RESEARCH PAPER

Antisense inhibition of a pectate lyase gene supports a role for pectin depolymerization in strawberry fruit softening

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Abstract

Cell wall disassembly in softening fruits is a complex process involving the cumulative action of many families of wall-modifying proteins on interconnected polysaccharide matrices. One strategy to elucidate the in vivo substrates of specific enzymes and their relative importance and contribution to wall modification is to suppress their expression in transgenic fruit. It has been reported previously that inhibiting the expression of pectate lyase genes by antisense technology in strawberry (Fragaria × ananassa Duch.) fruit resulted in prolonged fruit firmness. This suggested that pectin depolymerization might make a more important contribution to strawberry fruit softening than is often stated. In this present study, three independent transgenic lines were identified exhibiting a greater than 90% reduction in pectate lyase transcript abundance. Analyses of sequential cell wall extracts from the transgenic and control fruit collectively showed clear quantitative and qualitative differences in the extractability and molecular masses of populations of pectin polymers. Wall extracts from transgenic fruits showed a reduction in pectin solubility and decreased depolymerization of more tightly bound polyuronides. Additional patterns of differential extraction of other wall-associated pectin subclasses were apparent, particularly in the sodium carbonate- and chelator-soluble polymers. In addition, microscopic studies revealed that the typical ripening-associated loss of cell–cell adhesion was substantially reduced in the transgenic fruits. These results indicate that pectate lyase plays an important degradative role in the primary wall and middle lamella in ripening strawberry fruit, and should be included in synergistic models of cell wall disassembly.

Key words: Cell wall, Fragaria, fruit ripening, pectate lyase, pectinases, strawberry.

Introduction

The ripening of fleshy fruits is commonly accompanied by pronounced softening, which has been mechanistically linked with both enzyme-mediated (Brummell and Harpster, 2001; Brummell, 2006) and non-enzymatic (Dumville and Fry, 2003) cell wall degradation. Recent evidence suggests that ripening-related changes in fruit cell turgor pressure also have a critical influence on fruit firmness (Saladie et al., 2007), but to date far more studies have focused on changes in wall composition, architecture, and associated wall-modifying proteins (Vicente et al., 2007). A number of specific changes in wall structure appear common to most ripening fruits and a general model for cell wall disassembly can be widely applied. However, more detailed studies of wall metabolism have revealed clear examples of interspecific variability (Brummell and Harpster, 2001; Rose et al., 2003). Some of these can be correlated with differences in the texture of the ripe fruit that can, for example, be classified as soft and melting, or crisp.

Strawberry (Fragaria × ananassa Duch.) is included in a group of fruits that develop a soft melting texture during growth and ripening. This coincides with numerous
modifications of the primary cell wall and degradation of the middle lamella, including an increase in pectin solubilization, depolymerization of xyloglucan, and cell wall swelling (Redgwell et al., 1997b; Koh and Melton, 2002). The swelling phenomenon in turn correlates in vitro with pectin metabolism and possibly loosening of the xyloglucan–cellulose network in vivo (Redgwell et al., 1997b; Brummell, 2006). A number of other studies have reported particular aspects of wall restructuring in ripening strawberry fruit (e.g. Lara et al., 2004; Roshli et al., 2004) and, in addition to the widely reported increase in pectin solubilization, several have described hemicellulose depolymerization (Huber, 1984; Nogata et al., 1996; Rosli et al., 2004). It has been suggested that cellulose is not degraded during strawberry ripening and thus may not play a significant role in fruit softening (Koh et al., 1997; Koh and Melton, 2002) and recent reports showed that the inhibition of several endoglucanase genes [from glycosyl hydrolase family 9 (GH9)] did not perceptibly affect fruit firmness (Woolley et al., 2001; Palomer et al., 2006). However, it should be noted that assays to evaluate cellulose levels have typically targeted only levels of crystalline cellulose and significant degradation of para-crystalline cellulose may occur (Rose et al., 2003), which is much more difficult to measure. Moreover, the in vivo substrates of plant GH9 enzymes have still not been determined (Urbanowicz et al., 2007) and the mismatch between substrates and enzyme activities in this case clearly complicates data interpretation.

