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Strawberry Fruit Softening: Role of Cell Wall Disassembly and its Manipulation in Transgenic Plants

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ABSTRACT

Strawberry is the most economically important soft fruit characterized by its delicious flavour, intense colour and soft texture. The rapid loss of firmness during strawberry ripening is the main determining factor of its short shelf life, and great losses occur by bruising, oversoftening and subsequent fungal infections during postharvest transportation and storage. Cell wall disassembly and the reduction of cell to cell adhesion due to middle lamella dissolution have been recognized as the main causes of fruit softening. In the case of strawberry fruit, cell wall disassembly during ripening is characterized by a solubilization of pectins, a slight depolymerization of covalently bound pectins, a loss of galactose and arabinose, as well as a reduction in the hemicellulosic content. The genetic manipulation of genes encoding proteins involved in wall disassembly has been explored as a biotechnological approach to reduce fruit softening. Thus, the silencing of fruit specific pectate lyase or polygalacturonase genes, both acting on demethylated pectins, by antisense transformation increased significantly the firmness of ripe strawberry fruit without affecting other fruit quality parameters. By contrast, the down-regulation of the expression of genes involved in the processing of hemicellulosic polymers, such as endo-β-1,4-glucanase or expansin genes, did not modify fruit firmness. Other cell wall genes, e.g. β-xylosidase, β-galactosidase, α-arabinofuranosidase, have been cloned in strawberry fruit, and their expression analyzed along fruit development, but their function on fruit softening has not been assessed yet by transgenic approaches. Overall, these results indicate that pectin processing during ripening is a critical factor in the softening of strawberry fruit, and the manipulation of this process by down-regulating pectinase genes a successful approach to improve this trait. This review summarizes recent progress on strawberry fruit softening, focusing in the role of cell wall disassembly and genes involved in that process.

Keywords: cell wall, *Fragaria* × *ananassa*, fruit ripening, fruit shelf life, fruit softening, pectin, postharvest
Abbreviations: **CDTA**, *trans*-1,2-diaminocyclohexane-tetraacetic acid; **CNR**, colorless non-ripening; **EGase**, endoglucanase; **GalA**, galacturonic acid; **HGA**, homogalacturonan; **NMR**, nuclear magnetic resonance; **PG**, polygalacturonase; **PL**, pectate lyase; **PME**, pectin methyl esterase; **RIN**, ripening-inhibitor; **RG**, rhamnogalacturonan; **PAW**, phenol:acetic acid:water

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INTRODUCTION

Strawberry, *Fragaria* × *ananassa* Duch., belongs to the genus *Fragaria* of the Rosaceae family, and is one of the most economically important small fruits. World production and consumer demands for strawberry have increased steadily since the 1970s, reaching over 4 million tons in 2009 (FAOSTAT 2009). This colourful and delicious fruit has also a high nutritional value with essential minerals, organic acids, vitamins and antioxidant compounds, benefits to human health (Hannum 2004).

Texture is a key quality character for fleshy fruits. Strawberry belongs to the group of soft fruits which are characterised by a high and rapid loss of firm texture during the ripening process, acquiring a melting texture in few days after ripening. This fast softening is the major source of commercial losses and results in a short shelf life and susceptibility to diseases (Perkins-Veazie 1995). It is estimated that a 5-30% of strawberry yield is loss due to oversoftening and/or fungal decay. Furthermore, the rapid rate of strawberry softening influences the frequency of harvest and limits transportation and storage of harvested fruits.

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Invited Review

Therefore, improvement of strawberry texture to reduce spoilage during ripening and postharvest is of great commercial importance. This topic constitutes one of the main objectives of strawberry breeding programs, using both, traditional and biotechnological approaches (Faedi *et al*. 2002; Graham 2005; Mercado *et al*. 2007). Fruit texture is a complex trait depending on numerous factors such as tissue composition and architecture, cell shape, turgor pressure, mechanical properties of the cell wall and the strength and extension of adhesion areas between adjacent cells (Harker *et al*. 1997; Bourne 2002). It is generally accepted that the cell wall disassembly and the reduction of cell to cell adhesion, as result of middle lamella dissolution, are the main factors that cause fruit softening (Brummell and Harpster 2001; Brummell 2006). In general, cell wall modifications responsible for the softening involve the depolymerization of matrix glycans, the solubilization and/or depolymerization of pectins, and the loss of neutral sugars from side-chains of pectins (Brummell 2006; Goulão and Oliveira 2008). These changes induce the loosening of the xyloglucan-cellulose network and cell wall swelling, increasing wall porosity that may facilitate degradative enzymes to access to their substrate (Brummell 2006). These cell wall modifications are common to the ripening of most fleshy fruits, independently on its classification as climacteric or non-climacteric, although each species has specific patterns of cell wall disassembly. Strawberry ripening is characterized by an extensive dissolution of the middle lamella of the cortical parenchyma cells. In ripe fruits, cells appear separated by a considerable intercellular space and a little cell to cell contact area (Redgwell *et al*. 1997a; Santiago-Doménech *et al*. 2008). At the cell wall level, a moderate pectin solubilization and depolymerization and a slight reduction of the molecular weight of hemicellulosic polymers are general features of strawberry softening. However, these changes can vary depending on the cultivar and strawberry species considered, as discussed later.

