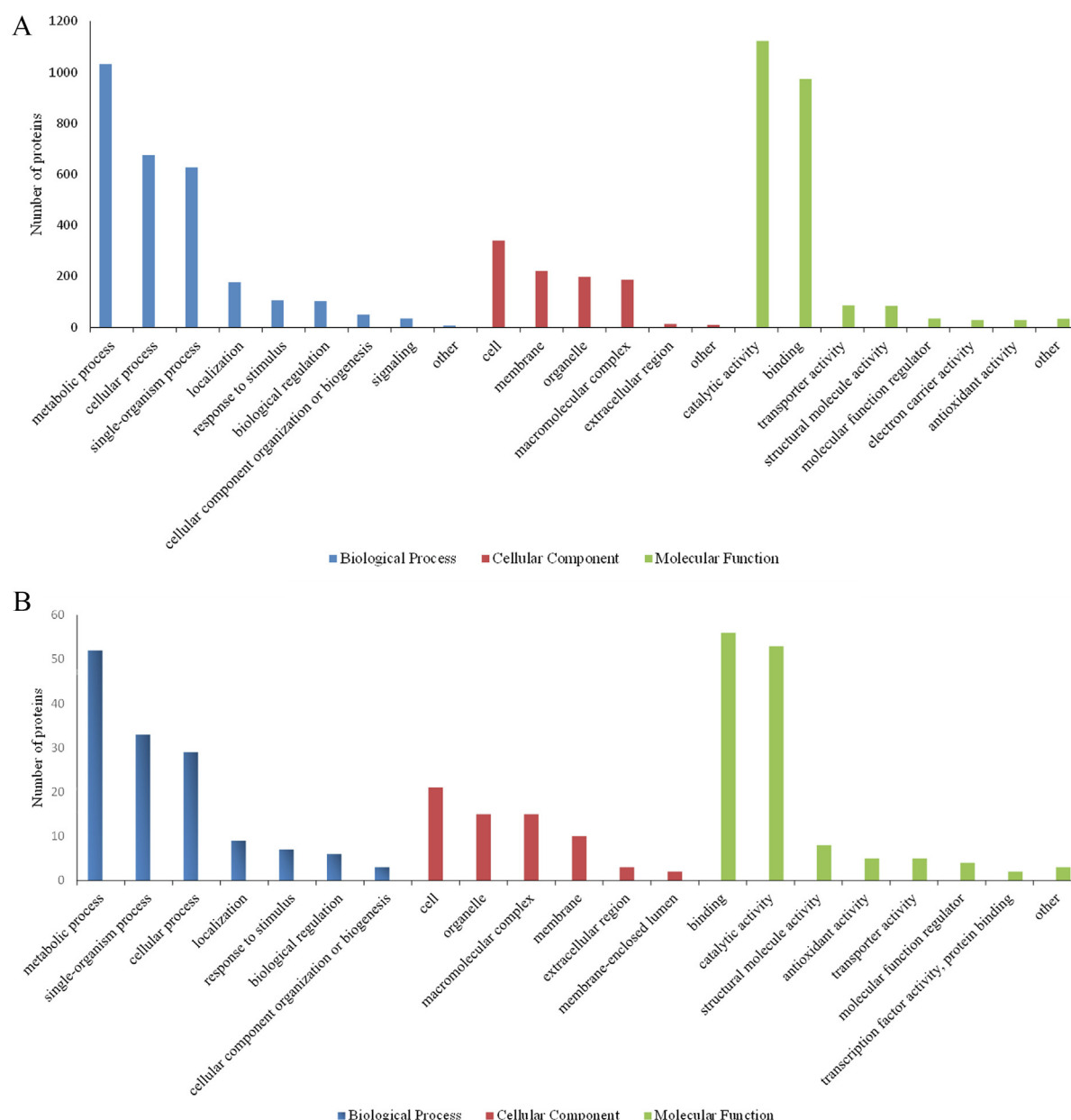


Fig. 1. Quantitative protein results from the Gene Ontology annotation classification. (A) Mature red stage compared with mature green stage (R/G). (B) NaCl-treated mature fruits compared with control mature fruits (N/R). The vertical axis indicates the number of proteins identified in the two comparison groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

metabolic process, single-organism process, and cellular process in both comparison groups. For cellular component, the cell, membrane, organelle and macromolecular complex classes were the most plentifully represented groups. The molecular functions of these proteins were concentrated on catalytic activity and binding in both the R/G and N/R comparisons. We also analyzed the subcellular locations of these proteins (Fig. S3, Table S2): among the 2607 differentially proteins identified from fruit in the red ripening period compared with fruit in the green ripening period (R/G), 35% were located at the chloroplast, 24% were located in the cytoplasm and approximately 18% proteins were located in the nucleus. Of the 153 differentially expressed proteins identified in NaCl-treated mature fruits and control mature fruits (N/R), most of the proteins were located at the chloroplast and cytoplasm, followed by nuclear and extracellular localization.

3.3. Functional enrichment analysis of differentially expressed proteins

We further employed KEGG analysis to determine whether these differentially proteins were significantly enriched in specific functions. In the comparison of R/G, the differentially expressed proteins were focused on 10 metabolic processes (Fig. 2A), including photosynthesis, glyoxylate and dicarboxylate metabolism, carbon fixation in photosynthetic organisms, glycine, serine and threonine metabolism, photosynthesis-antenna proteins, pentose and glucuronate interconversions, ubiquinone and other terpenoid-quinone biosynthesis, flavonoid biosynthesis, butanoate metabolism and cyanoamino acid metabolism. Among these metabolic pathways, proteins involved in pentose and glucuronate interconversions had significant changes, and were closely related to the degradation or synthesis of cell walls. For mature fruits under salt treatment compared to control mature fruits (N/R), we discovered that 2 metabolic processes were up- or down-regulated by KEGG enrichment analysis, linoleic acid metabolism and flavonoid

