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Field Performance of Transgenic Tomato with Reduced Pectin Methylesterase Activity

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Abstract. **Transgenic 'Rutgers' 37-81^ tomato *(Lycopersicon esculentum* Mill.) homozygous for a pectin methylesterase (PME) antisense gene, which lowers PME activity and increases levels of soluble solids, was compared to azygous (a segregating line of 37-81^ with 0 copies of the introduced gene) and wild-type 'Rutgers' in the field during Summer 1992 and 1993 to determine the effects of the introduced PME antisense gene on tomato plant growth, fruit set, fruit yield, and fruit processing attributes. Fresh and dry weight accumulation in transgenic plants was similar to wild-type 'Rutgers' and azygous 37-81^ lines during 1992 and 1993, indicating that the introduced PME antisense gene did not affect biomass accumulation. Transgenic plants showed an increase in fruit number and yield in 1992 compared to wild-type 'Rutgers' and azygous 37-81^, but no differences were observed among the three genotypes in 1993. Average fruit weight did not show significant differences among the three genotypes in 1992, but was lower in azygous and transgenic plants than wild-type plants in 1993. Transgenic fruit had higher soluble and total solids and higher pH than control fruit, but shelf life was somewhat shorter in transgenic fruit. Overall, these data indicate that introduction of the PME antisense gene, which improves the processing quality of tomatoes, does not adversely affect fruit yield or vegetative growth of plants.**

Modification of plant gene expression by antisense, co-suppression, or over-expression of metabolizing enzymes has provided powerful tools for improving crop plants (Finnegan and McElroy, 1994; Tabler, 1993). Several genes with great potential to modify fruit shelf life have been identified, including 1-aminocyclopropane-1-carboxylate (ACC) synthase, ACC deaminase, ACC oxidase, and polygalacturonase. Alteration in the expression of these genes during ripening has led to significant improvement in shelf life of tomatoes (Gray et al., 1992; Hamilton et al., 1990; Klee et al., 1991; Kramer et al., 1992; Oeller et al., 1991; Schuch et al., 1991). In addition to shelf life, processing characteristics, such as total and soluble solids, juice viscosity, lycopene levels and chemistry of pectins, are economically significant parameters of processing tomato cultivars. Breeding approaches have been used to improve processing potential of tomatoes, and success has been achieved in increasing soluble solids such as sugars and organic acids (Hewitt and Garvey, 1987; Rick, 1974; Stevens, 1986). However, little progress has been made in modifying quality of pectins present in tomato fruit. The chemistry of fruit pectins is an important factor in determining the processing quality of fruit juice, especially gelling characteristics. Higher molecular weight and increased degree of methyl-esterification have been suggested to improve quality of processed products made from fruit juice (Leach et al., 1993; Miers et al., 1967). Reduction in polygalacturonase (PG) activity in tomato using its antisense gene has been reported to improve tomato shelf life and increase Bostwick values of processed tomato juice (Kramer et al., 1992; Schuch et al., 1991).

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We have introduced an antisense gene for pectin methylesterase (PME) into tomato under the control of the constitutive cauliflower mosaic virus 35S (CaMV 35S) promoter to develop transgenic tomatoes with reduced PME activity (Tieman et al., 1992). PME, which demethoxylates pectins, is believed to be involved in degradation of pectic cell wall components by lowering the degree of methoxylation of fruit pectins, thus making pectin more susceptible to depolymerization by PG (Pressey and Avants, 1982). In an earlier report, we demonstrated that tomatoes with reduced PME activity have altered pectin chemistry, including higher degree of pectin esterification and increased pectin molecularweight (Tieman et al., 1992). Greenhouse-grown transgenic fruit expressing a PME antisense gene also show higher levels of soluble solids (Tieman et al., 1992).

