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Cell Wall Metabolism in Ripening Fruit[1]

VI. Effect of the Antisense Polygalacturonase Gene on Cell Wall Changes Accompanying Ripening in Transgenic Tomatoes

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Cell walls of tomato (*Lycopersicon esculentum* Mill.) fruit, prepared so as to minimize residual hydrolytic activity and autolysis, exhibit increasing solubilization of pectins as ripening proceeds, and this process is not evident in fruit from transgenic plants with the antisense gene for polygalacturonase (PG). A comparison of activities of a number of possible cell wall hydrolases indicated that antisense fruit differ from control fruit specifically in their low PG activity. The composition of cell wall fractions of mature green fruit from transgenic and control (wild-type) plants were indistinguishable except for *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid (CDTA)-soluble pectins of transgenic fruit, which had elevated levels of arabinose and galactose. Neutral polysaccharides and polyuronides increased in the water-soluble fraction of wild-type fruit during ripening, and this was matched by a decline in Na_2CO_3-soluble pectins, equal in magnitude and timing. This, together with compositional analysis showing increasing galactose, arabinose, and rhamnose in the water-soluble fraction, mirrored by a decline of these same residues in the Na_2CO_3-soluble pectins, suggests that the polyuronides and neutral polysaccharides solubilized by PG come from the Na_2CO_3-soluble fraction of the tomato cell wall. In addition to the loss of galactose from the cell wall as a result of PG activity, both antisense and control fruit exhibit an independent decline in galactose in both the CDTA-soluble and Na_2CO_3-soluble fractions, which may play a role in fruit softening.

Although the cell wall changes that result in fruit softening during ripening are still being unraveled (Brady, 1987; Fischer and Bennett, 1991), this process has been particularly well studied in tomato (*Lycopersicon esculentum*), where both solubilization of polyuronides and loss of Gal and Ara from the pectic fraction (Gross and Wallner, 1979; Gross, 1984; Seymour et al., 1990) have been implicated. Naturally occurring ripening mutants have been exploited in tomato (Gross, 1984; DellaPenna et al., 1987), but their usefulness has been hampered by the pleiotropic effects they exhibit. The development of transgenic tomato lines where the expression of a single cell wall-degrading enzyme is drastically reduced by incorporation of an antisense gene is particularly promising because it allows the role of specific enzymes to be evaluated. This approach suggests that neither PG (Smith et al., 1988) nor pectin methylesterase (Tieman et al., 1992) alone brings about fruit softening in tomato.

In this paper, we examine the cell wall changes in ripening tomato fruit in which transformation with the antisense gene for PG prevents the rise in PG activity characteristic of normal fruit. What is the in vivo role of PG in the ripening tomato fruit? Polyuronide levels would be expected to be dramatically affected by the presence or absence of PG. Changes in other wall components would imply a linkage of these components in the wall matrix to polyuronides or would suggest that modification of these polymers is triggered as a consequence of PG expression.

MATERIALS AND METHODS

Plant Material

Field-grown mature green, turning, and red tomato (*Lycopersicon esculentum* Mill.) fruit of transgenic plants (line CR3) with the antisense gene for PG and of control plants were supplied by Calgene, Inc. (Davis, CA). Ripeness stage was based on fruit color. Color was measured with a Minolta CR-200 reflectance colorimeter and is reported as the a* values from the L*a*b* uniform color space (CIELAB). a* indicates hue on a green (−) to red (+) axis. Mean a* values were −5.7, +1.0, and +17.6 for control green, turning, and red fruit, respectively, and −6.8, −0.59, and +14.6 for anti-sense green, turning, and red fruit, respectively. Outer pericarp, cut into segments (approximately 1 cm^2), was prepared from both control and antisense fruit of each stage and stored at −20°C until use.

A separate group of fruits, representing the color stages used for cell wall and enzyme analyses, was taken to determine the relationship between flesh color (a* value) and flesh firmness. Firmness was measured with a penetrometer on discs cut from the outer pericarp of two or three fruits at each color state (Campbell et al., 1990). Values for disc color and firmness are presented in Table I.