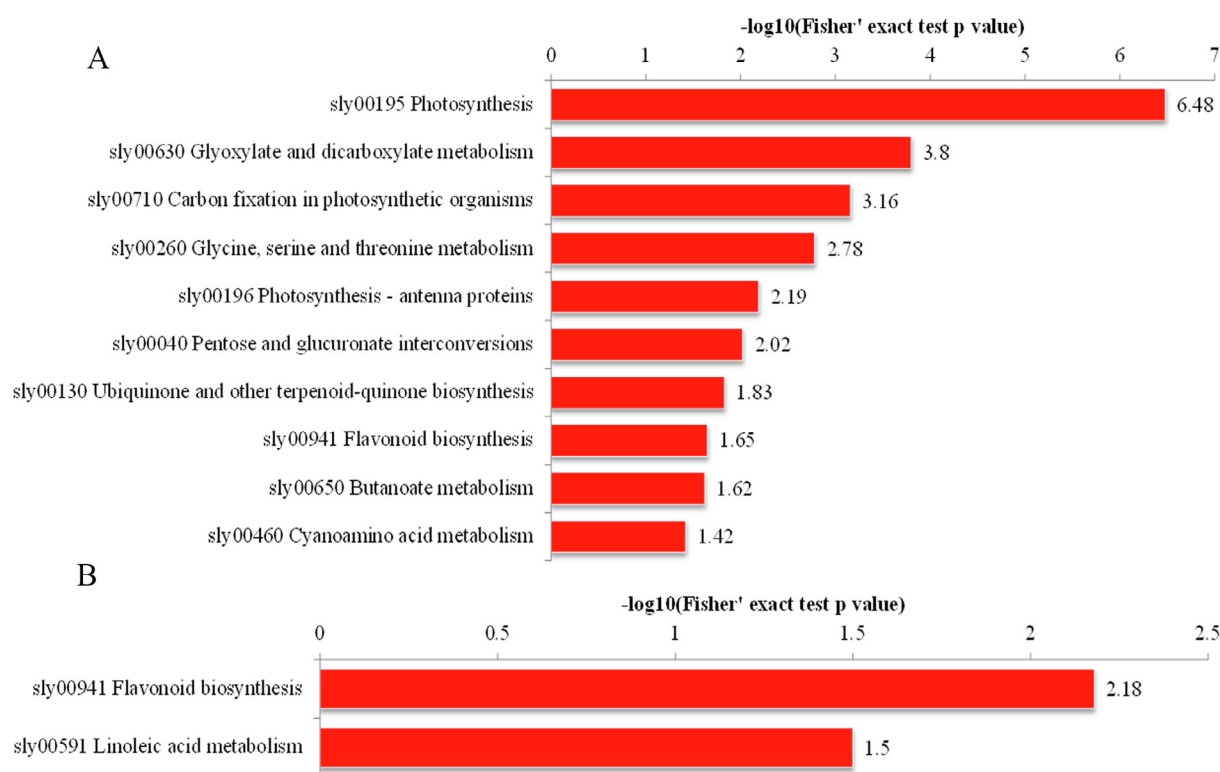


Fig. 2. KEGG pathway enrichment analysis of differentially significant proteins. (A) The enrichment analysis of the comparison between red mature and green mature tomato fruits (R/G). (B) The enrichment analysis of the comparison between mature fruits under salt treatment and control mature fruits (N/R). Pathway enrichment analysis verified that p-value less than 0.05 indicate significant change. For the negative logarithm ($-\log_{10}$) conversion of p-values obtained by the enrichment test, the larger the converted value, the more significant the enrichment of this functional type. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

biosynthesis (Fig. 2B). Between these two processes, linoleic acid metabolism is associated with the synthesis of volatile substances, and flavonoid biosynthesis plays an important role in the formation of fruit quality.

3.4. Metabolite profiles of tomato fruit at different ripening periods and in response to salt treatment at the red mature stage

We used untargeted metabolomics based on GC-TOFMS technology to obtain the metabolite profiles of mature green fruits, mature red fruits and mature red fruits under salt-treatment. A total of 244 metabolites were identified, of which 114 had annotation information (Table S3). We employed univariate statistical analysis to obtain the differential metabolites between the green ripening period and the red ripening period (R/G) and the changes in metabolic composition in NaCl-treated mature fruits relative to control mature fruits (N/R). The results suggested that the differential metabolites mainly included alkylamines, amino acids, carbohydrates, fatty acids, organic acids, and nucleotides (Table 1). Among these identified metabolites, the levels of L-tryptophan, L-valine, L-aspartic acid, trehalose, D-galactose, chlorogenic acid, alpha-tocopherol, and glycolic acid were significantly increased, whereas levels of sucrose, most amino acids and linoleic acid significantly were decreased in mature fruits relative to mature green fruits (R/G). We can conclude that some conditions related to metabolite quality had significantly changed during fruit development and ripening. Thirteen metabolites were the most significantly changed under salt treatment in red mature fruits relative to control mature fruits (N/R) (Table 2), including alkylamines (spermidine), amino acid (3-oxoalanine, L-alanine, L-tryptophan, L-methionine), carbohydrates (turanose, D-glucuronic acid), indoles (tryptamine, serotonin) and organic acids (pyruvic acid, malonic acid, 2-furoic acid, glycolic acid).

Changes in these metabolites could be responsible for the improvement in tomato fruit quality under NaCl treatment.

3.5. Metabolic pathway enrichment analysis of differential metabolites

Metabolic pathway enrichment was analyzed to select the changes in key metabolic pathways during fruit maturation (R/G) and the effects of salt treatment on metabolic process in mature fruits (N/R). Seven metabolic pathways experienced marked changes in the mature red stage relative to the mature green stage (R/G), including starch and sucrose metabolism, valine, leucine and isoleucine biosynthesis, beta-alanine metabolism, alanine, aspartate and glutamate metabolism, glyoxylate and dicarboxylate metabolism, pantothenate and CoA biosynthesis and glycine, serine and threonine metabolism (Fig. 3A). Among these metabolic processes, starch and sucrose metabolism play significant roles in sugar accumulation for fruits. Between NaCl-treated mature fruits and control mature fruits (N/R), these differential metabolites were mainly involved in alanine, aspartate and glutamate metabolism, pentose and glucuronate interconversions, arginine biosynthesis, citrate cycle (TCA cycle), ascorbate and aldarate metabolism and beta-alanine metabolism (Fig. 3B). The citrate cycle is the center of integrating the metabolism of three major substances: sugar, lipid and protein. These changes will provide effective information for the study of the mechanism of fruit quality improvement under salt treatment.

