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An Antisense Pectin Methylesterase Gene Alters Pectin Chemistry and Soluble Solids in Tomato Fruit

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Pectin methylesterase (PME, EC 3.1.11) demethoxylates pectins and is believed to be involved in degradation of pectic cell wall components by polygalacturonase in ripening tomato fruit. We have introduced antisense and sense chimeric PME genes into tomato to elucidate the role of PME in fruit development and ripening. Fruits from transgenic plants expressing high levels of antisense PME RNA showed <10% of wild-type PME enzyme activity and undetectable levels of PME protein and mRNA. Lower PME enzyme activity in fruits from transgenic plants was associated with an increased molecular weight and methylesterification of pectins and decreased levels of total and chelator soluble polyuronides in cell walls. The fruits of transgenic plants also contained higher levels of soluble solids than wild-type fruits. This trait was maintained in subsequent generations and segregated in normal Mendelian fashion with the antisense PME gene. These results indicate that reduction in PME enzyme activity in ripening tomato fruits had a marked influence on fruit pectin metabolism and increased the soluble solids content of fruits, but did not interfere with the ripening process.

INTRODUCTION

Pectin methylesterase (PME) is an enzyme found in all plants and in many plant pathogenic bacteria and fungi (Rexova-Benkova and Markovic, 1976; Huber, 1983a; Collmer and Keen, 1986). It catalyzes the deesterification of galactosyluronate methyl esters of pectin to their free carboxyl groups. PME activity has been detected in most plant tissues; however, the enzyme is particularly associated with ripening fruit, abscission zones, and maturing cell walls (Rexova-Benkova and Markovic, 1976; Sexton and Roberts, 1982; Huber, 1983a; Northcote, 1986). Increases in PME enzyme activity have been reported during ripening of several fruits, including tomato (Tucker et al., 1982; Harriman et al., 1991). Plant cell walls are highly complex structures composed of cellulose, hemicellulose, pectins, structural proteins, and other components (Varner and Lin, 1989). Pectins are primarily composed of polygalacturonic acid (homopolymers of [1→4]α-D-galactopyranosyluronic acid units with varying degrees of the carboxyl groups methylesterified) and rhamnogalacturonan I (a heteropolymer of repeating [1→2]α-L-rhamnosyl-[1→4]α-D-galactosyluronic acid disaccharide units) (Lau et al., 1985). It has been suggested that the carboxyl groups of pectins are highly methylesterified when they are synthesized, but the esters are later cleaved by pectin methylesterase in the cell wall (Kauss and Hassid, 1967; Roberts, 1990). The role of PME in plant cell growth and development is not yet clear. It has been suggested that demethoxylation is necessary for the formation of Ca^{2+} cross-bridges in pectins, leading to stabilization of the middle lamella (Fry, 1986; Northcote, 1986). It has been proposed that less methoxylated pectin may be more extensible, allowing plant cells to respond more rapidly to auxin or lowered pH during cell growth (Ricard and Noat, 1986).

Major transitions in fruit development and metabolism accompany the initiation of fruit ripening. In addition to alterations in pigment biosynthesis and production of volatile compounds, tomato fruit undergoes significant changes in texture during ripening. Although several cell wall hydrolases have been implicated in fruit softening, special attention has been paid to the degradation of polyuronides (Fischer and Bennett, 1991). The role of polygalacturonase during tomato fruit ripening has been extensively investigated at genetic, biochemical, and gene expression levels (Fischer and Bennett, 1991). PME may play a role in fruit softening by increasing in vivo susceptibility of pectins to polygalacturonase during ripening (Pressey and Avants, 1982; Seymour et al., 1987; Koch and Nevins, 1989). Unlike polygalacturonase, which is synthesized only during ripening, PME is produced during the development of tomato fruit (Tucker et al., 1982; Fischer and Bennett, 1991; Harriman et al., 1991). PME mRNA, protein, and enzyme activity are first detectable in 10- to 20-day-old fruit, and continue to accumulate until the turning stage of fruit ripening before declining (Harriman et al., 1991). At the turning stage of ripening, fruit contain about 20-fold higher levels of PME enzyme activity as compared to 10-day-old fruit (Harriman et al., 1991).

To gain insight into the role of PME in tomato fruit development, we introduced an antisense PME gene under the control of the cauliflower mosaic virus 35S promoter into tomato. We

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have identified transgenic plants with greatly reduced levels of PME expression (<10% of normal tomato cultivar Rutgers at the enzyme activity level) in fruits. The fruits from transgenic plants ripened normally, and temporal production of ethylene and accumulation of lycopene were not affected. Fruit expressing an antisense PME RNA did, however, show several other changes including higher degree of pectin methylesterification, increased pectin size, a decrease in EDTA-soluble pectins, and an increase in soluble solids.

RESULTS

Construction of the Chimeric PME Antisense RNA Gene

Figure 1 shows the chimeric PME antisense gene construct used in our study. We have previously isolated a fragment from a genomic library of tomato cultivar Cherry VFNT DNA in vector Charon 35 containing three PME genes in tandem repeat (Harriman and Handa, 1990). The introduced gene contains a 1.6-kb HincII-EcoRI fragment from one of these genes. This fragment contains 185 bp of the 5′ untranslated region, two introns of 207 and 89 bp, and the entire coding region for PME except for 85 bp at the 3′ end. This fragment was cloned in both sense (pTiPMES) and antisense (pTiPMEA) orientations under the control of the cauliflower mosaic virus 35S promoter in vector pKYLX7 (Schardl et al., 1987). pTiPMES and pTiPMEA were introduced into tomato cultivar Rutgers using *Agrobacterium tumefaciens*–mediated transformation with kanamycin resistance as a selectable marker (Fillatti et al., 1987). Genomic DNA from independent putative transformants (18 with the antisense RNA construct pTiPMEA, seven with the sense construct pTiPMES) was digested with EcoRI and analyzed by DNA gel blotting with pKYLX7 as a probe. The single EcoRI site in the chimeric sense and antisense gene constructs (Figure 1) allowed the determination of number of copies of chimeric genes introduced into independent transformants. Among the transformants containing the antisense RNA construct, three contained three copies, four contained two copies, and 11 contained a single copy of the introduced gene (data not shown). Among the transformants containing the sense construct, one contained three copies, one contained two copies, and three contained one copy of the introduced gene (data not shown). One putative transformant from the antisense construct and two putative transformants from the sense construct did not show the presence of a hybridizing DNA fragment. To further confirm the presence of the inserted PME gene (antisense or sense orientation) in these transformants, total genomic DNA was digested with XbaI and SacI and analyzed by DNA gel blotting using a PME cDNA insert from PET1 (Harriman et al., 1991) as a probe. All transformants which contained DNA fragments that hybridized to pKYLX7 also showed the presence of a new 1.6-kb fragment that hybridized to the PME cDNA insert from PET1 (data not shown).