By contrast, relatively more is known about the enzymology of ripening-related pectin modification and, as discussed by Koh and Melton (2002) and references herein, pectin solubilization can be ascribed to a range of different processes: pectin depolymerization; loss of calcium stabilized pectin gel structure (Knee et al., 1977; Lara et al., 2004); cleavage of linkages between pectins and hemicellulose (Nogata et al., 1996); and disentanglement of pectin complexes following the hydrolysis of arabinan and possibly also galactan side chains (Redgwell et al., 1997a; Trainotti et al., 2001; Koh and Melton, 2002). Of these processes, pectin depolymerization has sometimes been considered of minor importance in ripening strawberry fruit, due to the limited degree of depolymerization compared with that seen in some other fruits, which is consistent with the low or undetectable polygalacturonase (PG) activity (Huber, 1984; Nogata et al., 1996; Brummell, 2006). However, other studies have detected more substantial depolymerization of certain pectin fractions (Rosli et al., 2004), and a recent paper described a soft-fruited strawberry cultivar (cv. Toyonaka) with high PG activity (Villareal et al., 2007).

Another enzyme that may contribute to ripening-related pectin degradation is pectate lyase, which catalyses the cleavage of unesterified galacturonosyl linkages by a \( \beta \)-elimination reaction (Marín-Rodríguez et al., 2002). Several pectate lyase genes have been identified in different fruits, with expression patterns increasing during ripening (Domínguez-Puigjaner et al., 1997; Medina-Escobar et al., 1997; Nunan et al., 2001), but in general far less is known about pectate lyase activity, localization, regulation, or contribution to pectinolysis than PG (Brummell and Harpster, 2001). It has been reported previously that inhibiting the expression of a strawberry pectate lyase gene by antisense transformation resulted in firmer fruits with an extended post-harvest shelf-life (Jiménez-Bermúdez et al., 2002). Three fruit-specific pectate lyase genes with similar expression patterns have been described (Benítez-Burraco et al., 2003), and introduction of the antisense transgene resulted in the suppression of all three in ripe fruits (Benítez-Burraco et al., 2003). This therefore represents a potentially valuable experimental system in which to better understand the contribution of pectate lyases to ripening-related pectin degradation. A preliminary study of the cell wall composition of the pectate lyase antisense fruits suggested a lower degree of cell wall swelling and possibly pectin solubilization (Jiménez-Bermúdez et al., 2002), but a detailed analysis of wall structure was not described. The goal of this present analysis was to extend the study and evaluate more fully the consequences of pectate lyase suppression on strawberry fruit cell wall architecture and, particularly, the pectin polymer network. Data are presented that support a role for pectate lyase in pectin depolymerization and solubilization in the cell wall and/or middle lamella, and it is suggested that these enzymes should be included in models of synergistic ripening-related cell wall disassembly.

**Materials and methods**

*Plant material, growth conditions, and firmness measurements*

Control, non-transformed strawberry (*Fragaria ×ananassa* Duch. cv. Chandler) plants and three independent transgenic pectate antisense lyase lines (Apel 14, Apel 23, and Apel 39; described in Jiménez-Bermúdez et al., 2002) were grown in a greenhouse under natural light. Transgenic ripe fruits showed a strong reduction in pectate lyase mRNA levels, ranging from 90% in Apel 14 to 99% in Apel 23 and Apel 39. Fruits were harvested at different developmental stages [small green, G1; large green, G2; white, W; turning (meaning at least 25% surface red), T; totally red ripe fruit, R; and overripe fruit (ripe fruits stored 3 d at 25 °C in a growth chamber), OR], frozen in liquid nitrogen, and stored at −25 °C until used. Firmness was measured in fresh fruits using a hand penetrometer with 3.1 or 9.6 mm² end surface area cylindrical probes, puncturing twice, on opposite sides, per fruit. A minimum of 25 fruits per developmental stage was measured. Anthocyanin content was assayed in ripe fruits as previously described (Jiménez-Bermúdez et al., 2002) and expressed as the difference of absorbance between 530 nm and 657 nm per gram fresh weight.