Since higher soluble solids and improved pectin chemistry are important parameters in determining processing quality, we evaluated transgenic tomato plants expressing a PME antisense gene to determine whether improved processing qualities are maintained stably under field conditions without any detrimental effects on plant growth. PME is an ubiquitous enzyme found in most tissues of all plants and has been implicated in various growth and developmental processes (Huber, 1983; Northcote, 1986; Rexova-Benkova and Markovic, 1976; Sexton and Roberts, 1982). Since genes under the control of the CaMV 35S promoter are expressed in most plant tissues (Harpster et al., 1988; Odell et al., 1985), we also determined the effects of the introduced gene on other agronomically important traits, including plant fresh and dry weight accumulation, fruit set, and fruit ripening. We report here that lowered PME activity does not have any deleterious effect on plant growth and development, but slightly improves the total and soluble solid contents of ripe tomatoes.

Materials and Methods

Plant material. Wild-type 'Rutgers', transgenic 37-81^, and azygous 37-81^ lines were tested in field trials to determine the effects of lowered PME activity on various agronomic traits. Transgenic 37-81^ and azygous 37-81^ are segregating lines of the transformant 37-81^ containing two (homozygous) and0 (azygous)

copies, respectively, of the introduced PME antisense gene (Tieman et al., 1992). The original 37-81^ transgenic (T_1) parental line was obtained by introducing a PME antisense gene into wild-type 'Rutgers' using *Agrobacterium tumefaciens* -mediated transformation (Tieman et al., 1992). Transgenic 37-81^ and azygous 37-81^ lines are maintained by selfing after each generation. During the 1992 trials, T4 lines were used, while T5 seeds obtained from the 1992 trials were used for the 1993 studies. Purity of each population was established using a polymerase chain-reaction assay for the introduced gene.

Field trial design. Tomato plants were grown in the field at the O'Neall Memorial Research Farm (Tippecanoe County, Ind.) in a randomized complete block design with four replications for each cultivar. Each row contained 10 plants spaced 46 cm apart, and rows were spaced 9 1 cm apart. The plot consisted of an experimental row and two guard rows for each block, with the same line grown in the experimental and guard rows of each block. All fruit at the red-ripe stage were harvested weekly, and total fruit weight and fruit number from each row were determined. Average fruit weight was determined by dividing total fruit weight by total fruit number collected from each row. Random fruit samples were selected from each row at each harvest and used to determine pH, soluble solids, and total solids content. Individual whole fruit were ground in a blender and pH was determined. An aliquot of the homogenate was centrifuged in an Eppendorf microcentrifuge and the supernatant was used to determine soluble solids (°Brix) with a refractometer. Total solids content was determined by freeze drying an aliquot of the homogenate and dry weight was calculated as a percentage of fresh weight. At the end of the growing season, all plants from the experimental rows were harvested individually from the ground level and weighed immediately to determine plant fresh weight. Dry weight was determined after drying plant tissue at 50C until a constant weight was obtained.

Fruit grade and shelf life. Freshly harvested fruit were graded for quality as described by Gould (1992) and according to the following: grade A was free of blemishes with even color development, grade B had minor or uneven color development, grade C had some blemishes without any signs of deterioration, while grade D had major blemishes and deterioration. Fruit shelf life was determined by storing ripe fruit at room temperature (25 ± 2C). Fruit were considered unusable when the first signs of deterioration were observed.

PME activity and protein levels. PME activity and protein levels were determined by protein blotting with anti-PME antibodies as described earlier (Harriman et al., 1991; Tieman et al., 1992)

Statistical analysis. Data for the four experimental replicates were analyzed using SAS general linear model procedures (SAS Institute, Cary, N.C.).