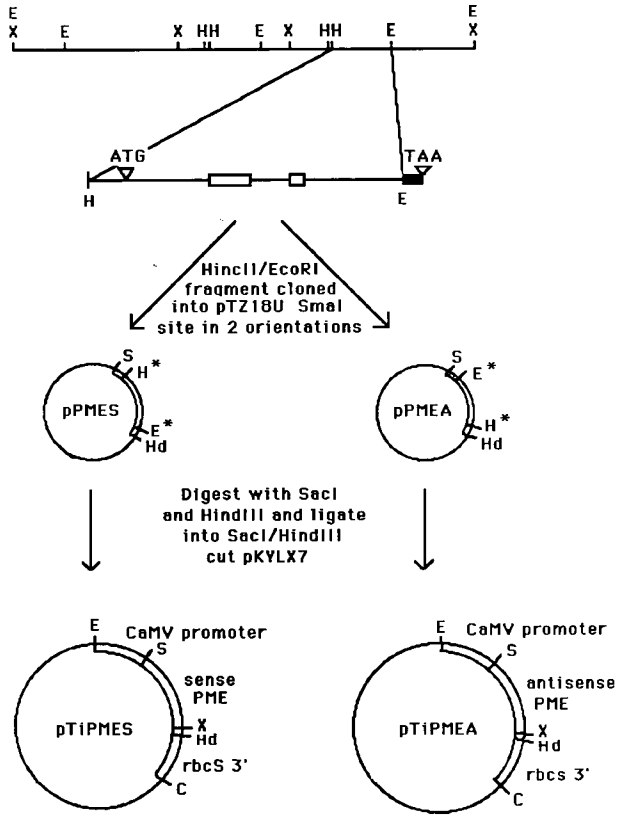

Figure 1. Structure of the Chimeric Genes Containing a Partial PME Gene in Sense and Antisense Orientations.

Shown at top is a partial restriction map of the PME genomic clone isolated from a genomic library of Cherry VFNT tomato DNA in vector Charon 35 that contains three genes for PME. A 1.6-kb HincII-EcoRI fragment from one of these genes was introduced into vector pKYLX7 under the control of the cauliflower mosaic virus (CaMV) 35S promoter in antisense (pTiPMEA) and sense (pTiPMES) orientations and followed by the rbcS 3′ termination sequence. The 1.6-kb fragment includes a part of the 5′ untranslated region, two introns (open boxes), and the entire coding region except for 85 bp at the 3′ end (black bar). E* and H* represent sites that were lost during blunt end ligation of the 1.6-kb HincII-EcoRI fragment to pTZ18U. Structures are not drawn to scale. C, ClaI; E, EcoRI; H, HincII; Hd, HindIII; S, SacI; X, XbaI.

Expression of the Introduced Sense and Antisense Chimeric Genes in Transgenic Plants and Their Effects on PME Gene Expression

Figure 2 shows that transgenic plants containing the PME antisense chimeric gene exhibited a wide range of PME enzyme activity and protein, mRNA, and antisense RNA levels in their fruits. PME enzyme activity in red ripe fruits from antisense transgenic plants ranged from about 7 to 40% of wild-type Rutgers fruits (Figure 2). Strand-specific probes were used to determine accumulation of PME sense RNA and antisense

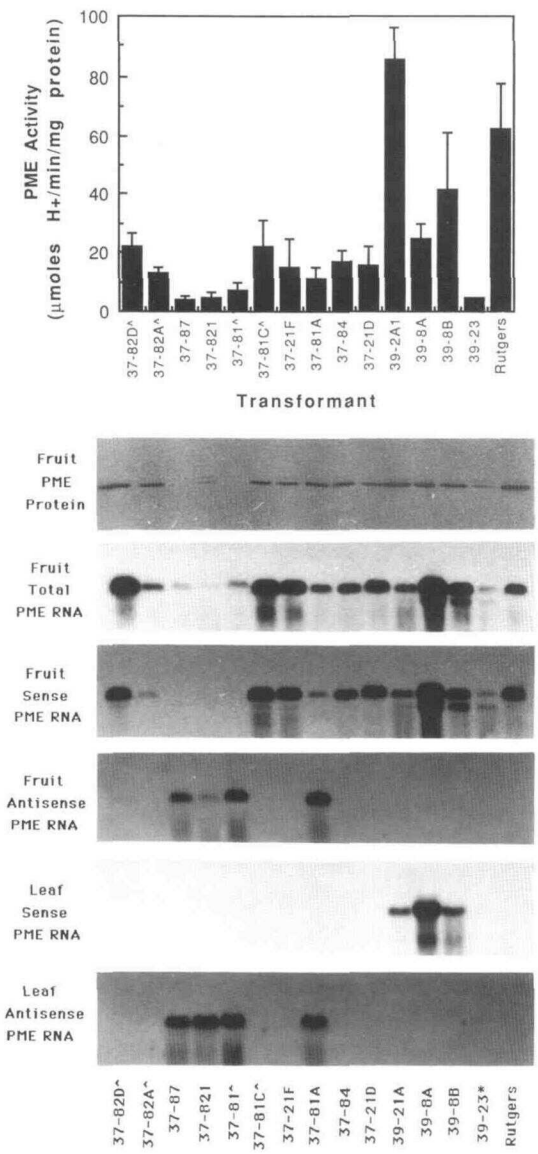

Figure 2. PME Enzyme Activity, Protein, and Antisense and Sense RNA Levels in Ripe Fruits of Independent Transgenic Plants Containing the Antisense or Sense Chimeric PME Genes.

Transgenic plants that begin with 37 contain the PME antisense chimeric gene, whereas those which begin with 39 contain the PME sense chimeric gene. Also shown is the expression of antisense and sense RNA from the introduced genes in the leaves of transgenic plants. PME enzyme activity (mean ± SE) was determined from at least four fruits for each independent transgenic plant. Plant 39-23* produced only one fruit. Immunoblot and RNA gel blot analyses were performed as described in Methods. RNA gel blots were probed with ^{32}P-labeled PME sense or antisense RNA probes transcribed from the 1.6-kb EcoRI-HincII DNA fragment using a Promega in vitro transcription system. Note the presence of two RNA bands in fruit containing the PME sense chimeric gene: the top band represents mRNA from the endogenous PME gene, whereas the lower band represents the partial mRNA made from the introduced PME gene.

RNA in fruits from the transgenic plants. Three transgenic plants expressing the antisense gene (37-87, 37-821, and 37-81^ that contained one, two, and one copies of the antisense PME gene, respectively) showed the largest reduction in PME enzyme activity (<10% of control Rutgers fruits), protein, and mRNA in red ripe fruits (Figure 2). The level of PME antisense RNA in fruits from 37-821 was less than that found in fruits from 37-87 and 37-81^. PME enzyme activity in red ripe fruits from three transformants expressing the sense strand varied between 40 to 140% of wild-type Rutgers fruits, but significant changes in PME mRNA and protein were not observed (Figure 2). Fruits from 39-8A, which contained three copies of the sense chimeric gene, showed the lowest PME enzyme activity of the three transformants tested. Transformant 39-23 showed severely reduced PME enzyme activity and abnormal vegetative growth, yielded only one fruit, and was not further investigated.

Expression of the introduced sense or antisense genes in leaves of transgenic plants was highly variable among plants (Figure 2). Leaves and fruits of transformants 37-87, 37-821, 37-81^, and 37-81A showed significant accumulation of a 1.9-kb RNA species that hybridized to an antisense PME RNA probe, whereas detectable levels of this hybridizable RNA were not present in leaves or fruits of other antisense transgenic plants or wild-type Rutgers plants. Leaves and fruits of all transgenic plants containing the sense chimeric gene accumulated a 1.6-kb RNA species which hybridized to a PME sense RNA probe, but much higher levels were observed in transformant 39-8A (Figure 2). The hybridizing RNA species present in fruits and leaves of transgenic plants containing the antisense chimeric gene is approximately 300 bp longer than that present in transgenic plants containing the sense chimeric gene. This is about the size of two introns present in the PME gene used in this study, indicating that two introns are processed both in fruit and leaf tissues only when present in the sense orientation.

