Postharvest Biology and Technology 112 (2016) 277–289

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Effect of postharvest nitric oxide treatment on the proteome of peach fruit during ripening

Ruoyi Kang[a], Li Zhang[b], Li Jiang[a], Mingliang Yu[c], Ruijuan Ma[c], Zhifang Yu[a,*]

[a] College of Food Science and Technology, Nanjing Agricultural University, Nanjing 210095, PR China
[b] College of Education and Humanity, Suzhou Vocational University, Suzhou 215104, PR China
[c] Institute of Horticulture, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, PR China

ARTICLE INFO

Article history:
Received 19 January 2015
Received in revised form 21 August 2015
Accepted 23 August 2015
Available online 12 September 2015

Keywords:
Nitric oxide
Proteome
Peach fruit
Ethylene
Antioxidant enzymes
TCA cycle

ABSTRACT

Nitric oxide (NO) is an important signalling molecule with diverse physiological functions in plants. This study investigated the protein profile changes of harvested peach fruit (*Prunus persica* L. cv. 'Xiahui NO.5') exposed to NO gas treatment. A total of 104 protein spots with abundance changes (>two-fold) in response to NO were observed, and the spots were further functionally classified into 7 categories, including energy and metabolism (30.77%), stress response and defence (25.00%), cell structure (8.65%), protein fate (8.65%), transport and transduction (6.73%), ripening and senescence (5.77%) and unclassified (13.46%). The effects of NO at the proteomic level in peach fruit are complicated and involve various biological processes. NO could induce an abundant increase of superoxide dismutase (SOD) and the ascorbate–glutathione cycle enzymes to promote the production of the complex 1-aminocyclopropane-1-carboxylic acid oxidase (ACO–NO–ACC), which affects the generation of ethylene. Also, NO could inhibit electron transport and oxygen consumption, enhance the tricarboxylic acid cycle (TCA) cycle and the glycolysis pathway, and NO could repress the loss of Ca^{2+} ions and other structural components that maintain the mechanical properties of cell structures.

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1. Introduction

Nitric oxide (NO) is an important bioactive signalling molecule with diverse physiological functions in phylogenetically distant species (Perazzolli et al., 2006). It has become evident that NO constitutes an important endogenous signal in cellular metabolism and in the modulation of hormonal responses, plant growth and developmental processes (Arasimowicz and Floryszak-Wieczorek, 2007). The use of NO on post-harvest fruit ripening has become a focus for many researchers because of the potential of NO to maintain fruit quality after harvest and to be a powerful tool for gaining insight on ripening processes (Manjunatha et al., 2010). Increasing numbers of studies indicate that the NO signal influencing fruit ripening is complicated. NO appears to play significant roles in the transit and storage of fruit commodities, and the effects of NO were linked to the inhibition of pectin depolymerisation (Zhang et al., 2011a), reduction of chilling injury (Singh et al., 2009), decrease in ethylene production (Zhu et al., 2006), and inhibition of phenolic metabolism (Zhu et al., 2009).

It was reported that NO could inversely regulate ethylene production in growing plants (García et al., 2010). Investigators presumed that NO is bound to ACC oxidase to form an ACC oxidase–NO complex, which is chelated by ACC to produce an ACC–ACC oxidase–NO complex and leads to a decrease in ethylene production (Zhu et al., 2006). NO-fumigated fruit showed decreased activities of exo- and endo-polygalacturonase (exo- and endo-PG), endo-1,4-β-D-glucanase (EGase) but maintained higher pectin esterase (PE) activity in pulp tissues during mango ripening and cool storage (Zaharah and Singh, 2011).

NO also plays a critical role in suppressing reactive oxygen species (ROS) (del Río et al., 2002). NO can act as a reaction cascade breaker and prevent oxidization damage by directly suppressing either the activities of ROS enzymes or the relevant signalling cascade when ROS is release under abiotic or biotic stresses (Rümer et al., 2009). NO treatment reduced the increases in membrane permeability and lipid peroxidation, delayed the increases in both the rate of O^{2-} production and H_2O_2 contents, and increased the activities of SOD, CAT and APX. These results indicate that NO positively maintains the balance between the formation and detoxification of ROS (Wu et al., 2012). Although research on NO in plants had gained significant advances in recent years, the role of the NO in plants remains unclear, especially in fruit ripening.

