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Synergistic action of antioxidative systems contributes to the alleviation of senescence in kiwifruit

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ABSTRACT

Fruit senescence has been reported to be initiated by reactive oxygen species (ROS) and directly affects the quality of post-harvest fruit. Antioxidative systems protecting fruits against ROS-induced damages have been studied, but it remains poorly understood whether antioxidative systems are involved in the regulation of kiwifruit senescence. This study shows that senescence of kiwifruit was accompanied by elevation in ROS levels and subsequent oxidative damage in membranes and proteins. Controlled atmosphere storage (CA, 1% O_2 plus 5% CO_2), which maintained the activity of antioxidative systems, effectively suppressed the accumulation of ROS, alleviated oxidative damage and finally delayed fruit senescence, suggesting the regulatory effect of antioxidative systems on kiwifruit senescence. To further determine the underlying molecular mechanisms, expression profiles of 25 oxidative stress-related genes were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) in kiwifruit stored in air and CA, respectively. Gene-expression patterns revealed that upregulated expression of *SOD3*, *CAT1*, *APX1*, *APX2* and *GR3* in the CA may predominantly contribute to the maintenance of antioxidative systems. Additionally, the synergistic action of antioxidative components is one of the determinants for the competence of the antioxidative systems. These findings may broaden understanding of the multifaceted mechanism of fruit senescence and underscore the positive effect of controlled atmosphere storage on the antioxidative systems in this process.

1. Introduction

Fruits are of major economic importance for human consumption as a nutrient source like flavor compounds, fiber, vitamins and antioxidants (Manning et al., 2006). Senescence of post-harvest fruit greatly affects the fruit sensory and nutritional quality (Tian et al., 2013), thus it is vital to elucidate the mechanism of fruit senescence. Accumulating evidence has shown that fruit senescence is modulated by multiple factors such as ethylene and ethylene signaling (Yoo et al., 2008), abscisic acid (Zhang et al., 2009), and MADS-box transcriptional factors (Hileman et al., 2006; Qin et al., 2012). A better understanding of the common regulatory mechanisms shared by both climacteric and non-climacteric fruits may create new opportunities for controlling fruit senescence. It has been clarified that reactive oxygen species (ROS) and oxidative damage are responsible for fruit senescence (Qin et al., 2009; Tian et al., 2013). However, the role of ROS-defensive mechanisms in fruit senescence remains largely unknown.

Antioxidative systems, which control the steady-state level of ROS under normal conditions, are composed of several ROS-scavenging enzymes, a number of low-molecular-mass antioxidants, and enzymes regenerating the active forms of antioxidants (Blokhina et al., 2003). Under unfavorable conditions, antioxidative systems may be overwhelmed, and ROS accumulation leads to increased organelle and cell damage (Foyer and Noctor, 2009; Yin et al., 2009). In response to stresses, certain mechanisms may activate antioxidative systems by a program of gene expression to control the ROS level (Møller and Sweetlove, 2010). It has been reported that pretreatment of seeds with H_2O_2 could induced the expression of stress protein and improve salt tolerance in wheat seedlings (Wahid et al., 2007). Treatment with paraquat is able to moderate the activities of ROS-scavenging enzymes and influence oxidative stress (OS) intensity under drought stress in cucumber leaves (Liu et al., 2009). Therefore, the final consequence of stress is dependent on the antioxidative systems. For the effect of antioxidative systems on the process of fruit senescence, it has been partly explained that the activities of oxygen-scavenging enzymes

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decrease during maturation in sweet orange (Huang et al., 2007), while the activity for ascorbate peroxidase (APX) and superoxide dismutase (SOD) in mitochondria increase during maturation and senescence in pepper fruit (Jimenez et al., 2002a). However, previous studies give ambiguous information about the role of antioxidative systems in fruit senescence, which needs to be further studied.

To delay fruit senescence, post-harvest application of ozone, oxalic acid, and controlled atmosphere (CA) storage are usually performed (Tian et al., 2002; Wang et al., 2009; Minas et al., 2012). Among them, CA storage at low temperature has been identified as a common method to maintain quality and extend shelf life, especially for climacteric fruits (Wang et al., 2005; Liu et al., 2013). Kiwifruit is a climacteric fruit that is sensitive to low concentrations of ethylene (Yin et al., 2010). CA storage was utilized to maintain kiwifruit firmness and increase storage life up to 120–140 d (Tonini et al., 1999). It has been reported that CA could reduce cell wall degradation (Ding et al., 2006), inhibit ethylene production (Gorny and Kader, 1996), maintain membrane fluidity and integrity (Zhang and Tian, 2010) and augment some antioxidative metabolism (Singh and Singh, 2013). Nevertheless, systematic study about the antioxidative systems in response to CA storage during fruit senescence remains largely unexplored. A better understanding of the physiological basis and molecular mechanisms involved in this process is of importance to elucidate the mechanisms of fruit senescence and improve management strategies for senescence and quality control. The present study focused on dynamic changes in ROS levels and oxidative damages, key enzymes and metabolites involved in oxidative metabolism, as well as expression profiles of gene families encoding corresponding enzymes. The results demonstrated the regulatory role of antioxidative systems in the process of fruit senescence.