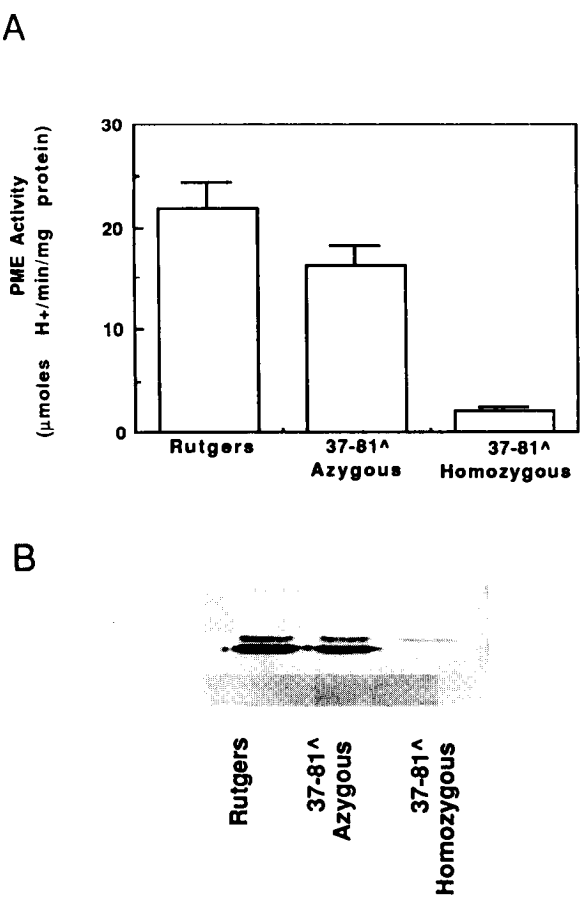

Fig. 1. Effects of the introduced PME antisense gene on PME gene expression. PME activity **(A)** and PME protein levels **(B)** in red ripe fruit from control 'Rutgers' and azygous 37-81^ plants and transgenic 37-81^ plants. For PME activity, bars represent means ± SE of 10 independent samples for each genotype. For PME protein, 10 µg total salt-extractable protein from ripe tomatoes was electrophoresed on 12% SDS-PAGE, blotted onto nitrocellulose and visualized using polyclonal fruit PME antibodies. The lower band represents the 34kD PME polypeptide (Tieman et al., 1992).

Results and Discussion

Effects of the introduced antisense PME gene on PME gene expression. PME activity and protein levels in transgenic 37-81^, azygous 37-81^, and wild-type 'Rutgers' fruit were determined to show that the introduced antisense PME gene lowered PME gene expression in field-grown transgenic tomatoes in a manner similar to that seen in greenhouse-grown fruit (Tieman et al., 1992). As shown in Fig. 1, PME activity was reduced in transgenic fruit to < 10% of wild-type or azygous 37-81^ control fruit. The levels of PME protein, as detected by immuno-blotting with PME antibodies, were also severely reduced in transgenic 37-81^ fruit. The 34kD PME isozyme was not detectable in fruit of transgenic 37-81^ plants. The levels of PME activity and protein in azygous 37-81^ fruit were similar to that of wild-type fruit, indicating that reduced PME gene expression in transgenic 37-81^ fruit was due to the presence of the introduced PME antisense gene. Since seeds of the T5 generation were used for these studies, these results demonstrate that the effects of the introduced antisense PME genes are stably maintained during sexual propagation of transgenic plants.

Effects of the introduced gene on plant biomass and fruit yield. PME has been proposed to be involved in several growth and developmental processes in plants, including cell wall growth, maturation, and extensibility; cation binding capacity and modulation of pH of cell walls; ion acquisition and balance; cell wall porosity; and formation of abscission zones (Baron-Epel et al., 1988; Fry et al., 1993; Grignon and Sentenac, 1991; Moustacas et al., 1991; Nari et al., 1991; Rexova-Benkovaand Markovic, 1976; Sexton and Roberts, 1982). To determine the effects of the introduced gene on biomass accumulation, plants were harvested at the end of the growing season and total gains in fresh and dry weight were determined. As shown in Table 1, no significant differences were observed in fresh or dry weight of plants between wild-type 'Rutgers', azygous 37-81^ and transgenic 37-81^ during 1992 and 1993 trials. The ratio of dry weight to fresh weight was not different

