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Difference in activity and gene expression of pectin-degrading enzymes during softening process in two cultivars of Chinese pear fruit

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ABSTRACT

Cell wall pectin-related physiological and molecular properties changes in two different cultivars of Chinese pear fruit, 'Jingbaili' and 'Yali', were investigated in this study. 'Jingbaili' pear fruit, which softened quickly, showed higher pectin fracture solubilization, while 'Yali' pear fruit, which maintained a firm texture, presented little change in the content of pectin fractions during storage. The activities of PG, β-GAL and α-ARF that involved in fruit softening were significantly higher in 'Jingbaili' pear fruit compared to 'Yali' pear fruit. Furthermore, the mRNA accumulation of the genes, such as *PG1, PG2, GAL4, ARF1* and *ARF2* that encoded above-mentioned cell wall hydrolase dramatically increased in 'Jingbaili' pear fruit during fruit softening. Thus, we concluded that 'Jingbaili' pear fruit may be more prone to degradation in cell wall pectin compared to 'Yali' pear fruit, suggesting that their different softening patterns determine the different modes of cell wall pectin disassembly between these two different types of Chinese pear fruit.

1. Introduction

Loss of flesh firmness is not only a sign of a stage of maturation, but it is also an evidence of the inevitable process of programmed ripening and senescence in many postharvest fruits. Several studies have demonstrated that fruit softening is predominantly accompanied by cell wall disassembly and middle lamella dissolution, which attenuates cell–cell adhesion, although it is widely believed that this process undergoes a series of complex physiological and biochemical changes (Brummell and Harpster, 2001; Brummell, 2006; Cosgrove, 2014; Goulao et al., 2008; Ng et al., 2013; Park and Cosgrove, 2015). This disassembly is mainly modified by pectin solubilization, neutral sugar loss, and xyloglucan depolymerization (Gwanpua et al., 2014; Posé et al., 2013; Sanudo-Barajas et al., 2009; Vicente et al., 2007), which results in fruit softening. These processes are thought to involve the coordinated and interdependent action of numerous cell wall-modifying enzymes, such as polygalacturonase (PG), β-galactosidase (β-GAL), α-arabinofuranosidase (α-ARF), pectin methylesterase (PME), pectate lyase (PL) and xyloglucan endotransglycosylase/hydrolase (XTH) (Brummell, 2006; Brummell and Harpster, 2001; Giovannoni, 2001; Goulao et al., 2008; Opazo et al., 2013).

In European pears, the main modification of fruit firmness loss has been shown to involve the solubilization of a large amount of arabinose and galacturonic acid (Ahmed and Labavitch, 1980). Murayama et al. (2006) demonstrated changes in the cell wall components of pears and that pectin solubilization reduced mealy texture during fruit softening. Hiwasa et al. (2004) concluded that the different softening behaviors in pears were attributed to differences in the activity and gene expression of PG. In addition, changes in β-GAL and α-ARF activities have been shown to be correlated with pear fruit softening (Mwaniki et al., 2005) and to change the cell wall composition, such as the presence of water soluble pectin (WSP), covalent soluble pectin (CSP) and hemicellulose in pear (Wei et al., 2009). Furthermore, the divergent expressions of *β-GALs* and *α-ARFs* exhibit characteristics of differential regulation during fruit softening in various pear cultivars (Mwaniki et al.,

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2005; Tateishi et al., 2001, 2005). These results indicate an overall process, including cell wall disassembly, modifying enzymes and related gene regulation, because any individual modification was insufficient for fruit softening, as reported by several other studies (Goulao et al., 2007, 2008; Gwanpua et al., 2014). Although some progress have been studied on cell wall disassembly of pear fruit, it is still important to further elucidate how the activities of these enzymes and the related gene expression coordinately contribute to cell wall degradation during fruit softening.

Two Chinese pear cultivars, 'Jingbaili' (*Pyrus ussuriensis* Maxim. cv. Jingbaili) and 'Yali' (*Pyrus bretschneideri* Rehd. cv. Yali), are climacteric types and belong to native pear genotypes in China. Among these genotypes, 'Jingbaili' pear fruit undergoes rapid softening during ripening (Gao et al., 2012), while 'Yali' pear does not show a clear loss of firmness even at the end of the ripening stage (Hiwasa et al., 2004). Thus, we examined the alterations in the content of cell wall pectin fractions, and the activities and expression of related genes of softening-associated enzymes (i.e., PG, β-GAL and α-ARF) to determine the correlation of cell wall solubilization with fruit softening behaviors and the difference in the physiological and molecular properties between 'Jingbaili' and 'Yali' pear fruit.

2. Materials and methods

2.1. Plant materials and treatments

Fruits of two pear cultivars, 'Jingbali' (*Pyrus ussuriensis* Maxim. cv. Jingbaili) and 'Yali' (*Pyrus bretschneideri* Rehd. cv. Yali), were harvested from a 25 years old orchard in Changli, Hebei Province, China. The 'Jingbaili' fruit were harvested at 110 day after full bloom (Average weight: 100.3 ± 6.2 g, TSS: 12.0 ± 1.1 %), and the 'Yali' fruit at 138 day after full bloom (Average weight: 174.8 ± 9.8 g, TSS: 10.5 ± 0.8 %). The fruits with uniform size and without mechanical injury, insects, and diseases were directly stored at 20 ± 1 °C, 75% relative humidity. The flesh were immediately frozen in liquid nitrogen and then stored at −70 °C for subsequent analysis.

2.2. Determination of fruit firmness

Fruit flesh firmness was measured at two equatorial regions of the peeled flesh using a fruit firmness-meter (Model: GY-4, Hangzhou, China). Ten fruits were sampled for each measuring point.