Inheritance of the Antisense RNA Gene

Transformant 37-81^, which contained one copy of the antisense chimeric gene, was chosen for further studies. Flowers of this transformant were self-pollinated, and plants from the resultant seeds were analyzed for inheritance of the antisense gene. The PME antisense gene segregated in this seed population yielding progeny with zero, one, and two copies of the inserted gene as detected by genomic DNA gel blot analysis as shown in Figure 3. Among the progeny analyzed, eight plants had not inherited the antisense chimeric gene, 12 were heterozygous, and six homozygous for the antisense chimeric gene. This indicates that the antisense gene was not lethal even in the homozygous state and that inheritance followed normal Mendelian segregation. As shown in Table 1, reduction in PME enzyme activity levels correlated with the presence of the antisense chimeric gene.

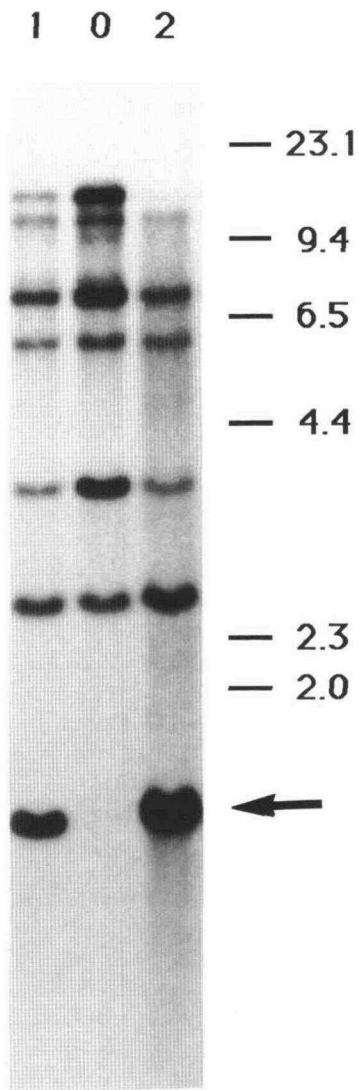

Figure 3. DNA Gel Blot Analysis of Genomic DNA from Leaves of Progeny of 37-81^.

Ten micrograms of total DNA was digested with XbaI and SacI, electrophoresed on a 0.7% agarose gel, blotted onto nylon membranes, and hybridized with a ^{32}P-labeled insert of a PME cDNA clone (Harriman et al., 1991). The 1, 0, and 2 shown at top represent progeny with one, zero, and two copies of the antisense gene. Shown at right are the lengths of DNA fragments from HindIII cut λ DNA. The arrow indicates the 1.6-kb DNA fragment from the introduced gene.

Changes in PME Gene Expression during Development of Transgenic Fruits

Figure 4 shows PME gene expression during fruit development and ripening in fruits from wild-type Rutgers, transformant 37-81^ (T_1), and segregants of 37-81^ homozygous (designated as T_3 homozygotes) for the antisense chimeric PME gene. As reported earlier, PME enzyme activity increased until the turning stage in Rutgers (Harriman et al., 1991), but in 37-81^ and its homozygous progeny it remained fairly constant throughout development. On a percentage basis, however, PME enzyme activity in 37-81^ (T_1 or T_3 homozygotes) fruits decreased gradually from 25% of normal Rutgers fruits at 25 days after flowering to <10% at the red ripe stage. PME protein, as determined by immunoblotting using alkaline phosphatase-conjugated antibodies, increased throughout ripening in Rutgers fruits, but was not detectable in fruits of 37-81^ and its homozygous progeny even when blots were overexposed (Figure 4). In addition to PME protein, our anti-PME antibodies recognized a minor polypeptide with a molecular weight slightly higher than PME (Harriman et al., 1991). Levels of this protein decreased somewhat during wild-type tomato fruit development and showed a similar pattern in 37-81^ (T_1 or T_3 homozygotes) fruits.

Strand-specific probes showed that only trace amounts of PME mRNA were present in 37-81^ fruits (T_1 or T_3 homozygotes) throughout fruit development. Fruits from transgenic plants, however, accumulated significant levels of PME antisense RNA. These levels were slightly higher in T_3 homozygous fruits (Figure 4). Fruits from transgenic plants expressing the antisense PME gene showed the expected expression of polygalacturonase at mRNA levels (Figure 4).

Table 1. Levels of Soluble Solids and PME Enzyme Activity in Red Ripe Fruits from Independent Transgenic Plants and Segregants of 37-81^

Fruit Type	PME Activity (% Rutgers)	Soluble Solids (% Rutgers)	Number of Fruits
Rutgers	100 ± 16	100 ± 4	5
37-87[a]	6 ± 1[b]	107 ± 1	5
37-821[a]	7 ± 2[c]	145 ± 9[b]	4
37-81^[a]	12 ± 2[b]	111 ± 9	5
37-21F[a]	24 ± 10[c]	112 ± 4	5
37-21D[a]	23 ± 7[b]	100 ± 5	6
37-84[a]	27 ± 5[c]	95 ± 4	4
37-81C^[a]	41 ± 9[c]	94 ± 5	4
37-82D^[a]	36 ± 5	93 ± 4	7
0[d]	99 ± 5	98 ± 3	3
1[d]	8 ± 2[c]	102 ± 1	3
2[d]	11 ± 2[b]	117 ± 3[c]	3

Data are presented as means ± SE of enzyme activity and soluble solids in fruits from each transformant.

[a] T_1 transgenic plants containing the antisense PME gene.

[b] Significantly different from wild-type Rutgers ($P < 0.01$).

[c] Significantly different from wild-type Rutgers ($P < 0.05$).

[d] T_2 segregants of 37-81^ with zero, one, and two copies of the antisense PME gene.

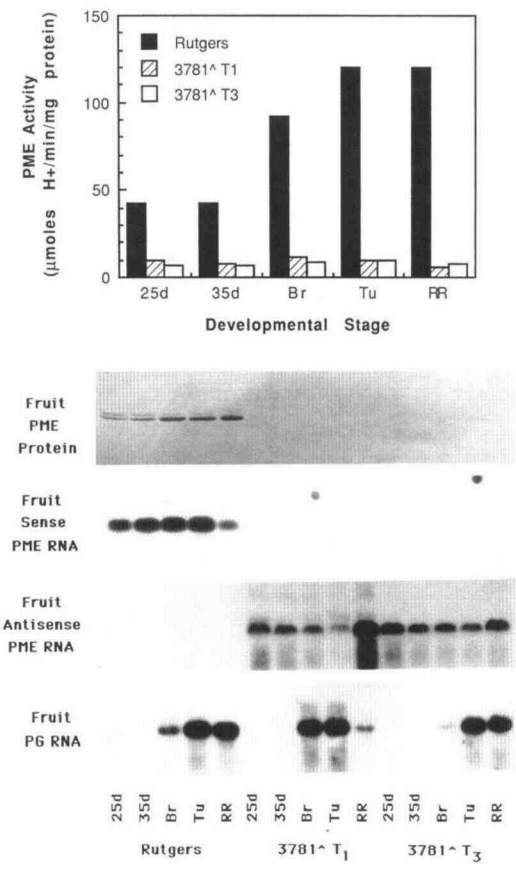

Figure 4. PME Gene Expression at the Enzyme Activity, Protein, mRNA, and Antisense RNA Levels in Developing Fruits from Tomato Cultivar Rutgers, 37-81^ (T_1), and 37-81^ (T_3 Homozygotes).

