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Comparative proteomics analysis of differentially accumulated proteins in juice sacs of ponkan (*Citrus reticulata*) fruit during postharvest cold storage

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ABSTRACT

Comparative proteomics analysis was carried out in 'Egan No.1' ponkan (*Citrus reticulate* cv. Egan No.1) fruit during low temperature storage. Commercially mature fruit were harvested, stored at 4 °C, and sampled four times at one month intervals. Two-dimensional gel electrophoresis and MALDI-TOF–TOF MS were performed to examine the protein changes during the postharvest storage period. Results showed that 74 proteins were differentially regulated, from which 56 proteins were identified by blasting against NCBInr (green plant) and EST_viridiplantae databases. All identified proteins were then classified into functional classes according to known biosynthetic pathways, including C-compound and carbohydrate metabolism, amino acid metabolism and response to storage environmental stimuli. In addition, subcellular location and time-dependent accumulation trends of differentially accumulated proteins associated with fruit quality were analyzed. To understand the relationships between these differentially accumulated proteins and fruit quality changes, composition of organic acids (malic acid, citric acid and quinic acid) and soluble sugars (fructose, glucose and sucrose) were investigated. Possible mechanisms responsible for fruit quality change in ponkan fruit during storage are discussed.

1. Introduction

During postharvest storage of citrus fruit, quantitative and qualitative changes affecting fruit quality occur, and physiological and environmental factors affecting postharvest properties have been extensively studied (Asrey et al., 2008). Storage life of citrus differs widely in different varieties, loose-skin mandarins being the most perishable with a shorter storage life than oranges and other citrus types (Shi et al., 2008). However, ponkan (*Citrus reticulata* Blanco), the most important commercial easy-peeling genus of the tangerine family in China, is an obvious exception. Ponkan fruit are usually harvested in late November and can be stored well until the following May.

To maintain quality of citrus fruit during postharvest storage, temperature, relative humidity, and atmospheric components of the environment are usually given most attention (Pasentsis et al., 2007). Although complex genetic, biochemical and physiological pathways may collectively control and regulate fruit postharvest quality, previous studies have mainly concentrated on expression analysis of quality-related genes (e.g. Malladi and Burns, 2008). However, DNA and mRNA levels do not always correlate well with protein levels due to post-transcriptional and post-translational processes. Moreover, proteins play critical roles in functional performance and there is far less information available on their functional products than their gene profiles. Only a few studies have been carried out to correlate changes of proteins with fruit quality of citrus, and those studies mainly focused on preharvest fruit development (Katz et al., 2007), albedo tissue during cold storage (Lliso et al., 2007) and pigment metabolism (Alos et al., 2008). To date, no quality-related proteomic analyses of juice sacs during postharvest storage of citrus fruit has been published.

Two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry (MS) offers a comprehensive proteomic approach (Jorrin et al., 2007). In fruit under postharvest conditions, it has been successfully applied, for example, to identify novel pear fruit proteins (Pedreschi et al., 2007). In recent years, interest in proteomics of plant foods such as peach (Chan et al., 2007; Qin et al., 2007), chickpea (Pandey et al., 2008) and green bean (Lee et al., 2009), has increased. On the other hand, although several differentially accumulated proteins involved in development or fruit maturity have been reported using 2-DE-based proteomic approaches (Katz et al., 2007; Lliso et al., 2007; Alos et al., 2008), there is still a lack of information about the proteins associated with quality in juice sacs of stored citrus fruit.

In a preliminary study on ponkan fruit, we had found several genes (citrate synthase, malate dehydrogenase, and isocitrate

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lyase) differentially expressed at the mRNA level, and components of organic acid and soluble sugar pools changed in response to postharvest storage time and conditions. However, the changing trends of the transcriptional profiles were not consistent with the physiological changes of sugars and acids. Therefore, we investigated fruit quality changes under storage conditions at the protein level, of which there is little information available. In the current study, we developed specific protein extraction and 2-DE protocols for citrus fruit juice sac tissues, and analyzed proteins involved in the fruit quality of ponkan fruit using a comparative proteomic approach. Moreover, to understand the relationships between protein and fruit quality, we further investigated the contents of organic acids (malic acid, citric acid and quinic acid) and soluble sugars (fructose, glucose and sucrose) in all samples.

2. Materials and methods

2.1. Sample collection and storage

Ponkan fruit (*C. reticulata* cv. Egan No.1) were harvested at commercial maturity (two-thirds of the whole peel has turned from green to orange) from the National Center of Citrus Breeding Orchard in Huazhong Agricultural University (Wuhan, China). All fruit were stored at optimal conditions of 4 °C and 85–90% relative humidity. Juice sacs from ten fruit bulked were harvested as one sample, and four samples were taken at 30 d intervals from November 20, 2007 to February 18, 2008. Samples were filtered to remove segment membranes and seeds. They were then immediately frozen in liquid nitrogen and stored at −80 °C until use.

