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Inheritance and effect on ripening of antisense polygalacturonase genes in transgenic tomatoes

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Abstract

The role of the cell wall hydrolase polygalacturonase (PG) during fruit ripening was investigated using novel mutant tomato lines in which expression of the PG gene has been down regulated by antisense RNA. Tomato plants were transformed with chimaeric genes designed to express anti-PG RNA constitutively. Thirteen transformed lines were obtained of which five were analysed in detail. All contained a single PG antisense gene, the expression of which led to a reduction in PG enzyme activity in ripe fruit to between 5% and 50% that of normal. One line, GR16, showed a reduction to 10% of normal PG activity. The reduction in activity segregated with the PG antisense gene in selfed progeny of GR16. Plants homozygous for the antisense gene showed a reduction of PG enzyme expression of greater than 99%. The PG antisense gene was inherited stably through two generations. In tomato fruit with a residual 1% PG enzyme activity pectin depolymerisation was inhibited, indicating that PG is involved in pectin degradation *in vivo*. Other ripening parameters, such as ethylene production, lycopene accumulation, polyuronide solubilisation, and invertase activity, together with pectinesterase activity were not affected by the expression of the antisense gene.

Introduction

During ripening, fruit undergo changes in physiology and biochemistry that affect colour, flavour, and texture. In tomatoes these changes include an increase in ethylene production characteristic of the ripening of climacteric fruit, and an accumulation of the red pigment lycopene and the cell wall hydrolase polygalacturonase (PG). PG protein is not detected in unripe fruit but accumulates during ripening due to *de novo* enzyme synthesis [37]. Current evidence suggests that PG is developmentally regulated at the level of transcription [28]. PG mRNA is not detected in extracts from green fruit but the steady-state level increases during ripening reaching a peak in orange or early red fruit before declining [9, 20]. Genomic clones encoding the PG gene of tomato have been iso-

lated and characterised [3, 23]. Analysis of these clones indicates that there is only one PG gene per haploid genome.

The precise role of PG during ripening is at present unknown. In tomato PG accumulation is closely correlated with softening [17, 14]. In contrast, there is little correlation between softening and the activities of other cell wall degrading enzymes such as pectinesterase [6, 24, 25], or cellulase [18, 6]. Furthermore, fruit of the *rin* mutant do not soften significantly during ripening and produce little PG [32, 34, 37]. These observations have led to the suggestion that PG is primarily responsible for softening during tomato ripening. However, other enzymes and non-enzymic processes may also be important in softening [38].

During ripening significant breakdown of pectin in tomato pericarp can be observed as an increase in the amount and a decrease in the molecular weight of soluble polyuronides [19, 25]. PG action has been implicated in this pectin breakdown, since purified PG degrades isolated cell walls *in vitro* [31], and induces changes in cell wall ultrastructure characteristic of ripening when applied to unripe pericarp [8]. However, polyuronide degradation *in vivo* is likely to be much less extensive than that observed *in vitro* [25, 26] and the relationship between PG activity and pectin degradation remains unclear.

Recently, work with transgenic plants has led to the role of PG in softening being questioned [30, 11]. Fruit of plants engineered to express antisense PG RNA, have been shown to develop only 10% of the normal level of PG during ripening [30, 28]. Despite this reduction, no difference in the compressibility of the fruit was observed [30]. However, PG is produced in large quantities in ripening fruit and it is possible that an insufficient reduction in protein was obtained to affect softening. Giovannoni *et al.* [11] have shown that in *rin* fruit transformed with a chimaeric PG gene under the direction of an ethylene/propylene-inducible promoter there is no significant effect on fruit compressibility when PG is induced. However, since *rin* is a pleiotropic mutation, several aspects of fruit ripening are affected. Consequently, the lack of softening in transgenic *rin* fruit may be due to the reduced expression of other genes necessary for PG action.

It has also been suggested that PG may have a role in the initiation of tomato ripening by inducing changes in compartmentalisation of proteins [32], or by stimulating ethylene production leading to ripening [5, 1]. Ethylene appears to have a co-ordinating role in fruit ripening [39], but the increase in endogenous ethylene production is detected prior to that of PG [4, 12].

We have previously described the production of transgenic tomato plants expressing a PG antisense gene which have reduced levels of PG activity [30]. In this paper we describe the production of further transgenic plants with reduced PG activity due to the presence of the antisense gene. We also present a detailed analysis of the fruit ripening characteristics of the selfed progeny of one of these plants, some of which produce only 1% of the normal level of PG. The role of PG in pectin degradation and fruit ripening is discussed.