Biotechnological attempts to reduce fruits softening using transgenic plants have followed two main strategies. Firstly, in climacteric fruits, the suppression of ethylene production through transformation with antisense sequences of genes responsible for its synthesis prevents the ripening process as a whole, including softening (Hamilton *et al.* 1990; Oeller *et al.* 1991). However, when fruits are treated with ethylene to induce ripening the shelf life is usually as short as in wild-type fruits. Moreover, this strategy is not feasible to non-climacteric fruits as the strawberry. More recently, the genes underlying the tomato mutants phenotypes *RIPENING INHIBITOR* (*RIN*) and *COLOURLESS NON RIPENING* (*CNR*), which fail to ripen and are not responsive to ethylene, have been cloned (Vrebalov *et al.* 2002; Manning *et al.* 2006). Both genes encode transcription factors that are located upstream to ethylene biosynthesis in the ripening regulatory network. It has been suggested that these regulators might be conserved between both climacteric and non-climacteric fruits. Supporting this hypothesis, a ripening related strawberry gene homolog to tomato *RIN* gene has been cloned (Vrebalov *et al.* 2002). However, the genetic manipulation of these regulatory genes in tomato causes pleiotropic effects, reducing fruit quality, or even leads to lethality (Matas *et al.* 2009).

A second strategy employed in different fruits has been based on reduction of the rate of softening by transgenic manipulation of the expression of genes encoding cell wall modifying enzymes. This alternative to the prevention of ripening might extend shelf life allowing the normal development of other ripening events, such as accumulation of sugars, volatiles or pigments. This approach could be applied to climacteric and non-climacteric fruits, and it could also be commercially desirable for fruit processed products where improved integrity is an important quality trait, as yoghurt or jam. The main limitation of this transgenic approach is that most cell wall proteins are present as multi gene families, and therefore, the suppression of a single gene can be complemented by another related gene. Additionally, cell wall processing is the result of the coordinated expression of several families of genes and unpredictable unintended changes may occur. Moreover, the complex structure, composition and interactions among the different polymers of the cell wall determine that confirmation of activity *in planta*, or substrate specificity, is lacking for most genes encoding cell wall-modifying proteins. Despite these limitations, the manipulation of cell wall related genes has been extensively explored in tomato, the model system for transgenic analysis of fruit softening and ripening, with limited success (Brummell and Harpster 2001), although a recent work describes a significant shelf life extension by suppressing N-Glycan processing enzymes (Meli *et al.* 2010). A reduction in softening has also been achieved in strawberry fruits by silencing pectinases (Jiménez Bermúdez *et al.* 2002; Quesada *et al.* 2009a) and the quality of processed fruits was also improved in these transgenic plants (Sesmero *et al.* 2007, 2009). In spite of these promising results, inhibiting or delaying textural changes in ripe strawberry fruit while maintaining other quality attributes still remains as a major challenge to breeders and fruit physiologists. In this paper, we review recent insights into mechanisms of tissue softening in strawberry, focusing on the cell wall disassembly taking place during ripening, and enzymes and genes involved in that process.

CELL WALL MODIFICATIONS DURING STRAWBERRY RIPENING

Primary cell wall

The flesh of most fruit is composed of parenchyma cells which have a thin primary wall. In the most accepted model, the type I primary cell wall is fundamentally a network of cellulose microfibrils coated with and cross-linked together by matrix glycans. The spaces in the cellulose-matrix glycan network are filled by highly hydrated pectins, forming also a network, held together and cross-linked with other wall components by different types of bonds (Carpita and Gibeaut 1993).

Cellulose is the single component of the microfibrillar phase, and it is formed by long linear chains of *c*. 8000 D-glucose residues linked by β-(1→4) linkages. Approximately, 36 parallel chains are associated by extensive hydrogen bonds forming a microfibril (Taylor 2008). The internal region of microfibrils is crystalline and excludes water, whereas the external layers are more amorphous, and matrix glycans may be interwoven with the glucan chains (Pauly *et al*. 1999). Matrix glycans or hemicelluloses are composed predominantly of neutral sugars and do not contain galacturonic acid (GalA). Xyloglucans, the most abundant glycan, are linear chains of (1→4)-β-D-glucan, like cellulose, but with numerous xylose residues regularly spaced (Carpita and Gibeaut 1993). The glucan backbone of xyloglucan binds tightly to the surface of cellulose microfibrils, and also these molecules can span the distance between adjacent microfibrils linking them together.

The fundamental cellulose-xyloglucan framework represents about 50% of the wall mass, and it is embedded in a more soluble matrix of polysaccharides, glycoproteins, low-molecular weight compounds and ions (Willats *et al*. 2001). Pectin is the most abundant class of macromolecule within this matrix, representing about 30% in primary cell wall, although fruits are usually enriched in pectins, accounting for up to 60% in many species (Redgwell *et al*. 1997a). Furthermore, pectin is the most abundant polymer in the middle lamella, regulating intercellular adhesion (Willats *et al*. 2001). The principal component of pectins is D-galacturonic acid that form the backbone of three polysaccharide domains present in all pectin species: homogalacturonan (HGA), rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II). HGA is a linear homopolymer of GalA linked by α-(1→4) glycosidic bonds, and is thought to contain 100-200 GalA residues. HGA is synthesized in the Golgi apparatus and deposited in the cell wall with the 70-

80% of GalA residues methyl-esterified at the C-6 carboxyl (Willats *et al*. 2001). The removal of this methyl ester groups *in muro* allows the cross-linking of HGA chains by calcium, and the formation of gels. RG-I is formed by a backbone of the disaccharide (1→2)-α-L-rhamnose-(1→4)-α-D-galacturonic acid, with 20-80% of rhamnose residues substituted by side chains of neutral sugars. The number of lateral residues varies from a single glycosyl residue to 50 or more, producing a highly variable family of polysaccharides. Arabinans, galactans and arabinogalactans are the most common side chains, although other sugars and acids, such as L-fucose, D-glucose, D-mannose, D-xylose, and D-glucuronic acid, are sometimes found (Van Buren 1991). The highly branched RG-I is known as "hairy" pectin in comparison with the "smooth" region of HGA. Pectins are classically depicted as an extended chain of HGA and RG-I regions, although an alternative model has been suggested where HGA are side chains of RG-I (Vincken *et al*. 2003). The third pectin domain is RG-II, a branched polymer containing a HGA backbone of 9 GalA residues substituted by 4 heteropolymeric side chains of consistent length. RG-II glycosyl sequence is highly conserved in all vascular plants, and it is present in the cell wall as dimmers covalently cross-linked by a borate diester (O'Neill *et al*. 2004).

