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Research Paper

Stimulatory involvement of abscisic acid in wound suberization of postharvest kiwifruit

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ABSTRACT

Wound-induced suberization is an essentially protective healing process for fruit to reduce water loss and avoid infecting. However, cognate mechanisms that regulate this process are little known. To expand our knowledge of suberization induced by wounding, a wound-healing investigation together with metabolite profiling study was conducted in postharvest kiwifruit (*Actinidia deliciosa*). The development of suberization in wounded fruit was demonstrated by autofluorescence observation and toluidine blue staining at 1–4 day (d) after wounding. Activities of phenylalanine ammonia-lyase (PAL), cinnamyl-alcohol dehydrogenase (CAD) and peroxidase (POD) in wound-healing tissue were enhanced by abscisic acid (ABA). The constituent analysis of suberin including polyphenolics (SPP) and polyaliphatics (SPA) proved that exogenous ABA increased the content levels of total phenols, total flavonoids and alkanes, alkenes, alcohols, alkane acids, olefine acids, esters, glycerides and vitamin E in wound-healing tissue. Results suggested that ABA stimulated suberization through the activation of PAL, CAD and POD to accelerate wound-healing of wounded kiwifruit.

1. Introduction

In the case of wounding and damage to the surface, plant would elicit series of physiological responses. Wound healing plays an important role in maintaining postharvest quality and shelf life. Complete healing requires a complexly structural and physiological progress at the wound surface and is followed by the establishment of a functional suberin (Dastmalchi et al., 2015). Wound-induced suberin is analogous to cutin, but consists of slightly longer chain length fatty acids as well as a polyphenol domain (Beisson et al., 2012; Pollard et al., 2008; Schreiber, 2010).

Suberin appearing in barrier tissues of plants is a kind of lipid polyester and plays key roles in plant including structural support, water control and defense against stimuli from environment (Barros et al., 2015; Boerjan et al., 2003; Lendzian, 2006). For example, suberized cells accumulate in consecutive layers to build fortification in plant with secondary growth. Studies on suberin have shown that suberin is a cell wall-associated biopolymer, composed of suberin polyphenolic (SPP) and suberin polyaliphatic (SPA) as two main domains (Bernards, 2002; Bernards and Lewis, 1998).

The synthesis of suberin involves three essential enzymes including phenylalanine ammonia-lyase (PAL), cinnamyl-alcohol dehydrogenase (CAD), and peroxidase (POD) (Bonawitz and Chapple, 2010; Whetten and Sederoff, 1995). PAL is considered as a critical enzyme regulating suberin accumulation (Bate et al., 1994; Elkind et al., 1990) and has long been recognized as a highly important enzyme in wound-healing and particularly wound-induced suberization (Bernards et al., 2000; Lulai et al., 2008; Tao et al., 2016). It catalyzes the first step of the phenylalanine metabolism pathway which generates various phenylpropanoid products including cinnamic acid, cumaric acid and caffeic acid etc. The accumulation of these phenolics as SPP would contribute to form suberin (Bernards, 2002; Graça and Pereira, 2000b; Graça and Santos, 2007; Lulai, 2007; Lulai and Morgan, 1992). CAD functions in the last step of the monolignol biosynthesis pathway prior to polymerization in suberization, catalyzing the reduction of cinnamaldehydes to cinnamyl alcohols (Mansell et al., 1974; Weng and Chapple, 2010). POD, catalyzing the last step in suberin synthesis, plays an important role in cell wall-related defense through phenol polymerization and suberization (Fernandez-Perez et al., 2015a; Fernandez-Perez et al., 2015b; Quiroga et al., 2000; Sitbon et al., 1999).

Through partial depolymerization by enzymatic and chemical methods, the composition analysis of soluble oligomers by mass spectrometry has been reported (Rocha et al., 2001; Wang et al., 2010). By these methods, fatty acids, alkanes, alcohols, dimeric esters, long-chain ω-hydroxyacids, α, ω-diacids and glycerol were found in cork suberin (Graça and Santos, 2006), potato periderm (Graça and Pereira, 2000a) and tomato wound-healing layer (Tao et al., 2016).