PME enzyme activity, protein, mRNA, and antisense RNA levels were determined as given in Figure 2. Also shown is the expression of polygalacturonase in developing fruits from these genotypes. Polygalacturonase mRNA was detected on RNA gel blots using a polygalacturonase cDNA (pSB1) insert as a probe (Biggs and Handa, 1988). Abbreviations for stages of fruit development are as follows: 25d and 35d, 25 and 35 days after flowering; Br, breaker; Tu, turning; RR, red ripe fruits.

Effect of Reduced PME Expression on Ripening Parameters

Figure 5 shows the rate of ethylene production, lycopene accumulation, and chlorophyll degradation in Rutgers and 37-81^ T_3 homozygous fruits. During the ripening process, 37-81^ (T_3 homozygotes) fruits showed ethylene production patterns identical to Rutgers fruits. Ethylene was first detected in breaker stage fruit, increased until the ripe stage, then decreased in both wild-type and transgenic fruits (Figure 5). Lycopene accumulation and degradation of chlorophyll in ripening 37-81^ (T_3 homozygotes) fruits was similar to that of the Rutgers fruit (Figure 5). Although slightly higher levels of lycopene and chlorophyll were observed in red ripe fruits of 37-81^ (T_3 homozygotes) fruits, these changes in pigments were not significant at $P < 0.05$. These results, along with the phenotype of fruits from transgenic plants shown in Figure 6, indicate

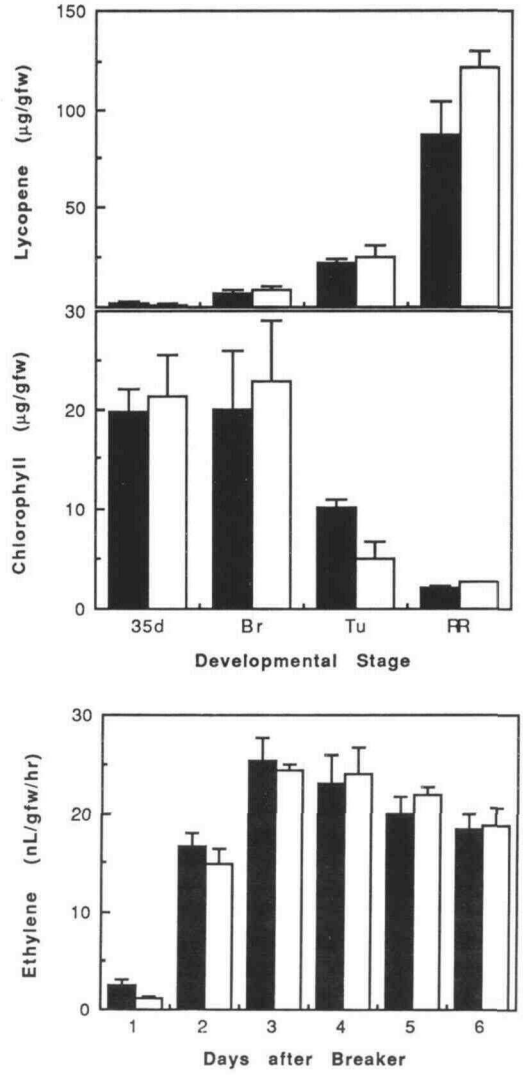

Figure 5. Effects of Reduced PME Gene Expression on Ripening Parameters.

Levels of lycopene and chlorophyll and rates of ethylene production in Rutgers and 37-81^ T_3 homozygous fruits are shown. Bars represent means ± SE of levels of chlorophyll, lycopene, and ethylene from independent fruits. Statistically significant differences in lycopene, chlorophyll, and ethylene between wild-type and 37-81^ transgenic fruits were not observed at any stage of fruit development. Black bars, Rutgers; open bars, 37-81^ T_3 homozygotes.

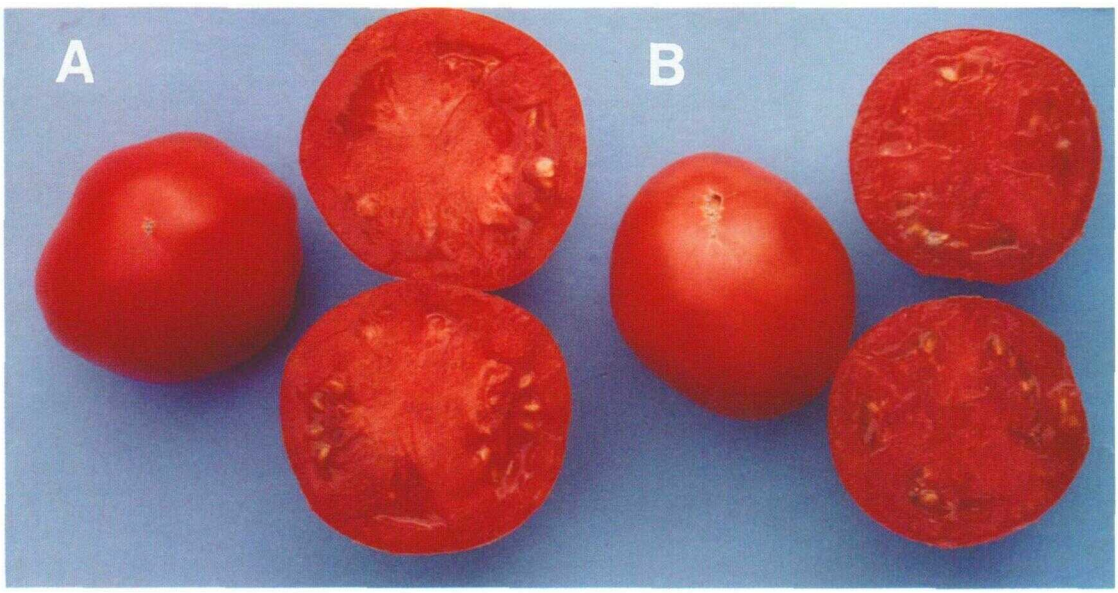

Figure 6. Phenotype of Wild-Type Rutgers and Transgenic 37-81^ T_3 Homozygous Fruits.

(A) Red ripe fruits from tomato cultivar Rutgers.
(B) Red ripe fruits from tomato transgenic line 37-81^ (T_3 homozygote).

that fruits with significantly reduced levels of PME enzyme activity ripen normally.

Effects of Reduced PME Expression on Degree of Pectin Methylesterification and Levels of Total and Soluble Pectins

The degree of methylesterification of cell wall pectins was significantly increased by introduction of the antisense PME gene as shown in Figure 7A. Total polyuronides from pericarp of 37-81^ (T_3 homozygotes) showed a 20 to 40% higher degree of methylesterification as compared to wild-type Rutgers throughout fruit ripening (Figure 7A). The effect of antisense PME on degree of methylesterification was most pronounced during the ripening process, when pectin degradation was greatest in wild-type fruit. At the red ripe stage the degree of pectin methylesterification was about 50% in the fruits from transgenic plants, compared to about 40% observed in the parental Rutgers fruits (Figure 7A).