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Abbreviations: CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid; CDTA-F, CDTA-soluble fraction; CO_3-F, 0.1 M Na_2CO_3-soluble fraction; CWM, cell wall material; PG, polygalacturonase; Rha, rhamnose; WSF, water-soluble fraction.

Table I. *Tomato flesh color (means of a* values of five replicates) and flesh firmness (means of five replicates; values are g force) for ripening control and antisense tomato fruits*

Ripeness Stage	Control Fruit		Antisense Fruit	
	Color	Firmness	Color	Firmness
Mature green	−6.2	283	−6.6	284
Turning	−0.1	180	−0.2	182
Red	17.9	24	18.4	164

Protein Extraction and Enzyme Assays

Salt-extracted enzyme fractions were prepared from triplicate 25-g samples of outer pericarp tissue of control and antisense fruit of each stage by the method of DellaPenna et al. (1987) except that 5 mM mercaptoethanol was substituted for 1 mM DTT throughout. Protein was assayed by the method of Bradford (1976). PG activity was measured by following the generation of reducing groups during a 30-min incubation of 50 μL of enzyme extract (5 μg of protein) with 200 μL of polygalacturonic acid (1 mg/mL) at 30°C using the method of Gross (1982). α-Arabinosidase, β-galactosidase, and α-galactosidase were assayed by incubating 50 μL of enzyme extract with 200 μL of 3 mM *p*-nitrophenyl glycoside in 50 mM acetate buffer, pH 4.7, for 1 h at 30°C (16 h for α-arabinosidase) and measuring A_{400} after the addition of 1 mL of 1 M Na_2CO_3.

Cell Wall Preparation, Fractionation, and Analysis

Triplicate 15-g portions of outer pericarp tissue from both control and antisense fruit at each maturity stage were heated in a boiling water bath for 10 min with 50 mL of 95% ethanol. (We have found that this treatment eliminates autolytic generation of reducing end groups from subsequent cell wall preparations.) Boiled discs were allowed to cool and then were homogenized (Polytron). Insoluble material was collected on a sintered glass funnel, washed with acetone until it was decolorized, and was then air dried. Portions (100 mg) of this CWM were slurried for 4 h at room temperature with 30 mL of 0.01% NaN_3 and centrifuged, and the supernatant (WSF) was removed. The pellet was then slurried sequentially with 30 mL of each of the following for 1 d at room temperature: (a) 0.05 M CDTA in 0.05 M acetate buffer, pH 6; (b) 0.1 M Na_2CO_3, 1 mg/mL $NaBH_4$; and (c) 4 N KOH, 1 mg/mL $NaBH_4$. After each extraction, the slurry was centrifuged, the supernatant was removed (and neutralized with glacial CH_3COOH in the case of 4 N KOH) and dialyzed exhaustively for 2 d at 4°C against several changes of distilled H_2O, and the volume of each dialysate was recorded. Aliquots of each fraction were assayed for neutral sugars by the anthrone method (Dische, 1962) and for uronic acids by the *m*-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen, 1973). A further aliquot was freeze-dried and hydrolyzed with 2 N TFA for 1 h at 121°C, and the resulting sugars were then converted to alditol acetates (Blakeney et al., 1983) and analyzed using a Perkin-Elmer 8320 gas chromatograph fitted with a DB-23 (J & W Scientific) capillary column (30 m × 0.25 mm, i.d.) using H_2 as carrier gas and with the oven temperature programmed from 200° to 213°C at 1°C/min. Integration of the flame-ionization detector signal was provided by a Perkin-Elmer (Sigma 10) data system. The hemicellulose fraction solubilized by 4 N KOH was assayed for xyloglucan (Kooiman, 1960) using as standard tamarind xyloglucan prepared from seeds of *Tamarindus indica* L. (Hayashi and Maclachlan, 1984). The α-cellulose pellet remaining after the sequential extraction was dried and weighed, and a 5-mg portion was analyzed for total uronic acids (Ahmed and Labavitch, 1977).