Earlier studies on exogenous abscisic acid (ABA) suggested that ABA

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might be referred to the regulation of wound-healing in potato tuber (Espelie and Kolattukudy, 1985; Soliday et al., 1978). Further, ABA has been correlated with enhanced suberin accumulation in *Arabidopsis* root (Efetova et al., 2007), potato tuber (Lulai et al., 2008) and tomato fruit (Tao et al., 2016).

Kiwifruit is susceptible to physical injury during harvest, transportation and storage, which most likely cause postharvest deterioration. A time series of microscopic observation, activity measurement of several suberin synthesis-related enzymes including PAL, CAD and POD, suberin composition analysis were carried out in present study to investigate the physiological and chemical characters of wound-healing progress and the influence of ABA on suberization induced by wounding in kiwifruit.

2. Materials and methods

2.1. Plant materials and treatments

Kiwifruits (*Actinidia deliciosa* cv. Xuxiang) were harvested at commercial harvest stage (firmness 60.5 ± 2.0 N and soluble solids content 8.0 ± 0.2%) and were selected on the basis of shape and size uniformity without defects from a standard orchard located in Fuyang District, Hangzhou, China. Harvested fruits were immersed in the solution of 0.5% (v/v) sodium hypochlorite for 3 min to surface-disinfect, washed with deionized sterile water followed by surface-dried at room temperature.

Afterwards, kiwifruits were cut in half lengthwise with a sterilized scalpel to produce wounding. Divide randomly wounded fruits into two groups and treat with 0.5 mM ABA (abscisic acid, ≥90%, Aladdin Industrial Inc., China) and deionized water (control), respectively, in a vacuum container (SHZ-D III, Mingyuan Instrument Co., Ltd., China) at 0.05 MPa for 30 s. After treatment, fruits were placed in a 20 °C incubator (HWS, Ningbo Southeast Instrument Co., Ltd., China) in dark to heal for 4 days.

2.2. Microscopy observation

Suberization development was observed with wax-embedded sections of healing tissue. Healing tissue (about 1.0 cm wide, 1.0 cm long and 0.5 cm deep) was isolated with a razor blade from wound-healing fruit at the indicated time. Tissue blocks were immediately fixed in FAA solution of formalin: acetic acid: 95% ethanol: water (3:1:10:7, v/v/v/v), and then processed as follows. Tissue blocks were dehydrated before embedded in Paraplast Plus (Sigma). Sections of 5 μm thickness were obtained using a microtome (RM2235, Leica Microsystems Co., Germany). Sections were deparaffinized in xylene (10 min) twice and in a gradient ethanol series until being miscible with water. Some sections then were air dried and directly observed under fluorescence microscope (DM4000B LED, Leica Microsystems Co., Germany) using fluorescence excitation filter at 470–495 nm (Tao et al., 2016). The others were stained in toluidine blue O solution (0.05%) in benzoate buffer (pH 4.4) for 45 min and rinsed with clear water before observation under white light according to Hallett and Sutherland (2005). The staining and autofluorescence micrographs were obtained under 5× and 20× magnification (LEICA DFC450C, Leica Microsystems Co., Germany), respectively. Suberization rates were illustrated through quantification of staining and fluorescence intensity according to Kesanakurti et al. (2012), which was calculated with corresponding density through analyzing by ImageJ software (W. S. Rasband, National Institutes of Health, America). Healing tissues were also stored at −80 °C for following experiments.

2.3. Determination of PAL, CAD, and POD activity

PAL activity was determined by UV-1800 spectrophotometry according to Koukol and Conn (1961) with slight modifications. Frozen healing tissue samples of 1.0 g from each replicate were ground by using 10 mL of 0.1 M ice-cold sodium borate buffer (pH 8.8), containing EDTA (2 mM), β-mercaptoethanol (5 mM), and 4% (w/v) polyvinylpyrrolidone (PVP), which was then centrifuged at 8000 × *g* for 30 min at 4 °C. Then, 1 mL of the resulting supernatant was mixed with 3 mL of 0.1 M, pH 8.8 sodium borate buffer and 0.5 mL of 40 mM L-phenylalanine dissolved in 0.2 M, pH 8.8 sodium borate buffer. The mixture was kept at 37 °C in a water-bath for 90 min, after which 0.2 mL of 6 M HCl was added to terminate the reaction. The amount causing a change of 0.01 in absorbance per min at 290 nm was defined as one unit (U) of PAL activity. The activity of PAL was expressed in terms of U min^{-1} g^{-1} FW.

