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Control of Ripening

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Control of ripening

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SUMMARY

Ripening of fleshy fruits involves major changes in physiology and biochemistry that alter their colour, flavour, texture, aroma and nutritional value. These changes affect all cell compartments and require the expression of new genes encoding enzymes that catalyse reactions essential for the development of quality attributes. In climacteric fruits, such as tomato, ethylene functions as a hormone to stimulate changes in gene expression required for ripening. Molecular cloning experiments have led to the isolation of cDNAs encoding many ripening proteins. This has enabled the identification and manipulation of novel plant genes encoding enzymes involved in cell wall texture change, carotenoid biosynthesis, ethylene synthesis and the identification of gene control regions involved in fruit-specific, ripening-specific, and ethylene-regulated gene expression. Antisense and partial sense gene techniques have been developed to generate genetically modified plant lines in which specific genes have been permanently inactivated. These fundamental studies have led to production and evaluation of genetically modified tomato lines with improved colour, texture, storage life, and processing characteristics. Zeneca Seeds has established a new business division, the aim of which is to utilize these techniques for the development of improved fruit and vegetable varieties. In collaboration with Petoseed, Zeneca Seeds is in the process of transferring the genes leading to quality improvement of tomatoes to Petoseed's elite tomato germplasm. The primary focus is on the development of improved processing hybrids. These are being evaluated in collaboration with Hunt Wesson, a large and diversified tomato processing company. It is planned that products based on this research will be introduced in the USA in 1995.

1. INTRODUCTION

The tomato has been a popular organism for genetic studies and within the last 20 years has become the model system for fundamental studies on fruit ripening. The physiology and biochemistry of tomato ripening is of particular interest because it involves many fundamental processes, including the synthesis and action of hormones, the biosynthesis of carotenoids and formation of chromoplasts, metabolism of starch, sugars and acids, a concerted rise in respiration, and modifications to the structure and composition of the cell wall (Hobson & Grierson 1993). The commercial importance of the crop is shown by the fact that world tomato production (by weight) has now overtaken bananas, pome fruits, and grapes, and in 1990 was 6.9×10^7 tonnes (see Hobson & Grierson 1993). The scientific interest is to understand how the entire metabolism of the fruit is transformed to generate the quality attributes associated with ripening. This is now known to be stimulated by the hormone ethylene, and to require the regulated expression of many genes (Gray *et al.* 1992).

We described previously initial work leading to the cloning and identification of ripening cDNAs and genes (Grierson *et al.* 1986*a*). In this article we shall describe the use of transgenic tomato plants to characterize ripening-specific gene control regions and to generate targeted mutations to identify new genes and to probe their function *in planta*. These studies have identified genes for previously uncharacterized enzymes and improved our understanding of the control of ripening of climacteric fruits in general (Grierson 1992). As the research progressed, it provided techniques for the improvement of tomato by directed plant breeding and has led to the development of a range of tomato lines with improved commercial characteristics.

2. IDENTIFICATION OF RIPENING GENES

Several ripening genes have now been identified (table 1). The first to be characterized encoded polygalacturonase (PG), which hydrolyses α-1,4 linkages in the polygalacturonic acid component of the cell walls of ripening fruit. There are three different isoforms of PG but they all appear to arise from post-translational modification of a single polypeptide, which is synthesized *de novo* during ripening. In earlier studies (Bird *et al.* 1988) it was shown that a 1.4 kb DNA sequence from the 5′ flanking region of the PG gene was sufficient to direct ripening specific expression of the

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Table 1. *Identification and manipulation of tomato ripening genes*

cDNA	encoded protein	gene promoter	antisense inhibition	co-suppression	over-expression
TOM6	polygalacturonase Grierson *et al.* (1986*a*)	Bird *et al.* (1988)	Smith *et al.* (1988, 1990*a*)	Smith *et al.* (1990*b*)	—
F1	polygalacturonase Sheehy *et al.* (1987)	Rose *et al.* (1988)	Sheehy *et al.* (1988)	—	—
PG16	polygalacturonase DellaPenna *et al.* (1986)	—	—	—	*rin* mutant Giovannoni *et al.* (1989)
PE1	pectinesterase Ray *et al.* (1988)	—	Hall *et al.* (1993)	—	—
PET1	pectinesterase Harriman *et al.* (1992) Tieman *et al.* (1992)	—	Tieman *et al.* (1992)	Tieman *et al.* (1992)	—
TOM13	ACC oxidase Hamilton *et al.* (1990, 1991)	—	Hamilton *et al.* (1990) Picton *et al.* (1993)	—	—
tACC2 tACC4	ACC synthase Rottman *et al.* (1991)	Rottman *et al.* (1991)	Oeller *et al.* (1991)	—	—
VV4A VV4B	ACC synthase Van der Straeten *et al.* (1990)	—	—	—	—
E8	unknown: affects C_2H_4 synthesis Penarrubia *et al.* (1992)	Cordes *et al.* (1989) Giovannoni *et al.* (1989)	Penarrubia *et al.* (1992)	—	—
TOM5	phytoene synthase Bird *et al.* (1991)	Ray *et al.* (1992)	Bird *et al.* (1991)	Fray & Grierson (1993)	Fray & Grierson (1993)
TOM75	membrane channel Fray *et al.* (1993)	—	—	—	—
2A11 (TOM4)	homology to storage protein, Bowman/Birk inhibitor Pear *et al.* (1989) (Fray, personal comm.)	Pear *et al.* (1989) van Haaren & Houck (1991)	—	—	—
— (TOM96)	invertase, Klann *et al.* (1992) (Ray, personal comm.)	—	—	—	—
E17	proteinase inhibitor Margossian *et al.* (1988)	—	—	—	—
TOM66	heat shock protein: expressed during ripening Fray *et al.* (1990)	—	—	—	—