RESULTS

Enzyme Activities

Figure 1A shows control fruit exhibiting the typical dramatic increase in PG activity that accompanies ripening in tomato fruit. In contrast, PG activity in fruit containing the antisense gene for PG never increased beyond the low level found in green fruit (Fig. 1A). It seemed possible that PG action might impinge on the development of the activity of other hydrolases, so the activities of a number of glycosidases were assayed in control and antisense fruit. As ripening progressed in control fruit, there was a modest decline in α-arabinosidase and α-galactosidase activities (Fig. 1, B and C), whereas there was a slight increase in β-galactosidase activity (Fig. 1D). Enzyme activities of antisense fruit were not significantly different from those of control fruit except in the case of α-arabinosidase and β-galactosidase of mature green fruit, where activities were lower, a difference that may be significant.

Gross Changes in Major Wall Fractions

Cell walls were extracted to give three pectin-rich fractions—WSF, CDTA-F, and CO_3-F. The WSF is thought to represent wall polymers solubilized in vivo but remaining in

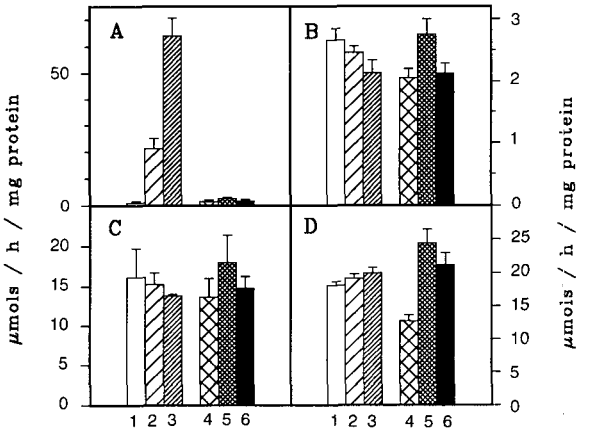

Figure 1. Salt-solubilized activities of PG (A), α-arabinosidase (B), α-galactosidase (C), and β-galactosidase (D) of control and antisense tomatoes at the green, turning, and red stages. Mean ± SD of three replicates. Units are expressed in μmol of *p*-nitrophenol except for A, which is in μmol of galacturonic acid reducing equivalents. 1, Control green; 2, control turning; 3, control red; 4, antisense green; 5, antisense turning; 6, antisense red.

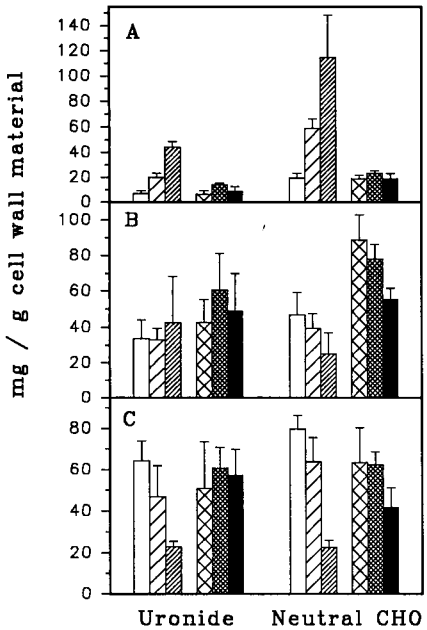

Figure 2. Quantities of uronide and neutral carbohdyrate solubilized from control and antisense tomato fruit CWM at the green, turning, and red stages by water (A), 0.05 M CDTA (B), and 0.1 M Na_2CO_3 (C). Mean ± SD of three replicates. Symbols as in Figure 1.

the apoplast and that can be obtained by water extraction of the purified cell wall. Residual enzyme activity can also enrich this fraction, but such autolysis can be prevented in tomato by a hot ethanolic pretreatment (Koch and Nevins, 1989), and a similar approach was used in this study. In both control and antisense fruit, the WSF comprised about twice as much neutral sugar residues as uronic acids. These polymers increased 5-fold in the control fruit, but in the transgenic fruit in which PG expression is impaired there was no such increase (Fig. 2A).

The CDTA-F is generally considered to represent ionically bound pectins (Selvendran and O'Neil, 1987). During ripening, no significant changes in polyuronide levels occurred in this fraction of either control or antisense fruit (Fig. 2B). There was, however, a marked decline in its neutral sugar content (probably representing pectin side chains), and this trend was evident in both antisense and control fruit. Interestingly, these neutral sugars were about twice as abundant in the polymeric CDTA-F from antisense fruit compared with control fruit of equivalent ripeness (Fig. 2B).

