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Comparative proteomic approaches to analysis of litchi pulp senescence after harvest

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ABSTRACT

Litchi fruit (*Litchi chinensis* Sonn.) is highly perishable after harvest. The shelf life is only 4–6 days under ambient temperature storage conditions, which has restricted the development of the litchi industry to a considerable extent. To investigate the molecular mechanisms of litchi fruit senescence, comparative proteomic analysis was carried out on litchi pulp. After two-dimensional gel electrophoresis (2-DE), 64 spots were significantly differentially expressed, 61 of which were successfully identified using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC–ESI–MS/MS). All of the identified proteins were classified according to biological process, molecular function, and cellular component using Blast2GO. Results showed that those proteins were mainly involved in signal transduction, cell wall metabolism, primary and secondary metabolism, energy metabolism. Specifically, many up-regulated proteins were involved in auxin/ethylene regulation, which suggested that auxin and ethylene might cooperate to regulate litchi pulp senescence. Histone deacetylase and DNA methyltransferase might involve the down-regulation of proteins related to reactive oxygen species (ROS) scavenging, glycolysis, tricarboxylic acid cycle, and ATP synthesis in litchi senescence. A higher proportion of differentially expressed proteins were up-regulated and these were involved in a range of processes including cell wall organization or biogenesis, anaerobic respiration, protein degradation, lipid degradation. All of those proteins might accelerate fruit softening, deterioration and senescence. This study is the first to carry out proteomic analysis of the regulation of litchi fruit senescence.

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1. Introduction

Litchi, as a popular tropical and subtropical fruit, has high commercial value on the international market due to its exotic flavor and nutritional qualities (Zhang et al., 2015). However, litchi fruit is highly perishable after harvest. The shelf life is only 4–6 days under ambient temperature storage conditions, which has significantly restricted the development of the litchi industry. Litchi fruit senescence presents as pericarp browning, water loss and aril breakdown. Aril breakdown is the main symptom of litchi pulp senescence. In this process, fruit weight decreases gradually, fruit color changes from redness to brownness, fruit firmness reduces gradually, and compounds indicative of spoilage are gradually accumulated (Jiang et al., 2006). All of these changes seriously reduce the market value of litchi.

Litchi is a non-climacteric fruit for which ethylene does not play a central role in fruit senescence. There has not yet been exact evidence about fruit senescence regulation in non-climacteric fruit. Abscisic acid has been reported to be likely involved in grape senescence (a non-climacteric fruit), while ethylene was reported to be involved in non-climacteric fruit (Sun et al., 2010), but neither of these plant hormones had a central role in fruit ripening and senescence. Previous studies on litchi fruit senescence mainly focused on the physiological changes and development of the preservation measures to prolong shelf life. Jiang et al. (2007) reported that adenosine triphosphate (ATP) plays an important role in litchi fruit senescence. Energy deficiency accelerated litchi fruit senescence and senescence was delayed in ATP-treated fruit (Yi et al., 2008). Pure oxygen gas treatment inhibited litchi fruit senescence effectively and it also increased the accumulation of ATP (Duan et al., 2004). However, little information was available on the regulatory mechanism of litchi pulp senescence, especially at a global proteomics level.

Proteomics provides a powerful tool to explain the molecular mechanism involved in fruit biology by investigating the global changes at the protein level. Two-dimensional gel electrophoresis (2-DE) coupled with liquid chromatography-electrospray ionization-tandem mass spectrometry (LC–ESI–MS/MS) as a mature and stable proteomics approach has been successfully used to identify a large number of

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fruit-ripening and senescence-related proteins (Angel Huerta-Ocampo et al., 2012). But no proteomics information relating to litchi fruit senescence mechanism has been reported. In this study, three samples were prepared during the fruit pulp senescence process; 2-DE coupled LC–ESI–MS/MS was used for the comparative proteomic analysis of the regulatory mechanism of litchi pulp senescence.

2. Materials and methods

2.1. Plant material

Commercial mature litchi (*Litchi chinensis* Sonn.) fruit cv. "Guiwei" were obtained from an orchard in Guangzhou, Guangdong province of China. Fruits of uniform shape and color were disinfected with 0.1% Sportak solution for 3 min, air dried for 2 h, and then stored at 25 °C with 85–90% relative humidity. Pulp tissue was collected 1 day, 3 days, and 6 days after harvest, immediately frozen in liquid nitrogen, and then stored at −80 °C for protein extraction. Thirty fruit were sampled for each replicate and three biological replicates were conducted independently.

2.2. Total soluble solids (TSS) measurement

Pulp tissue from 30 fruit was homogenized in a grinder and the supernatant was collected to analyze the total soluble solids (TSS), which were assayed using a hand refractometer (J1-3A).

2.3. Respiration rate measurement

The respiration rate was measured according to the method of Huang et al. (2012). Ten fruits were sealed inside a 4.2 L glass jar for 2 h at 20 °C. Then, 1 mL aliquots of headspace gas which was withdrawn from the jars was injected into a gas chromatograph (GC-9A; Shimadzu, Kyoto, Japan) using a thermal conductivity detector (TCD) and a Poropak N column (Shimadzu).

2.4. Protein extraction from litchi pulp

Ten grams of frozen pulp were used for protein extraction according to the method described by Yun et al. (2010). The concentration of the proteins was determined using a Bio-Rad Protein Assay Kit (Bio-Rad, USA).