Materials and methods

Plant material

Tomato plants (*Lycopersicon esculentum* Mill cv. Ailsa Craig) were grown in glasshouses, or in controlled environment chambers as described previously [13].

Tomato transformation

The PG antisense gene was constructed by removing a 730 bp *Hinf* I fragment from the 5′ end of the PG cDNA clone (pTOM6) [15], including 50 bp of untranslated region, and cloning it in the reverse orientation between cauliflower mosaic virus (CaMV) 5′ and nopaline synthase (nos) 3′ sequences [30]. It was then cloned into the plant transformation vector, Bin 19, which was then mobilised to *Agrobacterium tumefaciens* strain LBA 4404. Transformation of tomato stem segments was per-

formed as described previously [3]. Separate transformation events were designated 'T' and numbered sequentially. Individual plants were designated 'GR' and also numbered sequentially.

Genomic Southern blotting

Genomic DNA was extracted from frozen leaves by the method of Raeder and Broda [22]. Approximately 10 μg DNA was digested with restriction enzymes, fractionated in a 0.8% agarose gel, and blotted by the method of Chomczynski and Qasba [7]. Membranes were prehybrised in 5 × SSPE (0.75 M NaCl, 50 mM $NaH_2PO_4 \cdot H_2O$, 5 mM EDTA) 1% SDS, 100 μg ml^{-1} denatured herring sperm DNA at 65 °C for 4 hours, and hybridized in the same buffer containing probe for 18 hours at 65 °C. The probe was either nick-translated or oligo-labelled [10] *Pst* I insert of pTOM6 (PG cDNA) [15]. The membranes were washed in 0.1 × SSC, 1% SDS at 65 °C and exposed to X-ray film.

Ethylene production

Fruit were placed in gas-tight jars at 20 °C for 1–2 hours after which time a 1 ml sample was removed for ethylene measurement by gas chromatography [12].

Enzyme extraction and assay

Enzymes were extracted as described previously [34]. PG and invertase were assayed in 50 mM sodium acetate pH 3.8, 150 mM NaCl, 0.5% polygalacturonic acid and 50 mM sodium acetate pH 4.6, 150 mM NaCl, 200 mM sucrose respectively. Reducing groups formed were measured by the arsenomolybdate method of Nelson [21]. Pectinesterase (PE) was assayed in 150 mM NaCl, 0.5% pectin [36]. The level of 5 mM NaOH required to maintain the pH at 8.0 was measured using a Radiometer automatic titrator.

Pigment extraction and assay

Pigment was extracted from pericarp by vigorously homegenising in a 6 : 4 mixture of hexane and acetone and scanned in a spectrophotometer. Lycopene at 1 mg ml^{-1} has an absorbance of 320 at 502 nm [33].

RNA extraction and northern blotting

RNA was extracted from tomato fruit pericarp [13] and leaf tissue [29] as described previously. 10 μg samples of glyoxal denatured total RNA were fractionated in 1.2% agarose gels prior to blotting onto nylon membranes (Hybond-N, Amersham). PG sense- and antisense-specific probes were synthesised using a TransProbe T kit (Pharmacia) [30]. Hybridization was performed at 65 °C in 10× NTE (0.3 M NaCl, 2 mM EDTA, 60 mM Tris-HCl pH 8.0), 1% SDS, 200 μg ml^{-1} denatured salmon sperm DNA, 5 × Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA). Membranes were washed at 65 °C in 0.2 × NTE, 0.1% SDS. PG mRNA was quantified from autoradiographs of the northern blot using a Joyce Loebl Chromoscan densitometer.

Cell wall polyuronide determinations

Tomato acetone-insoluble solids (AIS) were prepared from individual tomato fruit as described by Seymour *et al.* [25]. Soluble polyuronides were extracted from AIS by incubation with constant stirring in 50 mM sodium acetate pH 4.5, 40 mM EDTA, for 4 hours [27]. After filtration soluble polyuronides were determined using the *m*-hydroxydiphenol method [2], specific for uronic acids. The weight-average (apparent) M_r values of these polyuronides were determined by low-speed sedimentation equilibrium as described previously [27] except that a 5 mW He-Ne laser light source was used. At the low loading concentrations used (0.4 mg ml^{-1}) the effects of non-ideality are likely to be small (see Table 2.2 in

[16]) and hence the apparent M_r values are likely to be only a few percent underestimates of the true values. The samples were analysed in a 50 mM phosphate/chloride buffer pH 6.8, containing 50 mM NaCl, 5 mM EDTA. In an attempt to exclude the effects of aggregation selected samples were also analysed in 100 mM phosphate/chloride buffer pH 6.8 containing 1 M NaCl, 5 mM EDTA. No differences were observed between the two methods.