The CO_3-F, which is considered to be a more highly esterified population of the more tightly bound pectins (Selvendran and O'Neil, 1987), exhibited changes (Fig. 2C) that in many ways are the inverse of those seen in the WSF (Fig. 2A). Mirroring the rise in water-soluble polyuronides as control fruit ripen, there was a dramatic decline in polyuronides in this fraction (Fig. 2C). This decline in CO_3-F polyuronides (40 mg/g CWM) was matched quantitatively by the increase in water-soluble polyuronides. Antisense fruit in which there was no increase in WSF polyuronides (Fig. 2A) correspondingly showed no decline in CO_3-F polyuronides

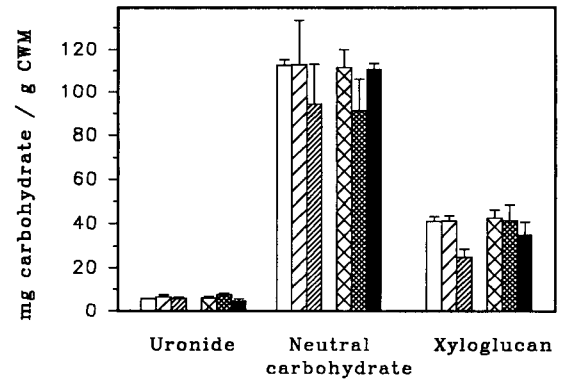

Figure 3. Hemicellulosic uronide and neutral carbohydrate solubilized from control and antisense tomato fruit CWM at the green, turning, and red stages by 4 M KOH. Mean ± SD of three replicates. Symbols as in Figure 1.

(Fig. 2C). The increase in WSF neutral sugar seen in control fruit (Fig. 2A) was also paralleled by a decline in neutral sugar in the CO_3-F polymers (Fig. 2C). Although there was no increase in WSF neutral sugar in ripening transgenic fruit (Fig. 2A), there was a marked decline in neutral sugar of the CO_3-F polymers of these fruit (Fig. 2C).

In addition to the pectic fractions, changes in the 4 N KOH-soluble hemicelluloses and the residual α-cellulose fraction were examined. Levels of total hemicellulosic neutral sugars did not change in any significant way during ripening of either the control or transgenic fruit (Fig. 3). Uronic acids are present in relatively small amounts in this fraction and showed no change with ripening. Xyloglucans, assayed by iodine binding (Kooiman, 1960) and constituting one-third of the total hemicelluloses, did show a decline in the transition from turning to red in control fruit, but this change was not evident in the transgenic fruit (Fig. 3). The small amount of uronic acid remaining in the α-cellulose fraction declined with ripening in the control fruit but showed no change (Fig. 4) in the antisense fruit.

Changes in Neutral Sugar Composition

Analysis of the neutral sugar composition of the WSF reveals that Rha, Ara, and Gal, and to a lesser extent Glc and Xyl, are increasingly solubilized as ripening proceeds in con-

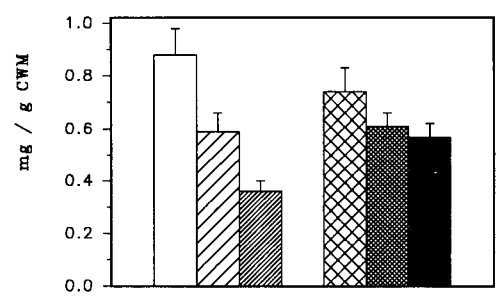

Figure 4. Uronide levels in the α-cellulose fraction from control and antisense tomato fruit CWM at the green, turning, and red stages. Mean ± SD of three replicates. Symbols as in Figure 1.