Table 2. *Effect of PG gene flanking regions on ripening-specific expression of the CAT reporter gene in transgenic tomatoes*

(The range of CAT activity for each construct is given in parentheses. Unpublished results of F. Nicholass.)

construct	number of plants analysed	number of plants with ripening-specific CAT activity	CAT activity in ripe fruit (units, mg^{-1} protein)
pCB1 (1.4 kb 5′)	11	3	0.9 (0.7–1.2)
pCB19 (5 kb 5′)	18	11	459 (24–1040)

chloramphenicol acetyl transferase (CAT) gene in transgenic plants. Quantitative measurements of the expression levels of the PG promoter-CAT constructs showed they were relatively low compared to the endogenous PG gene and further gene control regions were sought. This search demonstrated (table 2) that sequences located within a 5 kb 5′ PG gene flanking region were capable of directing 1000-fold higher levels of expression of the CAT reporter gene during ripening of transgenic fruit. Initial results suggest that they give consistently high levels of transgene expression, with fewer position effects.

Other ripening cDNAs or genes that have been characterized include those for a second wall-modifying enzyme (pectinesterase), enzymes involved in ethylene synthesis (ACC synthase, ACC oxidase, E8), a phytoene synthase which is required for synthesis of carotenoids in fruit, invertase, and a putative membrane channel (table 1).

3. INHIBITION OF GENE EXPRESSION WITH ANTISENSE AND SENSE GENES

We tested whether it was possible to inhibit the expression of endogenous genes using antisense and sense contructs. Transgenic tomato plants were produced containing an inverted 730 b.p. 5′ fragment of the PG cDNA (Grierson *et al.* 1986*b*), under control of the CaMV 35*S* transcription promoter and flanked by a 3′ nopaline synthase (*nos*) gene fragment. Measurement of enzyme activity in a number of transgenic plants demonstrated that PG was, indeed, down-regulated (figure 1) and the inhibition varied between different transformants (Smith *et al.* 1988). PG activity was found to be inhibited by up to 90% in some plants containing an antisense gene inserted at a single site. There was a corresponding reduction in the amount of PG mRNA, while other aspects of ripening, such as the synthesis of ethylene, lycopene, or other cell wall modifying enzymes, were unaffected (Smith *et al.* 1988, 1990*a*). The antisense genes were shown to be stably inherited and PG activity was found to be inhibited by 99% or more in plants homozygous for the antisense construct (Smith *et al.* 1990*a*). The pattern of inheritance of the antisense gene followed Mendelian principles. Fruit from plants that failed to

