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Wound-induced H_2O_2 and resistance to *Botrytis cinerea* decline with the ripening of apple fruit

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ABSTRACT

Fruit ripening is a developmental process and is associated with increased susceptibility to mechanical injury, which favours *Botrytis cinerea* infection. Using 'Gala' apples harvested at different stages of ripening, we demonstrated that wounding can activate initial H_2O_2 accumulation and wound healing ability to defend against *B. cinerea* penetration. Delaying the harvest date attenuated those responses. Superoxide dismutase, peroxidase and catalase, which are all involved in H_2O_2 metabolism, were differentially activated by wound stress depending on the stage of fruit maturity. Mature fruit were less able to respond to wounding by increasing phenylalanine ammonia lyase and peroxidase activity, which are associated with reduced phenolics and lignin content in local wound sites. The reduced response in late-harvested fruit contributes to the fruit ripening-induced loss of wound healing ability and increases susceptibility to *B. cinerea*. In addition, the rapid increase of H_2O_2 content immediately after wounding in early-harvested fruit was followed by increased phenylalanine ammonia lyase and peroxidase activity. In late-harvested fruit, the reduced ability to increase phenylalanine ammonia lyase and peroxidase activity in response to wounding was consistent with ripening-reduced generation of H_2O_2 early after wounding, leading to reduced resistance to *B. cinerea*. Thus, H_2O_2 accumulation in response to wounding is modulated by fruit maturity and is required for efficient wound healing and resistance to *B. cinerea*.

1. Introduction

Botrytis cinerea causes gray mold disease in a broad range of fruit types, including apples, and is one of the most severe postharvest diseases. In most cases, this pathogen requires a wound in the epidermis to enter susceptible tissue and initiate infection (Spotts et al., 1998). Mechanical injury, caused during harvesting and postharvest handling, provides an optimal locus for infection. The increased susceptibility to mechanical damage caused by fruit ripening, which leads to biochemical changes such as enhanced respiration and oxidative stress, cell wall breakdown and reduced protein synthesis, may lower resistance to pathogen penetration (Imaseki, 1985; Torres et al., 2003). Though plant tissue can induce multiple defense strategies to overcome wound stress and prevent further pathogen invasion, these wound-induced defense responses may be modulated by fruit ripening.

The early phase of a plant's response to wounding is usually accompanied by the generation of hydrogen peroxide, H_2O_2 (Orozco-Cańdenas and Ryan, 1999). The presence or accumulation of H_2O_2 at the wound surface has been postulated to play an important role in plant defense (Wu et al., 1995; Rea et al., 2002). In addition to its oxidative potential in killing or inhibiting the growth of pathogens, H_2O_2 has been implicated in reinforcing the cell wall around wounds by the oxidative cross-linking of apoplastic structural protein and lignin and suberin polymerization. H_2O_2 also serves as a second messenger in induction of defense genes (Wu et al., 1997). Despite its importance in wound healing, H_2O_2 accumulation by wounding may depend on development in the organism. In 'Golden Delicious' fruit, a two week harvest delay resulted in a lower increase in H_2O_2 levels after wounding, which was associated with higher susceptibility to *Penicillium expansum* (Torres et al., 2003). In potato tubers, age-induced loss in the ability of tubers to produce a burst of superoxide radicals is coincident with reduced capacity to develop a wound barrier effective against pathogen infection (Kumar and Knowles, 2003). Moreover, superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and/or peroxidase (POD, EC 1.11.1.7), which involved in H_2O_2 metabolism, are differentially activated by wound stress depending on plant growth stage (Chandru et al., 2003). Thus, we hypothesized that wound-induced H_2O_2 and associated enzymes that play an important role in wound-defense processes are also regulated by fruit ripening.

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The wound healing response results in the production of wound periderm, which was thought to be lacking in fruit after harvest (Simons and Aubertin, 1959; Skene, 1981). However, the accumulation of phenolics and lignin-like materials strengthen the cell wall around wounds in mature fruit and effectively protect the underlying tissue from invasion by pathogens (Lakshminarayana et al., 1987). Phenylalanine ammonia lyase (PAL; EC 4.3.1.5) is a key enzyme in the first step of the phenylpropanoid pathway, which is directly involved in the synthesis of phenols and lignin (Yao and Tian, 2005). Apples, a typically climacteric fruit, release autocatalytic ethylene as the fruit ripens. Since PAL can be induced by ethylene, more mature fruit with higher ethylene production may induce higher PAL activity and gain increasing resistance to pathogens. However, Torres et al. (2003) showed that late harvest mature fruit were more susceptible to *P. expansum*, which was associated with lower wound-induced H_2O_2. In potatoes, older tubers with increased accumulation of ethylene lack the ability to form superoxide radicals on wound surfaces and are less efficient at up-regulating PAL in response to wounding (Kumar and Knowles, 2003). These results raise questions about whether decreased resistance to pathogens in more mature apple fruit is a result of lower wound-induced H_2O_2 levels, leading to reduced wound healing ability, or rather the H_2O_2 serves as a signal to induce the expression of PAL. Thus, we examined the effects of fruit ripening in 'Gala' apples on ethylene production, PAL activity, and wound-induced H_2O_2, and analyzed their impact on wound healing ability and resistance to *B. cinerea*.