To analyze the ripening-related changes in the cell wall, isolated walls are sequentially extracted to produce fractions enriched in a specific component. Usually, these extractions are performed with: 1) PAW (phenol-acetic acid-water) or water, yielding pectins freely soluble in the apoplast and solubilized by *in vivo* processes; (2) chelating agents, usually CDTA, solubilizing pectins ionically bound to the cell wall, thought to derived mainly from the middle lamella; (3) sodium carbonate, releasing pectins covalently bound to the primary wall; (4) weak alkali, such as 1 M KOH, which solubilizes matrix glycans loosely bound in the wall; (5) strong alkali, 4 M KOH, releasing matrix glycans tightly bound in the wall, mainly xyloglucans. The residue after this sequential extraction is mainly composed of cellulose. The solubilization of pectins into different fractions occurs during ripening, and for example, sodium carbonate-extractable pectins in an immature fruit may be extracted from ripe fruit in the water or chelator-soluble fraction (Rose *et al*. 2003).

Cell wall disassembly in strawberry ripening

A few numbers of studies have analyzed cell wall modifications during development and ripening of strawberry fruit. However, the different experimental procedures used for cell wall extraction and fractionation and the different genotypes analyzed make difficult the achievement of a clear picture of this process.

Cell wall and cellulose contents slightly decreased during ripening in all cultivars studied, independently of its firmness (El-Zoghbi 1994; Redgwell *et al*. 1997a; Koh and Melton 2002; Rosli *et al*. 2004). The loss of cell wall material is more pronounced in cortical than pith tissues (Koh and Melton 2002), but this parameter does not seem to be related to fruit firmness (Figueroa *et al*. 2010). As is generally observed in most fruits, cellulose networks remain unaltered during ripening, although NMR studies indicate that strawberry contains an exceptionally small amount of crystalline celullose (Koh *et al*. 1997).

The hemicellulose content also diminished during strawberry ripening in cultivars with contrasting firmness (Koh and Melton 2002; Rosli *et al*. 2004). Regarding the molecular weight of these polymers, controversial results have been obtained. In an early work, Huber (1984) reported a significant depolymerization of hemicellulosic polymers during ripening of fruits from cv. 'Dover'. By contrast, Rosli *et al*. (2004) did not observe hemicellulose changes in 'Pajaro' fruits and only a very slight depolymerization in firm (cv. 'Camarosa') and soft fruits (cv. 'Toyonaka'). Nogata *et al*. (1996) also detected a reduction of the high molecular weight polymers in hemicellulose fractions, although these changes were more prominent during fruit growth than at ripening stage.

The solubilization of pectins is the most consistent feature of strawberry softening. Knee *et al*. (1977) observed that from 7 to 21 days after petal fall over 90% of the polyuronides were cell wall bound, and then declined to 30% at ripening. The percentage of pectins extracted with EDTA also increased up to day 21 and diminished thereafter. Huber (1984) showed that the polyuronide content in cell wall extracts treated with Na_2EDTA increased from 30% in undeveloped fruit to 65% in ripe fruit. The increase of soluble pectin occurred between large green and pink fruit, but full ripe red fruit showed a content of Na_2EDTA soluble pectins similar to pink fruit. A more detailed characterization of cell wall fractions, as described in the above section, has been reported by several authors (Koh and Melton 2002; Rosli *et al*. 2004; Figueroa *et al*. 2010). Koh and Melton (2002) showed that the Phenol-Hepes and Water cell wall fractions, both containing soluble pectins, increased in a cell wall basis from unripe to ripe fruit, as well as the polyuronide content in both fractions. By contrast, CDTA and Na_2CO_3 fractions, containing quelated and covalently bound pectins, respectively, decreased during ripening. Both fractions also contained a less amount of polyuronides. A similar trend in the behaviour of water and 50 mM HCl fractions was observed by Rosli *et al*. (2004) when comparing three *F.* × *ananassa* cultivars with contrasting firmness, and by Figueroa *et al*. (2010) when comparing this species with *F. chiloensis*. HCl extracted pectins, however, does not full correspond with sodium carbonate fraction (Prasanna *et al*. 2007). Contrary to the results of Koh and Melton (2002), Figueroa *et al*. (2010) did not observe significant changes in quelated polymers during ripening. Interestingly, the largest changes in water-soluble and covalently-linked polymers occurred in the transition from green to turning stages, both in *F.* × *ananassa* (cv. 'Chandler') and *F. chiloensis* (Figueroa *et al*. 2010). Finally, Redgwell *et al*. (1997a) also observed an increase in the content of uronic acid in PAW fraction during the ripening of strawberry fruit, although this increment was moderate in comparison with other fruit such as avocado or kiwifruit. Therefore, it seems clear that pectin solubilization occurs at the expense of the pectin fraction covalently bound to the cell wall.