Table 1. Field evaluation of transgenic 37-81^, azygous 37-81^, and wild-type 'Rutgers' tomato plants during Summer 1992 and 1993.[z]

Genotype	Plant fresh wt (g)	Plant dry wt (g)	Plant dry wt (%)	Fruit yield/ plant (g)	Avg fruit weight (g)	Fruit/ plant (no.)
			1992			
Rutgers	1930 ± 199	392 ± 30	20.9 ± 0.6	3075 ± 220	94.6 ± 5.0	32.6 ± 2.0
Azygous 37-81^	2539 ± 330	474 ± 31	20.6 ± 1.4	3496 ± 430	88.3 + 2.9	39.8 ± 5.1
Transgenic 37-81^	2098 ± 66	401 ± 20	19.8 ± 0.9	3980 ± 160	89.4 ± 2.5	44.6 ± 1.4
			1993			
Rutgers	872 ± 104	183 ± 18	21.2 ± 0.7	2510 ± 349	100.3 ± 3.0	24.9 ± 3.2
Azygous 37-81^	754 ± 50	150 ± 13	19.9 ± 0.9	2338 ± 67	80.1 ± 3.5	29.2 ± 0.7
Trangenic 37-81^	817 ± 16	157 ± 4	19.3 ± 0.6	2157 ± 250	78.8 ± 3.2	27.2 ± 2.1

[z]Numbers reported represent means ± SE of four experimental rows of 10 plants each. Transgenic 3781^ is homozygous for the introduced PME antisense gene, while azygous 37-81^ is a segregating line with 0 copies of the introduced gene.

among the three genotypes for the two growing seasons. Additionally, we observed no phenotypic differences among the three genotypes. Lack of effect of the introduced gene on biomass accumulation and plant phenotype is likely due to the fact that we have used a PME gene, which is expressed only in fruit tissues (Harriman et al., 1991). The presence of multiple isoforms of PME in tomato and other crops has been demonstrated in various plant tissues (Gaffe et al., 1994; Sajjanantakul and Pitifer, 1991). We have shown earlier that accumulation of the PME isoform present in the vegetative tissues of tomato plants is not inhibited by the expression of a fruit specific PME antisense gene (Gaffe et al., 1994).

Total fruit yield per plant from transgenic 37-81^ was not significantly different from wild-type or azygous 37-81^ control plants in 1993, but higher fruit yield compared to 'Rutgers' was observed for transgenic 37-81^ in 1992 (Table 1). In 1992, fruit number per plant was higher in the transgenic 37-81^ than in the 'Rutgers' control, but was not significantly different from the azygous 37-81^ (Table 1). However, in 1993 significant differences in fruit number per plant among the three genotypes were not