Results

Analysis of individual transgenic plants expressing anti-PG RNA

Tomato plants were transformed with a PG antisense gene (JR16A), designed to express anti-PG RNA constitutively [30]. Thirteen separate transformants were selected on the basis of kanamycin resistance, five of which have been grown to maturity and analysed further. A Southern blot of *Xba* I-digested leaf DNA from these transformants was probed with labelled PG cDNA (Fig. 1). In addition to the endogenous fragments (Fig. 1, lane 1) a single additional fragment of varying size was observed in DNA from transformed plants (Fig. 1, lanes 2–6). A plant containing an antisense gene would be expected to give two additional bands: a fragment larger than 908 bp, depending on site of insertion, and a small internal fragment of 374 bp. The small fragment was not detected in this blot. In a separate Southern blot of *Eco* RI and *Hind* III digested DNA probed with PG cDNA, all transgenic plants were shown to contain fragments of 1075 bp and 460 bp representing fragments internal to the inserted DNA (data not shown). Analysis of ripe fruit showed that PG activity was reduced in all five antisense transformed plants and varied from 5% to 51% of normal (Table 1).

Inheritance of the antisense gene

Eleven progeny produced by selfing one individual transformant, GR16, were studied. A

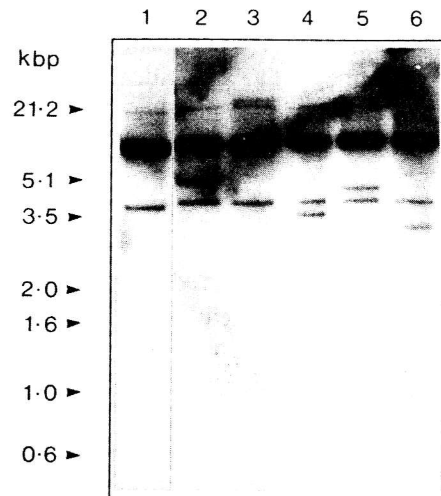


Fig. 1. Genomic Southern blot of DNA from 5 individual primary transformants containing the JR16A antisense construct. Approximately 10 μg of genomic DNA was digested with *Xba* I and probed with the *Pst* I insert of pTOM6 (PG cDNA). Lane 1, untransformed plant; lane 2, T30; lane 3, T26; lane 4, T27; lane 5, T29; lane 6, T41. In addition to the endogenous bands lanes 2 to 5 each contain a single additional hybridizing fragment.

Southern blot of *Eco* RI and *Hind* III digested leaf DNA from each plant was probed with labelled PG cDNA. Plants with inserted antisense genes contained two additional bands (1075 bp and 460 bp) that were not present in those of untransformed plants. The size of the larger fragment was reported previously as 1230 bp on the basis of restriction analysis of the

Table 1. PG activity in ripe fruit of JR16A transformants.[1]

Transformed plant line	Number of plants assayed	mean % normal PG activity
T26	3	10
T27	3	36
T29	3	5
T30	2	36
T41	1	51

[1] Separate transformation events were designated T and numbered sequentially. PG activity is the mean from between 2 and 4 fruit per plant, extracted and assayed individually.

CaMV promoter [30]. This has been corrected to 1075 bp following sequencing. Plants were allocated to three groups on the basis of intensity of the antisense bands in comparison with the endogenous PG bands in the same track: those without antisense fragments, those with low hybridisation and those with high hybridisation to the antisense gene (Fig. 2A). These groups corresponded to three classes of plant identified on the basis of PG activity in ripe fruit (Table 2). Selfed progeny without inserted DNA had normal PG activity, those with low-intensity hybridisation had approximately 20% of normal PG activity, and those with high hybridisation had about 1% of PG activity. This demonstrates that the antisense gene segregated with the reduced PG phenotype in the F_1 generation. Futhermore, plants with 1% PG activity may be homozygous for the PG antisense gene, those with 20% PG activity hemizygous and those with normal PG activity have not inherited the antisense gene. Ten selfed progeny of a 1% PG plant, GR98, also produced fruit that had a 1% PG phenotype confirming it as homozygous for the inserted antisense gene.