This consensus on the role of pectin solubilization during strawberry ripening does not exist for pectin depolymerization. For a long time since Huber report (1984), no role for enzymatic hydrolysis in pectin solubilization was assumed. He did not found depolymerization of chelated pectins, leading to the general view that depolymerization does not play a major role in strawberry fruit softening. However, Redgwell *et al*. (1997a) showed a slight depolymerization of PAW pectins, and more recent papers confirm that depolymerization of covalently bound pectins occurs in the ripening of fruit from cv. 'Toyonaka' (Rosli *et al*. 2004) and 'Chandler' (Santiago-Doménech *et al*. 2008; Figueroa *et al*. 2010). Finally, Figueroa *et al*. (2010) indicate that the higher softening of *F. chiloensis* is not related to pectin depolymerization but to some other process that solubilise pectins at an earlier developmental stage than in *F.* × *ananassa*.

The content of neutral sugars does not change significantly during strawberry ripening (Gross and Sam 1984; Redgwell *et al*. 1997b; Koh and Melton 2002; Figueroa *et al*. 2010). Arabinose, xylose and galactose are the major neutral sugars in unripe and ripe strawberry fruit. During ripening, a loss of arabinose and galactose occurs, while the amount of xylose increased, and rhamnose, fucose, manose and glucose remain unchanged (Redgwell *et al*. 1997b). Most galactose and arabinose were associated with pectin polymers attached to the cell wall after 4 M KOH extraction (Redgwell *et al*. 1997b), and this fraction and the 4 M KOH-residue supported the highest loss of both neutral sugars (Redgwell *et al*. 1997b; Koh and Melton 2002). According to Redgwell *et al*. (1997b) the loss of neutral sugars

Table 1 Summary of experimental results derived from transgenic strawberry plants in which softening related genes have been suppressed.

Protein family	Cell wall target	Gene	Effect of gene suppression	References
Pectate lyase	Demethylated HGA	*plC*	Increased fruit firmness and extended shelf life	Jiménez-Bermúdez *et al*. 2002; Youssef *et al*. 2009
Polygalacturonase	Demethylated HGA	*FaPG1*	Increased fruit firmness and extended shelf life	Quesada *et al*. 2009a; García-Gago *et al*. 2009a
Pectin methylesterase	Methylated pectins	*FaPE1*	Firmness not evaluated. Increased fungal pathogen resistance	Osorio *et al*. 2008
Endo-β-(1,4)-glucanase	Xyloglucan	*FaEG1*	No effect on firmness	Wolley *et al*. 2001; Palomer *et al*. 2006
		FaEG3	No effect on firmness	Mercado *et al*. 2010
Expansin	Xyloglucan-Cellulose network	*FaExp2*	No effect on firmness. Significant growth alterations	García-Gago *et al*. (personal communication)

did not correlate with softening.

In conclusion, despite the different works performed to correlate cell wall changes with fruit firmness, a straightforward relation between wall metabolism and texture has not been found, probably because of the limited number of genotypes surveyed. Overall, the most characteristic cell wall modification during strawberry ripening is the solubilization of pectins which supposedly occurs at the expense of the polyuronides covalently linked to the cell wall. Several hypotheses have been proposed to explain this solubilization. Huber (1984) pointed out to the synthesis of a modified more freely soluble form of pectins during ripening. Koh and Melton (2002) postulated that solubilization could be associated with the disentanglement of pectins in the cell walls due to the degradation of arabinan side chains or to the cleavage of linkages between pectins and hemicelluloses. Finally, Rosli *et al*. (2004) and Santiago-Doménech *et al*. (2008) indicated that pectin solubilization could be due to a slight depolymerization of covalently linked pectins.

CELL WALL GENES AND STRAWBERRY SOFTENING

Cell wall disassembly during fruit ripening is mediated by genes encoding enzymes and proteins able to modify the different polysaccharide moieties, especially matrix glycans and pectins. These enzymes have been reviewed in different papers (Fischer and Bennett 1991; Fry 2004; Goulão and Oliveira 2008; Wong 2008). Cell wall modifying enzymes are usually classified in pectolytic, able to cleave or modify the nature of pectin polysaccharides, and non-pectolytic, responsible for hemicellulose modifications. Several of these genes have been cloned in strawberry, and, for a few of them, their role on softening analyzed through genetic modification (**Table 1**). Most of these genes are fruit-specific and its expression repressed by auxin, a common feature of ripening-related genes in strawberry (Manning 1994). Main cell wall enzyme activities will be discussed in the next sections, focusing in those genes where functional analyses are available.

Pectate lyase

Pectate lyases (PLs) were firstly described as pathogen-secreted extracellular enzymes that help pathogenesis by cleaving polygalacturonate blocks in the plant host cell-wall (Davis *et al*. 1984). In plant genomes, many genes that show similarity with PL genes from pathogens have been isolated and characterized. The abundance of ESTs for these genes in tomato and the presence of PL-like transcripts in several fruits would indicate that these enzymes may have a more important role in ripening than previously suspected (Marín-Rodríguez *et al*. 2002), specially in those fruits with low levels of polygalacturonase activity. This enzyme catalyzes the cleavage of glycosidic bonds of unsaturated regions of pectins by a β-elimination reaction, at a pH optimum of 8-11. PL requires the presence of calcium ions and generates oligosaccharides with de-esterified galacturonosyl residues at their non-reducing ends. Different methods for measuring PL activity have been reported, but as far as we know, the presence of PL activity in fruit has only been demonstrated in banana and mango (Marín-Rodríguez *et al*. 2003; Payasi and Sanwal 2003; Chourasia *et al*. 2006).

Three ripening-related PL genes, *plA* to *plC*, have been identified in cultivated strawberry (Medina-Escobar *et al*. 1997; Benítez-Burraco *et al*. 2003). The three genes show a similar pattern of gene expression, being detected in fruit but not in vegetative tissue, and up regulating their expression during ripening. *F. chiloensis* fruit also express a PL gene during ripening (Figueroa *et al*. 2008).