2. Materials and methods

2.1. Fruit and treatment

'Hongyang' kiwifruit (*Actinidia chinesis* cv. Hongyang) were obtained at commercial maturity from an orchard of the Edan Company in the DuJiangYan city of Sichuan Province, China. The fruit were sorted based on size and the absence of physical injuries or infections, then pre-cooled at 0 °C for 24 h.

In the present study, kiwifruit were divided into two groups, in which the first group was stored in the controlled atmosphere (CA, 1% O_2 + 5% CO_2 at 0 °C), while the second group was stored in air at 0 °C as a control. The control fruit were placed in plastic boxes (45 cm × 30 cm × 25 cm), wrapped in perforated polyliners (0.02 mm thickness, with 5 holes of 20 mm diameter on upper and side surfaces) to maintain relative humidity at approximately 95%. Fruit were sampled after stored for 30, 60 and 90 d, and immediately divided into two lots. One lot was used to determine quality properties. The flesh of 15 fruit were cut into small slices, pooled quickly and immediately frozen in liquid nitrogen, and then stored at −80 °C for further studies. The other lot with 20 fruit was transferred to room temperature (25 °C) to investigate shelf life. Shelf life was defined as the period during which kiwifruit possess marketable characteristics at room temperature (25 °C) following cold storage.

2.2. Measurement of firmness and soluble solids content

Firmness was determined on opposite peeled cheeks of each fruit using a hand-held firmness tester (FT-327, Italy), equipped with a 10 mm plunger tip and expressed as Newtons (N). Soluble solids content (SSC) was measured with a pocket refractometer (PAL-1, Japan) and expressed as "%".

2.3. Levels of reactive oxygen species and oxidative damages

Superoxide production rate was measured by monitoring the nitrite formation from hydroxylamine in the presence of O_2^-, as described by Song et al. (2009). Corrections were made for O_2^- production in the absence of hydroxylamine hydrochloride. The result was expressed as nmo1 kg^{-1} s^{-1}. The quantification of H_2O_2 was based on a ferrous oxidation/xylenol orange assay. The detection was performed with Hydrogen Peroxide Assay Kit (Beyotime, China) by measuring the absorbance at 560 nm on Synergy™ Mx Multi-Mode Microplate Reader (BioTek, USA). The content of H_2O_2 was calibrated to a standard curve with known concentrations of H_2O_2 and expressed as μmol kg^{-1}.

The oxidative damage of lipids was assessed as the content of malonaldehyde (MDA) after reaction with thiobarbituric acid (TBA) by HPLC according to the method of Matamoros et al. (2010). A C18 column (5 μm, 250 mm × 4.6 mm, Intersil ODS-3, GL Sciences, Japan) was used on the UltiMate® 3000 HPLC systems (Dionex), and the elution buffer was 5 mM potassium phosphate buffer (pH 7.0) containing 10% acetonitrile and 0.6% tetrahydrofuran. The $(TBA)_2$–MDA adduct was detected at 532 nm and calibration curves were made using 1,1,3,3-tetraethoxy-propane (Sigma). The final content of MDA was expressed as μmol kg^{-1}.

The oxidative damage of proteins was estimated as the levels of protein carbonylation. The extraction of total proteins was performed as described by Saravanan and Rose (2004). Carbonylation of proteins were measured with the OxyBlot Protein Oxidation Detection Kit (Chemicon International, Billerica, MA). Proteins were derivatized to 2,4-dinitrophenylhydrazone (DNP) by incubation with 2,4-dinitrophenyl-hydrazine (DNPH). To monitor the equal loading of samples, Coomassie Brilliant Blue (CBB) R-250 was used to stain the proteins in the gels.

2.4. Determination of total antioxidant capacities

Frozen kiwifruit flesh (3.0 g) were extracted with 6 ml of ethanol–acetone–water (5:3:2, v/v/v) for 15 min. After centrifugation, the residue was extracted twice using the same conditions. The three supernatants were combined and then stored at −20 °C until used for analysis.

Antioxidant capacity determined by radical cation $ABTS^{\bullet+}$ (ABTS) was performed with Total Antioxidant Capacity Assay Kit with a Rapid ABTS method (Beyotime, China). The absorbance at 405 nm was measured, Trolox was used as a reference standard, and the results were expressed as moles of Trolox per mass of fruit flesh, mol kg^{-1}. Antioxidant capacity determined by $DPPH^{\bullet}$ free radicals (DPPH) was performed as the method of Brand-Williams et al. (1995). Results were expressed as moles of Trolox per mass of fruit flesh, mol kg^{-1}. The determination of oxygen radical absorbance capacity (ORAC) was performed as described by Du et al. (2009). The fluorescence was measured every 166 s for 97 min with a 485 nm excitation filter and a 535 nm emission filter, and results were expressed as moles of Trolox per mass of fruit flesh, mol kg^{-1}. Ferric reducing antioxidant power (FRAP) was carried out with Total Antioxidant Capacity Assay Kit with FRAP method (Beyotime, China). The absorbance at 595 nm was determined and calibration was against a ferrous iron standard curve. Results were expressed as moles of Fe^{2+} per mass of fruit flesh, mol kg^{-1}.

2.5. Determination of antioxidative enzyme activities

All the enzymes were extracted at 4 °C. Activity of superoxide dismutase (SOD) was assayed according to the method of Wang