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Thaumatin-like proteins and their possible role in protection against chilling injury in peach fruit

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ABSTRACT

Peaches are highly perishable; they ripen and deteriorate quickly at ambient temperature, and cold storage is used to slow these processes. The cell wall protein composition of two peach cultivars, and total protein composition were examined at harvest and after cold storage (3 weeks, 5 °C) by two-dimensional polyacrylamide gel electrophoresis. The two peach cultivars used were 'Oded', a white-, melting-flesh, cling-stone, early season cultivar resistant to chilling injury, and 'Hermoza', a white-, melting-flesh, free-stone, mid-season cultivar susceptible to chilling injury. Following storage, peptides in the cell wall with molecular masses ranging from 18 kDa to 60 kDa were identified by amino acid sequence to be thaumatin-like protein 1 precursor and thaumatin-like protein 2 precursor. qRT-PCR analysis revealed that the thaumatin-like protein 1 precursor transcript accumulated significantly in both cultivars during storage. However, after 1 and 2 weeks of cold storage at 5 °C the thaumatin-like protein 1 precursor transcript levels were significantly higher in the chilling injury-resistant peach 'Oded' than the susceptible peach 'Hermoza'. This early accumulation of the thaumatin-like protein 1 precursor transcript in the resistant peach suggests that thaumatin-like protein 1 precursor (and perhaps thaumatin-like protein 2 precursor) might be involved in protecting against chilling injury. Although thaumatin-like proteins accumulated to high levels in cell walls of chilling injury-sensitive 'Hermoza', the kinetics of transcript accumulation suggest that the early appearance of the transcript for this protein family might be involved in shielding the fruit from the dramatic cell wall-structure changes that accompany the onset of chilling injury in stone fruit, and that result in woolliness development.

1. Introduction

Peaches are highly perishable; they ripen and deteriorate quickly at ambient temperature (Lurie and Crisosto, 2005). Therefore, low temperature storage (0–5 °C) is a common strategy used to slow the ripening processes as well as decay development during storage and/or shipment (Crisosto et al., 1999; Lurie and Crisosto, 2005). However, if susceptible varieties of peach, nectarine, and other stonefruit such as plum and apricot are held too long at a low temperature, they will not ripen properly when rewarmed and will develop chilling injury (CI) (Crisosto et al., 1999; Zhou et al., 2000a, 2001; Crisosto and Labavitch, 2002; Brummell et al., 2004b; Manganaris et al., 2005, 2006).

In peach fruit that develop CI symptoms, cell wall modifications have been extensively studied (Zhou et al., 2000a,b,c; Brummell et al., 2004a,b). Long-term cold storage of susceptible peaches is known to result in dramatic changes in the cell wall properties. The manifestation of CI in peaches includes defective cell wall disassembly and the development of a dry, woolly rather than soft, juicy texture (Lurie and Crisosto, 2005). Recent studies have shown considerable changes in transcription in cold-stored peaches compared to unstored fruit (Gonzalez-Aguero et al., 2008; Ogundiwin et al., 2008; Tittarelli et al., 2009; Vizoso et al., 2009). This leads to the hypothesis that changes in cell wall proteins may be associated with the cold-induced development of woolliness, the most ubiquitous symptom of CI in peach.

Proteomics is a powerful tool for studying and identifying global changes in structure and abundance of plant proteins in response to developmental and environmental signals (Rampitsch and Srinivasan, 2006; Shi et al., 2008). There has been an increasing trend in use of proteomic methods in the field of fruit and vegetable physiology over the last few years (Rocco et al., 2006; Hjernø et al., 2006; Pedreschi et al., 2008). However, little research has utilized proteomic approaches in the field of postharvest physiology (Pedreschi et al., 2008; Shi et al., 2008), although, proteomic approaches utilizing two-dimensional polyacrylamide gel elec-

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trophoresis (2D-PAGE) together with mass spectrometry have been used to study plant responses to low temperature stress (Cui et al., 2005; Amme et al., 2006).

Proteomic methods have been tested and employed successfully to study different plant organelles including cell walls (Feiz et al., 2006), chloroplasts, peroxisomes and mitochondria (Peltier et al., 2000; Fukao et al., 2002; Heazlewood et al., 2003). Proteomic examination of plant cell walls is extremely challenging because of contamination with polysaccharides, polyphenolics and cytosolic proteins (Feiz et al., 2006). To prevent contamination of cytosolic proteins stringent washes must be used as part of the extraction procedure.

Thus far, to our knowledge no one has employed a proteomic strategy to study low temperature stress in cell walls of peach, and no proteomic data of cell wall proteins are yet available. Therefore, the present study provides the first fundamental cell wall proteomic analyses of the cold-stored peach fruit tissue. In the present study, and as part of our ongoing efforts to understand physiological and molecular responses of peach fruit to cold storage, we used 2D-PAGE analysis joined with reverse HPLC microspray mass spectrometry (HPLC/MS/MS) to examine changes in the cell wall proteins and total proteins of peach fruit subjected to cold storage. The 2D-PAGE results were also confirmed with quantitative reverse transcriptase-PCR (qRT-PCR) analysis.

2. Materials and methods

2.1. Plant material and treatments

'Hermoza', a white fleshed, free-stone, melting-flesh, mid-season peach susceptible to CI, and 'Oded', a white fleshed, cling-stone, melting-flesh, early season peach resistant to CI, were sampled at harvest, harvest plus 3 d ripening at 20 °C, after 1, 2 and 3 weeks storage at 5 °C, and 3 weeks storage at 5 °C plus 3 d ripening at 20 °C. At each time of sampling, fruit were cubed, weighed and flash frozen in liquid nitrogen, and stored at −80 °C. Five fruit were sampled per treatment.