As shown in Figure 7B, during ripening levels of chelator-soluble uronic acids were about 0.10 to 0.14 μmol/mg cell wall lower in 37-81^ (T_3 homozygotes) pericarp than in Rutgers pericarp. Levels of total uronic acids in transgenic pericarp cell walls showed a similar decrease (about 0.07 to 0.13 μmol/mg cell wall) as compared to Rutgers pericarp cell walls (Figure 7B). Because the levels of EDTA-insoluble uronic acids in transgenic and Rutgers pericarp were similar, the observed decrease in uronic acid content in transgenic pericarp as compared to Rutgers pericarp was primarily due to lower levels of EDTA-soluble pectins.

Effect of Reduced PME Expression on Depolymerization of Pectin Polymers

To determine the effects of PME enzyme activity on pectin depolymerization, chelator-soluble polyuronides extracted from pericarp of 37-81^ (T_3 homozygotes) and Rutgers fruits at mature green and red ripe stages were size fractionated on a Sepharose CL4B column (DellaPenna et al., 1990). As shown in Figure 8A, the chromatographic profiles of polyuronides from mature green fruits of 37-81^ (T_3 homozygotes) were similar to those of Rutgers mature green fruit. The majority of the polyuronides eluted near the void volume, indicating high molecular weight (Figure 8A). Chelator-soluble polyuronides from red ripe pericarp of both Rutgers and 37-81^ (T_3 homozygotes) exhibited a decrease in size as compared to chelator-soluble polyuronides from mature green fruits. However, red ripe 37-81^ (T_3 homozygotes) pericarp contained higher levels of intermediate size polyuronides than red ripe Rutgers pericarp, indicating that during ripening the polyuronides in 37-81^ (T_3 homozygotes) pericarp were not depolymerized to the extent of those of Rutgers pericarp (Figure 8B). Interestingly, the proportion of the high molecular weight EDTA-soluble pectins (fractions 42-54 in Figure 8) in pericarp both at the mature green and red ripe stages were lower in 37-81^ (T_3 homozygotes) as compared to wild-type Rutgers.

Effect of PME Enzyme Activity on Fruit Solids and Soluble Solids

Pericarp and whole fruits from 37-81^ (T_3 homozygotes) and Rutgers were analyzed for solids (percent dry weight) and soluble solid levels. Fruits from transgenic plants showed significantly higher levels of soluble solids as compared to Rutgers fruits. As shown in Figure 9A, the effects of the antisense PME gene on soluble solid levels were significant after the fruits reached their mature size, i.e., between the mature green and red ripe stages. Soluble solid levels in pericarp were about 15% higher throughout the ripening process in 37-81^ T_3 homozygous fruits than Rutgers fruits (Figure 9A). Although the trait of reduced PME enzyme activity segregated with the antisense PME gene in progeny of 37-81^, significant increases in soluble solids were observed only in fruits from plants homozygous for the antisense PME gene (Table 1). Pericarp from independent transformants expressing less than 25% of the PME enzyme activity of Rutgers fruits showed some increase in soluble solids, which was significant only for fruits from transformant 37-821, which contains two copies of the antisense PME gene (Table 1). This result is consistent with the increase in soluble solids observed in segregants of 37-81^. Homozygous T_2 progeny contained about 17% higher levels of soluble solids (Table 1). Further characterization of soluble solid levels in fruits from independent transgenic plants, especially after segregation analyses, is needed to determine a relationship between PME enzyme activity and soluble solid levels in fruits. As shown in Figure 9B, whole fruit soluble solid

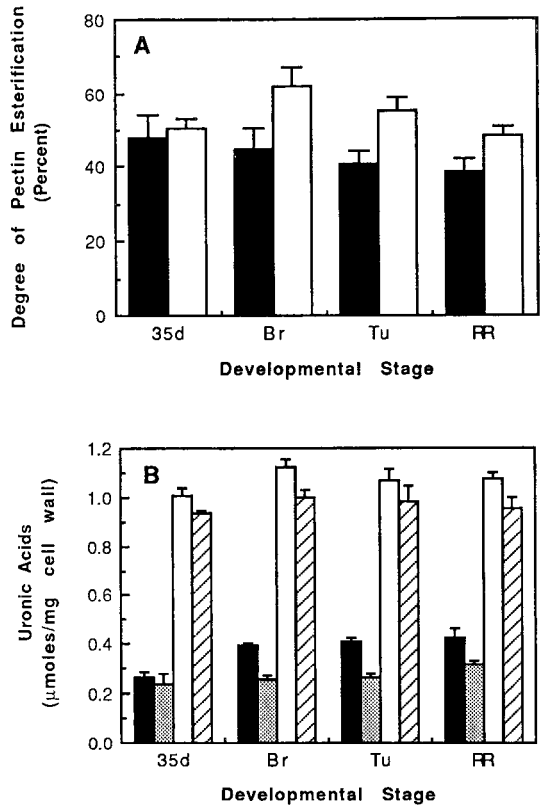

Figure 7. Degree of Pectin Methylesterification and Levels of Chelator-Soluble and Total Uronic Acids in Fruits from Rutgers and Transgenic Line 37-81^ (T_3 Homozygote).

(A) Degree of pectin methylesterification (means ± SE) in Rutgers (black bars) and 37-81^ T_3 homozygous (open bars) fruits.
(B) The levels of total and chelator-soluble uronic acids in Rutgers and 37-81^ (T_3 homozygote) fruits. Hatched and stippled bars, total and EDTA-soluble uronic acids, respectively, in 37-81^ (T_3 homozygote); open and black bars, total and EDTA-soluble uronic acids, respectively, in Rutgers. Bars represent levels (means ± SE) of chelator-soluble and total uronic acids. Cell walls were isolated as described in Methods. Abbreviations for stages of fruit development are as given in Figure 4.

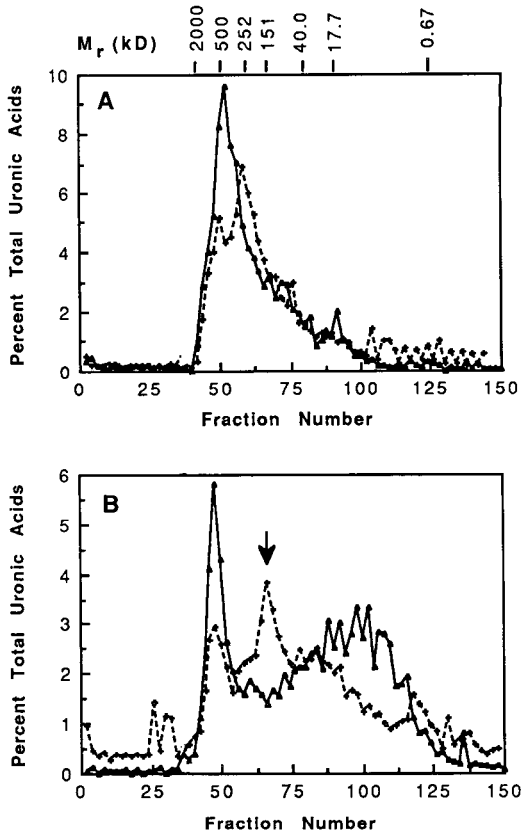

Figure 8. Gel Filtration Chromatographic Analysis of EDTA-Soluble Polyuronides from Rutgers and 37-81^ T_3 Homozygous Fruits.