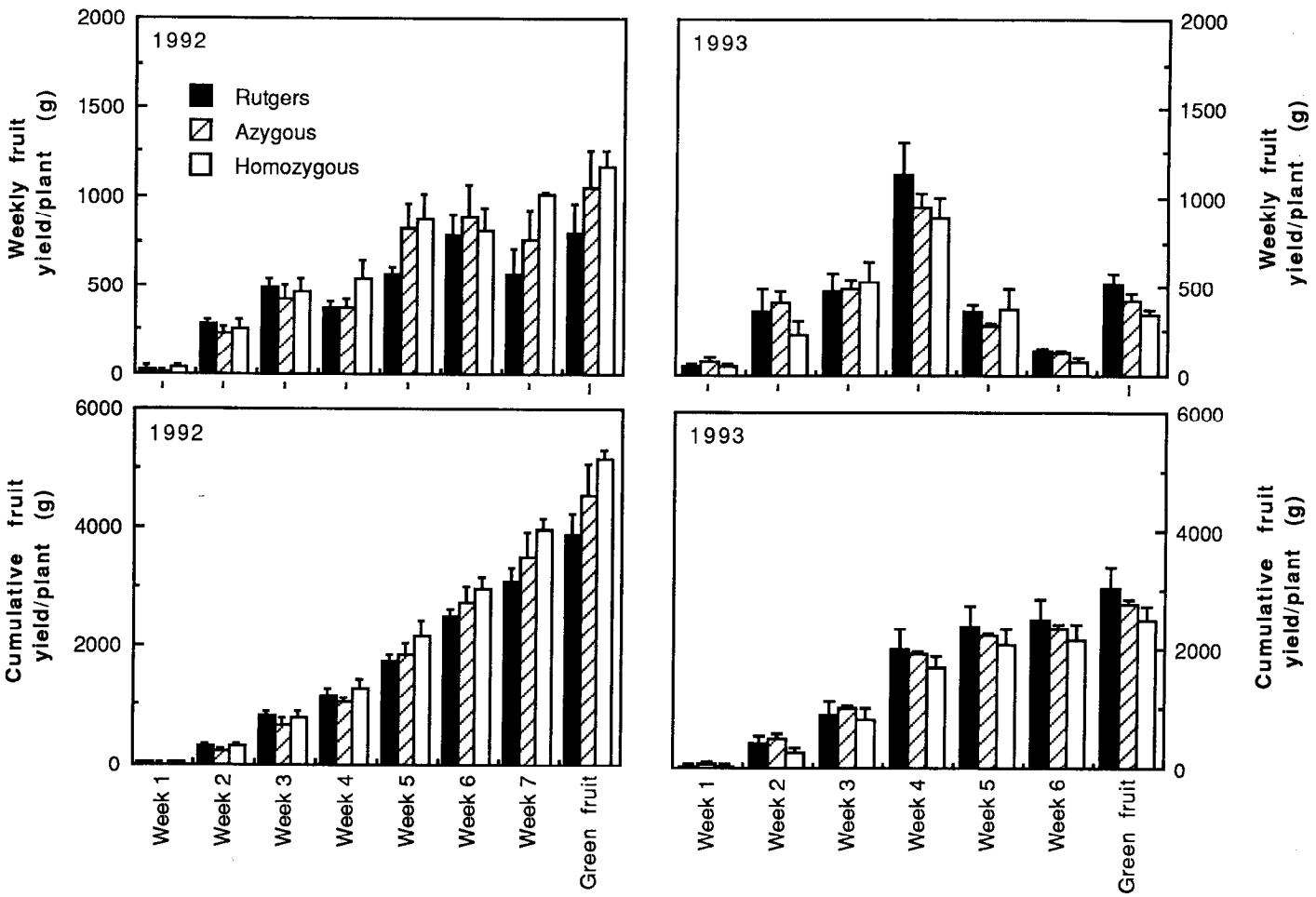

Fig. 2. Weekly and cumulative fruit yields from wild-type 'Rutgers', azygous 37-81^, and transgenic 37-81^ at successive harvests during 1992 and 1993. Cumulative fruit yield is the sum of all weekly harvest yields from the first weekly harvest until and including the harvest week indicated. Bars represent means ±SE of four replicates with 10 plants each.

observed. During the 1993 harvest average fruit weight from the transgenic 37-81^ and azygous 37-81^ was similar but lower than the wild-type 'Rutgers' control (Table 1).