3.6. Co-analysis of proteomics and metabolomics

To analyze the relationship of mutual adjustments between proteins and small metabolic molecules, proteomics and metabolomics were integrated, and the differentially expressed proteins and differentially detected small molecules were compared with the KEGG database to

Table 1

Differential metabolites between the mature red stage and the mature green stage (R/G) using univariate statistical analysis.

Class	Name	HMDBID	KeggID	p	FC
Alkylamines	Ethanolamine	HMDB00149	C00189	2.70E-06	0.5
	Putrescine	HMDB01414	C00134	2.80E-03	0.5
	Spermidine ▲	HMDB01257	C00315	4.10E-02	1.7
Amino Acid	Glycine	HMDB00123	C00037	2.50E-06	0.2
	3-Aminoisobutanoic acid	HMDB03911	C05145	7.90E-06	0.5
	L-Tryptophan ▲	HMDB00929	C00078	2.20E-05	6.6
	Beta-Alanine	HMDB00056	C00099	3.40E-05	0.3
	L-Valine	HMDB00883	C00183	8.00E-05	0.1
	L-Alanine ▲	HMDB00161	C00041	1.10E-04	0.3
	L-Alloisoleucine	HMDB00557	NA	2.60E-04	0.4
	L-Aspartic acid	HMDB00191	C00049	4.70E-04	58.4
	Methylcysteine	HMDB02108	NA	1.30E-03	3
	L-Leucine	HMDB00687	C00123	2.70E-03	0.3
	3-Nitrotyrosine	HMDB01904	NA	4.40E-03	0.4
	L-Homoserine	HMDB00719	C00263	2.10E-02	0.7
	L-Phenylalanine	HMDB00159	C00079	2.40E-02	0.4
	L-Threonine	HMDB00167	C00188	3.30E-02	0.7
	Urea	HMDB00294	C00086	3.60E-02	0.6
Carbohydrates	Trehalose ▲	HMDB00975	C01083	4.60E-05	6.3
	D-Xylose	HMDB00098	C00181	2.00E-04	1.6
	Fructose 6-phosphate	HMDB00124	C00085	2.40E-03	0.7
	Glucose 6-phosphate	HMDB01401	C00092	3.70E-03	2
	Alpha-Lactose	HMDB00186	C00243	5.90E-03	0.4
	Sucrose	HMDB00258	C00089	7.90E-03	0.6
	Isomaltose	HMDB02923	C00252	1.20E-02	1.9
	Turanose	HMDB11740	C19636	2.70E-02	3.1
	D-Galactose	HMDB00143	C00984	3.00E-02	0.5
Fatty Acids	Linoleic acid	HMDB00673	C01595	4.00E-02	0.8
Hormone	Normetanephrine	HMDB00819	C05589	8.50E-05	5.5
Nucleotide	Guanosine	HMDB00133	C00387	3.50E-04	4.1
	Cytidine	HMDB00089	C00475	5.70E-04	1.9
	Uridine	HMDB00296	C00299	1.60E-02	1.4
	Adenosine	HMDB00050	C00212	4.20E-02	1.4
Organic Acids	Glyceric acid	HMDB00139	C00258	1.40E-07	0.4
	Pyruvic acid ▲	HMDB00243	C00022	1.20E-05	0.5
	Pyroglutamic acid	HMDB04230	C05942	4.30E-05	0.2
	Suberic acid	HMDB00893	C08278	4.30E-05	0.2
	Chlorogenic acid	HMDB03164	C00852	1.40E-04	3
	L-Lactic acid	HMDB00190	C00186	7.60E-04	0.2
	Hydroxypropionic acid	HMDB00700	C01013	8.20E-04	3.3
	Hypotaurine	HMDB00965	C00519	1.50E-03	1.7
	Malonic acid ▲	HMDB00691	C00383	3.10E-03	0.6
	L-Pipecolic acid	HMDB00716	C00408	5.80E-03	3
	Succinic acid	HMDB00254	C00042	2.00E-02	0.5
	Pyrophosphate	HMDB00250	C00013	3.10E-02	1.5
	Glycolic acid ▲	HMDB00115	C00160	4.10E-02	0.9
Vitamin	Alpha-Tocopherol	HMDB01893	C02477	6.00E-03	2.2
NA	2-Hydroxybenzoic	NA	NA	3.00E-03	7.1

Note: A p-value < 0.05 was considered significant, and fold change (FC) < 1 indicates that the metabolite had significantly decreased, while fold change (FC) > 1 indicates that the metabolite had significantly increased. ▲ represents differentially expressed metabolites that change together at different ripening stages and under salt treatment.

obtain the corresponding protein pathways. Through proteomics and metabolomics integration, we identified 49 metabolic pathways that showed changes at the mature red stage compared to the mature green stage and 22 metabolic pathways that showed changes in salt-treated mature fruits compared to control mature fruits (Table S4). We speculated that some metabolic pathways related to nutritional quality changed in both comparison groups, such as starch and sucrose metabolism, biosynthesis of amino acids, galactose metabolism,

Table 2

Differential metabolites between NaCl-treated mature fruits and control mature fruits using univariate statistical analysis.