2.2. Total protein extraction and quantification

Samples were grinded into a fine powder in the presence of liquid nitrogen. Proteins were then extracted from the frozen powder (6 g) using the method of Isaacson et al. (2006) with a little modification to remove impurities. As citrus fruit contain significant amounts of pectic acid, phenolic compounds and other interfering substances which can be co-extracted with proteins (King et al., 1994), a phenol extraction protocol was adapted for this study. Protein pellets were then dried with nitrogen gas, resuspended in 0.6 mL of isoelectric focusing (IEF) solubilization buffer containing 8 M urea, 4% CHAPS, 65 mM DTT and 0.2% ampholine pH 4–7 (Bio-Rad, Hercules, CA, USA). The mixture was centrifuged at 10,000 × *g* for 30 min to remove insoluble polymers. Sample protein concentrations were measured using the Bio-Rad RC DC protein assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

2.3. Isoelectric focusing with immobilized pH gradient and second-dimensional gels

Isoelectric focusing was carried out with 1 mg of protein. Aliquots of protein were diluted with IEF solubilization buffer, and 250 μL of solution was used to passively rehydrate the 17 cm pH 4–7 IPG strip overnight. IEF was conducted in a Protean IEF Cell (Bio-Rad) at 20 °C and with a 50 μA per strip limit, according to the following migration program: demineralization at 250 V (linear ramping) for 30 min, at 1000 V (rapid ramping) for 1 h, voltage boosting at 10,000 V (linear ramping) for 5 h, focusing at 10,000 V (rapid ramping) for 80,000 V h. At IEF completion, strips were equilibrated for 15 min in 6 mL equilibration buffer (6 M urea, 20% glycerol, 2% SDS, 0.375 M Tris–HCl pH 8.8), 2% DTT was added followed by alkylation in 6 mL 2.5% iodoacetamide in equilibration buffer for 15 min. The second-dimensional SDS-PAGE was performed with 15% acrylamide gels using Protean II Xi (Bio-Rad). Gel electrophoresis was first conducted at 80 V for 0.5 h then at 180 V for 5.5 h. Three replicate gels were run for every sample.

2.4. Protein staining and image analysis

After SDS-PAGE, gels were fixed for 1 h in the presence of 40% ethanol and 10% acetic acid. Gels were then washed with ultra pure water and stained overnight in a solution containing 20% methanol, 0.12% (w/v) CBB G-250, 10% o-phosphoric acid and 10% (w/v) ammonium sulfate. The next day, gels were washed five times in ultra pure water for 1.5 h each before they were scanned. Spots on the gel were quantified with PDQuest 2-D analysis software, version 7.4 (Bio-Rad). To obtain the highest gel matching, an automated and manual matching function was used in lieu of the automated routine. Quantity of a protein spot was calculated based on the volume. A quality score of <30 was adopted to define low-quality spots, which were eliminated in further analysis (Bhushan et al., 2007). To account for quantitative variations in intensity of protein spots between samples, the spot densities were normalized as a percentage of the total densities of all spots on the corresponding gel. Those spots showing more than 2-fold density difference when compared with those from other samples were excised for MS protein identification. Experimental molecular mass and p*I* were calculated from digitized two-dimensional electrophoresis images using standard molecular mass marker proteins as references.

2.5. In-gel tryptic digestion

In-gel digestion was performed as described by Koy et al. (2003). The CBB-stained protein spots were excised from the gel, sliced into 1 mm^3 and destained overnight at room temperature with 200 μL of 50 mM NH_4HCO_3 in 40% ethanol. After stain removal, the liquid was discarded. Gel slices were then washed with 50 μL of 50 mM NH_4HCO_3 twice. Subsequently, NH_4HCO_3 buffer was removed and replaced by 150 μL of acetonitrile, then incubated with acetonitrile for 10 min. After repeating the acetonitrile incubation step twice, the gels were dried in a vacuum centrifuge for 30 min. Subsequently, an aliquot (5 μL) of trypsin solution (75 ng/μL in 50 mM NH_4HCO_3) was added to each sample followed by addition of 50 mM NH_4HCO_3 and solubilizing agent. The mixture was then incubated at 30 °C for 16 h.

2.6. MS analysis and database searching

Each sample (1 μL) was mixed with an equal volume of the HCCA (α-cyano-4-hydroxycinnamic acid) matrix solution. The mixture was then immediately spotted onto a 384-well MALDI target plate and dried at room temperature. Tandem MALDI mass spectrometry was conducted using a 4800 MALDI-TOF/TOF (Applied Biosystems, Framingham, MA, USA) mass spectrometer. MS spectra were acquired using a total of 1200 laser shots per spot in the positive ion reflector mode over the full-scan spectrum. The 6 most abundant precursor ions were selected for MS/MS scans. MS data were processed with the internal calibrants (tryptic autodigestion products) to determine a maximum of 6 precursors for MS/MS per spot. The resulting peak lists were submitted for database sequence searches using MASCOT v2.1.03 software (Matrix Science, UK). Data were searched against the EST_viridiplantae viridiplantae_20090508 and NCBInr 20090521 protein database. The search was performed taking green plants as taxonomy, and the other parameters were the enzyme trypsin, one missed cleavage, fixed modifications of carbamidomethyl, variable modifications of oxidation, peptide tolerance of 100 ppm, fragment mass tolerance of ±0.5 Da, peptide charge of 1+. Only results of peptides with MS/MS $^*P < 0.05$ confidence were reported.