* Corresponding author. Fax: +86 25 84395618.
E-mail address: yuzhifang@njau.edu.cn (Z. Yu).

http://dx.doi.org/10.1016/j.postharvbio.2015.08.017
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As for peach fruit ripening, there have been physiological, biochemical and proteomics studies, and researchers have determined the main functional proteins during peach fruit ripening were involved in substance and energy metabolism, stress response and defences, and cell structure and signal transduction (Jiang et al., 2014; Palma et al., 2011; Prinsi et al., 2011; Zhang et al., 2011b, 2012a). These proteins were shown to have significant roles during peach fruit development and ripening, and the proteins were differentially regulated according to species and maturity (Prinsi et al., 2011) and various postharvest treatments, such as heat (Lara et al., 2009; Zhang et al., 2011b), cold (Zhang et al., 2010), 1-MCP or ethephon (Zhang et al., 2012b), and SA (Chan et al., 2007). These proteomic studies help to elucidate the physiological mechanisms of peach fruit development and ripening, and their findings offer theoretical evidence for better quality control of post-harvest peach fruit in practice.

This study analysed and identified differentially accumulated proteins in NO-treated peach fruit to explore the molecular mechanism of NO in peach fruit ripening. Of interest was whether the biological processes and functions of differentially accumulated proteins were similar to the processes in previous physiological and biochemical reports, and the goal of this study was to determine whether the pattern of protein profile changes after NO treatment was similar to that of other postharvest treatments, such as 1-MCP, heat and cold.

2. Experimental

2.1. Fruit material and treatments

Commercially matured peach fruit (*Prunus persica* L. cv. 'Xiahui NO.5') with a mean firmness of 12.84 N and a mean soluble solids content (SSC) of 13.76% were obtained on June 10, 2012 from a peach orchard at the Jiangsu Academy of Agricultural Science, which is located in Nanjing, Jiangsu Province, China. This variety matures early in the season, has melting white flesh with a clingstone, and excellent quality. It is one of most favourable cultivars in Jiangsu Province. Harvested fruit (CK0) were immediately delivered to the laboratory and sorted on the basis of uniform size and the absence of visual defects. The fruit were divided into two groups containing thirty fruit per replicate (30×3) and were immediately exposed to the following treatments: (a) for nitric oxide treatment, peach fruit were sealed in desiccators under 10 $\mu L\,L^{-1}$ nitric oxide gas (Nanjing special gas Factory Co., Ltd., China) for 3 h. To prevent the accumulation of CO_2, a 1% (w/v) KOH (Sinopharm Chemical Reagent Beijing Co., Ltd., China) solution was placed inside the desiccators. After ventilation for 30 min, the peach fruit were held at room temperature (20–25 °C) with 80–90% relative humidity for 1, 3 and 5 days (NT1, NT3 and NT5, respectively). (b) The control group was placed directly into storage to ripen without any treatment for 1, 3 and 5 days (CK1, CK3 and CK5, respectively). The fruit mesocarp from each treatment was immediately frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.2. Protein extraction and quantification