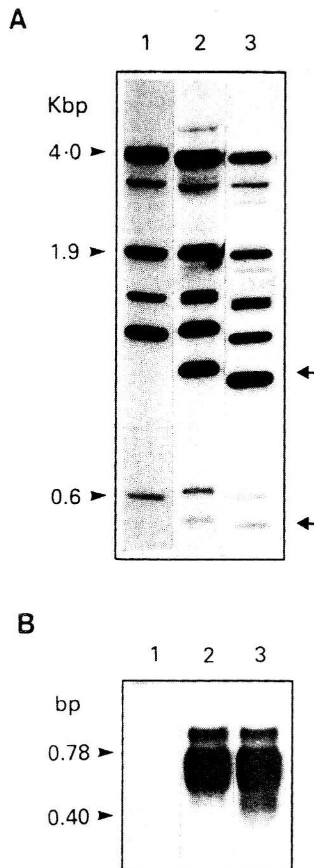


Fig. 2. Inheritance and expression of antisense genes in GR16 selfed progeny. A. Genomic DNA was extracted from 11 F_1 plants. Data from a representative plant expressing normal (GR106), 20% (GR104), or 1% (GR103) PG activity is presented here. Approximately 10 μg DNA was digested with *Eco* RI and *Hin*d III and probed with the *Pst* I insert of pTOM6 (PG cDNA). Lane 1, GR106; lane 2, GR104; lane 3, GR103. DNA from plants containing the antisense gene have additional fragments (indicated by arrow) of 1075 bp and 460 bp. B. Total leaf RNA (10 μg) from 11 F_1 plants was analysed by northern blot probed with an antisense strand-specific probe. Data from GR106, GR104, and GR103 are presented.

Table 2. PG activity in ripe fruit of GR16 (T26) progeny.[1]

Plant	Mean % normal PG activity	Antisense gene copy number (estimated from Southern blot)
GR100	111	0
GR102	86	0
GR106	111	0
GR95	20	1
GR96	26	1
GR101	16	1
GR104	27	1
GR98	<0.5	2
GR99	0.85	2
GR103	1.3	2
GR105	0.85	2

[1] Individual transformed plants were designated GR and numbered sequentially. PG activity is the mean from 2 fruit per plant harvested at 7 days post breaker, extracted and assayed individually.

PG antisense gene expression

In order to determine whether or not the level of antisense PG RNA was correlated with the degree of reduction of PG activity, leaf RNA was extracted from 11 F_1 plants of GR16 and investigated by northern analysis. When probed with an antisense-specific probe no relationship between