**Cell wall isolation**

Cell walls from green (G2) or ripe (R) fruits were isolated according to Redgwell et al. (1992), with some minor modifications. Briefly,
10–15 fruits were powered in liquid nitrogen and 20 g 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, Nottingham, UK). The pellet was resuspended in 20 ml of water and the supernatant recovered as above. This step was performed twice. All supernatants were combined and dialysed (mol. wt cut-off 8000) against distilled water for 5 d at 4 °C. After dialysis, the PAW extract was centrifuged at 23 000 g for 20 min, and the supernatant was concentrated in a rotary evaporator to ~5 ml, and finally freeze dried. This extract constitutes the PAW fraction. The residue from the first centrifugation, containing the cell wall material (CWM), was incubated overnight in 20 ml 90% DMSO to solubilize starch. The extract was then centrifuged at 4000 g, the pellet washed twice with 20 ml distilled water, and the CWM recovered after freeze-drying. A minimum of three independent extractions per line and developmental stage were performed.

Cell wall fractionation

The CWM was sequentially extracted with water, 50 mM trans-1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid (CDTA) in 50 mM sodium acetate (pH 6), followed by 100 mM Na2CO3 containing 0.1% NaBH4 to provide pectin-enriched fractions, and then with 1 M KOH and 4 M KOH to generate hemicellulose-enriched fractions. The procedure was as follows: CWM from the three extractions was pooled and 90 mg was incubated overnight in 45 ml of distilled water on an orbital shaker. The extract was then centrifuged at 6000 g for 15 min and then the process was repeated with another 45 ml of water. The supernatants were combined, filtered through a GF/C (Whatman, UK) glass-fibre filter, and dialysed against water in the same conditions as described for PAW extract. After dialysis the extract was concentrated with a rotary evaporator and finally freeze dried. Water-insoluble residue was washed twice with distilled water and extracted with the next reagent, following the same procedure to obtain the CDTA-, Na2CO3-, and KOH-soluble fractions. At least three independent fractionations were performed per CWM sample.

The uronic acid (UA) content in the different fractions was estimated using the assay of Blumenkrantz and Asboe-Hansen (1973), as modified by Van den Hoogen et al. (1998), and total sugars by the orcinol-sulphuric method (Dubois et al., 1956).

Anatomical sections of fruit parenchyma

Small cortical tissue fragments were obtained from ripe fruits of control and transgenic lines, a minimum of five independent fruits per genotype being processed. Cortical tissue corresponded to cylinders obtained from the zone of maximum diameter of the fruits. Tissue samples were fixed in Bouin reagent (Panreac Quimica, Spain) for 24 h and dehydrated in an increasing concentration series of ethanol:water to 95%. Embedding was performed in Historesin (Historesin Embedding Kit, Leica) following the manufacturer’s instructions. When polymerized, 4-μm-thick sections were obtained with a microtome, mounted on glass slides, and stained with 0.2 g l−1 ruthenium red for 2 h.

Size exclusion chromatography

A manually poured column (0.8×95 cm) of Sepharose CL-6B (Sigma-Aldrich Química SA, Spain) was used to fractionate the polymers present in the PAW and water fractions. Gel medium was equilibrated with 0.2 M acetate buffer, pH 5, and cell wall extracts (10–14 mg) were dissolved in 1 ml buffer, loaded on the column, and eluted at 14 ml h−1. Two millilitre fractions were collected and assayed for UA and total sugar contents. Similarly, manually poured columns (0.5×45 cm) of Sepharose CL-2B were used to fractionate material in the CDTA, Na2CO3-, and the two KOH fractions. In the case of the CDTA extract, gel medium was equilibrated in the same buffer as above, but for the other extracts the columns were equilibrated with TRIS–HCl 0.1 M buffer, pH 8.5. Samples were dissolved in equilibration buffer (6–8 mg ml−1) and 200 μl loaded on the column, then eluted at a 14 ml h−1 flow rate. One millilitre fractions were collected and assayed for UA and total sugar contents.

Neutral sugar composition of the polymers present in the 4 M KOH extract was determined by GC-MS of their alditol acetates following hydrolysis in 2 M trifluoroacetic acid as described in Fry (1988).

Statistical analyses

The SPSS software package (SPSS Inc., Chicago, IL, USA) was used for all the statistical analyses. Mean comparisons between control and transgenic samples were performed using a t-test, p=0.05. When data did not fulfil the requirement for this parametric test, a Mann–Whitney U-test was employed to determine differences. Unless stated otherwise, data are presented as means and the bar represents standard deviations.