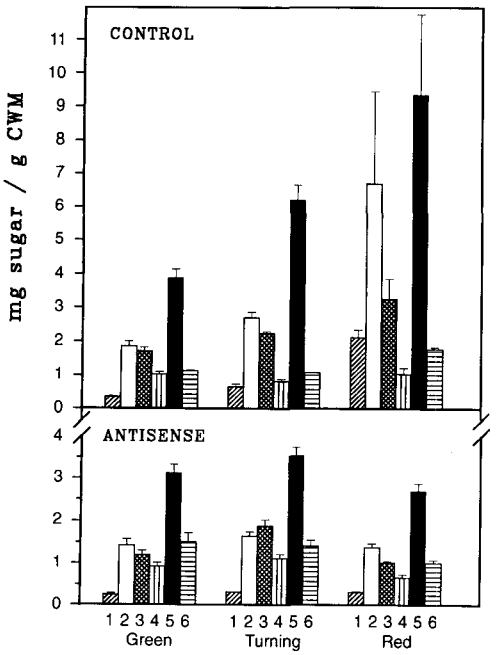

Figure 5. Sugar composition of water-soluble pectin from control and antisense tomato fruit CWM at the green, turning, and red stages. Mean ± SD of three replicates. 1, Rha; 2, Ara; 3, Xyl; 4, Man; 5, Gal; 6, Glc.

trol tomato fruit (Fig. 5). The amount and sugar composition of the WSF of green antisense fruit are no different than that of control mature green fruit but, unlike control fruit, there is no change in this fraction with ripening; the water-soluble pectins of mature green, turning, and red fruit were virtually identical in amount and composition (Fig. 5).

In the CDTA-F, only the level of Gal changes appreciably with ripening in the control fruit (Fig. 6). There may be a slight increase in Gal levels in this fraction as fruit reach the turning stage, but what is significant is the marked decline in Gal in the red fruit. This loss of Gal is also seen in the antisense fruit, and, possibly, there is also some decline in the amount of Ara; but what is most interesting is the elevated level of both Gal and Ara in the mature green antisense fruit relative to control fruit (Fig. 6). These higher Ara and Gal levels undoubtedly account for the higher neutral carbohydrate levels measured in the CDTA-F of antisense fruit relative to that of control fruit (Fig. 2B).

The most dramatic changes in the composition of the CO_3-F are seen as the fruit go from the turning to red stage (Fig. 7). In antisense fruit, only one sugar changes in this fraction, and that is Gal, which declines during ripening to one-third the starting level found in the green fruit (Fig. 7). In normal fruit, however, Rha, Ara, and Gal all decline with ripening, with the decline in Gal being disproportionately large.

Notwithstanding the variability of the data, the results of the compositional analysis of the hemicellulose (4 N KOH-soluble) fraction (not presented) do suggest a decline with ripening in Gal, Xyl, and Glc, the constituent sugars of the xyloglucan component of this fraction. Nonetheless, from the results of the iodine-binding xyloglucan assay such a decline

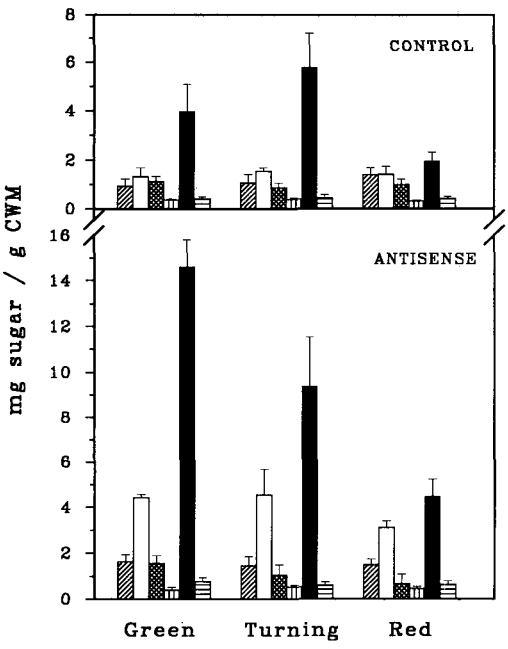

Figure 6. Sugar composition of CDTA-soluble pectin from control and antisense tomato fruit CWM at the green, turning, and red stages. Mean ± SD of three replicates. Symbols as in Figure 5.