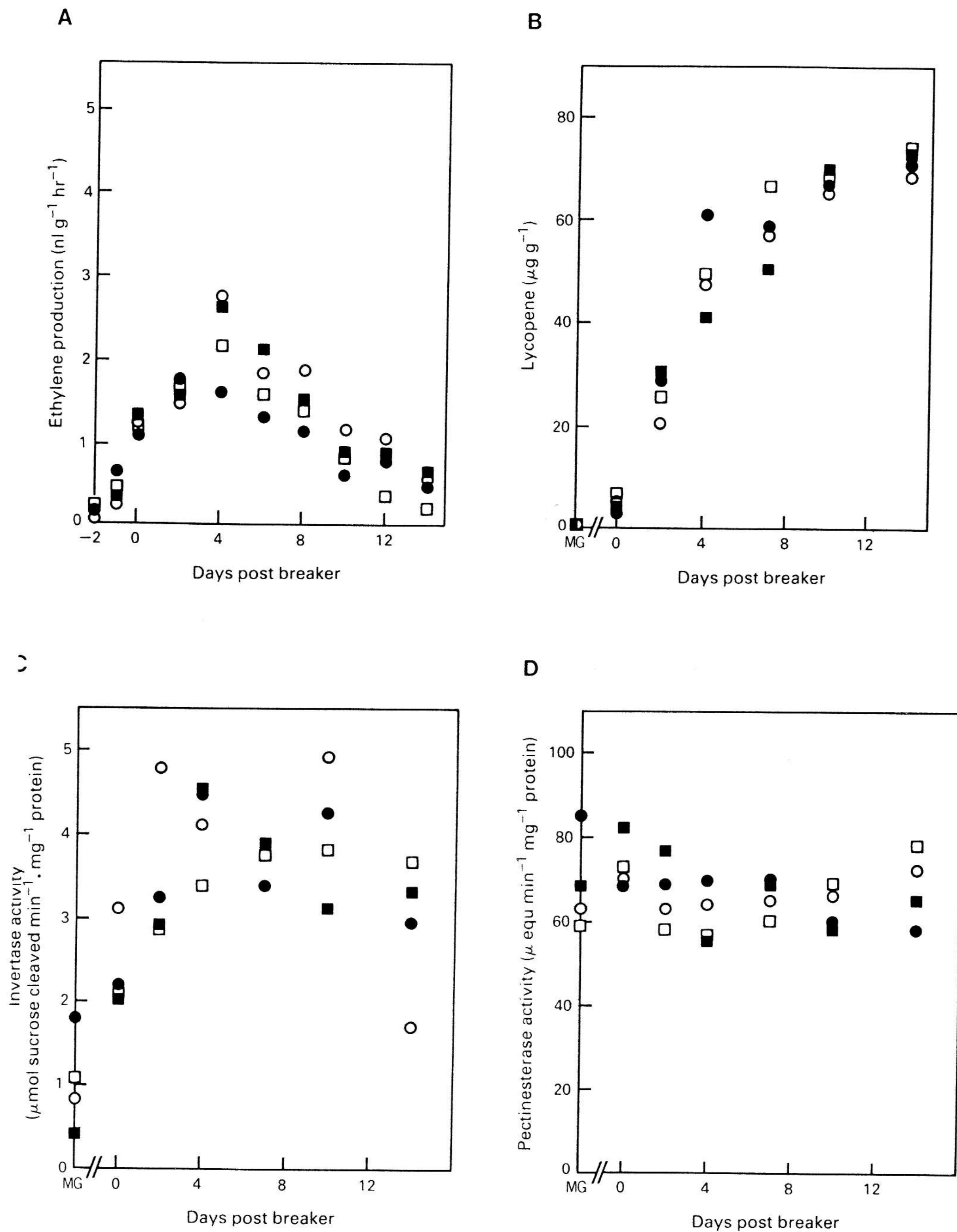


Fig. 3. Ethylene production, lycopene accumulation, and the activities of invertase and pectinesterase during ripening of selected GR16 progeny containing 0, 1, or 2 antisense genes. Fruit were either tagged at the first visible sign of ripening, harvested at various days thereafter, and assayed for enzyme activity or lycopene accumulation, or were harvested when mature green and their ethylene production monitored during ripening off the vine. Untransformed (○); GR102 (100% PG) (●); GR95 (20% PG) (□); GR105 (1% PG) (■). A. Ethylene production. B. Lycopene accumulation. C. Invertase activity. D. Pectinesterase activity. MG: mature green.

the amount of antisense PG RNA in leaves and the reduction in PG activity in fruit was observed (Fig. 2B). However, plants with 1% of normal PG activity appeared to contain at least one additional low molecular weight band on northern blots. The exact identity or significance of this band is not yet clear.

Biochemical ripening characteristics

Representatives of each of the three groups of GR16 progeny (GR102: 100% PG; GR95: 20% PG and GR105: 1% PG) were selected for more detailed study and their ripening behaviour compared to that of normal fruit. The presence of an active antisense PG gene was found to have no effect on ethylene production (Fig. 3A), lycopene accumulation (Fig. 3B), invertase activity (Fig. 3C), or pectinesterase activity (Fig. 3D) during ripening. In contrast, a reduction in PG activity was apparent throughout ripening of GR95 and GR105 fruit (Fig. 4A).

PG mRNA analysis

The amount of PG mRNA present in normal fruit and the three types of GR16 progeny was determined by northern analysis. RNA samples extracted at various stages of ripening were probed with a strand-specific probe and the intensity of a band corresponding to PG mRNA was quantified (Fig. 4B). In normal fruit PG mRNA was not detected in green pericarp but increased during ripening, reaching a peak seven days after the breaker stage before declining. In GR105 fruit a similar profile was obtained but the peak PG mRNA level was only 1.5% that of normal fruit. In separate experiments measurements of RNA from GR102 and GR95 fruit at selected points during ripening indicated that in GR102 the