Results

Stability of the phenotype and ripening stage

Figure 1A shows the consistency and stability of the ‘firmer fruit’ phenotype that was previously reported for pectate lyase antisense transgenic strawberry lines (Jiménez-Bermúdez et al., 2002). During several growing seasons, transgenic fresh fruits were consistently significantly firmer than control fruits during ripening. The present study focused on the cell walls of red ripe (R) fruit at harvest and so special care was taken when sampling the fruits to avoid differences in ripening stage which might influence the results. Figure 1B shows the mean values for anthocyanin content, which was used to monitor ripening in control and transgenic fruits. Anthocyanin levels were similar in control and transgenic lines 23 and 39, and slightly higher in line 14, indicating that transgenic fruits sampled for cell wall analysis were harvested at equivalent ripening stages.

Cell wall yield and fractionation

Cell wall content (dry mass CWM) per 100 g of fruit fresh weight ranged from 0.92 g to 1.43 g. The highest value (transgenic line 23) was statistically different from the control (P=0.95) while the other two transgenic lines were similar to control fruits (average = 1.0±0.1 g per 100 g fresh fruit weight). Cell wall polymers were not only present in the CWM extract but were also detected in the PAW fraction, and so, after dialysis and drying, the content of this fraction was related to cell wall weight and presented together with the amounts of the different fractions that were sequentially extracted from the CWM (Fig. 2). The mean values of PAW contents were lower in the three transgenic lines and the differences were statistically significant for lines 23 and 39. A similar trend
was observed when the PAW contents were related to fresh fruit weight. This lower content of PAW fraction can be interpreted as reflecting lower solubilization of cell wall polymers in the transgenic fruits. In all the genotypes the PAW fraction polymer content was higher in red than in green fruit, but the ripening-related increase was less in the transgenic lines (128%, 27%, and 142% for Apel lines 14, 23, and 39, respectively) than in the control fruit (427%), which further supports the idea of a lower solubilization in the transgenic fruits.

Figure 2 also shows the yield of the different CWM fractions. The pool of all the fractions obtained, including the remaining material after 4 M KOH extraction, represented on average 76±4% (w/w) of the initial cell wall weight used for sequential extraction, suggesting low losses during processing. The most abundant fraction was the CWM solubilized with 50 mM CDTA. Statistical differences between control and the three transgenic lines were only observed for the water-extracted fraction, where the amount was higher from the control cell walls.

**Pectin content and solubilization**

In all the genotypes, the highest content of UA was found in the PAW extracts, with mean values lower for the three transgenic lines, although the differences were only statistically significant for line 23 (Fig. 3A). Among the various cell wall fractions, the highest UA levels were...
present in the CDTA fraction and the lowest amounts in the ‘hemicellulosic’ 4 M KOH fraction. The amount of pectin (inferred from UA abundance) was significantly higher in the CDTA and sodium carbonate (CO3) extracts from the cell wall of all three transgenic lines than from those of the control fruits (Fig. 3A). It is noteworthy that the weights of these two fractions were not quantitatively different between control and transgenic lines in Fig. 2. The ratio of more soluble (PAW+water fractions) to bound (CDTA+CO3 fractions) pectins was significantly higher in the control (3.0) than the transgenic lines (1.8), and this difference was even higher if the soluble fraction was related to the covalently bound fraction (CO3) alone (8.2 and 4.0 in control and transgenic lines, respectively). Total sugars were also measured in the different cell wall fractions but no significant differences were found between control and transgenic lines (results not shown). Interestingly, the UA/sugar ratios were higher for the three transgenic lines in PAW, water, CDTA, carbonate, and 1 M KOH fractions, indicating that the cell wall fractions were enriched with pectins in the case of transgenic fruits (Fig. 3B). The only exception to this trend was in the CDTA fraction of the Apel 39 line. Collectively, these results suggest a lower pectin solubilization in the transgenic Apel lines.

This interpretation of decreased pectin solubilization was supported by microscopic evidence (Fig. 4) since a denser pectin staining was observed between cells in cortical tissues from transgenic fruits than in control fruits. Additionally, fewer intercellular spaces were observed in the transgenic fruit tissues, whose cells showed greater cell adherence than those in sections from control fruit. This apparently greater cell to cell adhesion in the transgenic samples suggests a greater integrity of the pectin-rich middle lamella.