Although relative fruit and vegetative yields were similar among the three genotypes for the same year, they differed between the 2 years. To investigate the basis of this finding, we examined fruit fresh weight and fruit number per plant for each weekly harvest during the growing season. During 1992, fruit yield for all genotypes tested increased until the fourth weekly harvest and then remained similar until the last harvest, with many green fruit still remaining on the plants (Fig. 2). This resulted in a continuous increase in cumulative yield during the 1992 harvests. However, in 1993, fruit yield for all three genotypes increased until the fourth weekly harvest and then declined during the fifth and sixth weekly harvests (Fig. 2). This resulted in a slight increase in cumulative yield during later harvests in 1993 (Fig. 2). The patterns of weekly fruit yield and cumulative fruit yield for each year were similar in all three genotypes, except for the seventh weekly harvest of 1992 when higher fruit yield was observed for transgenic 37-81^ compared to wild-type 'Rutgers' (Fig. 2). Also, cumulative fruit yield for the 1992 harvest, after including green fruit, was higher fortransgenic 37-81" compared to 'Rutgers' (Fig. 2). Fruit number for the three genotypes showed a similar pattern during both years, except that in 1992 a higher fruit number was observed in transgenic 37-81^ than in wild-type 'Rutgers' during the seventh weekly harvest (Fig. 3). For all genotypes in 1992, fruit number increased with each weekly harvest, while in 1993 it peaked on the fourth weekly harvest and then declined. It is likely that differences in field performance during 1992 and 1993 were due to the climatic conditions. There were many cool, cloudy days during the time of fruit set in 1993 (data not shown), and this may have contributed to decreased yield. However, for the same year all growth parameters were similarly affected for all three genotypes, suggesting that the introduced antisense gene has no deleterious effect on plant and fruit growth even under very different climatic conditions.

Variable patterns for average fruit weight for the three genotypes were observed for the 1992 and 1993 growing seasons, Average fruit weight increased until the third weekly harvest of 1992 before declining and remained constant during the first and second weekly harvests of 1993 before declining (Fig. 3). In 1992, only the sixth weekly harvest showed some decrease in average fruit weight from transgenic 37-81^ compared to 'Rutgers' and azygous 37-81^ controls. However, for most weekly harvests in 1993 the average fruit weight from transgenic 37-81^ and azygous 37-81^ was lower than wild-type 'Rutgers' (Fig. 3). Although no difference in the average fruit weight was observed between transgenic 37-81^ and azygous 37-81^, the average fruit weight of these genotypes was significantly lower than wild-type 'Rutgers' during the 1993 harvest (Table 1). This may be due to tissue culture-induced mutations, including somaclonal variations, that have been reported in many plant species (Maliga, 1984). Collectively, our results indicate that the presence of the antisense PME gene does not affect fruit number or average fruit weight adversely.