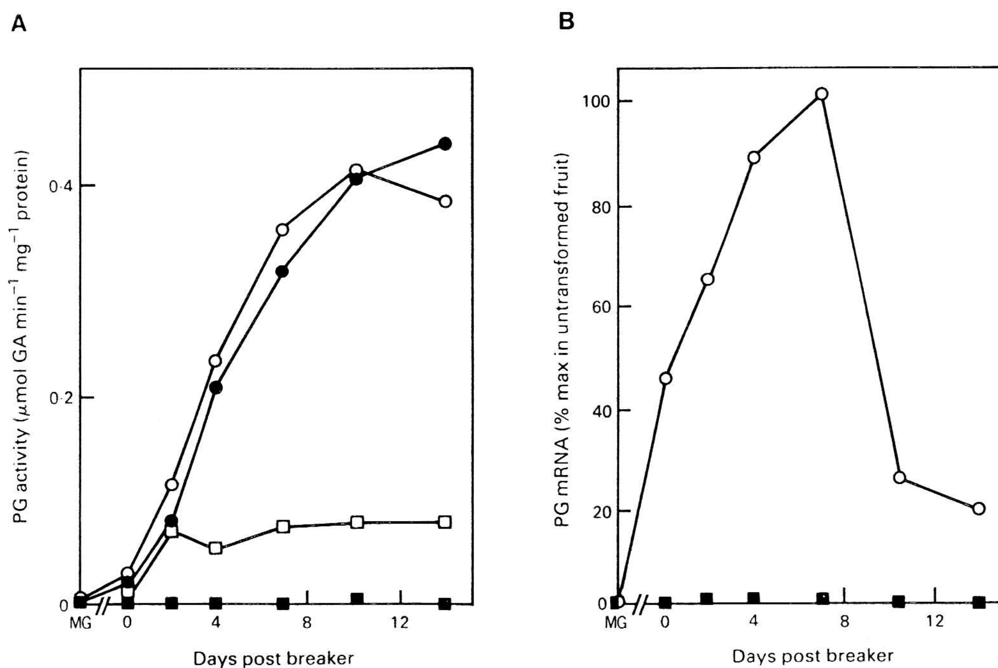


Fig. 4. PG activity and mRNA content during ripening of selected GR16 progeny containing 0, 1, or 2 antisense genes. Fruit were tagged at the first visible sign of ripening and samples picked at various stages during ripening. Untransformed (○); GR102 (100% PG) (●); GR95 (20% PG) (□); GR105 (1% PG) (■). A. PG activity in cell wall protein extracts. B. PG mRNA content of pericarp tissue. MG: mature green.

amount of PG mRNA was the same as that in untransformed fruit, whereas in GR95 maximum expression was 10 to 20% of the untransformed level (data not shown). Thus the reduction in PG activity during ripening of antisense fruit is a reflection of a reduction in the steady-state level of PG mRNA.

PG isoenzyme analysis

There are three isoenzyme forms of PG (PG1, PG2a, and PG2b) that are structurally related [34, 35, 40]. In normal fruit PG1 is the dominant form during early stages of ripening (Fig. 5, lane 1), but accounts for only about 10% of total PG in ripe fruit (Fig. 5, lane 2). However, in GR95 and GR105 fruit the majority of PG was in the PG1 isoenzyme form throughout ripening (Fig. 5, lanes 5 to 8).

Pectin analysis

The level of soluble polyuronides (recovered from acetone-insoluble solids) during tomato ripening were unaffected by the antisense RNA induced reduction in PG enzyme activity (Fig. 6A). In order to determine the effect of substantially reduced PG activity on pectin depolymerisation,

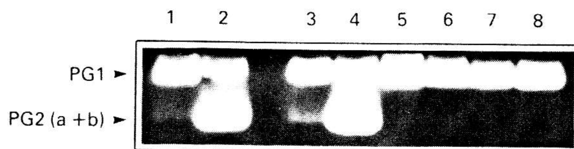
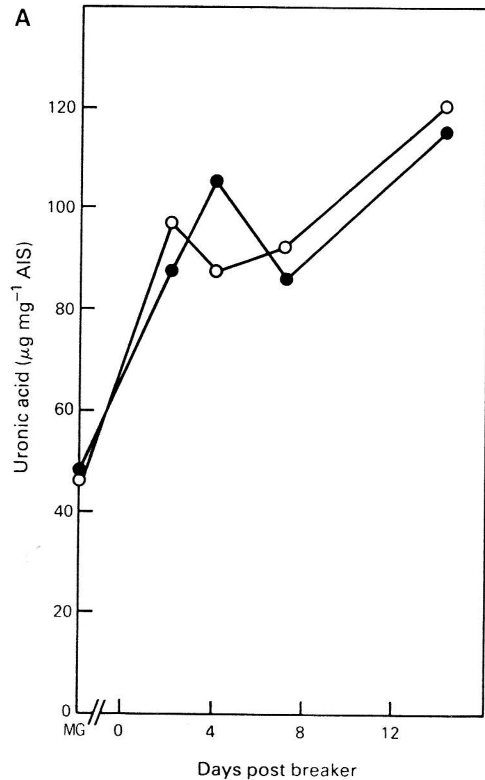