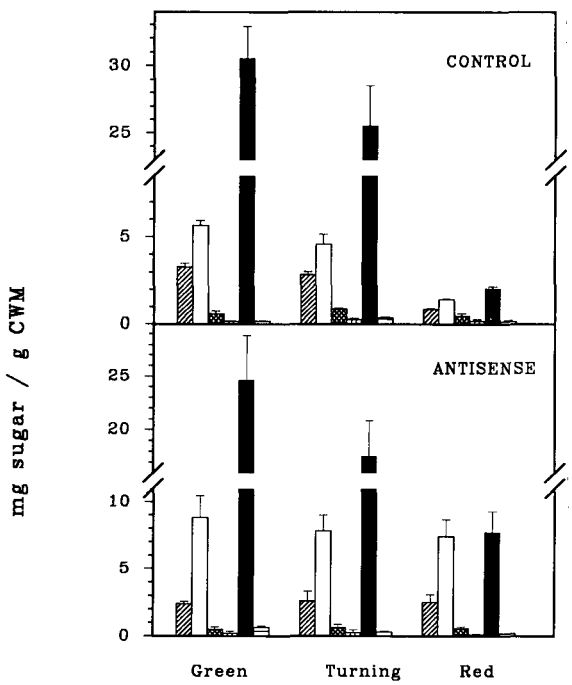

Figure 7. Sugar composition of Na_2CO_3-soluble pectin from control and antisense tomato fruit CWM at the green, turning, and red stages. Mean ± SD of three replicates. Symbols as in Figure 5.

was evident only in control fruit (Fig. 3). A more rigorous appraisal would probably recognize only the loss of Gal at the turning to red transition as statistically significant, and it may be that this is a reflection of the Gal loss seen at this same stage in the CO_3-F and CDTA-F pectins.

DISCUSSION

Transgenic tomato lines carrying the antisense PG gene vary in their efficiency at suppressing PG expression. The restriction of PG activity in antisense red fruit to levels typical of mature green fruit, as described here (Fig. 1A), is consistent with earlier studies of the effect of the antisense PG gene (Sheehy et al., 1988; Smith et al., 1988). From a theoretical standpoint, transformation with the antisense gene for PG should affect PG, and only PG, expression. On the other hand, it is conceivable that PG action on the cell wall may release fragments that, acting as elicitors, trigger the expression of other cell wall-modifying enzymes. In a token attempt to rule out this possibility and so simplify the interpretation of any compositional differences encountered in antisense and control fruit, three glycosidases were examined. These were assayed by their effect on the appropriate nitrophenyl-glycosides, an approach that, although convenient, may not truly reflect cell wall-directed hydrolysis (Pharr et al., 1976). The slight decline in α-arabinosidase and α-galactosidase activities and marginal increase in β-galactosidase activity found (Fig. 1, B–D) are similar to those previously reported (Wallner and Walker, 1975; Campbell et al., 1990). Generally, glycosidase activities were similar in control and transgenic plants, supporting a specific effect of the antisense PG gene on PG. However, the significantly lower α-arabinosidase and β-galactosidase activities seen in mature green transgenic fruit (Fig. 1, B and D) cannot be ignored. The transgenic fruit also showed consistently higher levels of CDTA-soluble neutral polymers (Fig. 2B), and compositional analyses suggest that these elevated levels are attributable to higher than normal levels of Ara and Gal (Fig. 6). Antisense PG mRNA is expressed in immature green transgenic tomato fruit even before PG mRNA is expressed (Sheehy et al., 1988), but how this could depress the expression of α-arabinosidase and β-galactosidase or other enzymes related to pectic Ara and Gal is not clear.

Our results show that whereas control fruits exhibit an increase in water-soluble pectins as ripening proceeds and PG levels rise, the antisense fruit, which show little PG activity, also fail to solubilize pectin (Fig. 2A). In contrast to this, a previous study of ripening in fruit of transgenic tomato with the antisense gene for PG revealed pectin solubilization as extensive as that seen in normal fruit (Smith et al., 1990). Similarly, fruit of the "Never-ripe" tomato mutant, which also shows little PG activity during ripening, exhibited a rise in water-soluble pectin similar to that in wild-type fruit (Seymour et al., 1987). These contradictory results are probably attributable to the inclusion, in both studies, of EDTA, a potent pectin extractant, in the medium used to solubilize the water-soluble pectins. The effect of this would be to pool true water-soluble pectins with chelator-soluble pectins and make PG-linked solubilization more difficult to detect.

