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Proteomics approach reveals mechanism underlying susceptibility of loquat fruit to sunburn during color changing period

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ABSTRACT

The objective of this work was to investigate why loquat fruit peels are more sensitive to high temperature and strong sunlight, making them highly susceptible to sunburn, during the color changing period (CCP). Two dimensional gel electrophoresis (2-DE) of the fruit peel proteins was performed over three developmental periods, namely green fruit period (GFP), color changing period and yellow ripening period (YRP). Fifty-five protein spots with at least 2-fold differences in abundance were successfully identified by MALDI-TOF–TOF/MS. The identified proteins were divided into categories related to heat-shock response, stress response and defense, energy metabolism, photosynthesis and protein biosynthesis. The results showed that expression of proteins related to anaerobic respiration and photorespiration were increased while the proteins related to ROS scavenging, polyamine biosynthesis, defense pathogens and photosynthesis were decreased during CCP under heat stress. Our findings provide new insights into the molecular mechanism of loquat fruit susceptible to sunburn during CCP.

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1. Introduction

Sunburn is a common physiological disease that manifests as yellow or brownish spots on the sunlight-exposed side of fruits such as apple (Felicetti & Schrader, 2009), loquat (Deng et al., 2012), grape (Krasnow et al., 2010) and pomegranate (Weerakkody, Jobling, Infante, & Rogers, 2010). Sunburn results in significant loss in fruit quality and production. High temperature and strong light are the main external factors that cause fruit sunburn. High temperature, especially swift increase in temperature, plays a major role in initiation of sunburn when sunlight intensity reaches a certain threshold (Krasnow et al., 2010).

Approximately 2% of genes in the plant genome are altered by high temperature (Lim et al., 2006), resulting in significant changes in physiological and biochemical processes in plants (Fan, Song, Forney, & Jordan, 2011; Qu, Ding, Jiang, & Zhu, 2013). Previous research showed that antioxidant enzymes, such as ascorbate peroxidase (APX) and catalase (CAT), act primarily as ROS scavengers in plants under heat stress (Gill & Tuteja, 2010). Furthermore, Zonia and Munnik (2006) reported that signal transduction played an important role in plants under heat stress. In addition, heat stress factor was found to play a thermo-tolerance protein under heat stress in plants (Koyro, Ahmad, & Geissler, 2012).

Proteomics is a powerful tool to observe molecular and physiological changes in plant cells under various stresses. Several previous proteome studies identified a large number of heat stress-responsive proteins in plants (Jagadish et al., 2010; Ray et al., 2011) such as longan (You et al., 2012) and rice (Lee et al., 2007). Wang, Ma, Song, Shu, and Gu (2012) identified 42 differentially expressed proteins that involved in 13 cell metabolic processes during soybean seed development and maturity under high temperature and humidity stress. Zhang et al. (2011) found 30 proteins induced by heat treatment in post-harvest peach. Lee et al. (2007) indicated that HSPs played an important role in the resistance of plants to heat stress. Koussevitzky et al. (2008) reported that some wheat proteins involved in starch synthesis, such as glucose-1-phosphate adenyltransferase, were down-regulated under heat stress. However, the expression of S-adenosylmethionine synthetase increased

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under heat stress in barley (Majoul, Bancel, Triboï, Ben Hamida, & Branlard, 2003). Furthermore, studies have shown that high temperature coupled with strong sunlight causes protein denaturation, membrane damage, decrease in photosynthesis and injury to the photosynthetic apparatus (Hu, Guo, Shen, Guo, & Li, 2009).

Loquat (*Eriobotrya japonica* (Thunb.) Lindl.) is an important fruit in Asia that ripens in late spring and early summer. Sunburn due to high temperature and strong light damages a large quantity of loquat fruits every year. This has serious impact on fruit quality, which translates to significant economic loss for farmers. Some studies have investigated the symptoms of sunburn in loquat fruits and devised protective methods (Wu & Zhong, 2011), but very few studies have examined the molecular and physiological mechanism of sunburn in loquat fruit. The present study investigates the proteome of loquat fruit peel under conditions of high temperature and strong light stress during different fruit developmental periods in order to reveal how sunburn initiates cellular damage and why loquat fruit peel is particularly vulnerable to high temperature and strong light stress during color changing period (CCP).

2. Materials and methods

2.1. Plant materials and stress treatment

'WDYDB' loquat (field GenBank number: PPM0056), one of the loquat varieties susceptible to sunburn (Felicetti & Schrader, 2009), was planted in the National Field GenBank of Loquat in Fuzhou, China. The fruits were harvested during 3 different fruit developmental periods, namely green fruit period (GFP), CCP and yellow ripening period (YRP), from 7-year old loquat trees with heat treatment of 40 °C for 90 min under natural sunlight intensity from 1128.5 to 1312.8 $\mu mol\ m^{-2}\ s^{-1}$. Control fruits were grown under ambient temperature (28 ± 2 °C) and ambient sunlight intensity during the same time. Harvested treatment and control fruits were randomly divided into three groups. Each group has 6 fruits. Sun-side of the fruits was peeled and the peels were immediately frozen in liquid nitrogen, stored at −80 °C for further analysis. Three independent biological replicates were performed.