Sepharose CL4B chromatographic profiles of chelator-soluble polyuronides isolated from cell walls of Rutgers (solid lines) and 37-81^ T_3 homozygous (dashed lines) fruit pericarp.
(A) Cells walls isolated from mature green fruits of Rutgers and 37-81^ (T_3 homozygotes) plants.
(B) Cell walls isolated from red ripe fruits of Rutgers and 37-81^ (T_3 homozygotes) plants. The arrow indicates the intermediate size pectins that increase in 37-81^ red ripe fruits.

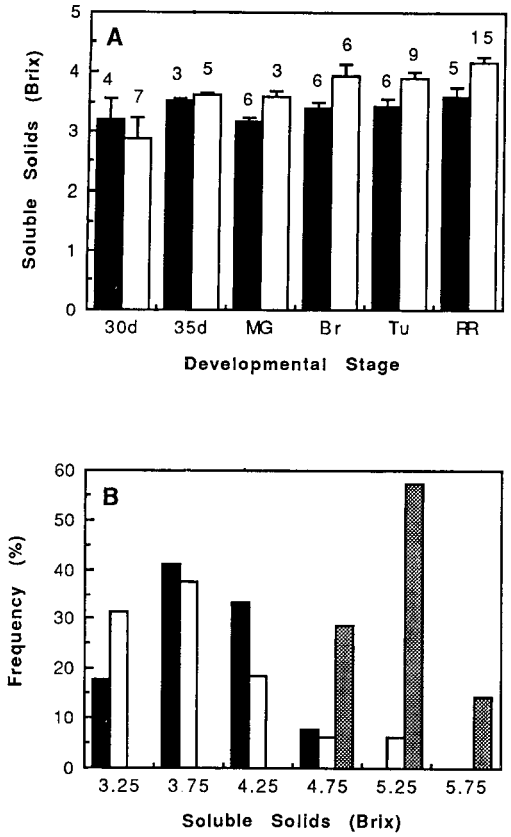

Figure 9. Effect of Reduced PME Enzyme Activity on Fruit Soluble Solids.

Data shown in **(A)** and **(B)** are from separate experiments.
(A) Levels of soluble solids in pericarp of Rutgers (black bars) and 37-81^ T_3 homozygous (open bars) fruits during ripening. Bars represent levels (means ± SE) of soluble solids at each stage of ripening. Numbers above bars indicate the number of fruits analyzed. Soluble solids in 37-81^ T_3 homozygous fruits were significantly ($P < 0.05$) higher than wild-type Rutgers fruits at mature green (MG), breaker, turning, and red ripe fruit stages.
(B) Frequency distribution for whole fruit soluble solid content of red ripe fruits from Rutgers (black bars) and segregants of 37-81^ with zero (open bars) and two (shaded bars) copies of the introduced gene. X-axis labels indicate the midpoint value of the range. Number of fruits analyzed for Rutgers, 37-81^ with zero copies, and 37-81^ with two copies were 39, 16, and 23, respectively. The differences in fruit soluble solid levels between 37-81^ segregants with two copies of the antisense gene and wild-type Rutgers or 37-81^ segregants with no copies were highly significant ($P < 0.001$).

content also showed an increase in 37-81^ (T_3 homozygotes) as compared to Rutgers fruits. The Brix values in 37-81^ (T_3 homozygotes) fruits ranged between 4.7 and 5.8 (mean ± SD; 5.25 ± 0.33), whereas in Rutgers fruits it ranged between 3.0 and 5.0 (mean ± SD; 3.89 ± 0.48). The Brix values in fruits from segregants containing zero copies of the antisense gene ranged between 3.05 and 5.3 (mean ± SD; 3.87 ± 0.62) and were similar to parental Rutgers fruits (Figure 9B). Similar differences in Brix values were observed in T_4 plants (D. M. Tieman and A. K. Handa, unpublished results).

Reduced PME enzyme activity in fruits from transgenic plants did not show a significant effect on the total solids (percent dry weight) present in red ripe pericarp tissue or whole fruits. Rutgers pericarp tissue contained about 9.6% ± 1.1% solids, whereas 37-81^ (T_3 homozygotes) contained about 9.6% ± 0.3% solids. Whole red ripe fruits contained 9.1% ± 1.4% solids in Rutgers as compared to 9.3% ± 0.4% in 37-81^ T_3 homozygous fruits. Although some of the observed increase in soluble solids could be due to pectin that remained soluble in fruits from transgenic plants, preliminary analysis of soluble neutral sugars in the presence and absence of invertase treatment indicated that levels of sucrose were higher in pericarp of fruits from transgenic plants. In addition, HPLC analysis of neutral sugars in the soluble fraction showed the presence of low molecular weight polysaccharides in the transgenic pericarp that were undetectable in the parental Rutgers pericarp (D. M. Tieman and A. K. Handa, unpublished results). Further identification and quantification of neutral sugars in the fruits from transgenic plants are in progress. These results, along with the decrease in chelator-soluble pectins, suggest that the increase in degree of pectin methylesterification due to lowered PME enzyme activity may lead to a repartitioning of carbohydrates in the fruit cells.

DISCUSSION

Patterns of PME Gene Expression in Fruits from Transgenic Plants Containing the Antisense and the Sense PME Chimeric Genes

Fruits from all tested antisense transgenic plants showed a severe reduction in PME enzyme activity. Based on the accumulation of PME protein, mRNA, and antisense RNA, these transformants could be divided into several categories: (1) transformants 37-87 and 37-81^ that did not have detectable levels of PME protein, showed trace amounts of PME mRNA, and accumulated high levels of PME antisense RNA; (2) transformant 37-821 that contained two copies of the antisense gene, did not contain detectable levels of PME protein, and had a trace amount of PME mRNA, but did not accumulate high levels of PME antisense RNA in fruits; (3) transformant 37-81A that accumulated high levels of PME antisense RNA and low levels of PME mRNA, but contained significant levels of PME protein; (4) transformant 37-82A^ that did not accumulate high levels of PME mRNA or antisense RNA, but contained PME protein; and (5) transformants 37-82D^, 37-81C^, 37-21F, 37-84, and 37-21D that did not accumulate PME antisense RNA and showed wild-type levels of accumulation of PME mRNA and protein.

The mechanism(s) responsible for the varied PME gene expression patterns in transgenic plants expressing the antisense

gene is unclear. However, variable effects of the introduced antisense RNA genes in ripening tomatoes have been observed earlier (Sheehy et al., 1988; Smith et al., 1988; Hamilton et al., 1990; Bird et al., 1991; Oeller et al., 1991). Part of the observed heterogeneity could be due to the influence of bordering sequences on the expression of the introduced gene (van der Krol et al., 1988). Interference with splicing of introns during the maturation of mRNA has been suggested as one of the possible mechanisms by which introduced antisense genes affect the production of a particular protein (van der Krol et al., 1988). We have used an antisense gene construct containing two introns (Figure 1) that were not processed when present in antisense orientation, but were excised when present in the sense orientation (Figure 2). The positional effects of insertion might be responsible for some of the observed variability in PME gene expression in independent transgenic plants expressing antisense RNA. Other mechanisms, including rapid degradation of antisense RNA and mRNA hybrids, effects on transcription rate, and inhibition of mRNA transport to the cytoplasm, have been suggested by which antisense RNA inhibits the production of a protein (van der Krol et al., 1988).