Fig. 5. PG isoenzyme content during ripening of selected GR16 progeny containing 0, 1, or 2 antisense genes. Fruit were tagged at the first visible sign of ripening and samples picked at 2 and 10 days postbreaker. Cell wall proteins were separated in a non-denaturing polyacrylamide gel and stained for PG activity. Lane 1, 1.5 μg protein from untransformed day 2 fruit; lane 2, 0.5 μg protein from untransformed day 10 fruit; lane 3, 1.5 μg protein from GR102 day 2 fruit; lane 4, 0.5 μg protein from GR102 day 10 fruit; lane 5, 4.0 μg protein from GR95 day 2 fruit; lane 6, 4.0 μg protein from GR95 day 10 fruit; lane 7, 50 μg protein from GR105 day 2 fruit; lane 8, 50 μg protein from GR105 day 10 fruit.



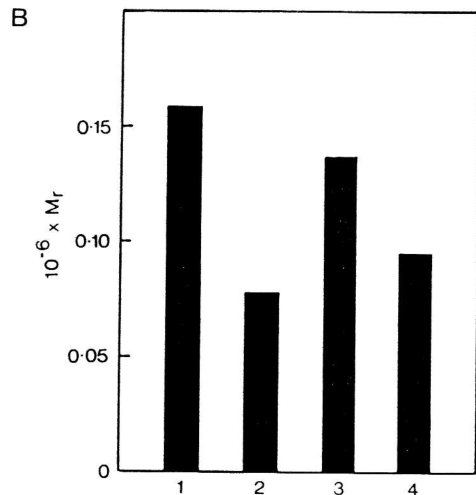


Fig. 6. Polyuronide solubilisation and weight-average M_r during ripening of selected GR16 progeny containing 0, 1, or 2 antisense genes. A. Soluble polyuronide content of acetone insoluble pericarp extracts during ripening of untransformed (●), and GR105 (1% PG) (○). Each point is the mean from 3 fruit assayed individually. B. Weight-average M_r of soluble polyuronides from antisense and untransformed unripe fruit, and from 14 day post breaker untransformed fruit and fruit containing 1 (GR95), or 2 (GR105) antisense genes. 1, untransformed and antisense unripe fruit; 2, untransformed ripe fruit, 3, GR105 ripe fruit (1% PG); 4, GR95 ripe fruit (20% PG). Each point is the mean from 3 fruit assayed individually.

the weight-average M_r of pectin fragments was determined (Fig. 6B). Samples from green fruit of antisense and normal plants had a similar M_r average of 158 000. As ripening progressed pectin breakdown occurred resulting in an increase in amount (Fig. 6A), but a decrease in size (Fig. 6B) of soluble polyuronide fragments. In normal and GR102 fruit 14 days after the breaker stage the polyuronide fragment size had fallen to a M_r average of 80 000. By contrast, after 14 days the M_r average was 95 000 in GR95 and 135 000 in GR105 fruit. These results show that whilst the *in vivo* solubilisation of pectin was unaffected by substantially reduced PG levels, depolymerisation of pectin was largely prevented.

Discussion

We have previously reported the analysis of a transgenic tomato plant in which PG expression had been down-regulated using antisense RNA [30]. We have now extended our investigations and shown that the down regulation observed previously is a general feature of plants containing this antisense gene. In five independent transformants we have observed a significant down-regulation of PG enzyme activity, ranging from 50% to 95% inhibition. The variation in effectiveness of antisense RNA in different transformants may be due to differences associated with the site of insertion of the antisense gene. It was not due to the copy number of the gene in different transformants since we have shown that it was present as a single copy per diploid genome in the five plants examined. When the antisense gene copy number was doubled by selfing the original GR16 transformant a 99% reduction in the endogenous PG expression was achieved (Fig. 4).