2.2. Total soluble protein extraction, and 2-DE analysis

Protein extraction of loquat peels was modified from our reported method (You et al., 2012). Briefly, 4 mL ice-cooled extract buffer (0.7 M sucrose, 100 mM Tris–HCl pH 8.0, 50 mM L-ascorbic acid, 100 mM KCl, 50 mM disodium tetraborate decahydrate, 1% (v/v) Triton X-100, 2% (v/v) β-mercaptoethanol and 1 mM PMSF) was directly added to 1 g loquat peels. After extraction, the protein pellets were solubilized in lysis buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (w/v) DTT and 0.5% (v/v) pH 4–7 IPG buffer). The protein concentration was determined according to the Bradford method (1976), using bovine serum albumin as standard. Two-dimensional gel electrophoresis (2-DE) was performed according to the method of You et al. (2012). A total of 1.3 mg protein was loaded onto IPG strip (24 cm, pH 4–7) during rehydration for 12 h at room temperature. The first dimension, isoelectric focusing (IEF), was performed following the manufacturer's instructions (GE Healthcare, Uppsala, Sweden). The second dimension was performed on 12.5% polyacrylamide gels using an Ettan DALT Six Electrophoresis Unit (GE Healthcare, USA) after IEF. Subsequently, the gels were stained with Coomassie brilliant blue (CBB) G250 and the images were scanned for quantitative analysis using the ImageMaster 2D Platinum Trial software. Protein spots with more than 2-fold differential expression were selected with good repeatability ($P \leqslant 0.05$). At least three biological replicates were performed for each treatment condition.

2.3. MS identification of proteins

Analysis of protein was done by MALDI-TOF–TOF/MS mass spectrometry, followed by search of mass spectrometry data in the protein database to match known proteins. Combined MS and MS/MS spectra were submitted to MASCOT (V2.1, Matrix Science, London, U.K.) by GPS Explorer software (V3.6, Applied Biosystems) and searched with the following parameters: National Center for Biotechnology Information (NCBI) database (release date: 2010.07.01), trypsin digest with one missing cleavage, none fixed modifications, MS tolerance was set at 100 ppm; MS/MS tolerance of 0.6 Da. MASCOT protein scores (based on combined MS and MS/MS spectra) greater than 75 were considered statistically significant ($P \leqslant 0.05$). The individual MS/MS spectrum with statistically best (confidence interval > 95%) ion score (based on MS/MS spectra) was accepted.

2.4. Western blot confirmation

Immunoblot analysis was used to examine the expression of loquat peel proteins following the method of Wang et al. (2014). The proteins (50 μg per lane) were separated with 12.5% SDS–PAGE and subsequently transferred onto nitrocellulose membranes. Subsequently, the membranes were blocked for 2 h with 5% skimmed milk and then incubated with rabbit polyclonal antibody raised against HSP70 and alcohol dehydrogenase (ADH) at 1:5000 dilutions for 2 h. The membrane was washed (3 × every 15 min) with TBST buffer (0.01 M TBS, 0.1% Tween-20, pH 7.6) and then incubated with a secondary goat anti-rabbit IgG conjugated with horseradish peroxidase at 1:1000. Immunoblot signals were detected with DAB (Boster, Wuhan China). The intensities were quantified using Quantity software (Bio-Rad, USA).

2.5. Enzyme activity assays

Loquat tissue (1 g) was ground into powder in liquid N_2. Dehydroascorbate reductase (DHAR) activity was assayed essentially using the method of Hossain and Asada (1984). The activity was detected by measuring the increase in the absorbance at 265 nm due to the GSH-dependent production of ascorbate. Alcohol dehydrogenase (ADH) activity was performed according to Shimomura and Beevers (1983). One unit of ADH activity was defined as an increase of 0.1 in absorbance per minute at 340 nm under the assay conditions.

2.6. Statistical analysis

Statistical analysis was performed by one-way analysis of variance and the Student–Newman–Keuls test between treatments. Data are presented as mean ± standard deviation (SD) of three replicates (three independent replicates of each treatment). *P*-value less than 0.05 ($P < 0.05$) was considered statistically significant.

3. Results

3.1. Protein profiles of 2-DE

Protein profiles of loquat peels were analyzed using the ImageMaster software. The results showed that there were at least 1000 protein spots in each 2-DE gel. The fruit peel protein matching rates during GFP, CCP and YRP were 83.5%, 82.7% and 85.9% compared with control, respectively. These results indicated that the experiments were reproducible. A total of 68 proteins showed more than 2-fold ($P < 0.05$) differential expression, of which 27 were down-regulated and 40 were up-regulated, while 1 protein

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