Variable patterns of PME gene expression were also observed in transgenic plants containing the sense chimeric gene. Transformant 39-8A, which contained three copies of the introduced gene and accumulated high levels of truncated PME mRNA both in fruit and leaf tissue, showed the maximum reduction in PME enzyme activity (Figure 2). Transformant 39-21A, which did not show accumulation of truncated PME mRNA in fruit, did not show a decrease in PME enzyme activity. Transformant 39-8B, which showed intermediate accumulation of truncated PME mRNA, contained less PME enzyme activity in fruits as compared to the control. Cosuppression of homologous genes in *trans* has been reported in transgenic petunia petals containing either chalcone synthase or dihydroflavonol-4-reductase (Napoli et al., 1990; van der Krol et al., 1990). At present it is not possible to conclude that the observed effects of the introduced sense chimeric gene are due to cosuppression of homologous genes in *trans*.

Effects of PME Antisense Chimeric Gene Dosage on PME Gene Expression during Fruit Development

Analysis of progeny from transformant 37-81^ showed that the introduced gene segregated in normal Mendelian fashion and the reduced PME enzyme activity in fruits from transgenic plants was due to the presence of the antisense PME gene (Table 1). Progeny homozygous for the antisense gene, derived from self-pollination of transformant 37-81^, had levels of PME enzyme activity similar to those in the heterozygous state. Progeny inheriting no copies of the antisense gene had high levels of PME enzyme activity. Although the steady state levels of PME antisense RNA in T_3 homozygous fruits were higher than heterozygous fruits during development and ripening, no further reduction in PME enzyme activity in T_3 homozygous fruits as compared to T_1 heterozygous fruits was observed (Figure 4). In addition, PME-specific activity (units per milligram of protein) in heterozygous and homozygous fruits from transgenic plants remained relatively constant throughout development and ripening (Figure 4). However, the gene dosage of the antisense PME gene did have an effect on soluble solids (Table 1). It is possible that PME enzyme activity detectable in the 37-81^ fruits could be due to the presence of another isozyme which was not affected by the introduced PME antisense RNA gene. The presence of multiple molecular forms of PME in tomato fruit has been reported previously (Pressey and Avants, 1972; Tucker et al., 1982), and the activity of one of these forms increases during ripening (Tucker et al., 1982). Because antibodies or DNA probes specific for these isozymes are not available, it is not possible to determine which of these isozymes are not affected by the introduced antisense RNA gene. The three distinct tomato PME genes that we have isolated (Harriman and Handa, 1990) hybridize to the same RNA.

Effects of Reduced PME Gene Expression on Pectin Chemistry

During tomato fruit ripening the degree of pectin esterification decreases from mature green to the red ripe stage (Huber and Lee, 1986; Koch and Nevins, 1989). Reduction of PME enzyme activity causes a significant increase in degree of methylesterification of pericarp pectins of 37-81^, especially during ripening stages of fruit development. The levels of EDTA-soluble polyuronides were lower in 37-81^ (T_3 homozygotes) pericarp cell walls as compared to Rutgers pericarp cell walls. The levels of EDTA-insoluble pectins were similar in transgenic and wild-type fruits. These results indicate that ionic binding of pectin to cell walls is affected by PME enzyme activity. This is expected because the higher degree of methylesterification of pectin would decrease the level of interpectate calcium cross bridges in cell walls resulting in a decrease in EDTA-extractable pectins from fruit cell walls. The remaining pectins are presumably bound to fruit cell walls by other means and cannot be released by removal of calcium.

Depolymerization of pectins during fruit ripening has been well characterized (Huber, 1983b; DellaPenna et al., 1990). Red ripe pericarp from 37-81^ (T_3 homozygotes) showed higher levels of intermediate size pectins than Rutgers pericarp. Both 37-81^ (T_3 homozygotes) and Rutgers fruits showed depolymerization of pectins during ripening, but Rutgers red ripe pericarp contained higher levels of low molecular weight pectins. These results indicate that although polyuronide degradation is occurring in 37-81^ (T_3 homozygotes) pericarp, the action of polygalacturonase is partially inhibited, presumably due to the increased methylesterification of the pectin. Inhibition of pectin depolymerization has also been seen in tomato fruits expressing polygalacturonase antisense RNA. However, in these fruits levels of chelator-soluble pectins remained

similar (Smith et al., 1990). Synergism between PME and endopolygalacturonase has been suggested to play a role in depolymerization of pectin during tomato fruit ripening (Pressey and Avants, 1982; Seymour et al., 1987; Koch and Nevins, 1989). This is consistent with our results of higher molecular weight pectins and higher degree of methylesterification of pectins in fruits from transgenic plants (Figures 7 and 8).

Effects of Reduced PME Enzyme Activity on Soluble Solids

Soluble solids are the water soluble components of tomato fruit and account for approximately 75% of the total dry weight of ripe fruit (Hewitt and Garvey, 1987). Sugars and organic acids are the major organic constituents of ripe tomato fruit. They contribute not only to sweetness and sourness of fruit, but also are associated with overall flavor intensity (De Bruyn et al., 1971; Stevens et al., 1977; Jones and Scott, 1983). We have observed, though unexpectedly, that inhibiting PME gene expression by antisense RNA caused a marked increase in the soluble solid content of tomato fruit (Figure 9). The effects of the antisense gene on soluble solid levels were more pronounced in mature green and ripening tomato fruits than in immature tomato fruits. Several independent lines of evidence suggest that the observed effects on soluble solids were due to inhibition of PME enzyme activity. First, levels of soluble solids in fruit homozygous for the PME antisense chimeric gene were higher than Rutgers fruit throughout the ripening process (Figure 9A). Second, fruit with higher soluble solids segregated with the antisense chimeric gene and reduced PME enzyme activity in progeny of 37-81^ (Figure 9B and Table 1). Finally, higher levels of soluble solids were observed in T_1, T_2, T_3, and T_4 generations of transformant 37-81^. Collectively these results indicate that increased soluble solids in transgenic plants containing the antisense PME chimeric gene are associated with reduced PME enzyme activity and not simply the result of tissue culture effects or an unrelated mutation. These results should be interpreted with caution, however. Factors such as environment, stresses, age of plant, yield per plant, and cultural practices can influence levels of soluble solids in tomato fruit (Davies and Hobson, 1981). It is also possible that the inserted gene might have inactivated or influenced some other gene(s) involved in accumulation of total soluble solids in fruit. This seems unlikely because fruits from transformant 37-821, which has low PME enzyme activity, showed an increase in soluble solids (Table 1). Field trials of transgenic plants are necessary to establish the efficacy of these results.

Because large differences in percent soluble solids exist between the cultivated and wild tomato species, interspecies breeding has been used to improve levels of soluble solids in cultivated tomato varieties (Rick, 1974; Hewitt and Garvey, 1987). Restriction fragment length polymorphisms linked with genes associated with soluble solid content in tomato fruit have been identified (Osborn et al., 1987; Tanksley and Hewitt, 1988; Paterson et al., 1990). Effects of two of these restriction fragment length polymorphism segments, marked by Aconitase-2 (*Aco*-2) and chlorophyll *a/b* binding polypeptide-4 (*Cab*-4), on soluble solids were variable depending upon the genetic background in which they were placed (Tanksley and Hewitt, 1988). Both of these markers map on chromosome 7. The PME gene has been placed on chromosome 7, but is about 22 and 31 centimorgans apart from *Aco*-2 and *Cab*-4, respectively (Alpert et al., 1990). PME gene expression in *Lycopersicon chmielewskii* fruits, which have high levels of soluble solids, has not yet been examined (Rick, 1974). It will be interesting to determine what relationship, if any, exists between the levels of PME enzyme activity and soluble solid content in fruits from *L. chmielewskii* and cultivated tomato species containing quantitative trait loci from *L. chmielewskii*.