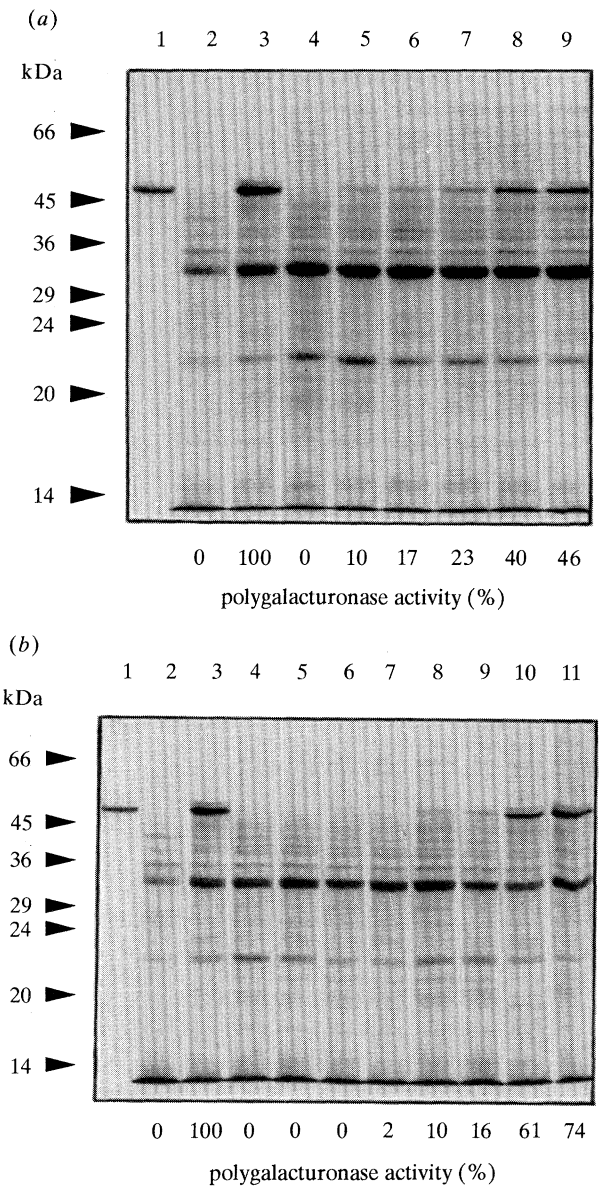

Figure 1. Inhibition of tomato polygalacturonase accumulation during ripening of transgenic fruit containing an antisense or sense gene. Cell wall proteins were extracted from unripe or ripe wild-type tomatoes (lanes 2 and 3) and fractionated by SDS-polyacrylamide gel electrophoresis, together with extracts from a range of ripe transgenic fruit containing different levels of PG enzyme activity (lanes 3–11). The gels were stained with Coomassie Blue to reveal protein bands and compared to purified tomato PG (lane 1). (*a*) Transgenic fruit containing truncated antisense PG genes. (*b*) Transgenic fruit containing truncated sense PG genes. The level of PG activity measured by enzyme assay is shown beneath each lane. (After Smith *et al.* 1993.)

inherit the transgene had normal levels of PG mRNA and enzyme during ripening (Smith *et al.* 1990a). This indicated that the antisense gene did not permanently modify the endogenous gene, as it could be recovered by segregation, thus ruling out the possibility of gene inactivation by homologous recombination. Sheehy *et al.* (1988) who used a full length PG cDNA in an antisense construct showed that the rate of transcription of the endogenous PG gene was unaffected in antisense plants, although the steady-state level of the

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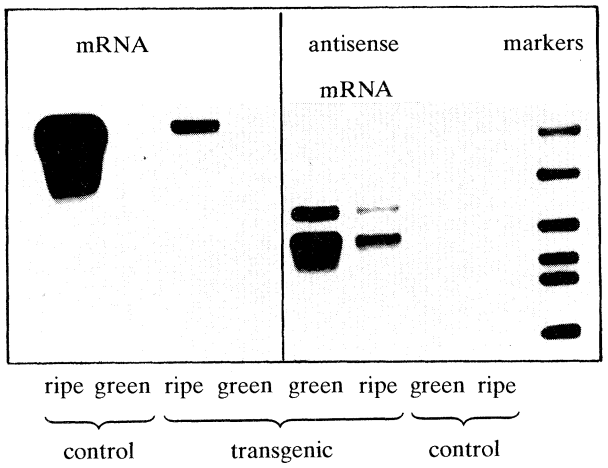

Figure 2. Effect of PG antisense genes on the accumulation of PG mRNA during ripening of tomato fruit. RNA was extracted from unripe and ripe wild-type tomatoes (control) and from transgenic fruit containing antisense genes in which there was a major reduction in PG enzyme, fractionated by agarose gel electrophoresis, blotted onto a nylon membrane and hybridized with either a sense-specific or antisense-specific single stranded RNA probe. The two PG antisense RNA bands are believed to arise by RNA processing at fortuitous poly A-addition signals in the antisense transcript. (After Smith *et al.* 1988.) Note the reduction in PG sense and antisense RNA during ripening of transgenic fruit.

mRNA that finally accumulated was reduced. Even short (150 b.p.) segments of the PG cDNA have been found to be effective in antisense constructs (Schuch 1991).

The mechanism of action of antisense genes is not clear. In our experiments, the 35*S* promoter caused antisense PG mRNA to accumulate in several different organs, including unripe fruit. During ripening, when PG mRNA is normally transcribed from the endogenous gene, we noted a reduction both in the PG mRNA and the antisense RNA (figure 2) (Smith *et al.* 1988). In some plants containing an ineffective antisense gene, due presumably to an unfavourable site of insertion, antisense RNA failed to accumulate in unripe fruit and there was no effect on the accumulation of transcripts from the endogenous PG gene during ripening. These results indicated that transcription of the endogenous gene and the transgene are necessary for the inhibition of gene expression to occur. As the transcripts do not accumulate to normal levels when an effective antisense gene is present, it has been assumed that some interaction accurs to render the sense and antisense RNA unstable. There is not, however, any unequivocal evidence for this.