Strong evidences for an important role of PL genes in strawberry softening have been obtained by their manipulation in transgenic plants. Jiménez-Bermúdez *et al*. (2002) showed that the inhibition of pectate lyase gene in strawberry by antisense transformation resulted in fruits firmer than control at the ripe stage, not affecting other fruit characteristics such as color, soluble solids or anthocyanin content. Cell wall analysis of the PL silenced lines revealed that transgenic fruits showed a lower degree of in vitro cell wall swelling and pectin solubilization, and a decreased depolymerization of more tightly bound polyuronides, quelated and covalently bound pectins (Jiménez-Bermúdez *et al*. 2002; Santiago-Doménech *et al*. 2008). The characteristic ripening-associated loss of cell-cell adhesion was also substantially reduced in transgenic fruits (Santiago-Doménech *et al*. 2008). More recently, Youssef *et al*. (2009) obtained transgenic strawberry lines with low levels of *plC* expression through transformation with this gene in sense orientation. The introduction of extra copies of *plC* resulted in a co-suppression of the gene, and, as previously observed in antisense transgenic plants, transgenic fruits were significantly firmer than control. Interestingly, a correlation between gene expression and fruit firmness was deduced from these results, since co-suppressed lines showed a lower *plC* down-regulation and were slightly softer than antisense lines (Youssef *et al*. 2009).

As should be expected, the reduction of softening in transgenic PL fruits improved the postharvest shelf life and the textural properties of processed fruits. After a 7 days period of storage at 5°C, antisense PL fruits were firmer and also showed a lower softening rate than controls (García-Gago *et al*. 2009a). Regarding fruit derived product, transgenic fruits were more resistant to the cooking process than control when processed into jam (Sesmero *et al*. 2007). Therefore, transgenic jams were enriched in berry fragments that were also firmer than berries in control jams. It is noteworthy that berry content is one of the most important quality attribute in jam. The physical characteristics of juices made from control and transgenic PL fruits have also been analyzed (Sesmero *et al*. 2009). Transgenic juices were more viscous than control, supposedly due to the higher content of large particles in the solid fraction of juices (Sesmero *et al*. 2009).

In summary, these results indicate that pectate lyase genes play an important role in the biochemical changes that occur in primary wall and middle lamella during strawberry fruit ripening, and should be included in synergistic models of cell wall disassembly.

Polygalacturonase

Polygalacturonases (PGs) were first identified in the sixties and have been suggested to be involved in the disassembly of pectin that accompanies many stages of plant development, particularly those that require cell separation (Hadfield and Bennett 1998). PGs catalyze the hydrolytic cleavage of galacturonic linkages, and can be exo- or endo-type. The exo-PGs remove single galacturonic acid residues from the non-reducing end of de-esterified polygalacturonic acid, while endo-type PGs, the most frequent in fruits, cleave these linkages at random. These enzymes have been extensively studied in tomato where PG protein and mRNA levels, as well as enzyme activity, correlate with fruit ripening and softening (Brummell and Harpster 2001). Tomato PG was the first cell wall hydrolase examined by transgenesis at the end of the 1980s (Sheehy *et al*. 1988; Smith *et al*. 1988). Transgenic tomato fruits expressing an antisense PG sequence resulted in 99% suppression of PG mRNA. This silencing reduced pectin depolymerization but not pectin solubilization during ripening (Smith *et al*. 1990). Despite this change, fruit softening was not significantly altered, but an extended shelf life of overripe fruits and a reduction on postharvest decay in transgenic fruits was observed (Kramer *et al*. 1992). These results led to the hypothesis that PG activity alone is not sufficient to affect texture, but it contributes significantly to tissue deterioration in the later stages of ripening (Hadfield and Bennet 1998; Giovannoni 2001).

In strawberry fruit, PG activity is low when compared with other fruits such as tomato, peach or avocado, which showed a peak of PG activity during ripening (Hadfield and Bennett 1998). Although early works were unable to detect PG activity in strawberry (Neal 1965; Barnes and Patchett 1976; Huber 1984; Abeles and Takeda 1990), many other researchers found a low level of PG activity in ripe fruit of several strawberry cultivars (Nogata *et al.* 1993; Lefever *et al*. 2004; Villarreal *et al*. 2008; Quesada *et al*. 2009a). In 'Toyonaka' fruits, Nogata *et al*. (1993) partially purified three different PG activities, two of them exo-PGs and one endo-acting. One of the exo-enzymes, decreased consistently from small green to overripe fruit stages (Nogata *et al*. 1993). Contrary to these results, other authors have found an increase on total PG activity during strawberry ripening (El-Zoghbi 1994; Villarreal *et al*. 2008). Furthermore, Lefever *et al*. (2004) found a negative correlation between fruit firmness and PG activity in several strawberry varieties evaluated for their resistance to processing. More recently, Figueroa *et al*. (2010) showed a contrasting behaviour in *F.* × *ananassa* and *F. chiloensis* fruits. While in the first case PG activity increased during ripening, *F. chiloensis* fruits displayed a significant reduction in PG activity, more pronounced in the transition from the green to the turning stage.