2.5. Two-dimensional gel electrophoresis (2-DE) and image analysis

Two-dimensional gel electrophoresis was carried out with 2 mg of litchi pulp protein according to Yun et al. (2010) using a 17 cm IPG strip (pH 5–8, Bio-Rad). Gels were stained with Coomassie blue (CBB). At least three gels of independent biological replicates were run for each sample. PDQuest 2-DE analysis software, version 8.0 (Bio-Rad), was used to analyze the image. In brief, an automated and manual matching function was used to obtain the highest gel matching. Data were normalized using the total quantity of valid spots on the corresponding gel to account for quantitative variations in the intensity of protein spots between samples. The normalized intensity of spots on three independent biological replicate 2-DE gels was averaged. Spots with a more than two-fold change between two samples were analyzed by Student's t-test and $P < 0.05$ was considered to indicate significant changes in abundance. All of these spots were excised for protein identification.

2.6. In-gel protein digestion and protein identification by LC–ESI–MS/MS

Protein spots were cut from gels, washed twice using 25 mM NH_4HCO_3 and 50% acetonitrile separately (1 h each time), and dehydrated by the addition of 500 μL of acetonitrile (ACN). Disulfide bonds were cleaved for spots by incubating the samples for 60 min at 56 °C with 200 μL of 10 mM DTT in 25 mM NH_4HCO_3 buffer, and alkylation of cysteines was performed by the addition of 200 μL of 55 mM iodoacetamide in 25 mM NH_4HCO_3 buffer and incubation of the samples for 45 min at room temperature in darkness. The spots were then dehydrated again with 500 μL of acetonitrile after being washed by 25 mM NH_4HCO_3 twice. After that, Trypsin (Promega) solution (10 ng·μL^{-1} in 25 mM NH_4HCO_3 buffer) was added for 37 °C overnight digestion which was stopped by the addition of 5% formic acid (FA). Finally, the extracts were dried under the protection of N_2. Samples were reconstituted in 3 μL of 0.1% trifluoroacetic acid (TFA) prior to MS analysis.

For LC–ESI–MS/MS, the digested peptide sample was desalted using a Strata X column (Phenomenex), vacuum-dried, and then resuspended in a 200 μL volume of buffer A (2% ACN, 0.1% FA). After centrifugation at 20,000 *g* for 10 min, the supernatant was recovered to obtain a peptide solution with a final concentration of approximately 0.5 μg·μL^{-1}; 10 μL supernatant was loaded on a LC-20 AD nanoHPLC (Shimadzu, Kyoto, Japan) by the autosampler onto a 2 cm C18 trap column. The peptides were then eluted onto a 10 cm analytical C18 column (inner diameter 75 μm) packed in-house. The samples were loaded at 15 μL FA·min^{-1} for 4 min, then a 91 min gradient was run at 400 nL·min^{-1} starting from 2% to 35% of buffer B (98% ACN, 0.1% FA), followed by a 5 min linear gradient to 80%, and maintenance at 80% of buffer B for 8 min, and finally return to 2% in 2 min.

The peptides were subjected to nanoelectrospray ionization followed by tandem mass spectrometry (MS/MS) in an LTQ Orbitrap Velos (Thermo, USA) coupled online to the HPLC. Intact peptides were detected in the Orbitrap at a resolution of 60,000. Peptides were selected for MS/MS using the collision-induced dissociation (CID) operating mode with a normalized collision energy setting of 35%. Ion fragments were detected in the LTQ. A data-dependent procedure that alternated between one MS scan followed by ten MS/MS scans was applied for the ten most abundant precursor ions above a threshold ion count of 5000 in the MS survey scan with the following Dynamic Exclusion settings: repeat counts, 2; repeat duration, 30 s; and exclusion duration, 120 s. The applied electrospray voltage was 1.5 kV. Automatic gain control (AGC) was used to prevent overfilling of the ion trap; 1×10^4 ions were accumulated in the ion trap to generate CID spectra. For MS scans, the m/z scan range was 350–2000 Da. Raw data files acquired from the Orbitrap were converted into MGF files using Proteome Discoverer 1.2 (PD 1.2, Thermo) and the MGF files were searched. Protein identification was performed using the Mascot search engine (Matrix Science, London, UK, version 2.3.02).

For protein identification, a mass tolerance of 20 ppm was permitted for intact peptide massed and 0.1 Da for fragmented ions, allowing one missed cleavage in the trypsin digests, with Glu → pro-Glu (N-term Q), Oxidation (M), and Deamidated (NQ) as the potential variable modifications and Carbamidomethyl (C) as the fixed modification. The charge states of peptides were set to +2 and +3. Specifically, an automatic decoy database search was performed in Mascot by choosing the decoy checkbox in which a random sequence of the database is generated and tested for raw spectra as well as the real database. To reduce the probability of false peptide identification, peptides with ion scores greater than "identity" were counted as identified. Each identified confident protein contained at least one unique peptide. More information of peptide and spectrum for each identified protein was provided in Supplementary Excel S1.

2.7. Real-time quantitative RT–PCR verification

Total RNA was extracted from fruit pulp using a Qiagen RNeasy Kit, according to the manufacturer's instructions. 2 μg total RNA was reverse transcribed for first-strand cDNA synthesis using the RevertAid First Strand cDNA synthesis kit (Fermentas, Lithuania). Gene-specific primer pairs (Supplementary Table S1) were designed using the Primer

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