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The nanostructural characterization of strawberry pectins in pectate lyase or polygalacturonase silenced fruits elucidates their role in softening

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ABSTRACT

To ascertain the role of pectin disassembly in fruit softening, chelated- (CSP) and sodium carbonate-soluble (SSP) pectins from plants with a pectate lyase, *FaplC*, or a polygalacturonase, *FaPG1*, downregulated by antisense transformation were characterized at the nanostructural level. Fruits from transgenic plants were firmer than the control, although *FaPG1* suppression had a greater effect on firmness. Size exclusion chromatography showed that the average molecular masses of both transgenic pectins were higher than that of the control. Atomic force microscopy analysis of pectins confirmed the higher degree of polymerization as result of pectinase silencing. The mean length values for CSP chains increased from 84 nm in the control to 95.5 and 101 nm, in antisense *FaplC* and antisense *FaPG1* samples, respectively. Similarly, SSP polyuronides were longer in transgenic fruits (61, 67.5 and 71 nm, in the control, antisense *FaplC* and antisense *FaPG1* samples, respectively). Transgenic pectins showed a more complex structure, with a higher percentage of branched chains than the control, especially in the case of *FaPG1* silenced fruits. Supramolecular pectin aggregates, supposedly formed by homogalacturonan and rhamnogalacturonan I, were more frequently observed in antisense *FaPG1* samples. The larger modifications in the nanostructure of pectins in *FaPG1* silenced fruits when compared with antisense pectate lyase plants correlate with the higher impact of polygalacturonase silencing on reducing strawberry fruit softening.

1. Introduction

Strawberry (*Fragaria* × *ananassa* Duch.) is the most economically important edible soft fruit, which is characterized by its delicious flavour, intense colour, soft texture and high nutritional value. Besides its economic importance, several authors have proposed strawberry as a model for the study of the ripening process in non-climacteric fruits (Posé et al., 2011). The fast softening of this fruit determines its short post harvest life, which results in large loses due to over-softening, bruising and subsequent fungal infections that generally are associated with this process.

It is generally accepted that textural changes during ripening of fleshy fruits, mainly a decrease in firmness, are caused by a reduction of cell to cell interaction due to the dissolution of the middle lamella, a loosening of the primary cell wall and a reduction in cell turgor (Goulao & Oliveira, 2008; Mercado, Pliego-Alfaro, & Quesada, 2011). However, this last process is less well studied and is difficult

Abbreviations: AFM, atomic force microscopy; APEL, antisense pectate lyase plants; APG, antisense polygalacturonase plants; CDTA, cyclohexane-trans-l,2-diamine tetraacetate; CSP, chelated soluble pectins; FTIR, Fourier transform infrared spectroscopy; HGA, homogalacturonan; L_N, number-average contour length; L_W, weight-average contour length; PDI, polydispersity index; PG, polygalacturonase; PL, pectate lyase; RGI, rhamnogalacturonan I; SEC, size exclusion chromatography; SSP, sodium carbonate soluble pectins.

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to separate from the previously mentioned changes in cell wall structure. Amongst the different components that form the cell wall, polyuronides are the polymers most likely to be extensively modified during ripening. This involves pectin solubilization, i.e. an increase in the content of polyuronides loosely bound to the wall, and depolymerization and the loss of neutral sugars from pectin side-chains (Brummell, 2006; Goulao & Oliveira, 2008; Paniagua et al., 2014). These changes are due to the coordinated action of cell wall modifying enzymes, such as polygalacturonase (PG), pectate lyase (PL), pectin methyl esterase, β-galactosidase or α-arabinofuranosidase, which are generally encoded by ripening-related genes (Brummell & Harpster, 2001; Goulao & Oliveira, 2008; Mercado et al., 2011). Amongst these enzymes PG (EC 3.2.1.15) has been the most studied because certain fruits, e.g. tomato, peach or avocado, possess relatively high levels of PG activity, which correlate with the rate of the softening process (Brummell & Harpster, 2001). PG was also the first hydrolase to be examined using transgenic methods in tomato (Sheehy, Kramer, & Hiatt, 1988; Smith et al., 1988). However, the minor effect of PG silencing on tomato softening led to the view that PG-mediated pectin disassembly during ripening makes only a small contribution to fruit softening (Brummell & Harpster, 2001; Hadfield & Bennett, 1998). More recent studies on strawberry, apple and papaya have challenged this hypothesis, suggesting a key role for pectin modifications in fruit softening (Atkinson et al., 2012; Fabi et al., 2014; Jiménez-Bermúdez et al., 2002; Quesada et al., 2009; Youssef et al., 2009, 2013).