The protein extraction was conducted according Tal Isaacson's protocol (Isaacson et al., 2006). Frozen fruit tissue (5 g) was ground to powder with liquid nitrogen, and 5 mL of phenol saturated with Tris–HCl (pH 7.5) was added. The mixture was shaken for 30 min at 4 °C, before the homogenate was centrifuged (Eppendorf Freezing Centrifuge, Germany) at $5000 \times g$ for 30 min at 4 °C and the upper phenolic phase was collected. An equal amount of phenol extraction buffer was added to the collected phenolic phase, and the upper phenolic phase was collected and precipitated overnight by adding five volumes of cold 0.1 mol L^{-1} ammonium acetate in methanol to the collected phenol phase and storing the samples at −20 °C for 1 h. The mixture was centrifuged at $5000 \times g$ at 4 °C for 30 min, and the pellets were collected. After washing the pellets twice with ice-cold methanol and twice with chilled acetone, the pellets were air dried at room temperature and dissolved overnight at 4 °C in lysis buffer (1 mg pellets in 100 μL lysis buffer [9 mol L^{-1} urea, 4% (w/v) CHAPS, 1% (w/v) DTT, 1% (v/v) pH 3–10 NL IPG buffer (GE Healthcare, USA)]). The samples were then centrifuged at $15{,}000 \times g$ for 15 min at room temperature. The supernatant was collected and centrifuged again. The supernatant contained the protein solution. The concentrations of the protein extracts were determined with the Bradford method (Bradford, 1976) and stored at −80 °C for isoelectric focusing electrophoresis (IEF).

2.3. 2-DE and staining

Sample aliquots containing 200 μg of proteins were mixed with fresh rehydration buffer [9 mol L^{-1} urea, 4% (w/v) CHAPS, 1% (w/v) DTT, 1% (v/v) pH 3–10 NL IPG buffer (GE Healthcare), and a trace amount of bromophenol blue (BPB)] to a total volume of 450 μL and then were applied to 24 cm, pH 3–10 NL dry strips. Isoelectric focusing was performed on a IPGphor Isoelectric Focusing System (GE Healthcare, USA) at 20 °C with the following steps: rehydration at 50 V for 12 h (step), 500 V for 1 h (step), 1000 V for 1 h (step), 10,000 V for 1 h (gradient), and 10,000 V for 10 h (step). The strips were subsequently equilibrated for two periods of 15 min with 1% (w/v) DTT and 2.5% (w/v) iodoacetamide in equilibration buffer [6 mol L^{-1} urea, 50 mmol L^{-1} Tris–HCl (pH 8.8), 30% (v/v) glycerol, 2% (m/v) SDS]. Following equilibration, the strips were run on 12% homemade gels with the Ettan Six vertical set (GE Healthcare, USA) at 15 °C for 45 min at 100 V, and the gel was subsequently run at 200 V for 6–8 h (until the BPB band reached the bottom of the gel). There were three replicates for each treatment.

2.4. Image acquisition and data analysis

The stained gel was scanned by an image scanner (GE Healthcare, USA) at a resolution of 300 dots per inch. All gel images were processed with PDQuest 8.0 software (Bio-Rad, USA) using three steps: spot detection, volumetric quantification, and matching. The differences in protein spot volume between the treatment and the control were calculated as fold ratio. A threshold of $p \leq 0.05$ (Student's t-test) and fold change ≥ 2 or ≤ 0.5 was used to identify protein spots with significant changes.

2.5. Protein in-gel digestion and identification by MALDI-TOF/TOF

The protein spots were de-stained with 25 mM NH_4HCO_3 in 50% ethanol for 2 h at 40 °C. The proteins were reduced with 10 mmol L^{-1} DTT in 50 mmol L^{-1} NH_4HCO_3 and alkylated in 55 mmol L^{-1} iodoacetamide in 50 mmol L^{-1} NH_4HCO_3 for 1 h at room temperature. The proteins were digested overnight at 37 °C by adding 15 mL of trypsin (Promega, Madison, USA). The resulting peptides were extracted by washing the gel pieces with 0.1% trifluoroacetic acid in 67% acetonitrile (ACN). The tryptic peptide masses were analysed by an ABI 5800 MALDI-TOF/TOF Plus mass spectrometer (Applied Biosystems, Foster City, USA). The data were acquired in a positive MS reflector using a CalMix5 standard to calibrate the instrument (ABI5800Calibration Mixture). Both the MS and MS/MS data were integrated and processed with the GPS Explorer V3.6 software (Applied Biosystems, USA) using default parameters. Based on combined MS and MS/MS spectra, the proteins were successfully identified based on a 95% or higher confidence interval of their scores in the Mascot V2.3 search engine (Matrix Science Ltd., London, U.K.). The following search

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