CAD activity was analyzed using a modification of the previous method (Mansell et al., 1974) by UV-1800 spectrophotometry. Frozen samples (0.5 g) were extracted with 4 mL of Tris–HCl buffer (pH 7.5, 100 mM) containing β-mercaptoethanol (5 mM), 4% (w/v) polyvinylpyrrolidone (PVP) and EDTA (2 mM), followed by centrifugation at 4 °C at 8000 × *g* for 20 min. The reaction mixture consisting of 1 mL of 3 mM NADP+, 1 mL of 3.2 mM trans-cinnamic acid, 1 mL of 100 mM phosphate buffer (pH 6.5), and 1 mL of enzyme extract solution was incubated in a 37 °C water bath for 30 min. The amount causing a change of 0.001 in absorbance per min at 340 nm was defined as one unit (U) of CAD activity. The activity of CAD was expressed as U min^{-1} g^{-1} FW.

POD activity was assayed using a UV-1800 spectrophotometer according to the method described by Lee (1973) with appropriate modifications. Samples (1.0 g) were ground and mixed homogeneously in 4 mL sodium phosphate buffer (200 mM, pH 6.8), and then centrifuged for 30 min at 4 °C at 8000 × *g*. The supernatant was combined with 1 mL of 50 mM sodium phosphate buffer (pH 6.8), 200 μL of 10 mM H_2O_2, 3 mL of 25 mM guaiacol, and 1 mL of enzyme extract. The amount causing a change of 1.0 in absorbance per min at 470 nm was defined as one unit (U) of POD activity. The activity of POD was expressed in terms as U min^{-1} g^{-1} FW.

2.4. Determination of total phenols and flavonoids contents

Blended material (1.0 g) was used to extract total soluble phenols with 10 mL of 0.5% acetic acid and 70% acetone. Keep the mixture in the dark at 4 °C for 24 h, then centrifuge at 4 °C at 8000 × *g* for 30 min. Collect the supernatant for the determination of phenols and flavonoids contents. According to Folin–Ciocalteu's procedure (Scalbert et al., 1989), the content of total phenols was determined in extract. Briefly, mix 0.5 mL of extract with 2.5 mL of 1:10 diluted Folin–Ciocalteu's phenol reagent, add 2.0 mL of 7.5% (w/v) Na_2CO_3 and keep 5 min at 50 °C, followed by measuring absorbance at 760 nm. A standard curve of gallic acid was made to estimate phenol content and expressed results as mg of gallic acid equivalents (GAE) 100 g^{-1} FW.

Total flavonoids content was measured according to the method described by Cvek et al. (2007) with some modifications. Add and mix 3.7 mL of the above extract, 0.15 mL of 10% $AlCl_3{\cdot}6H_2O$ and 0.15 mL of 5% $NaNO_2$ in a 10 mL test tube. After 5 min, add 1 mL of 1 M NaOH and measure absorbance of the mixture at 510 nm. A standard curve (0–100 μg) of rutin as standard was made to calculate the total flavonoids content and expressed results as mg of rutin equivalents (RE) 100 g^{-1} FW.

2.5. Suberin extraction and GC/MSD analysis

Extract soluble metabolites from suberizing tissues in accordance to Broeckling et al. (2005) with slight modifications. Briefly, lyophilize frozen samples for 36 h and ground into powders in liquid nitrogen. Dry powders (10 mg) were transferred into glass vials (4 mL). Add methanol/chloroform/water (4:2:1, v/v/v) (2.1 mL) to the dry powders, together with internal standard as 40 μL of dotriacontane (1.0 mg mL^{-1}). Eddy the mixture thoroughly and incubate 1 h at 50 °C.