The expression of a second cell wall modifying enzyme, pectinesterase (PE), which removes the methyl groups from cell wall pectin, has also been inhibited using antisense genes (Hall *et al.* 1993; Tieman *et al.* 1992). Two main PE isoforms, or groups of isoforms, are expressed in tomato and although the ratios of these change during ripening, both are expressed in unripe fruit and neither is ripening-specific (Tucker *et al.* 1982). We used a cDNA fragment encoding a tomato fruit PE (Ray *et al.* 1988) to generate transformed plants containing PE antisense genes. As was found with PG, the PE antisense genes inhibited the accumulation of endogenous PE mRNA and enzyme (figure 3) (Tieman *et al.* 1992; Hall *et al.* 1993). However, not all fruit PE activity could be inhibited by the antisense genes. Furthermore, PE activity in leaves, stems and roots was largely unaffected, even though the antisense transcript accumulated in these organs (table 3). These

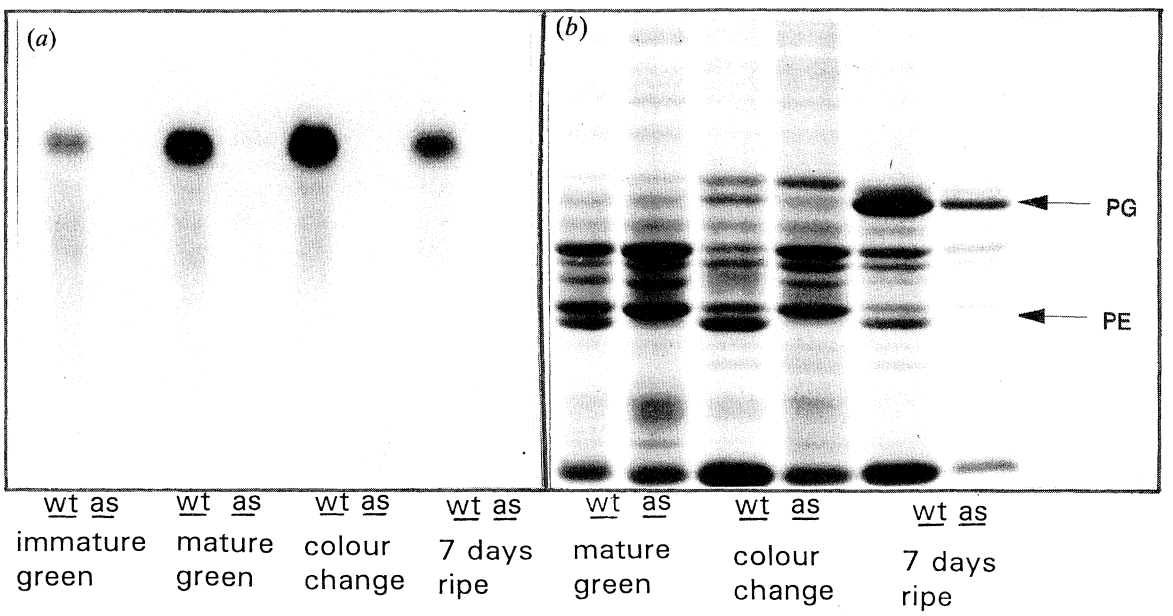

Figure 3. Inhibition by antisense genes of pectinesterase mRNA and enzyme synthesis in unripe and ripe tomatoes. Wild-type (control) and transgenic fruit containing PE antisense genes were assayed for the fruit-specific PE mRNA and protein by (*a*) northern blotting and (*b*) SDS-polyacrylamide gel electrophoresis. The positions of PG and PE proteins are shown by the arrows. (After Hall *et al.* 1993.)

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Table 3. *Not all pectinesterase activity is susceptible to antisense inhibition*

(All data points represent the mean of a minimum of three independent determinations ± the standard error of the mean. Data from Hall *et al.* (1993).)

	PE enzyme activity (μeq. H^+ g^{-1} fresh mass)	
sample	wild-type	PE antisense
red-ripe fruit, 7 d after breaker stage	36.1 ± 3.6	2.7 ± 0.6
breaker (colour change)	29.3 ± 9.2	3.8 ± 1.8
leaf	16.7 ± 1.6	21.5 ± 3.8
root	9.2 ± 2.8	10.9 ± 2.3

data indicate that there are at least two distinct classes of PE isoenzymes in the tomato plant. One type is highly expressed in fruit and it is this type that is susceptible to the homologous antisense gene. The residual enzyme activity in fruit, and that found in other organs, is immunologically distinct and the mRNA does not cross-hybridize to the known PE cDNA clone (Ray *et al.* 1988). We predict that to inhibit this second type of PE activity it will be necessary to clone cDNAs for the other isoform and to use these sequences to generate a new range of antisense plants.