2.2. Firmness and extractable juice measurements

Physiological parameters were measured following a protocol described by Zhou et al. (2000c). In brief, firmness was measured on two pared sides of each fruit using a penetrometer fitted with an 8-mm diameter plunger. After firmness was determined, the fruit was cut into halves and woolliness was estimated both by visual observation and organoleptically. Juicy fruit with no signs of woolliness were classed as healthy. The amount of expressible juice was determined by removing a tissue plug weighing about 2 g from each fruit with a cork borer, passing it through a 5 mL syringe into an Eppendorf tube, centrifuging and separately weighing the juice and solids (Lill and van der Mespel, 1988). Fifteen fruit were examined at each observation time for each cultivar.

2.3. Isolation of cell walls

The cell walls were isolated from 10 g of peach mesocarp stored at −80 °C following a protocol described by Feiz et al. (2006) with minor modifications. In short, the mesocarp was transferred into 50 mL of 5 mM acetate buffer, pH 4.6, 0.4 M sucrose and protease inhibitor cocktail (Roche Diagnostics GmbH, Germany) 1 tablet per 50 mL of the buffer. The mixture was ground in a blender (Osterizer, USA) for 2 min. After adding polyvinyl polypyrroliodone (PVPP; 0.5 g per 10 g of the tissue), the mixture was incubated in a cold room for 30 min while shaking. Cell walls were separated from soluble cytoplasmic fluid by centrifugation of the homogenate for 15 min at 1000 × *g* at 4 °C. The pellet was resuspended and recentrifuged in 15 mL of 5 mM acetate buffer, pH 4.6, containing first 0.6 M and then 1 M sucrose. The residue was washed with 200 mL of 5 mM acetate buffer, pH 4.6, on 30 μm pore size nylon net (Millipore, USA). The resulting cell wall fraction was ground in liquid nitrogen in a mortar and pestle prior to lyophilization. This process resulted in about 0.5 g of dry powder from 10 g of frozen tissue.

2.4. Cell wall protein extraction

The cell wall material from 10 g of frozen tissue was used for extracting the cell wall proteins using the method of Feiz et al. (2006) with slight adjustments. Proteins were extracted from the isolated cell walls by successive salt solutions in the following order: two extractions each time with 7.5 mL of $CaCl_2$ (5 mM acetate buffer, pH 4.6, 0.2 M $CaCl_2$ solution and 1 tablet of the protease inhibitor cocktail per 50 mL of the buffer), followed by two extractions with 7.5 mL of LiCl solution (5 mM acetate buffer, pH 4.6, 6 M LiCl and 1 tablet of the protease inhibitor cocktail per 50 mL of the buffer). After each incubation, the suspension was centrifuged for 15 min at 4000 × *g* and 4 °C. Supernatants were desalted and concentrated to a volume of 100–200 μL using 15-mL Amicon Ultra Centrifugal Filter Device (Millipore, USA) with a membrane cut-off of 3 kDa. This concentrate was mixed with two volumes of precooled absolute phenol, and precipitated overnight at −20 °C. The following day, after centrifugation, the pellet was air dried for 30–60 min and dissolved in immobilized pH gradient (IPG) re-swelling buffer containing 9 M urea, 3% CHAPS, 0.5% Triton X-100, 2% IPG buffer (pH 3–10). The whole extracted cell wall proteins from 10 g of the frozen fruit tissue was used to rehydrate 13-cm IPG strips (pH 3–10) (Amersham Biosciences, Sweden) for the 2D-PAGE analysis.

2.5. Total protein extraction

Total proteins were extracted from frozen fruit tissue with phenol–protein extraction protocol as described previously (Barent and Elthon, 1992). In brief, 5 g of tissue was frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. The frozen powder was suspended in 10 mL of protein extraction buffer [PEB; 700 mM sucrose, 50 mM Tris, 30 mM HCl, 2 mM dithiothreitol (DTT), 100 mM KCl and 5 mM Na_2EDTA] in a 50 mL centrifuge tube and then 10 mL of water-saturated phenol was added. The tube was sealed and shaken for 5 min at room temperature. The organic and aqueous phases were separated by centrifugation at 7000 × *g* for 10 min. The phenol phase was taken and 10 mL of the PEB and 2 mL of water-saturated phenol added, shaken and centrifuged as above. The phenol phase was re-extracted with an equal volume of the PEB and 1 mL of water-saturated phenol. Following centrifugation, the soluble proteins were precipitated from the phenol phase by adding five volumes of 0.1 M ammonium acetate in methanol (precooled at −20 °C prior to use). Protein precipitation occurred at −20 °C overnight. The following day, the solution was centrifuged for 10 min at 7000 × *g*. The pellets were washed three times with 0.1 M ammonium acetate in methanol and once with cold acetone. The pellets were air dried and solubilized in the IPG re-swelling buffer for (2D-PAGE). The protein content was determined with Quant-iT™ Protein Assay Kit (Invitrogen, USA) according to the manufacturer's instructions. Extracted total proteins (200 μg) was used to rehydrate 13-cm IPG strips (pH 3–10) (Amersham Biosciences, Sweden) for the 2D-PAGE analysis.

2.6. Two-dimensional polyacrylamide gel electrophoresis

The 2D-PAGE was performed according to the IPG principles and methods of Amersham Biosciences (Piscataway, USA) as described