Class	Name	HMDBID	KeggID	p	FC
Alkylamines	Spermidine ▲	HMDB01257	C00315	3.80E-03	0.6
Amino Acid	3-Oxalanine	HMDB11602	NA	2.00E-02	1.6
	L-Alanine ▲	HMDB00161	C00041	2.00E-02	1.3
	L-Tryptophan ▲	HMDB00929	C00078	4.50E-02	0.8
	L-Methionine	HMDB00696	C00073	4.90E-02	0.7
Carbohydrates	Turanose ▲	HMDB11740	C19636	2.60E-02	1.7
	D-Glucuronic acid	HMDB00127	C00191	5.00E-02	0.7
Indoles	Tryptamine	HMDB00303	C00398	3.60E-02	2
	Serotonin	HMDB00259	C00780	3.60E-02	2.3
Organic Acids	Pyruvic acid ▲	HMDB00243	C00022	8.70E-04	0.6
	Malonic acid ▲	HMDB00691	C00383	2.90E-02	0.7
	2-Furoic acid	HMDB00617	C01546	4.30E-02	1.4
	Glycolic acid ▲	HMDB00115	C00160	4.80E-02	1.2

Note: A p-value < 0.05 was considered significant and fold change (FC) < 1 indicates that the metabolite had significantly decreased, while fold change (FC) > 1 indicates that the metabolite had significantly increased. ▲ represents differentially expressed metabolites that change together at different ripening stages and under salt treatment.

phenylpropanoid biosynthesis, glycine, serine and threonine metabolism, biosynthesis of secondary metabolites and others.

Fig. 4 illustrates the network of relationships between proteins and metabolites. In the R/G comparison, we found that a metabolic molecule can integrate with multiple proteins and that proteins can also associate with multiple metabolic molecules when involved in a co-pathway. Among these, glycine, L-alanine, spermidine, D-xylose and sucrose were associated with many proteins and can be involved in multiple metabolic pathways. Thus, their interactions can affect fruit development and maturation. Between salt-treated mature and control mature fruits (N/R), spermidine and multiple proteins share common metabolic pathways. Consequently, we speculated that spermidine may play an important role in response to salt treatment. Apart from this finding, beta-fructofuranosidase had regulated relationships with trehalose, alpha-lactose and myoinositol, and they were closely associated with carbohydrate metabolism. Hence, understanding the relationships between proteins and metabolic molecules has great significance in studying the improvement of fruit quality under salt treatment.

4. Discussion

4.1. Carbohydrate metabolism is involved in fruit ripening and salt response

Soluble sugar contents obviously change with fruit development and ripening, and are related to hardness and volatile aroma, which are important factors affecting fruit quality. Fructose and glucose are the major soluble sugars in mature tomato fruit (Lu et al., 2010), and sucrose synthase, acid invertase and sucrose phosphate synthase are the vital enzymes participating in sucrose metabolism and play an important role in sugar accumulation in fruit (Jing et al., 2018). Li et al. (2016) suggested that some proteins participate in the suc-suc cycle, including sucrose phosphate synthase, sucrose synthases and hexokinases, which were increased during fruit ripening. In this study, sucrose synthase and beta-fructofuranosidase were up-regulated, and the sucrose content significantly decreased in the mature red stage relative to the green stage (Table S5, Fig. S4), and these changes may explain why fructose and glucose account for the most soluble sugars in tomato fruit maturity. Adverse environments, such as salt and drought stress, affect sugar accumulation in fruits, and Sato, Sakaguchi, Furukawa, and Ikeda (2006) found that salt treatment increased the contents of fructose and glucose in tomato. In the present study, beta-fructofuranosidase was up-regulated in NaCl-treated mature fruits