Three ripening-related polygalacturonase genes have been cloned in strawberry: *FaPG1*, also know as *spG* (Redondo-Nevado *et al*. 2001), *FaPG2* or *B4* (Salentijn *et al*. 2003), and *T-PG* (Villarreal *et al*. 2008). The three genes are upregulated during ripening and their expression is repressed by auxins (Villarreal *et al*. 2008; Quesada *et al*. 2009a; Villarreal *et al*. 2009). Interestingly, *T-PG* is almost identical to *FaPG1* gene but it shows a deletion of 85 bp that causes a frame shift and produces an inactive protein (Villarreal *et al*. 2008). According to these authors, firmer cultivars expressed preferentially *T-PG* during ripening, whereas softer cultivars showed higher expression of *FaPG1*and lower levels of *T-PG*. The differential expression of these two genes could be therefore related to fruit softening. More recently, Villarreal *et al*. (2009) found that the expression of both *FaPG1* and *T-PG* genes can also be regulated by ethylene, in spite of the non-climacteric nature of this fruit. Comparative studies of two different strawberry species, *F. chiloensis* and *F.* × *ananassa*, have also shown that the PG transcript level correlates with the progress of softening in both species (Figueroa *et al*. 2008). *F. chiloensis* fruits displayed higher levels of PG transcripts at earlier stages of development than *F.* × *ananassa*, suggesting that the faster softening of these fruits could be related to the earlier expression of cell wall degrading genes (Figueroa *et al*. 2008). However, these results are contradictory, since *F. chiloensis* showed a clear reduction on total PG activity during ripening (Figueroa *et al*. 2010).

The role of *FaPG1* on fruit softening has been assessed by genetic transformation (Quesada *et al*. 2009a; García-Gago *et al*. 2009b). These authors obtained several transgenic lines, cv. 'Chandler', expressing an antisense sequence of *FaPG1* under the control of the constitutive promoter *CaMV35S*. Fruits from selected transgenic lines showed a 90-95% decrease in the *FaPG1* transcript level, and were significantly firmer than control at ripening. Moreover, the postharvest behaviour of these transgenic lines was improved, and a decrease on the rate of softening was observed when transgenic fruits were stored for several days at low temperature (Quesada *et al*. 2009a; García-Gago *et al*. 2009a). The expression of *FaPG2* gene was not modified in transgenic fruits as could be expected because of the low homology between the two genes. Further analysis of ripe fruit showed that PG activity was only partially reduced, suggesting that *FaPG2* or other undiscovered PG genes were responsible for this remaining PG activity in strawberry. At the cell wall level, the firmer fruit phenotype in silenced *FaPG1* plants was associated with a reduction on pectin solubilization and an increase on pectins covalently bound to the cell wall (Quesada *et al*. 2009a). Although in general, changes in the cell wall induced by the silencing of *FaPG1* were similar to those above described in antisense PL plants, transgenic PG fruits at the ripe stage were slightly firmer than PL fruits (Quesada *et al*. 2009b; García-Gago *et al*. 2009a).

Pectin methylesterase

Pectins are secreted into the wall as highly methylesterified form, and later processed by pectinases such as pectin methylesterase (PME), which catalyze the demethylesterification of homogalacturonans, releasing acidic pectins and methanol (Micheli 2001; Pelloux *et al*. 2007). This enzyme plays a dual role in the cell wall. Its activity could give rise to blocks of free carboxyl groups, allowing the cross-linking of pectins by calcium bridges that contribute to cell wall stiffening. However, PME is also necessary for cell wall loosening, since PG or PL only cleaves bonds between galacturonic acid residues within blocks of de-esterified polygalacturonic acid. The most accepted hypothesis concerning the mode of action of PME is that this enzyme can act either randomly, promoting the action of other pectinases, or linearly, favouring the formation of calcium-mediated pectin gels (Micheli 2001). In plants, this enzyme is encoded by large multigene families whose members have different pattern of expression. The role of PME on fruit softening has been assessed in tomato by antisense suppression (Tieman *et al*. 1992; Gaffe *et al*. 1994). Despite a 90% reduction on PME activity, no effect on tomato fruit firmness was observed and, furthermore, fruit shelf life was reduced (Tieman *et al*. 1992). The alteration of the cation levels in the apoplast could be the responsible of the lower tissue integrity of these PME antisense fruits (Tieman and Handa 1994).

According to Barnes and Patchett (1976), PME activity increased during strawberry fruit development from the small green to the ripe stage, but then fell to its initial level in overripe fruit. Figueroa *et al*. (2010) also observed a maximum on PME activity at the turning stage and later decreased in ripe fruit at levels similar to those obtained in green fruit. However, Draye and Van Cutsem (2008) reported that PME activity decreased continuously from the green to the overripe stage. This result, however, is paradoxical, since the same authors reported an increase on the content of calcium-bound acidic pectin during ripening. Some discrepancies are also apparent in the results reported by Lefever *et al*. (2004). They found that firmer cultivars

contained lower PME activity than softer cultivars, with the exception of 'Camarosa' fruits that showed an extremely high PME activity, being one of the firmer genotype. Even more, a good negative correlation between PME activity and resistance to fruit cooking was deduced in that paper. However, when compared the degree of methylated pectin in a soft and a firm cultivar, water soluble pectin from the firm cv. contained a lower content of methylated pectins than the soft genotype, despite its low PME activity (Lefever *et al*. 2004). To entangle the role of PME on softening, vacuum infiltration of solutions containing PME and Ca^{2+} increases strawberry fruit firmness (Suutarinen *et al*. 2000; Duvetter *et al*. 2005), supposedly due to the formation of pectin gels that adds rigidity to the wall.