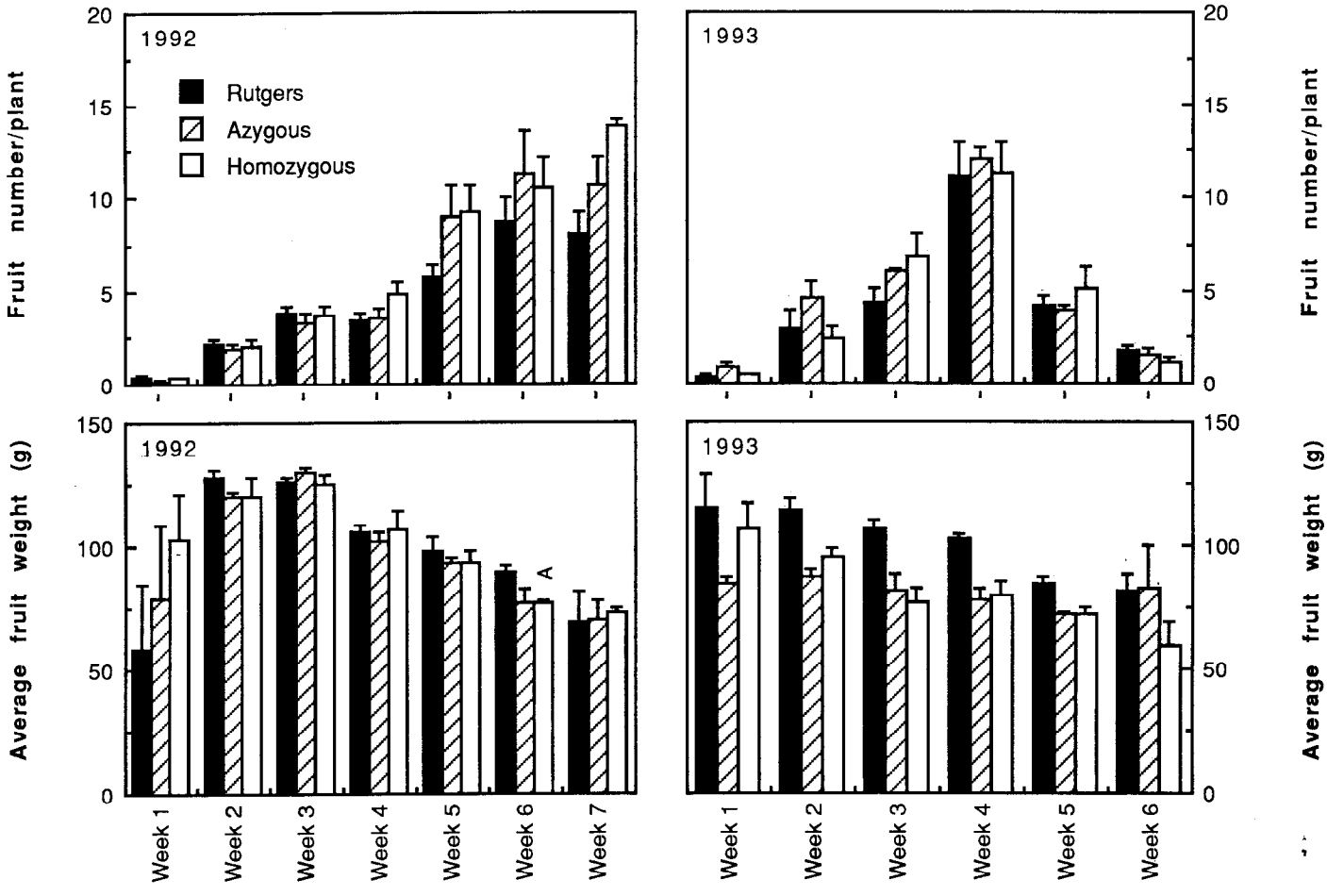

Fig. 3. Fruit number and average fruit weight from wild-type 'Rutgers', azygous 37-81^, and transgenic 37-81^ at successive harvests during 1992 and 1993. Bars represent means ± SE of four replicates with 10 plants each.

Table 2. Quality grade of wild-type 'Rutgers', azygous 37-81^, and transgenic 37-81" field-grown ripe tomatoes on the day of harvest.

Genotype	Total fruit (no.)	Mean fruit distribution by grade (% of total) ± SE[z,y]			
		A	B	C	D
Rutgers	965	19.9 ± 2.4	45.7 ± 4.3	25.3 ± 1.1	9.1 ± 2.4
Azygous 37-81	1153	23.2 ± 2.1	42.2 ± 3.9	29.1 ± 5.9	5.5 ± 0.7
Transgenic 37-81^	1090	18.7 ± 0.6	42.7 ± 0.7	29.8 ± 2.0	8.9 ± 1.5

[z]Fruit quality grade was determined as described in Materials and Methods. Grade A is the highest quality fruit, while grade D fruit would be rejected as unusable.
[y]No significant differences were found in the percentage of total fruit in a grade class among the three genotypes.

Table 3. Soluble and total solids and pH of the homogenate of field-grown wild-type 'Rutgers', azygous 37-81^, and transgenic 37-81^ fruit.[z,y]

Genotype	Soluble solids (°Brix)	Total solids (%)	pH
Rutgers	5.95 ± 0.03 (479)	7.61 ± 0.6 (288)	4.43 ± 0.01 (449)
Azygous 37-81^	6.00 ± 0.03 (373)	7.75 ± 0.07 (228)	4.41 ± 0.01 (360)
Transgenic 37-81^	6.16 ± 0.03 (488)	7.95 ± 0.05 (286)	4.47 ± 0.01 (450)

[z]Numbers represent means ± SE for all fruit analyzed.
[y]Numbers in parentheses represent the number of independent fruit samples analyzed.