Ripening-specific genes encoding PG or PL (EC 4.2.2.2) enzymes have been described in strawberry (Medina-Escobar, Cárdenas, Moyano, Caballero, & Muñoz-Blanco, 1997; Quesada et al., 2009; Villarreal, Rosli, Martínez, & Civello, 2008) and their roles in fruit softening evaluated by means of a functional approach. In previous studies, our research group obtained transgenic strawberry plants expressing antisense sequences of the *FaplC* gene, encoding a PL (Jiménez-Bermúdez et al., 2002; APEL lines) or the *FaPG1* gene, encoding a PG (Quesada et al., 2009; APG lines). Ripe fruits from both transgenic genotypes were significantly firmer than the wild type fruits. Based on their sequences, both genes encode putative endo-pectinases with a common target, de-esterified homogalacturonans (HGA), a major component of the primary cell wall and middle lamella. However, the mechanisms of action of PL and PG are different as are their optimum pH for enzymatic activity. Thus, PL cleaves HGA by β-elimination in the presence of divalent cations with an in vitro optimal pH ~8 (Marín-Rodríguez, Orchard, & Seymour, 2002). The PG degrades HGA by hydrolysis at acidic pH from 3.3 to 6.2 (Sénéchal, Wattier, Rustérucci, & Pelloux, 2014). Chemical analysis of cell wall extracts from APEL and APG transgenic fruits revealed that the silencing of both pectinases reduced middle lamella dissolution and pectin solubilization (Posé et al., 2013; Santiago-Doménech et al., 2008). Additionally, size exclusion chromatography results revealed higher molecular masses for the polymers present in the pectin fractions from the transgenic samples, which is consistent with a decreased depolymerization of these polyuronides.

In general, cell wall hydrolases involved in fruit softening are encoded by large gene families, within which a high degree of functional redundancy has been observed (Goulao & Oliveira, 2008; Vicente, Saladié, Rose, & Labavitch, 2007). It is unclear why a fruit invests energy on the simultaneous expression of PG and PL enzymes acting on the same pectin domain, both having a key role on strawberry softening. The enzymatic differences between PG and PL are not enough to explain this redundancy. If these enzymes act on different targets within the pectin matrix, the pectic chains of these two differently silenced transgenic lines might show different degrees of polymerization and branching. This type of structural modification can be characterized by atomic force microscopy (AFM) at the nano-structural level (Morris, Kirby, & Gunning, 2010). This technique has only recently been used to investigate pectin disassembly processes during fruit ripening (Paniagua et al., 2014). The main goal of this research was to analyze at the nano-structural level pectins from APG and APEL transgenic fruits to reveal the different effect of each enzyme in the pectin matrix and its implications on the mechanical properties of cell walls. Additional information has been obtained through the use of Fourier transform infrared spectroscopy (FT-IR) and size exclusion chromatography analysis (SEC). Based on previous studies, the present research has focused the nanostructural characterization of pectins which are ionically and covalently bound within the cell wall, since these fractions showed the most extensive changes as a result of *FaPG1* or *FaplC* genes silencing (Posé et al., 2013; Santiago-Doménech et al., 2008).

2. Material and methods

2.1. Plant material

Control, non-transformed, strawberry plants (*Fragaria* × *ananassa*, Duch., cv. 'Chandler'), transgenic antisense *FaplC* plants (line APEL39, described in Jiménez-Bermúdez et al. (2002) and Santiago-Doménech et al. (2008)), and antisense *FaPG1* plants (line APG29, described in Quesada et al. (2009) and Posé et al. (2013)) were grown in a greenhouse under a natural temperature and photoperiod regime. Transgenic ripe fruits showed a strong reduction in *FaplC* or *FaPG1* mRNA levels, higher than 95%. The quality of the ripe fruits at harvest was evaluated using only well-shaped fruits of uniform size and colouration, and weight higher than 5 g. Colour was estimated using a chromameter Minolta CR-400. Soluble solids were measured by using a refractometer Atago N1, and firmness by using a hand-penetrometer (Effegi) with a cylindrical needle of 9.62 mm^2 area. pH was measured in juices extracted from fruits. A minimum of 25 ripe fruits per line were evaluated. The fruits were harvested at the ripe stage, when fully red, frozen in liquid N_2 and stored at −30 °C until used.

2.2. Cell wall extraction and pectin fractionation

The cell walls were extracted from frozen ripe fruits following the protocol of Redgwell, Melton, and Brasch (1992) with some modifications, as described by Santiago-Doménech et al. (2008). Briefly, 10–15 frozen fruits were ground to a powder in liquid N_2 and 20 g were homogenized in 40 ml of PAW (phenol:acetic acid:water, 2:1:1, w:v:v). The homogenate was centrifuged at 4000 × *g* for 15 min and the supernatant filtered through Miracloth (Merck, Bioscience, UK). After centrifugation, the pellet obtained was treated with 90% aqueous DMSO to solubilize the starch. The extract was then centrifuged at 4000 × *g* and the pellet washed twice with distilled water. The water fraction was discarded, and the de-starched pellet, the cell wall material (CWM), was lyophilized and weighed.

Pectin fractions were obtained as described by Santiago-Doménech et al. (2008). CWM was washed overnight with deionised water, centrifuged at 6000 × *g* for 15 min and the pellet was sequentially extracted with 0.05 M trans-1,2-diaminocyclohexane-N,N,N′N′-tetraacetic acid (CDTA) in 0.05 M sodium acetate buffer, pH 6, followed by 0.1 M Na_2CO_3 containing 0.1% $NaBH_4$. CDTA extracted polysaccharides (CSP fraction) are those held in the cell wall by Ca^{2+}-mediated crosslinks with the extracts likely to arise primarily from the middle lamellae. Sodium carbonate solubilizes polysaccharides (SSP fraction) held in the wall by ester linkages (Brummell, 2006; Selvendran, 1985) and likely to arise mainly from the primary cell wall. Both pectin