In strawberry, four different PME genes, *FaPE1* to *FaPE4*, have been identified. One of them, *FaPE1*, is specifically expressed in fruit, showing an increasing expression during the ripening process up to a maximum in the turning stage (Castillejo *et al*. 2004). It was shown that the expression of *FaPE1* was induced by auxin at the onset of fruit ripening and down-regulated by ethylene during fruit senescence (Castillejo *et al*. 2004). This gene was expressed in the wild strawberry *F. vesca* under the control of the *CaMV35S* promoter (Osorio *et al*. 2008). Two transgenic lines with higher PME activity in fruit were selected. Cell wall analysis of these fruit showed a 20% reduction in the methyl esterification of soluble and quelated pectin. Interestingly, the mean molecular mass of the Na_2CO_3 soluble pectin fraction was significantly higher in the *FaPE1* overexpressing lines, when the contrary effect would be expected because of the higher susceptibility of de-methylated pectins to pectinase degradation. The effect of *FaPE1* expression on fruit firmness and postharvest shelf life was not reported by Osorio *et al*. (2008), but these fruits showed an enhanced resistance to *Botrytis cinerea* due to the constitutive expression of a pathogenesis-related gene, involved in the salicylic acid pathway. The activation of this pathogen defence response could be related to the lower degree of methyl esterification of oligogalacturonides (Osorio *et al*. 2008), small pectins that elicit different cellular responses, including fruit ripening (Dumville and Fry 2000).

Endo-β-1,4-glucanase

Plant endo-β-1,4-glucanases or EGases have been involved in processes in which cell wall weakening and cell separation take place, such as organ abscission and fruit softening. Their natural substrate within the cell wall is largely unknown, but it has been proposed that xyloglucans, integral and peripheral regions of non-crystalline cellulose and glucomannans are target of these enzymes (Brummell and Harpster 2001). In general, all fruits show EGase activity that increase during ripening (Brummell and Harpster 2001). However, the transgenic modification of several EGase genes in different fruits neither affected fruit softening nor modified hemicellulose metabolism (Brummell *et al*. 1999a; Harpster *et al*. 2002a, 2002b).

EGase activity increases significantly during strawberry fruit development, reaching a maximum level in overripe fruit (Abeles and Takeda 1990; El-Zoghbi 1994; Figueroa *et al*. 2010). Two EGase isoforms, encoded by two divergent genes, *FaEG1* and *FaEG3*, are responsible for this EGase activity (Harpster *et al*. 1998; Llop-Tous *et al*. 1999; Trainotti *et al*. 1999a, 1999b). The temporal expression pattern of both genes overlaps only partially. *FaEG1* is fruit specific and its expression starts at the white stage. By contrast, *FaEG3* is detected at earlier stages of fruit development, and also in growing vegetative tissues (Trainotti *et al*. 1999a). Additionally, *FaEG3* contains a putative cellulose-binding domain which apparently would make FaEG3 protein especially active against xyloglucans coating cellulose microfibrils (Trainotti *et al*. 1999b). Woolley *et al*. (2001) and Palomer *et al*. (2006) obtained transgenic strawberry plants containing antisense sequences of *FaEG1* gene. In both cases, fruit firmness, EGase activity and cell wall characteristics were similar in control and transgenic plants, in spite of the strong silencing of *FaEG1* achieved. As regard *FaEG3*, Mercado *et al*. (2010) down-regulated this gene by antisense transformation in cv. 'Chandler'. As previously observed for *FaEG1*, no effect on fruit firmness or EGase activity was detected in transgenic plants showing a 95% reduction in *FaEG3* transcript level. However, cell walls of transgenic fruit were enriched in hemicellulosic polymers and the molecular size of these polysaccharides was also slightly larger than in wild type. Overall, these results suggest that EGases do not play a significant role in fruit softening, although they can modify the extractability and/or molecular size of hemicelluloses.

Expansin

Expansins are proteins of unknown enzymatic activity that promote cell wall loosening and extension. Their mechanism of action or cell wall substrates has not been elucidated yet. It has been hypothesised that expansins disrupt noncovalent interactions between hemicellulose and cellulose microfibrils. This could have the effect of exposing previously inaccessible structurally important polymers to the action of ripening-associated cell wall hydrolases. This suggestion is consistent with the cellulose binding domain-like motif that is conserved among expansins (McQueen-Mason and Cosgrove 1994). These proteins were originally detected in growing tissues, where they play a role on cell elongation (Cosgrove 2000). However, expansin genes have also been detected in many other tissues, including those of ripe fruits. In tomato, fruit specific expansin genes seem to play a critical role on fruit softening. The down-regulation of *LeExp1* reduces fruit softening while the overexpression of this gene increases softening (Brummell *et al*. 1999b).

In the case of strawberry, seven expansin genes (*FaExp1* to *FaExp7*) have been isolated (Civello *et al*. 1999; Harrison *et al*. 2001; Dotto *et al*. 2006). Some of them are expressed in fruit, and the expression of *FaExp1*, *FaExp2* and *FaExp5* correlated with the ripening process (Harrison *et al*. 2001; Dotto *et al*. 2006). Furthermore, several studies using cultivars with different rates of softening showed higher transcript levels of these three expansin genes on the softer cultivars during ripening (Salentijn *et al*. 2003; Dotto *et al*. 2006). Most of the seven expansin genes are also expressed in *F. chiloensis*, although the expression pattern of some of them is slightly different to the one observed in cultivated strawberry (Figueroa *et al*. 2009).

Transgenic strawberry plants expressing low transcript levels of *FaExp2* by RNAi silencing have been generated (García-Gago *et al*. unpublished data). Firmness of ripe fruit was not modified in any transgenic line, and furthermore, most of the lines showed altered phenotypes, being the most common a reduced plant vigour and dwarf growth. These results suggest that *FaExp2* could be involved in cell growth rather than fruit softening. As far as we know, no other strawberry expansin gene has been assessed by transgenic technology.