**Analysis of the soluble fractions by gel permeation chromatography**

The profiles of the pectic polymers present in the PAW and water fractions are shown in Fig. 5. Size exclusion chromatography revealed that the PAW-soluble polyuronides formed a main peak of polymers with molecular mass >500 kDa, together with a tail of lower molecular-

mass material extending throughout the separation range of the column. Comparisons of profiles from the three transgenic fruits with the control sample suggested a slight increase in the average molecular mass of the soluble pectin in the transgenic samples. A more symmetrical population of water-solubilized polymers eluted mostly between 100 ml and 180 ml in all the genotypes, with an average molecular mass that was clearly lower than those solubilized with PAW. The profiles appeared similar, although three distinct peaks were observed in the control sample between approximately 20 kDa and 80 kDa, which were not resolved in the transgenic lines. The majority of the water-solubilized polyuronides migrated with apparent masses of 20–50 kDa.

**Analysis of the bound fractions by gel permeation chromatography**

A CDTA solvent was used, ostensibly to extract polymers that were ionically bound to the cell wall, and the resulting polyuronide profiles for the different genotypes are shown in Fig. 6 (left side). Polymers eluted throughout much of the fractionation range of the column, with most exhibiting a mass in excess of 2000 kDa. The main difference between control and transgenic lines was the presence of a substantial peak of high molecular-weight material in the control line, which was not apparent in wall extracts from the transgenic fruits. Two additional peaks were present in the control fraction (eluting at 18 ml and 23 ml) that were not evident in lines 14, 23, and 39.

The profiles obtained for polyuronides solubilized with Na2CO3 (CO3 fraction; Fig. 6, right side) showed the presence of a first peak eluting between 10 ml and 15 ml which corresponds to very high molecular-weight pectic polymers. The three transgenic lines showed a slight increase in the average molecular weight of this first peak and it was sharper and more pronounced in lines 14 and 23. A second group of pectic polymers (fractions eluting between 15 ml and 20 ml) was present in the three transgenic lines and essentially absent in the control, although a small shoulder was observed. The pool of polymers eluting between 20 ml and 30 ml represented at least 50% of the polyuronides present in this fraction and the profiles of the transgenic lines suggest that pectin processing is different in these fruits. It was observed that the CO3 fractions from the transgenic fruit had peaks (resolved at 13 ml and 18 ml) that were not present in the control fruit CO3 profiles, but that similarly eluting peaks were differentially present in the control fruit CDTA fraction. It should also be noted that in the CO3 transgenic profiles the elution time of the first peak suggested a higher molecular mass. These observations could be conceptually related with a reduced pectin depolymerization as a result of pectate lyase silencing. To gain further support for this hypothesis, the pectin profiles of the CO3 fraction were

![Fig. 4. Sections of cortical fruit tissue at the ripe stage of control and two independent transgenic lines, Apel 14 and 39, stained for pectin visualization. Bars represent 50 μm.](image-url)
analysed from green fruits collected prior to the decrease in firmness that occurs in the transition from green to white fruits (Fig. 7A). Increased fruit firmness was only observed in the transgenic fruits after this transition (Fig. 7B), and it was reasoned that the differences in cell wall must be minor at the green stage, and would not be related to the effects of the transgene, since the expression of the pectate lyases is ripening specific. The profiles obtained for the CO3 fractions from the cell wall of green fruits are shown in Fig. 8. The extracts from all four genotypes had two predominant populations of polyuronides, one of which eluted close to the first range mentioned for red fruits with a peak maximum at 10 ml, while the second of which comprised medium molecular-weight polymers. As expected, the profiles from the control and transgenic lines were similar, although line 39 had proportionally less high molecular-weight material. A comparison of the CO3 profiles from green fruits (Fig. 8) with those from red fruits (Fig. 6) revealed a reduction of the first peak (eluting between 8 ml and 12 ml) relative to the second (eluting between 18 ml and 30 ml) in both the control and transgenic lines 14 and 23 during ripening. However, these decreases were much more evident in extracts from the control fruits (50%) than from the transgenic lines (12% and 9% for lines 14 and 23, respectively). Additionally, the profiles of cell walls obtained from red transgenic fruits showed the presence of a pool of polymers eluted between 15 ml and 20 ml that was absent in the control cell wall of red fruits (Fig. 6).