The inheritance of the antisense gene has been observed through two generations. The gene is stable over this time, and more importantly, the antisense RNA effect in progeny plants is highly reproducible. In future experiments we will analyse the stability and effect of the antisense gene in succeeding generations in order to test the utility of this approach for the modification of crop plants.

With the exception of substantially reduced PG activity the antisense fruit apparently developed and ripened normally. There was no deviation from the normal change in activity of invertase, which increases during ripening (Fig. 3C), or pectinesterase (Fig. 3D), which is also involved in cell wall metabolism [38]. In addition there was no alteration in ethylene production (the earliest detectable sign of ripening) or the rate of lycopene accumulation. This argues against PG having a role co-ordinating ripening possibly by stimulating ethylene production [32, 1]. However, this possibility cannot be excluded entirely because of the residual 1% PG that persists in these fruit.

PG mRNA accumulates to 1–2% of the total poly(A)$^+$ RNA in normal fruit pericarp during ripening [9]. It is surprising how effectively antisense RNA controlled the expression of this abundant transcript. The mechanism by which inhibition of gene expression was achieved is not yet clear. One possibility is that transcription was inhibited. However, Sheehy *et al.* [28] have concluded that the rate of transcription of the PG gene was unaltered in plants in which another antisense construct was successfully used to control PG gene expression. Alternatively, antisense RNA may interfere with nuclear RNA processing or transport to the cytoplasm or may increase mRNA instability. A reduction in the level of PG mRNA accompanies reduced PG activity. This indicates that antisense RNA does not simply inhibit mRNA translation. No correlation was observed between the level of antisense transcript in leaves and the reduction of PG activity in the fruit, although there was clearly an effect of gene dosage on reduced activity in the hemizygous and homozygous plants (Table 2, Fig. 4). The multiple antisense transcripts may be due to premature termination as suggested previously [30].

Attempts to understand the role of PG in fruit ripening and softening have been hampered by the lack of single-gene PG mutants in tomato stocks. Mutants exhibiting reduced PG levels are available but are pleiotropic. Using the antisense RNA approach, we have generated reduced PG

mutants, in which other aspects of ripening are not affected. In these plants the major difference is the amount of depolymerisation of the soluble pectin produced in ripening fruit (Fig. 6). It is apparent therefore that one of the biochemical roles of PG during fruit ripening is to depolymerise pectic substances solubilised during ripening. The significance of depolymerisation of pectin in relation to fruit softening is unclear. No obvious differences in compressibility were found in fruit expressing 10% [30] or 1% PG levels. However, compressibility is only one aspect of softening. Changes in other physical parameters may also be involved in this complex process.

Our experiments indicate that the reduction in PG does not affect the total EDTA-soluble uronic acid levels (Fig. 6A). This suggests either that PG does not play a role in polyuronide solubilisation, or that the low PG levels present are sufficient to achieve normal levels of soluble polyuronides. The results of Giovannoni *et al.* [11] support the latter interpretation. In their experiments, *rin* fruit expressing 60% of normal levels of PG accumulate EDTA-soluble uronic acid comparable to those found in normal fruit. However, it is difficult to exclude the possibility that in their experiments the presence of considerable amounts of PG enzyme has led to *in vitro* solubilisation of pectin during the preparation of cell wall fractions [19, 25]. This point could be resolved by analysing the molecular weight distribution of the soluble polyuronide [25].

In the early stages of ripening PG1 is the dominant isoform that accumulates but it accounts for only about 10% of the total PG in fully ripe fruit. In antisense plants expressing 1% of normal activity, PG accumulated almost exclusively in the PG1 form throughout ripening. The antisense gene inhibited the formation of both PG1 (by approx. 90%), and PGs 2a and 2b (almost completely). This is consistent with the suggestion that the three isoenzymes result from post-translational modification of a polypeptide encoded by a single PG gene [3]. If PG has a role to play in the solubilisation of cell wall pectin, the observation that only the PG1 isoform was detected in GR105 suggests that a change from PG1 to PG2 isoforms may be a critical event in the switching between the solubilisation and depolymerisation functions. The presence of PG1 throughout ripening, although at a low level, may indicate that PG1 is required for solubilisation of pectin, whereas PG2, which is almost completely inhibited, may be responsible for pectin depolymerisation. Experiments using antisense fruit could clarify the role of PG in fruit cell wall metabolism if the amount of enzyme could be reduced further.

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