Other cell wall genes

Other cell wall related genes which could be involved in fruit softening have been identified and their expression analyzed in several strawberry cultivars. Martínez *et al*. (2004) cloned a cDNA encoding a putative β-xylosidase gene (*FaXyl*) from ripe fruit. The full-length *FaXyl* gene and its promoter region have been isolated more recently (Bustamante *et al*. 2006, 2009). β-xylosidase enzymes release single xylosyl residues from xylose containing oligosaccharides. Xylose is present in hemicelluloses (xyloglucan and mainly xylans) and pectins (xylogalacturonan). As most xylose in strawberry fruit appears in the hemicellulosic fraction (Koh and Melton 2002), it is most likely that *FaXyl* gene product acts on these cell wall polymers rather than pectins. Higher levels of *FaXyl* expression and β-xylosidase activity have been reported in 'Camarosa', a

firm cultivar, than in 'Toyonaka', a soft cultivar (Bustamante *et al*. 2006). Similarly, higher β-xylosidase activity was found in *F. chiloensis* than in *F.* × *ananassa*, cv. 'Chandler', also suggesting a correlation between this activity and fruit firmness (Figueroa *et al*. 2010). However, the pattern of *FaXyl* gene expression during fruit development and ripening differs among the different cultivars tested, making difficult the understanding of the role of this gene on softening.

As previously discussed, galactose and arabinose are the neutral sugars that most decreased during strawberry ripening. Both, β-galactosidase and α-arabinofuranosidase genes have been isolated in strawberry. α-Arabinofuranosidase activity was detected at all fruit developmental stages except in small green fruit (Rosli *et al*. 2009). This activity increased with fruit ripening, being the levels higher in a soft than in a firm cultivar. α-Arabinofuranosidase activity was also detected in *F. chiloensis* fruits, although in this case, the highest activity was observed in large green fruit (Figueroa *et al*. 2010). Three α-arabinofuranosidase genes (*FaAra1* to *FaAra3*) are responsible for this activity. The expression analysis of the three genes showed a complex pattern, being all them expressed during the whole fruit development, including the ripening stage. Apparently, none of them clearly correlated with softening, although considering the global expression of the three *FaAra* genes, the soft cultivar showed a higher expression than the firm cultivar (Rosli *et al*. 2009).

β-galactosidase activity increases during fruit development and remains high in ripe fruit, which is in accordance with the loss of galactose from cell wall (Trainotti *et al*. 2001; Figueroa *et al*. 2010). Three full length cDNAs encoding β-galactosidases (*Faβgal1* to *Faβgal3*) were isolated by Trainotti *et al*. (2001) from a library constructed in ripe fruit. The expression pattern of the three genes was complex, and only *Faβgal1* showed an increasingly high expression along the ripening process. Furthermore, *Faβgal1* and *Faβgal2* contain a lectin-like domain in its C-terminus to anchor itself to specific sugars. This domain could increase its efficiency in releasing galactose residues from cell wall polymers.

CONCLUDING REMARKS

Several authors have proposed strawberry as a model for the study of the ripening process in non-climacteric fruit. The significant advances achieved in our knowledge of genetic factors involved in strawberry fruit softening and other ripening components, such as aroma development, support this proposal. Nowadays, strawberry is probably the second fruit in importance, behind tomato, where more ripening-related genes have been assessed by transgenesis. Concerning softening, cell wall disassembly seems to be the most determinant factor in the loss of firm texture during ripening. This process involves the coordinate action of numerous enzymes, acting on different wall polysaccharides. Previous studies in tomato led to the view that the modification of a complex trait as fruit texture would be rather difficult through the manipulation of a single gene. However, strawberry fruit firmness was significantly improved by the silencing of pectinase genes (Jiménez-Bermúdez *et al*. 2002; Quesada *et al*. 2009a). Moreover, transcriptomic analysis of *FaPG1* down-regulated fruits showed minor changes in the expression of many other ripening genes, although a consistent increase in fruit firmness was obtained in these transgenic fruit (Quesada *et al*. 2009a). These results indicate that the transgenic manipulation of key cell wall genes has a potential interest to improve fruit texture.

At present, transgenic studies of fruit softening in strawberry have been restricted to few members of the main cell wall gene families, i.e. endo-β-glucanase, polygalacturonase, pectin methyl esterase, expansin. Interestingly, xyloglucan-endotransglycosilase/hydrolase (XTH) genes, which have an important role in xyloglucan metabolism (Goulão and Oliveira 2008), have not been reported yet in strawberry. New genes acting on different cell wall components should be tested to shed light on the softening process in strawberry fruit. XTH, β-xylosidase, β-galactosidase, α-arabinofuranosidase or enzymes degrading rhamnogalactunorane I are candidates to these future studies. Furthermore, considering the complexity of the cell wall and the coordinate action of the different genes involved in its processing, the simultaneous silencing of genes from different cell wall families appears as an essential tool to gain an insight into this process.

The transgenic manipulation of pectinase genes has proven to be successful to improve strawberry softening and to extend the shelf life of this extremely delicate fruit. From a commercial point of view, the main concern to this approach is the poor public acceptance of transgenic crops, particularly important in the case of fruits. To overcome this problem, some authors have proposed the use of native genes from the crop plant itself or from crossable species for genetic modification. The so called intragenic (Rommens 2004) or cisgenic (Schouten *et al*. 2006) approaches would be therefore comparable to classical breeding and should be exempted from the GMO regulation. The recent finding of a significant fruit firmness increase in strawberry plants transformed with a sense sequence of the *plC* gene (Yousseff *et al*. 2009) indicates that intragenic strawberry plants with improved fruit texture can be obtained, providing that the cell wall gene were conducted by a strong promoter to induce co-suppression. To fully accomplish this biotechnological approach, the search for ripening-specific promoters and the application of protocols for the elimination of marker genes are research areas that should be strengthened.

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