The KOH extracts were enriched in hemicellulose but also contained pectins, based on the levels of UA, and differences were observed in the UA/total sugar ratio between the control and the three transgenic lines (Fig. 3B). The profiles obtained for UA content in these two fractions in red fruits are shown in Fig. 9. The 1 M KOH profiles for control, line 14, and line 23 suggested relatively low amounts of residual polymers, but line 39 again exhibited different characteristics with significantly higher absorbance values and several well-defined peaks. Although the amount of pectin was low in the 4 M KOH fractions, the profiles indicated the presence of high molecular-weight polymers in the three transgenic lines that were absent in the control profile (10–13 ml and 18–20 ml elution volumes). These polymers may correspond to those observed in the CO3 fractions as they eluted in the same position.

Analyses of total sugar levels in the same fractions of the two KOH extracts (Fig. 10) revealed consistent
quantitative differences in that the peak heights were lower in the transgenic lines. An analysis of TFA-hydrolysable neutral sugars in the 4 M KOH fraction by GC-MS showed that glucose, xylose, and rhamnose predominated (with a molar ratio of 6:2.5:1, respectively) suggesting, as expected, a substantial enrichment in xyloglucan, but also the presence of rhamnogalacturonan pectins.

Discussion

The goal of this study was to characterize the effects on the strawberry fruit cell wall of suppressing the expression of ripening-related pectate lyase, and to relate pectin metabolism to the previously reported prolonged fruit firmness of the transgenic fruits. A sequential series of solvents was used to obtain fractions enriched in pectins that differed in how readily they were extracted. This approach can indicate their relative affinity with the wall and middle lamella, and thus provide an indirect measure of pectin macromolecular disassembly. For example, PAW is considered a poor solvent for pectic polysaccharides (Fry, 1988) unless they have been solubilized by in vivo processes within the wall (Redgwell et al., 1992, 1997b), while CDTA and sodium carbonate (CO3) are typically used to enrich for pectins that are ionically or covalently bound into the wall matrix, respectively.

The amount of material in the PAW fraction relative to the total cell wall was lower in all three transgenic lines, as was the UA content, although the ratio of UA:total sugars was not statistically different from the control fruit (Fig. 3). Additionally, the Apel lines showed less of a ripening-related increase in polysaccharide levels in the PAW fraction (Fig. 2) as well as an increase in average polymer size (Fig. 5). Similar results were obtained with the water-soluble fraction, which also contains more readily extractable polymers that interact relatively weakly with other cell wall components. The polyuronide molecular-weight profiles were similar in the control and transgenic profiles (Fig. 5), but the higher amount of material in the control water fraction (Fig. 2), which was pectin rich, based on the increased UA:total sugar ratio (Fig. 3), further suggests that pectins were less soluble from walls of the transgenic fruits.

Conversely, the CDTA and CO3 fractions comprise polymers that show a greater wall association. Clear quantitative differences between control and transgenic fruits were observed in the CDTA-soluble ionically bound pectin fraction (Fig. 3). This fraction is believed to be particularly enriched in homogalacturonan pectin from the middle lamella (Redgwell et al., 1992; Lara et al., 2004)

Fig. 6. Molecular mass profiles of polyuronides extractable by CDTA and sodium carbonate from fruit cell walls of control and three independent Apel transgenic lines. Profiles were obtained by size exclusion chromatography on Sepharose CL-2B. Fractions were assayed for uronic acid content. Uronic content of the fractions was normalized to the maximum absorbance value obtained in each profile. The elution volume for the blue dextran standard (2000 kDa) is shown on the figure.
and, relative to control cell walls, the UA content of this fraction was on average 52% higher in the three transgenic lines. Microscopic analyses also suggested reduced degradation of the middle lamella in the transgenic fruits (Fig. 4). In a preliminary study of the Apel cell walls using a different extraction protocol, a lower amount of CDTA-soluble polyuronides had previously been found in one of the transgenic lines (Jimenez-Bermudez et al., 2002). However, in that case, the starting material for the sequential extraction was an alcohol-insoluble residue, and it is likely that the chelator-soluble fraction contained pectins that were partitioned between the PAW and water fractions in the present study; hence the discrepancy.