In further experiments with PG, we showed that a *sense* transgene also efficiently inhibited the endogenous gene when expressed in transgenic plants (figure 1) (Smith *et al.* 1990*b*). As found for the antisense gene (Smith *et al.* 1988, 1990*a*), the sense construct which consisted of the 730 b.p. PG cDNA fragment inserted in sense orietation in pJR1 was expressed in unripe fruit. During ripening, there was a mutual reduction in the amount of mRNA that accumulated from the sense transgene and the endogenous PG gene (Smith *et al.* 1990*b*). The effect was specific for the PG gene and synthesis of lycopene and other aspects of ripening were unaffected. A similar down-regulation by sense genes was also described by Napoli *et al.* (1990) and van der Krol *et al.* (1990) for chalcone synthase and dihydro flavonol-4-reductase in petunia flowers. This specific gene inactivation, involving a sense transgene and an endogenous gene, has been termed 'co-suppression' (Jorgensen 1990). However, the effects of sense and antisense genes have several common features (Grierson *et al.* 1991) and antisense results could equally well be described by the term 'co-suppression'. The specificity of these effects indicates nucleic acid sequence recognition is involved and it has been suggested for sense genes that DNA–DNA interactions in some way cause down-regulation (Jorgensen 1990). In view of the similarities between sense and antisense effects, and taking into account the apparent requirement for transcription, it has been suggested that both sense and antisense genes could work by the production of antisense RNA (Grierson *et al.* 1991). It is also possible, however, that DNA–DNA or DNA–RNA interactions that occur during transcription could cause the observed inhibition of gene expression.

4. USE OF ANTISENSE TECHNIQUES FOR GENE IDENTIFICATION

The construction, screening, and analysis of ripening-related cDNA libraries revealed at least 20 clones many of which were of unknown function (Slater *et al.* 1985; Gray *et al.* 1992). We used an antisense approach to identify novel enzymes involved in the synthesis of ethylene (the ethylene-forming enzyme, now called ACC oxidase) and carotenoids (phytoene synthase) (table 1). The search for cDNA clones encoding enzymes required for ethylene synthesis was regarded as being of special interest, as ethylene plays a critical role in the ripening of all climacteric fruits and is also important for the senescence and abscission of leaves and flowers (Grierson *et al.* 1986*a*). Although neither of the two enzymes specific to the pathway (ACC synthase and ACC oxidase) had been purified, it proved possible to identify candidate clones using an indirect approach. It had been shown that TOM13 was expressed during ripening, wounding and in senescing leaves (Davies & Grierson 1989), situations in which ethylene synthesis is high. Hybrid-select translation and sequencing of cDNA and genomic clones showed TOM13 encoded a 35 kDa polypeptides with no obvious targeting signal or membrane-spanning regions, but did not reveal its function (Smith *et al.* 1986; Holdsworth *et al.* 1987, 1988). The significance of the correlation of TOM13 mRNA expression with ethylene synthesis was therefore tested by inhibiting its production in transgenic plants, using antisense genes. This caused a major inhibition of ethylene synthesis in ripening fruit and wounded leaves (figure 3). Inhibition occurred in an antisense gene-dosage-dependent manner and was correlated with a decrease in ACC oxidase activity (Hamilton *et al.* 1990). The suggestion that TOM13 encoded ACC oxidase posed some problems, however, as it had been widely believed that the enzyme, which had never been solubilized or purified, was membrane-associated and required intact membranes for activity (see John 1991). The fact that TOM13 encoded a 35 kDa polypeptide that was predicted to be soluble did not match with the expected properties of ACC oxidase. This problem was resolved by expressing TOM13 in *Saccharomyces cerevisiae* and showing that it conferred on the yeast the ability to convert ACC to ethylene with the same stereospecificity as that found previously for plant tissue sections (Hamilton *et al.* 1991). Expressing a TOM13-related mRNA in *Xenopus* oocytes also led to similar conclusions (Spanu *et al.* 1991). Antisense experiments with another cDNA (E8), that encodes a separate protein with some homology to ACC oxidase, revealed that preventing its production *stimulated* ethylene synthesis (Penarrubia *et al.* 1992). So far, the biochemical function of this protein is unclear.

ACC oxidase antisense fruit showed a delayed ripening phenotype, confirming the importance of ethylene in stimulating ripening. This inhibition was most pronounced when fruit were picked, although ripening could be partially restored to wild-type levels by supplying ethylene externally (Picton *et al.* 1993). It was also shown that leaf senescence in the same