Effects of the introduced gene on fresh fruit quality. Tomatoes were scored for quality grade on the day of harvest according to standard procedures (Gould, 1992). Although grade is a somewhat subjective measurement, all fruit were subjected to the same standards during visual inspection. As shown in Table 2, no significant differences in fruit quality among the three genotypes were found. Most fruit graded were found to be slightly imperfect (Grade B) but usable for processing. Between 5% to 10% of the fruit from each genotype was unusable.

Shelf life of field-grown tomatoes was determined by harvesting ripe fruit followed by storage at room temperature. Tomatoes were discarded when evidence of deterioration or desiccation was observed. 'Rutgers' and azygous 37-81^ fruit had a similar shelf life, while transgenic 37-81^ fruit had a slightly shorter shelf life than either 'Rutgers' or azygous 37-81^ fruit (data not shown). The reduced shelf life of transgenic fruit with reduced PME activity is consistent with our earlier result that PME plays an important role in determining tissue integrity during fruit senescence (Tieman and Handa, 1994). Transgenic fruit have significantly lower levels of bound Ca^{2+} (Tieman and Handa, 1994), which has been suggested to play a role in fruit firmness by contributing to the cohesiveness of the cell wall (Demarty et al., 1984; Fry, 1986; Grant et al., 1973). A reduction in cell wail integrity may also increase the susceptibility of the fruit to invading pathogens, resulting in decreased shelf life. Contrary to earlier speculation that PME plays a role in fruit softening by enhancing susceptibility of pectins to PG, our data indicate that PME is involved in maintaining integrity of fruit tissue during ripening and senescence (Tieman and Handa, 1994).

Effects of the introduced gene on tomato processing parameters. Several hundred field-grown fruit from transgenic 37-81^, azygous 37-81^, and wild-type 'Rutgers' were evaluated for increases in total and soluble solids and pH of the raw juice (Table 3). Whereas soluble and total solids in wild-type 'Rutgers' and azygous 37-81^ fruit were not significantly different, transgenic 37-81^ fruit showed significantly increased levels of soluble and total solids compared to the two controls. The Brix levels in transgenic 37-81^ fruit were 6.16 ± 0.03 compared to 5.95 ± 0.03 for 'Rutgers' and 6.00 ± 0.03 for azygous 37-81^. Total solids showed somewhat larger increases (4.5%) compared to soluble solids (3.5%) in transgenic 37-81^ fruit. Previously, we reported an increase in soluble solid levels but not in total solids in greenhouse-grown transgenic fruit pericarp (Tieman et al., 1992). It is not clear if this is due to the use of total fruit in the present study instead of only pericarp in the previous study. Also, several factors including environmental conditions, nutrition, stress, age of plant, yield per plant, and cultural practices can influence soluble and total solids levels in tomatoes (Davis and Hobson, 1981). Although the pH of raw juice from transgenic 37-81^ fruit was slightly higher than that of control wild-type and azygous fruit, increases in pH levels detrimental to processing were not observed (Table 3). A slightly higher pH was observed for raw juice of transgenic fruit may be explained by a reduction in free carboxylic groups in fruit pectins due to the increased degree of methoxylation (Tieman et al., 1992). Since the yield of tomato paste is directly proportional to solids levels, observed increases in soluble and total solids will have a significant impact on the processing performance of tomatoes (Gould, 1992). Overall, we have shown that reduction in fruit PME activity by the expression of its antisense gene has increased the processing quality of fruit without adversely affecting fruit yield or plant growth.

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