The mechanism responsible for increased soluble solids in fruits from transgenic plants with reduced PME enzyme activity is not clear. Some of this increase in soluble solids could be due to high methoxyl pectins which did not bind to cell walls. Little is known about the role of PME in plant growth and development. Ricard and Noat (1986) have proposed that interplay between PME enzyme activity and enzymes involved in cell wall expansion play an important role in cell wall extension and biosynthesis. This model, if true, could explain part of the increase in soluble solids in fruits from transgenic plants with reduced PME enzyme activity. Some precursors of cell wall biosynthesis would remain in the cytoplasm rather than being incorporated in the cell walls, thereby increasing the level of soluble solids. As stated earlier, we have seen accumulation of neutral oligomeric carbohydrates in the fruits from transgenic plants. Whether these represent the sugars which were not incorporated in the fruit cell wall remains to be seen. This model would, however, predict that the size of fruits from transgenic plants with reduced PME enzyme activity would be smaller than wild-type Rutgers fruit. Because several parameters influence fruit size in tomato, it is difficult to quantify changes in fruit size. We have not seen, however, an apparent change in the size of transgenic fruit. A large-scale greenhouse trial of transgenic tomato is in progress to determine the effect of reduced PME enzyme activity on fruit size.

METHODS

Plant Material

Tomato (*Lycopersicon esculentum* cv Rutgers) plants of the wild-type and transformed plants were grown under standard greenhouse conditions as described earlier (Biggs and Handa, 1989). Pericarp tissue was frozen in liquid nitrogen and stored at −80°C until use.

Plant Transformation

The 1.6-kb HincII-EcoRI fragment from a pectin methylesterase (PME) genomic clone (Harriman and Handa, 1990), isolated from a Charon 35 genomic library of Cherry VFNT tomato, was blunt ended and subcloned into the SmaI site of the polylinker region of vector pTZ18U (U.S. Biochemicals) in sense and antisense orientations. The HindIII-SacI

fragments from the resultant plasmids were cloned into the binary vector pKYLX7 (Schardl et al., 1987) (Figure 1) and transferred into *Agrobacterium tumefaciens* LBA4404 (Ooms et al., 1981) by triparental mating. Tomato cotyledons were transformed by cocultivation on tobacco feeder cells as described by Fillatti et al. (1987). Transformed plants were selected by growth on kanamycin-containing medium and confirmed by DNA gel blot analysis (Southern, 1975; Sambrook et al., 1989) with ^{32}P-labeled pKYLX7 or a 400-bp EcoRI-Sau3A fragment of the PME cDNA clone (Harriman et al., 1991).

PME Extraction and Enzyme Activity Assay

Total proteins from pericarp were extracted in 1 M NaCl, and PME enzyme activity was determined as described by Harriman et al. (1991). Total protein was determined by the method of Hartree (1972) using bovine serum albumin as a standard.

Immunoblot Analysis

Proteins extracted by 1 M NaCl from pericarp were electrophoresed on SDS polyacrylamide gels, immunoblotted to nitrocellulose, and detected with anti-PME antibodies and alkaline phosphatase-conjugated anti-chicken antibodies as described by Harriman et al. (1991).

RNA Hybridizations

Total RNAs from leaf and fruit pericarp tissues were extracted, electrophoresed on denaturing gels, and blotted to nitrocellulose as described earlier (Biggs and Handa, 1989). PME antisense and sense RNAs were detected using strand-specific probes of the 1.6-kb EcoRI-HincII fragment of the PME genomic clone cloned into pGEM3Z (Promega) and labeled with ^{32}P using a Promega in vitro transcription system using SP6 and T7 polymerases.

Genomic DNA Gel Blot Analysis

Genomic DNA was extracted from leaves by the method of Dellaporta et al. (1983). Ten micrograms was digested with the indicated restriction enzymes, electrophoresed on 0.7% agarose gel, denatured, and transferred to nitrocellulose as described for RNA gel blots. Blots were probed with ^{32}P-labeled pKYLX7 or a 400-bp EcoRI-Sau3A fragment of the PME cDNA clone PET1 (Harriman et al., 1991). Comparison of the hybridization intensities between the endogenous PME genes and the 1.6-kb DNA fragment from the introduced chimeric gene was used to identify heterozygous and homozygous segregants of 37-81^.

Ethylene Measurements

Tomatoes were enclosed in jars, and ethylene gas was collected and measured by gas chromatography as described earlier (Biggs et al., 1988).

Lycopene and Chlorophyll Determinations

Tomato pericarp tissue (1 g) was ground with a Tissuemizer homogenizer (Tekmar, Cincinnati, Ohio) and extracted with 1 volume 5:4 (v/v) hexane/acetone overnight at 4°C. Lycopene was determined as absorbance at 503 nm and chlorophyll at 665 nm and 649 nm (Handa et al., 1985).

Analysis of Pectins

To extract cell walls pericarp was preincubated in four volumes of ethanol at −80°C overnight before homogenizing with a Waring blender followed by homogenization with a Tissuemizer homogenizer. Homogenates were heated to 80°C for 20 min to inactivate cell wall–degrading enzymes, passed over a scintered glass funnel, washed with four volumes chloroform/ethanol (1:1) and four volumes acetone, and freeze-dried. The resulting ethanol-insoluble solids were used for determination of degree of esterification and extraction of EDTA-soluble pectins. The degree of esterification was determined by the method of Maness et al. (1990). EDTA-soluble pectins were extracted from ethanol-insoluble solids in 50 mM sodium acetate, 40 mM EDTA, pH 4.5, at 20°C for 4 hr and used for uronic acid assays and for column chromatography. Gel filtration column chromatography of pectic polysaccharides was performed as described by DellaPenna et al. (1990) using a Sepharose CL4B column (60 × 1 cm) and 0.4-mL fractions were collected. Fractions were analyzed for uronic acid content by the method of Filisetti-Cozzi and Carpita (1991). Each chromatographic profile was repeated three times with independently extracted samples with reproducible results. Estimation of the molecular mass of column fractions was made with blue dextran (2000 kD), branched dextrans 17.7 to 500 kD, and bromophenol blue (670 D) (Sigma). It should be noted that dextrans may not have the same conformation as pectic polymers; therefore, the values shown in Figure 8 are merely an estimation of molecular mass.

Determination of Total and Chelator-Soluble Pectins

Total uronic acids from ethanol-insoluble solids were determined by the method of Ahmed and Labavitch (1977). Chelator-soluble pectins were extracted from ethanol-insoluble solids as described by Huber (1983b), and uronic acids determined as described by Filisetti-Cozzi and Carpita (1991).

Determination of Soluble Solids

Either whole fresh fruits or frozen pericarp were homogenized in a Waring blender. An aliquot of homogenate was centrifuged in an Eppendorf microcentrifuge, and the supernatant was used to determine soluble solids (°Brix) with a hand-held refractometer (American Optical Corp., Buffalo, NY). As measured by refractometer °Brix detects reducing sugars and other soluble compounds (1 °Brix is approximately 1% [w/v] soluble solids). Total solids were determined by freeze drying either whole fruit or pericarp and determining dry weight as a percentage of fresh weight.

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