Chromatographic analyses of the polyuronide populations in the CDTA and CO3 fractions also suggested qualitative and quantitative differences between the control and transgenic fruits. The levels of high molecular-weight polymers in the CO3 fraction were substantially higher in Apel 14 and somewhat greater in the other two transgenic lines, and as described in Results, some peaks were differentially seen in the CDTA and CO3 profiles. For example, the CDTA control fruit extract had a profile with two peaks of high molecular-mass polymers (elution volumes 13 ml and 18 ml), which were diminished or absent in the transgenic profiles. However, peaks at similar elution volumes were extracted with Na2CO3 (Apel 14, Apel 23, and partially in Apel 39) or 4 M KOH (Apel 39). This suggests differential processing of the pectic polymers in the transgenic lines resulting in a lower solubilization of the highest molecular-mass group of polymers.

The substantial quantitative and qualitative changes in the various cell wall fractions found to occur in Na2CO3- and KOH-solubilized fractions lead to the proposal of a model whereby pectate lyase silencing leads to decreased depolymerization of the strongly bound pectins fractions, alters the interactions between various components of the pectic matrix, and increases the solubility of a subset of pectins. This may be either a direct consequence of reducing pectate lyase activity, or also indirectly by altering the action of other pectinolytic enzymes, such as PG and pectin methylesterase, for example by influencing access to their substrates. It is also noted that polyuronide accumulation continues during strawberry fruit ripening (Huber, 1984), and so it is possible that suppressing pectate lyase expression could affect wall composition and architecture by perturbing normal wall assembly. Immunolocalization studies have suggested that pectate lyase proteins are present in the primary walls of strawberry receptacle parenchyma cells (Benitez-Burraco et al., 2003), but the microscopy results shown here (Fig. 4) indicate that degradation of the middle lamella is significantly reduced in the transgenic fruits, which suggests that this is also the site of pectate lyase action. Moreover, a recent study of cell–cell adhesion in root parenchymatous tissue suggested that Na2CO3-soluble pectic polymers play an important role (Marry et al., 2006), which is congruent with the reduced depolymerization and solubilization of this wall fraction in the transgenic strawberry lines.

The cell wall analyses also suggested a decreased content of hemicelluloses in the three transgenic lines. Microscopic analyses also suggested reduced degradation of the middle lamella in the transgenic fruits (Fig. 4). In a preliminary study of the Apel cell walls using a different extraction protocol, a lower amount of CDTA-soluble polyuronides had previously been found in one of the transgenic lines (Jimenez-Bermudez et al., 2002). However, in that case, the starting material for the sequential extraction was an alcohol-insoluble residue, and it is likely that the chelator-soluble fraction contained pectins that were partitioned between the PAW and water fractions in the present study; hence the discrepancy.

Chromatographic analyses of the polyuronide populations in the CDTA and CO3 fractions also suggested qualitative and quantitative differences between the control and transgenic fruits. The levels of high molecular-

![Fig. 7. Fruit weight and firmness of control and transgenic lines during fruit development.](image-url)
between the major cell polysaccharide networks (Rose and Bennett, 1999; Vicente et al., 2007).

In summary, it has been demonstrated that the increased firmness of three independent transgenic lines of strawberry fruit exhibiting strong suppression of pectate lyase genes is correlated with consistent differences in pectin metabolism, polymeric interactions, and cell–cell adhesion. These results, as well as those reported by Rosli et al. (2004) and Villareal et al. (2007), suggest that enzymes involved in pectin depolymerization play a more
significant role in strawberry fruit softening than previously appreciated. Furthermore, this study emphasizes that pectate lyase, although far less studied than other pectinolytic enzymes such as PG and pectin methylesterase, is an important member of the likely synergistic suite of pectinases that contribute to cell wall disassembly.

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Fig. 10. Molecular mass profiles of cell wall polymers extractable by 1 M and 4 M KOH from fruit of control and three independent Apel transgenic lines. Profiles were obtained by size exclusion chromatography on Sepharose CL-2B. Fractions were assayed for total sugar content and presented as optical density values at 450 nm. The elution volume for the blue dextran standard (2000 kDa) is shown on the figure.
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