The solubilization of a substantial quantity of neutral carbohydrate in addition to polyuronide is instructive and supports the notion that neutral sugars are in some way linked to polyuronides in the intact wall. Our results show that Rha, Ara, and Gal, in particular, are solubilized along with galacturonan as ripening ensues (Fig. 5), and similar results have been obtained by treating tomato cell walls with purified PG (Pressey and Avants, 1982; Koch and Nevins, 1989). It has been suggested that this material released during ripening comprises two polymer types, one a low mol wt galacturonan and the other a larger, Gal-rich polymer in which neutral sugars predominate (Pressey and Himmelsbach, 1984).

Both the gross changes in polysaccharide levels in the pectic fractions and the compositional analyses support the view that the pectins solubilized by PG originate from the CO_3-F. The increases in water-soluble pectins in control fruit are matched by corresponding declines in Na_2CO_3-soluble pectins, whereas antisense fruit with depressed levels of PG fail to show this same rise and fall (Fig. 2, A and C). In looking at the sugar compositions of the pectic fractions, the link between these two is again confirmed. In ripening control fruit, the change in the CDTA-F is in one constituent sugar only, Gal (Fig. 6), whereas the water-soluble pectins show significant increases in Rha, Ara, and Gal (Fig. 5), the same sugars that decline in the CO_3-F (Fig. 7). Earlier work has also shown a decline in Na_2CO_3-soluble pectins with tomato ripening, but the omission of an initial water extraction makes it difficult to make further comparisons with these studies (Gross, 1984; Seymour et al., 1990). Lastly, although there are extremely low levels of uronide in the α-cellulose fraction, these, too, seem to be a target for PG, as evidenced by their sharp decline in control relative to antisense fruit (Fig. 4).

The usefulness of the transgenic plants in our study has been in helping to pinpoint those changes in cell wall composition that are a result of PG action. Previous studies have suggested that the antisense gene for PG has no effect on retarding ripening-related tomato fruit softening (as measured by tissue compressibility; Smith et al., 1988; Schuch et al., 1991). Poststorage evaluation of firmness (compressibility), however, indicates significant retardation of softening in antisense fruit (Kramer et al., 1992). In-depth rheological studies of ripening tomato fruit carrying the antisense PG gene must be undertaken to explain these apparently conflicting results.

Our analysis of softening (measured with a penetrometer) indicates little difference in softening as fruits change from green to turning, but shows substantial retardation of softening as antisense fruit change from turning to red. Thus, the biochemical changes that underlie softening should be of similar magnitude in both control and antisense fruit as they go from green to turning, but should be much greater in the control fruit for the turning to red transition. A striking uronide solubilization (at the expense of Na_2CO_3-soluble pectin) begins as control fruit turn color and continues into the red stage (Fig. 2, A and C). No such pattern develops for antisense fruit, which soften in parallel with the controls to the turning stage. Thus, pectin solubilization is insufficient to explain early aspects of softening.

In the CDTA-soluble pectins, the only significant change is a decline in Gal, especially as fruit go from turning to red, a feature also shared by the antisense fruit (Fig. 6). In the

Na_2CO_3-soluble pectins, the impression given is that an Ara-, Rha-, and, probably, Gal-containing uronide is metabolized by PG during ripening (Fig. 7). This would be analogous to the PG-dependent loss of pectic Ara from the walls of ripening pear fruit (Ahmed and Labavitch, 1980). However, the decline of Gal in this fraction is not only too large to be explained simply by PG solubilization of a Rha/Ara/Gal-containing polymer, but is also evident in antisense fruit, which, of course, show little PG activity. This PG-independent loss of Gal is common to both antisense and control fruit and may be an important event in softening. For some time it has been known that a β-galactosidase capable of hydrolyzing a β-1,4-galactan is present in tomatoes and that its activity increases during ripening (Pressey, 1983). The time is ripe to evaluate further this enzyme's role in fruit softening.

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