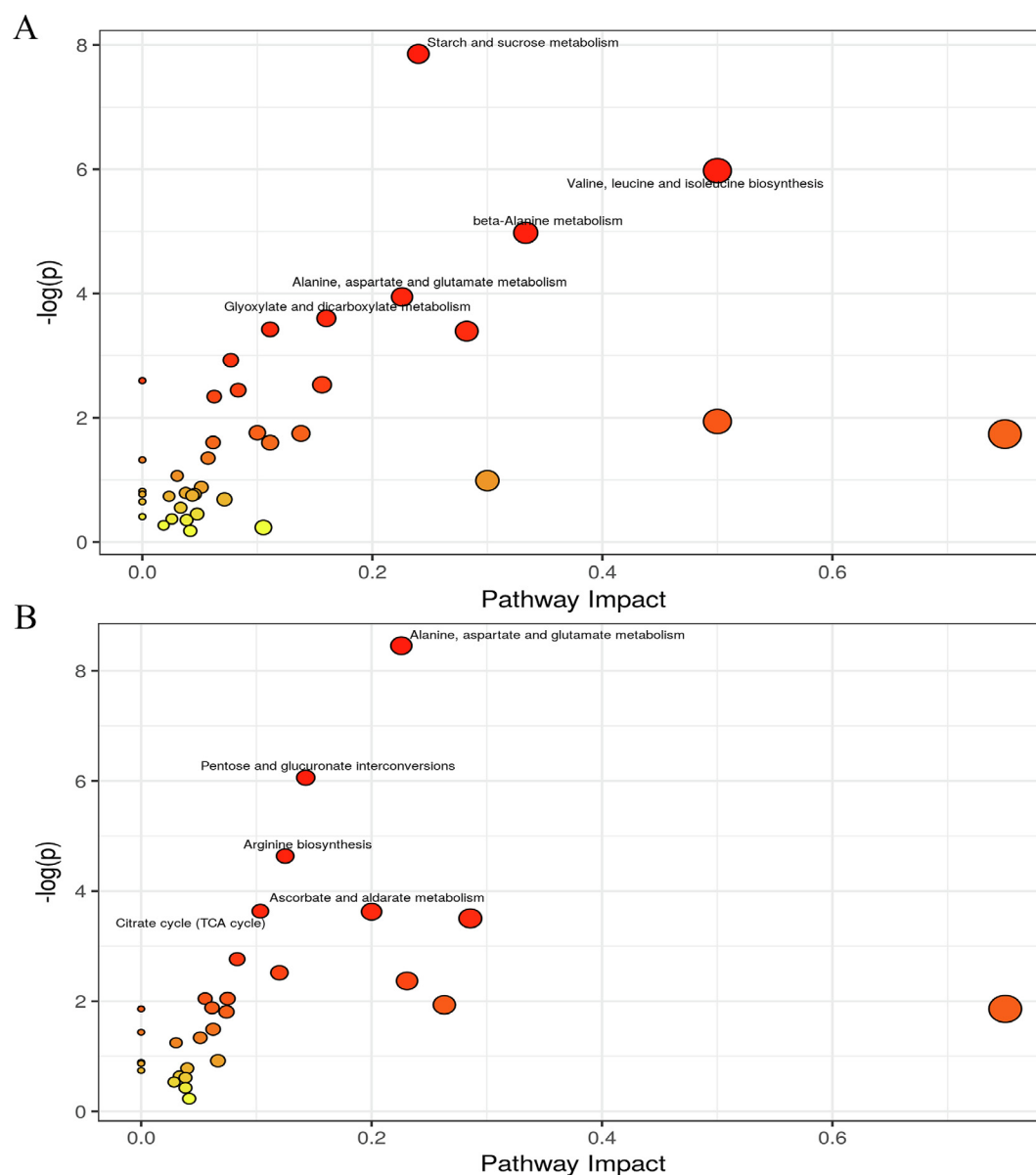


Fig. 3. Metabolic pathway enrichment analysis. (A) Comparison of the mature red stage with the mature green stage (R/G). (B) Comparison of NaCl-treated mature fruits with control mature fruits (N/R). $P < 0.05$ is the standard chosen for pathway significance, and the pathway impact value is used to measure the importance of a specific metabolic pathway. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Table S6, Fig. S4). Beta-fructofuranosidase can irreversibly catalyze the hydrolysis of sucrose to produce glucose and fructose. Studies have also demonstrated that antisense acid invertase gene in tomato and muskmelon can increase sucrose content (Klann, Hall, & Bennett, 1996; Yu et al., 2008). This may be the reason for the increase in sugar content under the salt treatment condition. The cell wall is composed of pectin, cellulose and hemicellulose, and changes in its structure and composition determine fruit firmness; in the process of fruit ripening, the cell wall gradually degrades, accompanied by reductions in firmness (Chylińska, Szymańska-Chargot, Deryło, Tchórzewska, & Zdunek, 2017). In the present study, the proteins involved in pentose and glucuronate interconversions, including pectinesterase, pectate lyase and polygalacturonase-2, were up-regulated at the red stage compared with the green stage (Table S5, Fig. S4). Pectate lyase and pectinesterase have been linked with pectin degradation (Marín-Rodríguez & Seymour, 2002; Zhu, Zhang, Liu, Chen, & Zhang, 2018). UDP-glucose 6-dehydrogenase participated in pectin synthesis, which was down-regulated during fruit maturation. D-xylosyl was one of the main

components of hemicellulose (Fan et al., 2018), and it is the main polysaccharide that forms cell walls. Our results showed that D-xylose was present in much higher levels in the mature red stage than in the green stage (Table 1), which could be the reason for the softening of the cell wall during tomato fruit ripening. Moreover, the main make-up of the cellulose system, including endoglucanase and beta-glucosidase, is up-regulated with fruit ripening. These changes make important contributions to cell wall softening. This study indicated that tomato fruit firmness was increased under salt stress (Fig. S6). Down-regulation of beta-galactosidase and endoglucanase may lead to an enhancement in tomato fruit hardness in salt-treated mature fruits (Table S6). Interestingly, this study also found that expansion was expressed in different patterns in the two comparison groups. Previously, research suggested that inhibition of the expression of Exp1 protein can delay fruit softening, but the fruit was much softer than the controls when the Exp1 protein was overexpressed (Brummell et al., 1999). Minoia et al. (2016) also proved *SLExp1* mutants play an important role in delaying fruit ripening and increasing fruit hardness. The combined effects of these