2.3. Determination of the fruit respiration rate

1.4 kg fruit each replicate was sealed in a container (volume: 9 L) for 0.5 h at 20 ± 1 °C. 10 mL sample of the headspace gas was withdrawn by a gas-tight syringe from each container through a septum stopper, and then injected into the infrared carbon dioxide analyzer (Model: HWF-1, Jintan, China). Three replicates for each measuring point, and the respiration rate was expressed as mg kg^{-1} h^{-1}.

2.4. Preparation and assay of the cell wall pectin fraction

Cell wall fractions were extracted according to methods previously described by Brummell et al. (2004) with slight modification. The powdered frozen flesh sample (3.0 g) was homogenized in 8 mL of 80% ethanol, stirred for 20 min at 80 °C and then centrifuged. The precipitate was washed with 80% ethanol and pure acetone three times and then sequentially immersed in 95% dimethyl sulfoxide (DMSO) for 12 h before centrifuging to remove the starch in the supernatants. Finally, the residue was dried until it was a constant weight at 45 °C in a ventilated oven, and the dried residue was considered to be the cell wall material (CWM). 50 mg CWM was extracted with distilled water and then centrifuged (15 min at 4000 × *g*). The supernatant was designated as the water-soluble pectin (WSP) fraction. The precipitate was sequentially dispersed with *trans*-1,2-diaminocyclohexane-*N*,*N*,N9,N9-tetraacetic acid (CDTA) and Na_2CO_3 containing 0.1% NaBH4 for 24 h to obtain CDTA-soluble pectin (CSP) fraction and Na_2CO_3-soluble pectin (NSP) fraction, respectively.. These three pectin fractions were quantified as uronic acid content using the carbazole method (Bitter and Muir, 1962) with three replications per sample.

2.5. Assay of pectin-degrading enzymes activities

Enzyme extraction was performed according to the method described by Brummell et al. (2004) and Wei et al. (2010). Three grams of frozen flesh sample was powdered and stirred in 6 mL of cold 12% polyethyleneglycol containing 0.2% sodium bisulphite. The homogenate was then centrifuged at 12,000× *g* for 20 min, and the pellet was washed with 4 °C aqueous 0.2% sodium bisulfite. The pellets were transferred into tubes containing 6 mL of cold 0.1 mol L^{-1} sodium acetate (pH 5.2), 100 mmol L^{-1} NaCl, 2% (V/V) β-mercaptoethanol, and 5% (W/V) polyvinylpyrrolidone (PVP) at 4 °C for 1 h. Following centrifugation, the supernatant was the crude extraction, which was used to assay the activities of PG (EC3.2.1.15), β-GAL (EC 3.2.1.23), and α-ARF (EC3.2.1.55). These studies were performed at 4 °C.

PG activity was determined as previously described by Gross (1982). 0.6 mL of enzyme extract was mixed with 3.0 mL of 0.5% polygalacturonic acid in 50 mmol L^{-1} sodium acetate buffer (pH 5.2) and was incubated at 37 °C for 12 h. Then the reaction mixture was added to 2 mL of borate buffer (0.1 mol L^{-1}, pH 9.0) and 0.5 mL of cyanoacetamide, boiled for 10 min and then cooled, and the absorbency was determined at 276 nm. Galacturonic acid was used as a standard and the controls for the boiled extract were run in reaction buffer. One unit of activity was defined as 1 μg of galacturonic acid released g^{-1} fresh weight (FW) min^{-1}. β-GAL and α-ARF activities were assayed as previously described by Brummell et al. (2004). The reaction mixture containing 1.0 mL of 0.1 mol L^{-1} sodium acetate (pH 5.2) and 2.0 mL of substrates (*p*-nitrophenyl-β-D-galactopyranoside and *p*-nitrophenyl-α-D-arabinofuranoside, respectively) were pre-incubated at 40 °C for 10 min, and 0.5 mL of enzyme extract was then added. The absorbency was read at 400 nm after the reaction, which was incubated at 37 °C for 20 min and terminated by the addition of 2.0 mL of 0.5 mol L^{-1} sodium carbonate. A calibration curve was obtained using free *p*-nitrophenol (PNP) as a standard. The unit of activity was expressed as nmol PNP g^{-1} FW min^{-1}. In all of the assays, experiments with boiled enzyme extract were used as the control. All the experiments were performed with three replications

2.6. RNA extraction and qPCR for PGs, GALs and ARFs

For the gene expression studies, total RNA was extracted from pear fruit using the modified CTAB method (Gasic et al., 2004). First-strand cDNAs were synthesized from DNase-treated RNA (0.5 μg) using a Takara RNA PCR Kit (AMV) Version 3.0 (TaKaRa Biomedicals, Japan). Quantitative PCR (q-PCR) was performed using a SYBR Premix Ex Taq™ (Perfect Real Time) Kit (TaKaRa Biomedicals, Japan) on a 7500 Real-Time PCR system (Applied Biosystems, USA). PCR primers for *PGs*, *GALs* and *ARFs* were designed using OMIGA 2.0 based on the sequences of *Pyrus bretschneideri* published in NCBI (Table 1). The q-PCR reaction was performed in a final volume of 25 μL, containing 12.5 μL of SYBR Green PCR Premix Ex Taq™, 1 μmol L^{-1} forward and reverse primers, and 10 ng of cDNA and was performed as follows: 10 s at 95 °C, 40 cycles of 95 °C for 5 s, and 60 °C for 34 s. To confirm the quality of product and primer specificity, the Tm of the amplification products was analyzed