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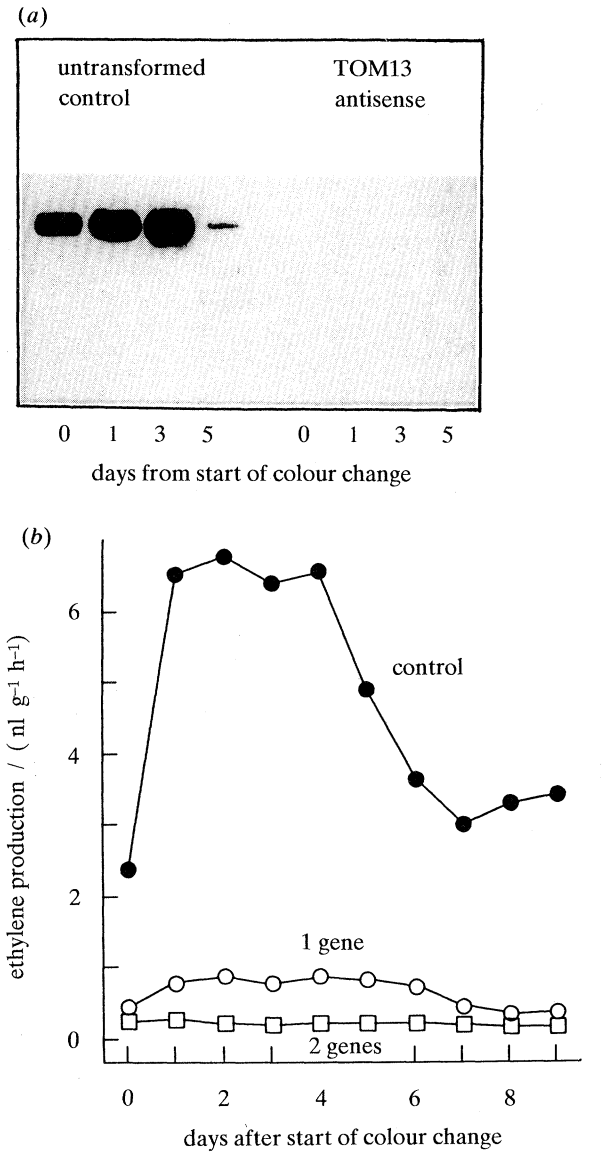

Figure 4. Inhibition by antisense genes of TOM13 (ACC oxidase) mRNA and ethylene synthesis during tomato ripening. Wild-type (control) and transgenic tomatoes containing TOM13 antisense genes were analysed for mRNA expression during ripening by (*a*) northern blotting and (*b*) ethylene synthesis. (After Hamilton *et al.* 1990.)

transgenic plants was delayed. Ethylene production can also be controlled by inhibition of ACC synthase. A spectacular inhibition of ripening was demonstrated by Oeller *et al.* (1991), who identified ACC synthase clones by immuno-screening of a cDNA expression library and generated transgenic tomatoes with 10 antisense genes. These fruit showed greatly reduced ripening, which could be restored by supplying ethylene, or its analogue propylene. Another approach to ripening control by reducing ethylene synthesis was demonstrated by Klee *et al.* (1991) who caused a decrease in the flux of ACC to ethylene by transforming tomatoes with a modified bacterial gene that metabolized ACC.

The next ripening-related gene to be identified was the phytoene synthase encoded by TOM5 (Bird *et al.* 1991). Fruit of transgenic plants containing TOM5 antisense genes were shown to turn yellow, not red, during ripening. This was due to the inhibition of phytoene synthesis, which is required for the production of the coloured carotenoids β-carotene and lycopene. The yellow colour that developed during ripening of fruits in which phytoene synthase expression was inhibited is due to the synthesis of an unrelated compound, naringenin chalcone, which in wild-type fruit is normally masked by the carotenoids. In addition to inhibiting fruit carotenoid biosynthesis, the TOM5 antisense gene also prevented the development of the normal yellow colour of the flower corolla (Bird *et al.* 1991), indicating that a similar or identical gene is expressed in tomato flowers. The phenotype of these TOM5 antisense mutants resembled that of the *yellowflesh* (r) mutant, which had been mapped to chromosome 3. Interestingly, RFLP mapping carried out by Mutschler and colleagues (Mutschler *et al.* 1988; Kinzer *et al.* 1990) had established that genes encoding TOM5 sequences were located on chromosomes 2 and 3. The *r* mutant, and a con-allelic mutant r^y, were shown to be deficient in production of the normal TOM5 mRNA. In *r*, the mRNA was truncated and contained an unrelated sequence at the 3′ end. Complementation of *r* was achieved by over-expression in transgenic plants of a wild-type TOM5 sequence under control of the CaMV 35*S* promoter. This restored the normal red colour due to carotenoid production during ripening (Fray & Grierson 1993). In some plants, the 35*S*-TOM5 sense gene did not lead to over-expression but co-suppressed a phytoene synthase gene normally expressed in *green* fruit. In such cases, fruit of the *r* mutant lacked carotenoids at the green stage and turned white, due to photobleaching. The normal yellow colour of the *r* fruit developed during ripening, when naringenin chalcone was produced (Fray & Grierson 1993). These results showed that the same gene construct was capable of causing over-expression or co-suppression in transgenic plants. It is likely that further studies on the differences in chromosomal location and sequence context of the transgenes in such plants may shed some light on the mechanism of co-suppression. Also, the ability to generate high levels of carotenoids in tomatoes may have particular advantages for the processing industry.

5. ASSESSMENT OF THE COMMERCIAL POTENTIAL OF GENETICALLY MODIFIED TOMATO LINES

So far, we have summarized the generation of several genetically modified tomato lines in which ripening related genes have been inhibited. We have evaluated fruit quality characteristics in large-scale replicated glasshouse trials to assess commercial potential. Tomato lines were grown under semi-commercial growing conditions over several growing seasons. Lines chosen for this work were homozygous for the introduced effect gene (table 4). Azygous lines and unmodified control lines of the same variety served as controls. Details of some of these experiments have already been published (Schuch *et al.* 1992; Murray *et al.* 1993).