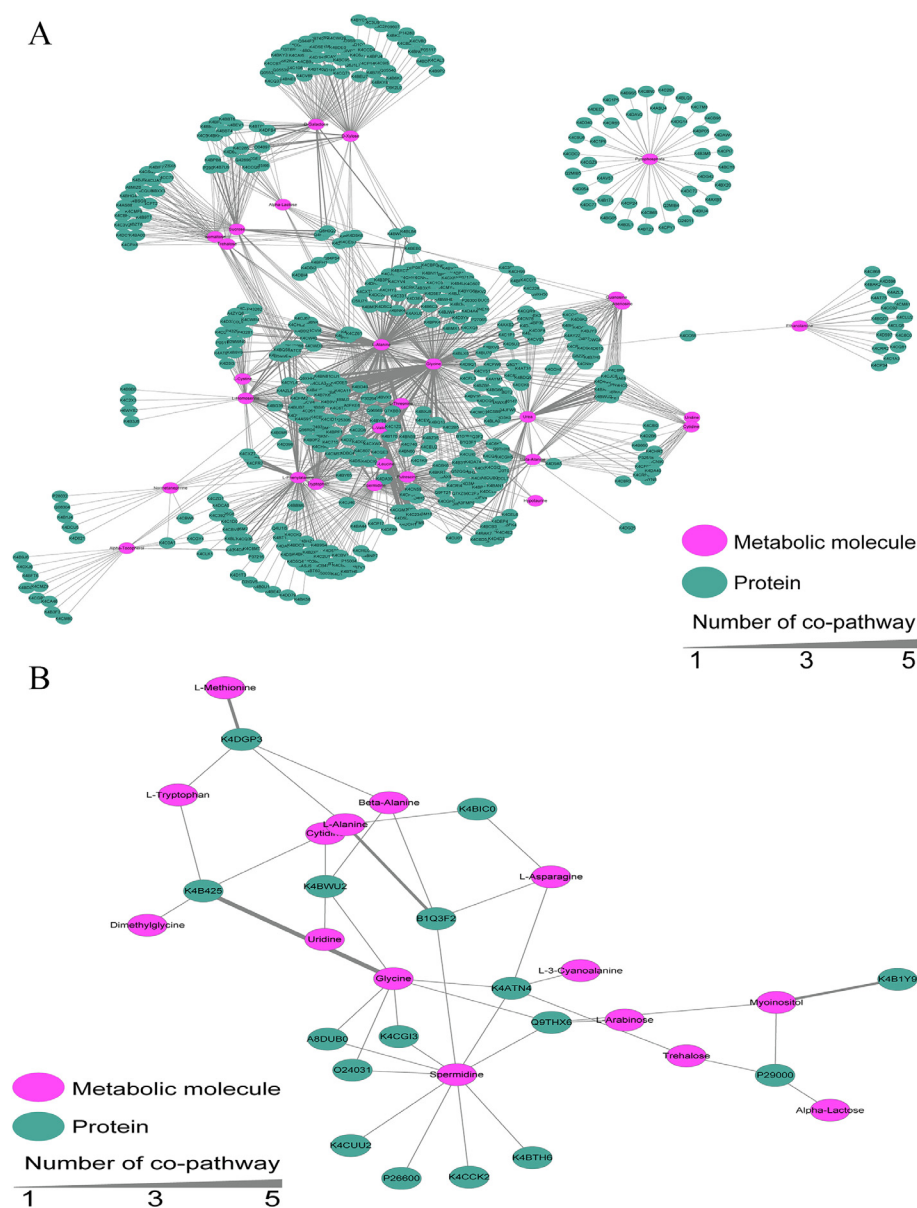


Fig. 4. Protein and compound networks based on pathways. (A) R/G, (B) N/R. Red represents proteins, green represents metabolic molecules. The line indicates that proteins and metabolic molecules are in a co-pathway; the thicker the line, the more pathways are the same. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

proteins may result in the change of fruit firmness under salt treatment, the mechanism of which needs further study.

4.2. Changes in amino acid metabolism in different fruit ripening stages and during salt treatment

Amino acids are the main parameter for evaluating the quality of tomato fruit. The content and component of amino acids are largely changed during fruit ripening, and it has been reported that aspartic acid and glutamate are expressed at higher levels in maturation stages (Snowden, Benjamin, Baxter, Smith, & Sweetlove, 2015). In the present study, we found that L-tryptophan, L-aspartic acid and methyl cysteine were significantly increased, while L-valine, L-alanine, L-leucine and L-phenylalanine were observably decreased at the mature red stage compared to the mature green stage (R/G). Among these amino acids, L-aspartic acid was increased 58.4-fold and was the most changed amino acid (Table S5, Fig. S5). It has been observed that the enzyme activity of glutamate decarboxylase was markedly decreased while γ -

aminobutyrate transaminase activity was increased in mature fruits relative to mature green fruits (Sorrequieta, Abriata, Boggio, & Valle, 2013). Similar results were also observed in the present study. Aspartate aminotransferase plays an important role in the synthesis or degradation of amino acids, and can catalyze the mutual conversion between keto acids and amino acids (Liu et al., 2017). Aspartate aminotransferase protein was up-regulated in red mature fruit compared with green mature fruit (Table S5, Fig. S4). Zhou et al. (2009) demonstrated that there are three coding genes for aspartate aminotransferase (*OsAAT*), and while overexpressing *OsAAT1* and *OsAAT2* could increase amino acid content, over-expressing *OsAAT3* did not significantly affect amino acid levels in rice. Therefore, the up-regulation of aspartate aminotransferase might play an important role in amino acid accumulation in the tomato ripening process. Amino acids are also involved in the synthesis of volatile aromas, which are responsible for fruit flavor; esters are the most important volatile aromas, and their synthesis is dependent on the expression of branched-chain-amino-acid aminotransferase (Sugimoto, Jones, & Beaudry, 2011). In

the present study, branched-chain-amino-acid aminotransferase protein expression in red mature tomato fruit was significantly up-regulated by salt treatment, implying that salt treatment could improve tomato fruit flavor. Levels of 3-oxoalanine and L-alanine were significantly increased in salt-treated mature fruits compared with control mature fruits (N/R) (Table S6, Fig. S5). Previous research proved that alanine is related to sweetness (Keutgen & Pawelzik, 2008). These results may explain the function of amino acid metabolism in tomato fruit quality improvement under salt treatment.

4.3. Fatty acid metabolism is involved in volatile aromatics formation and salt treatment affected fatty acid metabolism

The quality of tomato fruit is not only dependent on the accumulation of sugar, organic acids and amino acids but is also closely related with the volatile compounds, which mainly include esters, alcohol, aldehydes and terpenoids. The synthesis of volatile compounds occurs mainly during fatty acid metabolism and amino acid metabolism (Zhang et al., 2015). Lipoxigenase, alcohol dehydrogenase 2, allene oxide cyclase, and allene oxide synthase 2 are important enzymes involved in fatty acid metabolism. In the present study, lipoxigenase and alcohol dehydrogenase 2 were up-regulated in mature red fruits compared to mature green fruits (R/G), and only lipoxigenase was up-regulated by NaCl treatment in mature red fruits (Table S6). Lipoxigenase can be divided into 9-lipoxigenase and 13-lipoxigenase, which catalyze linolenic acid to 9-hydroperoxide and 13-hydroperoxide, respectively. The further reaction of 9-hydroperoxide produces (E, E)-2-4-noadial, while 13-hydroperoxide can further generate (Z)-3-hexen-1-ol, (Z)-2-hexen-1-ol and 1-hexanol via alcohol dehydrogenase (Zhang et al., 2015). Previous studies confirmed that lipoxigenase activity was enhanced with fruit development and ripening, such as in cucumber (Chen, Zhang, Hao, Chen, & Chen, 2015) and oriental melon (Guo et al., 2017). In addition, Chen et al. (2004) reported that the level of volatile matter was decreased in antisense lipoxigenase (*Tomlox*) transgenic tomatoes. The results indicated that linoleic acid was significantly decreased in the mature red stage compared with the green stage (R/G), which may be due to the up-regulation of lipoxigenase. Lipoxigenase was up-regulated in NaCl-treated mature fruits relative to control mature fruits (N/R), indicating that salt treatment induced linoleic acid metabolism (Table S6). KEGG pathway enrichment analysis also showed that the linoleic acid metabolic pathway was significantly changed in salt-treated mature fruits (Fig. 2B). Therefore, we speculated that salt treatment may increase the content of volatile aromatics by linoleic acid metabolism, which would then enhance the flavor of the fruit. Proteomics data illustrated that alcohol dehydrogenase was up-regulated 1.685-fold in mature fruits relative to immature fruits (R/G) (Table S5), and the activity of alcohol dehydrogenase was also improved in oriental melon with fruit development and ripening (Guo et al., 2017). However, alcohol dehydrogenase expression was significantly reduced during strawberry fruit ripening (Song et al., 2015). Because the expression of alcohol dehydrogenase is different in different fruit species, we can speculate that alcohol dehydrogenase has certain effects on the accumulation of volatile aromatics.