2. Materials and methods

2.1. Fruit

'Gala' apples (*Malus domestica* Borkh.) were obtained from a commercial orchard in Xuzhou (Jiangsu, China) and transferred within 24 h to our laboratory. Harvests were carried out on August 10 (H1, early), August 17 (H2, optimal) and August 24 (H3, late), 2010. Fruit were selected for uniform size and color without physical injuries or apparent infections. Harvest maturity indices were measured at each date.

2.2. Quality parameters

Firmness was measured on two opposite peeled sides using a penetrometer (FT 327, Facchini, Italy) with an 11 mm diameter probe. Soluble solids concentration (SSC) was determined by measuring the refractive index of the juice, and the data were expressed as a percentage, grams per 100 g fresh weight (FW). Acidity was measured as follows: 20 mL of pulp juice was diluted with 50 mL H_2O and titrated with 0.1 M NaOH solution to pH 8.10. The acidity was expressed in grams of malic acid per litre of juice. Starch Index was scored visually by using a 1–9 scale (1: high starch; 9: no starch), after staining an equatorial section with 0.22% (w/v) I_2–0.88% (w/v) KI solution. Data on maturity parameters represent the mean of 20 individual fruit. The same fruit were used to determine all the maturity indices.

2.3. Pathogen inoculum

B. cinerea was isolated from rotten apples during storage, and maintained on potato dextrose agar (PDA: infusion from potatoes 1000 mL, glucose 20 g, agar 15 g) at 4 °C. Fresh culture was grown on PDA plates at 25 °C before use. Spores were subsequently harvested by flooding the surfaces of 10-day old cultures with sterile distilled water and gently agitating the plates to dislodge the spores. The conidial suspension was prepared in 0.05% (v/v) Tween-80. Spore concentration was adjusted to 1×10^5 spores mL^{-1} with the aid of a haemocytometer.

2.4. Wounding, wound healing and pathogen inoculation

After harvest, 'Gala' apples were surface-sterilized with 2% sodium hypochlorite (v/v) for 2 min, rinsed with tap water and air-dried. All apples were wounded with a sterilized stainless-steel nail by making an injury 2 mm in diameter and 2–3 mm deep at two sides of each fruit, and incubated at 20 °C (85% RH) for 96 h. The healed wounds were then inoculated with 20 μL aqueous suspensions containing 10^5 conidia mL^{-1} *B. cinerea* and 0.05% (v/v) Tween-80 in each wound. Fresh wounds were inoculated immediately after wounding. The inoculated apples were stored at 20 °C, 85 ± 5% RH for ten days, after which the percentage of infected wounds and the lesion diameter caused by *B. cinerea* were measured. When the visible rot zone beyond the wound area on each fruit was more than 1 mm wide, it was counted as an infected wound. Ten apples (containing 20 wounds) were used for pathogen inoculation and the experiment was performed in triplicate.

2.5. Ethylene production after wounding

After each harvest, 'Gala' apples were wounded and incubated as described above. Ten fruit were enclosed in 10 L airtight jars for 2 h at 25 °C at the following time points: 0, 6, 12, 24, 48, 72 and 96 h after wounding. The headspace gas in the jars were sampled with a 1 mL plastic hypodermic syringe and injected into a gas chromatograph (model GC-14B, Shimazu, Japan) fitted with an alumina column at 70 °C and a flame ionization detector to assay the ethylene production. The rate of ethylene production was expressed as nL h^{-1} kg^{-1} FW. There were two replications for ethylene assays and the experiment was performed in triplicate.

2.6. Fruit tissue sampling

After harvest, 'Gala' apples were surface-sterilized and air-dried as described above. Four wounds were made at the equator of each fruit using the sterilized stainless-steel nail. Afterwards, all fruit were incubated at 20 °C (85% RH) for 96 h. Plugs of fruit tissue 1 cm diameter and 1 cm deep and centered around wounds were extracted using a cork borer from 10 fruit at 0, 6, 12, 24, 48, 72 and 96 h after wounding. Samples were mixed and frozen immediately in liquid nitrogen, then stored at −20 °C. Frozen samples were used for enzyme assays and measurements of protein, H_2O_2, total phenolic and lignin contents. There were two replications for each harvest date and the experiment was performed in triplicate.

2.7. Measurement of enzyme activity

All procedures of enzyme extraction were performed at 4 °C. For superoxide dismutase (SOD), 1 g of frozen tissue was ground with 5 mL 50 mM sodium phosphate buffer (pH 7.8) containing 0.1 mM ethylene diamine tetraacetic acid (EDTA) and 3% polyvinyl polypyrrolidone (PVPP) (w/v). For catalase (CAT), 1 g of frozen tissue was ground with 3 mL 100 mM sodium phosphate buffer (pH 7.0) containing 3% PVPP (w/v). For peroxidase (POD), 1 g of frozen tissue was ground with 3 mL 100 mM sodium phosphate buffer (pH 6.4) containing 3% PVPP (w/v). For phenylalanine ammonia lyase (PAL), frozen tissue (2 g) was ground with 5 mL of 100 mM sodium borate buffer (pH 8.7) containing 0.037% EDTA (w/v), 0.137% β-mercaptoethanol (v/v), and 3% PVPP (w/v). The extracts were then homogenized and centrifuged at 10,000 × *g* for 20 min at 4 °C. The supernatants were used for the enzyme assays.

SOD activity was determined by the method of Zhao et al. (2009) in a final volume of 3 mL, which contained 0.1 mL of