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Table 4. *Tomato lines evaluated in glasshouse trials*

gene	line	reduction of target gene		reference
PG	AC105		99%	Smith *et al.* (1988, 1990)
PE	AC295		90%	Hall *et al.* (1993)
PG/PE	AC101.16	PG	96%	unpublished
		PE	85%	
Acc oxidase	F1.11	ethylene	96%	Hamilton *et al.* (1990)
TOM 99	AC99A		>99%	unpublished
TOM 36	AC36A		>99%	unpublished

We have assessed the following fruit quality characters. For fresh market tomatoes: Brix, reducing sugars, acids, colour, dry matter, transport and handling characteristics, and in taste trials, acidity, sweetness and tomato flavour. For processed fruit: viscosity (Bostwick and serum), soluble solids, pH and flavour, and in taste experiments, sweetness and mouth texture. From these studies it has been possible to assign major quality improvements to several genes.

Low PG tomatoes have greatly enhanced processing characteristics when compared with controls (Schuch *et al.* 1991). Bostwick viscosity which is a measure of paste yield potential of a given tomato variety is increased by over 80% in tomato lines in which PG activity has been reduced by 99% (figure 5*a*).

Low PG tomato fruit samples had viscosity values much lower even in 'cold break' experiments than any of the 'hot break' samples where fruit extracts are heat treated to inactivate the cell wall modifying enzymes (Schuch *et al.* 1991). Cold break on the other hand does not involve this heat treatment step. Bostwick viscosity is influenced largely by the insoluble cell wall polymers. The increase in viscosity is attributable to the changes in the average molecular mass of tomato cell wall pectins determined in fresh fruit with low levels of PG (Smith *et al.* 1990). The conclusion which we have drawn from these experiments is that during the manufacture of tomato paste an increase in yield of paste should be achievable using low PG tomatoes.

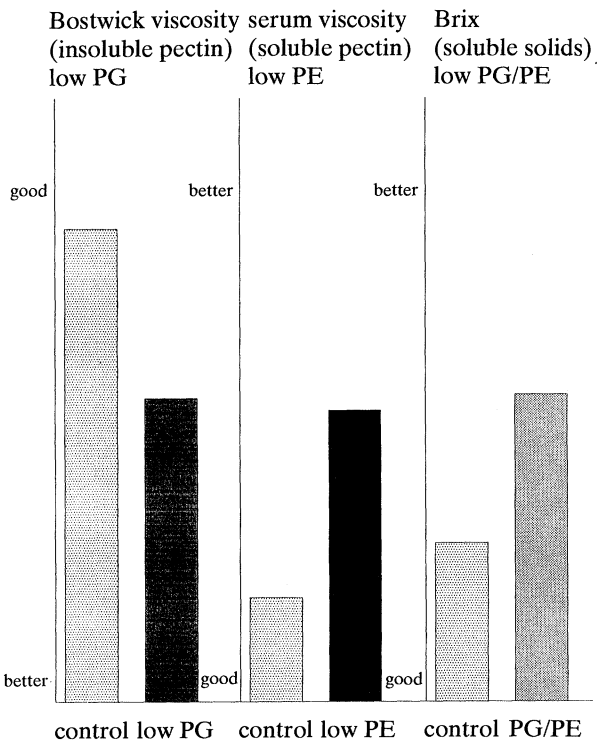

Figure 5. Relative processing performance of modified and unmodified fruit in three quality assays: Bostwick viscosity analysis of low PG fruit; serum viscosity of low PE fruit; and Brix (soluble solids) analysis of low PG and PE fruit.

Sheehy *et al.* (1988) have also generated tomatoes in which PG levels were reduced by 90%. These, together with lines with 99% reduction in PG activity, have been grown and assessed in field trials for agronomic and processing characteristics (Kramer *et al.* 1990). Kramer *et al.* (1992) reached the conclusion that a reduction in PG activity resulted in tomato juices and pastes with significantly better processing parameters such as Bostwick viscosity and Ostwald consistency (serum viscosity).

The fresh market attributes of low PG tomatoes have been assessed in detail as the hypothesis has been proposed that PG is the 'softening' enzyme of the tomato fruit (Hobson 1965). In early experiments no differences in fruit firmness were detected (Schuch *et al.* 1991). However, in repeat experiments small but statistically significant differences in fruit firmness during later ripening stages have been measured. Whether these small differences are sufficient for the production of a vine ripened product is doubtful in our minds, but may depend on variety. However, we have demonstrated that despite these small differences in firmness, damage of fruit is reduced leading to improved post harvest handling of low PG tomatoes (Schuch *et al.* 1991; Gray *et al.* 1992).