5. Conclusion

Based on combined proteomics and metabolomics analyses, a number of proteins and metabolites involved in the tomato fruit ripening process and salt response were identified, which are mainly related with the metabolism of carbohydrates, amino acids and fatty acids. These metabolisms globally affected the quality and flavor of fruit. Importantly, it was found that NaCl treatment induced the protein expression of beta-fructofuranosidase and lipoxigenase, which are responsible for the accumulation of sugar and volatile aromatics, respectively. Furthermore, NaCl treatment increased the accumulation of the sweet amino acid alanine and the expression of branched-chain-

amino-acid aminotransferase, which is a key enzyme involved in ester catalyzing synthesis. Additionally, NaCl treatment inhibited the expression of beta-galactosidase and endoglucanase, resulting in greater fruit firmness. Therefore, it can be concluded that salt treatment not only improves the nutrient and flavor quality but also increases the storage quality. These results provide a basic understanding of the fruit quality improvement of tomato grown in a saline substrate; however, the mechanism still needs further research.

CRedit authorship contribution statement

Huimeng Tang: Writing - review & editing. **Xu Zhang:** Writing - review & editing. **Biao Gong:** Writing - review & editing. **Yanyan Yan:** Writing - review & editing. **Qinghua Shi:** Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the National Key Research and Development Program of China (2018YFD1000800); the National Natural Science Foundation of China (31872954); the Shandong Province Modern Agricultural Technology System (SDAIT-05-05); the Project of Shandong "Double-First-Class" (SYL2017YSTD06).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2019.126009>.

References

- Baldina, S., Picarella, M. E., Troise, A. D., Pucci, A., Ruggieri, V., Ferracane, R., et al. (2016). Metabolite profiling of Italian tomato landraces with different fruit types. *Frontiers in Plant Science*, 7, 664.
- Brummell, D. A., Harpster, M. H., Civeello, P. M., Palys, J. M., Bennett, A. B., & Dunsmuir, P. (1999). Modification of expansin protein abundance in tomato fruit alters softening and cell wall polymer metabolism during ripening. *The Plant Cell*, 11(11), 2203–2216.
- Carrari, F., & Fernie, A. R. (2006). Metabolic regulation underlying tomato fruit development. *Journal of Experimental Botany*, 57(57), 1883–1897.
- Chen, G. P., Hackett, H., Walker, D., Andy, T., Lin, Z. F., & Grierson, D. (2004). Identification of a specific isoform of tomato lipoxigenase (*Tomlox*) involved in the generation of fatty acid-derived flavor compounds. *Plant Physiology*, 136(1), 2641–2651.
- Chen, S. X., Zhang, Z. Z., Hao, L. N., Chen, W. F., & Chen, S. Q. (2015). Profiling of volatile compounds and associated gene expression and enzyme activity during fruit development in two cucumber cultivars. *PLoS One*, 10(3), e0119444.
- Chylińska, M., Szymańska-Chargot, M., Deryło, K., Tchórzewska, D., & Zdunek, A. (2017). Changing of biochemical parameters and cell wall polysaccharides distribution during physiological development of tomato fruit. *Plant Physiology and Biochemistry*, 119, 328–337.
- Fan, W., Ge, G., Liu, Y., Wei, W., Liu, L., & Jia, Y. (2018). Proteomics integrated with metabolomics: Analysis of the internal causes of nutrient changes in alfalfa at different growth stages. *BMC Plant Biology*, 18(1), 78.
- Galli, V., Messias, R. D. S., Perin, E. C., Borowski, J. M., Bamberg, A. L., & Rombaldi, C. V. (2016). Mild salt stress improves strawberry fruit quality. *LWT – Food Science and Technology*, 73, 693–699.
- Gong, G., Yan, Y. Y., Zhang, L. L., Cheng, F., Liu, Z., & Shi, Q. H. (2019). Unravelling GSNOR-Mediated S-Nitrosylation and Multiple Developmental Programs in Tomato Plants. *Plant and Cell Physiology*. <https://doi.org/10.1093/pcp/pcz143>.
- Guo, X., Xu, J., Cui, X., Chen, H., & Qi, H. (2017). iTRAQ-based protein profiling and fruit quality changes at different development stages of oriental melon. *BMC Plant Biology*, 17(1), 28.
- Jing, X., Wang, H., Gong, B., Liu, S. Q., Wei, M., Ai, X. Z., et al. (2018). Secondary and sucrose metabolism regulated by different light quality combinations involved in melon tolerance to powdery mildew. *Plant Physiology and Biochemistry*, 124, 77–87.
- Keutgen, A. J., & Pawelzik, E. (2008). Contribution of amino acids to strawberry fruit quality and their relevance as stress indicators under NaCl salinity. *Food Chemistry*, 111(3), 642–647.
- Klann, E. M., Hall, B., & Bennett, A. B. (1996). Antisense acid invertase (*TIV1*) gene alters

- soluble sugar composition and size in transgenic tomato fruit. *Plant Physiology*, 112(3), 1321–1330.
- Lee, K. M., Oh, T. J., Kim, S. H., Kim, H. Y., Chung, H., Min, D. S., et al. (2016). Comprehensive metabolic profiles of mulberry fruit (*Morus Mlba Linnaeus*) according to maturation stage. *Food Science and Biotechnology*, 25(4), 1035–1041.
- Li, M. J., Li, D. X., Feng, F. J., Zhang, S., Ma, F. W., & Cheng, L. L. (2016). Proteomic analysis reveals dynamic regulation of fruit development and sugar and acid accumulation in apple. *Journal of Experimental Botany*, 67(17), 5145–5157.
- Li, X. L., Wang, C. R., Li, X. Y., Yao, Y. X., & Hao, Y. J. (2013). Modifications of Kyoho grape berry quality under long-term NaCl treatment. *Food Chemistry*, 139, 931–937.
- Liu, J. Y., Chang, M. C., Meng, J. L., Feng, C. P., Zhao, H., & Zhang, M. L. (2017). Comparative proteome reveals metabolic changes during the fruiting process in *Flammulina velutipes*. *Journal of Agricultural and Food Chemistry*, 65(24), 5091–5100.
- Liu, M. C., Liu, X. L., Hao, J., Gao, L. H., He, H. H., & Liu, L. W. (2010). Effect of simulated drought stress on plant growth, yield and fruit properties of tomato. *Acta Horticulturae*, 856, 193–202.
- Lu, S. W., Li, T. L., & Jing, J. (2010). Effects of tomato fruit under Na⁺-salt and Cl⁻-salt stresses on sucrose metabolism. *African Journal of Agricultural Research*, 5(16), 2227–2231.
- Manaa, A., Faurobert, M., Valot, B., Bouchet, J. P., Grasselly, D., Causse, M., et al. (2013). Effect of salinity and calcium on tomato fruit proteome. *Omics A Journal of Integrative Biology*, 17(6), 338–352.
- Marín-Rodríguez, M. C., & Seymour, G. B. (2002). Pectate lyases, cell wall degradation and fruit softening. *Journal of Experimental Botany*, 53(377), 2115–2119.
- Meng, C. Y., Yang, D. Y., Ma, X. C., Zhao, W. Y., Liao, X. Q., Ma, N. N., et al. (2016). *Journal of Plant Physiology*, 193, 88–96.
- Minoia, S., Boualem, A., Marcel, F., Troadec, C., Quemener, B., Cellini, F., et al. (2016). Induced mutations in tomato *SlExp1* alter cell wall metabolism and delay fruit softening. *Plant Science An International Journal of Experimental Plant Biology*, 242, 195–202.
- Riga, P., Anza, M., & Garbisu, C. (2010). Tomato quality is more dependent on temperature than on photosynthetically active radiation. *Journal of the Science of Food and Agriculture*, 88(1), 158–166.
- Rocco, M., D'Ambrosio, C., Arena, S., Faurobert, M., Scaloni, A., & Marra, M. (2010). Proteomic analysis of tomato fruits from two ecotypes during ripening. *Proteomics*, 6(13), 3781–3791.
- Rodríguezruiz, M., Mioto, P., & Palma, J. M. (2016). S-nitrosoglutathione reductase (GSNOR) activity is down-regulated during pepper (*Capsicum annuum* L.) fruit ripening. *Plant Science*.
- Sato, S., Sakaguchi, S., Furukawa, H., & Ikeda, H. (2006). Effects of NaCl application to hydroponic nutrient solution on fruit characteristics of tomato (*Lycopersicon esculentum* Mill.). *Scientia Horticulturae*, 109(3), 248–253.
- Snowden, C. J., Benjamin, T., Baxter, C. J., Smith, J. A. C., & Sweetlove, L. J. (2015). A tonoplast Glu/Asp/GABA exchanger that affects tomato fruit amino acid composition. *Plant Journal*, 81(5), 651–660.
- Song, J., Du, L., Li, L., Palmer, L. C., Forney, C. F., Fillmore, S., et al. (2015). Targeted quantitative proteomic investigation employing multiple reaction monitoring on quantitative changes in proteins that regulate volatile biosynthesis of strawberry fruit at different ripening stages. *Journal of Proteomics*, 126(22), 288–295.
- Sorrequieta, A., Abriata, L. A., Boggio, S. B., & Valle, E. M. (2013). Off-the-vine ripening of tomato fruit causes alteration in the primary metabolite composition. *Metabolites*, 3(4), 967.
- Sugimoto, N., Jones, A. D., & Beaudry, R. (2011). Changes in free amino acid content in “Jonagold” apple fruit as related to branched-chain ester production, ripening, and senescence. *Journal of the American Society for Horticultural Science American Society for Horticultural Science*, 136(6), 429–440.
- Szymanski, J., Levin, Y., Savidor, A., Breitel, D., Chappell-Maor, L., Heinig, U., et al. (2017). Label-free deep shotgun proteomics reveals protein dynamics during tomato fruit tissues development. *Plant Journal*, 90(2), 396–417.
- Yin, Y. G., Takehiro, T., Iijima, Y., Aoki, K., Shibata, D., Ashihara, H., et al. (2010). Metabolic alterations in organic acids and gamma-aminobutyric acid in developing tomato (*Solanum lycopersicum* L.) fruits. *Plant and Cell Physiology*, 51(8), 1300–1314.
- Yu, X., Wang, X., Zhang, W., Qian, T., Tang, G., Guo, Y., et al. (2008). Antisense suppression of an acid invertase gene (*MAI1*) in muskmelon alters plant growth and fruit development. *Journal of Experimental Botany*, 59(11), 2969–2977.
- Yue, R. Q., Lu, C. X., Han, X. H., Guo, S. L., Yan, S., Liu, L., et al. (2018). Comparative proteomic analysis of maize (*Zea mays* L.) seedlings under rice black-streaked dwarf virus infection. *BMC Plant Biology*, 18, 191.
- Zhang, Z., Mao, C., Shi, Z., & Kou, X. (2017). The amino acid metabolic and carbohydrate metabolic pathway play important roles during salt-stress response in tomato. *Frontiers in Plant Science*, 8, 1231.
- Zhang, J., Zhao, J., Xu, Y., Liang, J., Chang, P., Yan, F., et al. (2015). Genome-wide association mapping for tomato volatiles positively contributing to tomato flavor. *Frontiers in Plant Science*, 6, 1042.
- Zhou, Y., Cai, H., Xiao, J., Li, X., Zhang, Q., & Lian, X. (2009). Over-expression of aspartate aminotransferase genes in rice resulted in altered nitrogen metabolism and increased amino acid content in seeds. *Theoretical and Applied Genetics*, 118(7), 1381–1390.
- Zhu, G., Wang, S., Huang, Z., Zhang, S., Liao, Q., Zhang, C., et al. (2018). Rewiring of the fruit metabolome in tomato breeding. *Cell*, 172(1–2), 249–261.e12.
- Zhu, Z., Zhang, Y., Liu, J., Chen, Y., & Zhang, X. (2018). Exploring the effects of selenium treatment on the nutritional quality of tomato fruit. *Food Chemistry*, 252, 9.