Kramer *et al.* (1992) have demonstrated that tomatoes with reduced levels of PG activity (99% reduction) are significantly firmer than unmodified fruit during storage. In addition, laboratory tests have shown increased resistance to *Geotrichum candidum* and *Rhizopus stolonifer*, post-harvest fungal pathogenes. Based on these observations Calgene is now developing 'FlavrSavr' fresh market tomatoes which are expected to reach the US market later in 1993.

Low PE tomatoes have also been assessed. In these experiments the viscosity of the serum of the tomato fruit extract was greatly enhanced (figure 5*b*). This is largely due to the different methylation status of soluble pectin in the tomato (Hall *et al.* 1993). Small but significant changes in Bostwick viscosity

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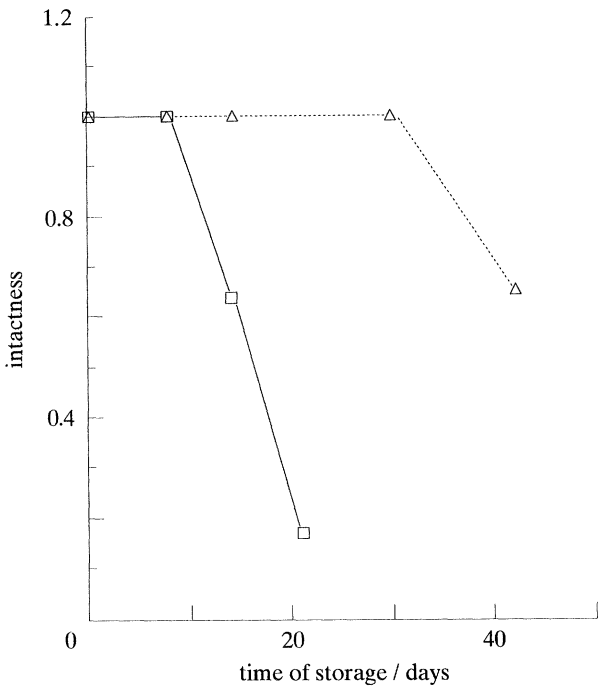

Figure 6. Increased storage of low ethylene fruit. Fruit from low ACC oxidase (triangles) and unmodified controls (squares) were collected at stage and stored for up to 40 days. Intactness of fruit was assessed visually. (Data after Murray *et al.* 1993.)

have also been measured (data not shown). However, the major effect of PE reduction is the increase in serum viscosity. Serum viscosity contributes to the glossy appearance of tomato paste. Therefore this attribute will be enhanced in low PE tomatoes.

We have combined the low PG and PE traits using two approaches. In one set of experiments a low PG–low PE line was generated via crossing of the parent low PG and PE lines. Selection for the presence of the effect genes combined with biochemical analysis allowed identification of lines in which PG and PE are reduced to a similar extent as in the single effect gene parents. We have also used transformation vectors containing both PG and PE sequences in sense (Seymour *et al.* 1993) and antisense orientation (unpublished data) to generate lines in which both cell wall genes have been down-regulated. These lines show the fruit quality characteristics of the single effect gene lines, and in addition, significant increases in soluble solids (Brix) (figure 5*c*). Thus, the combination of these two genes will allow us to improve one of the most important determinants of processing tomato quality.

To assess organoleptic characteristics of the genetically modified tomatoes we have carried out taste tests in the UK. The tests were carried out after review by an internal Zeneca Ethical Committe and consultation with the Advisory Committe for Novel Foods and Processes (ACNFP). Low PG tomatoes were considerably more granular in their mouth feel than unmodified tomatoes. Samples prepared by the cold break procedure from low PG fruit had the same Bostwick viscosity as those prepared by hot break from unmodified tomatoes. However, the mouthfeel distinguished these samples: the low PG sample was perceived to be more granular than the unmodified control. However, when the low PG–PE sample was compared to unmodified controls, the granularity of these samples could no longer be distinguished. Thus, reduction in PG and PE have different effects on textural characteristics which may influence product behaviour and taste and hence consumer preference.

Extensive studies have been carried out using the low ethylene tomatoes described earlier in this chapter. The slower ripening rate is visible as a reduction in the rate of accumulation of lycopene. We have also shown that in these tomatoes PG levels were normal (Hamilton *et al.* 1990; A. J. Murray, unpublished results). The greatest improvements in fruit quality characteristics which we have noted is the improved handling characteristics of low ethylene tomatoes (figure 6) (Murray *et al.* 1993).

Whether any of these modified tomatoes demonstrate other improvements in fruit quality needs to be determined in further trials with commercial cultivars.

6. EMERGENCE OF A COMMERCIAL VISION

Early on in the project the view had been that increased firmness could provide the fresh market industry with the ability to keep tomatoes on the vine until they have produced the maximal natural flavour. This concept and the data from the processing experiments led us in Zeneca Seeds to develop the idea that the genes which we have characterized and used to generate genetically modified fruits could add value to the tomato crop. This value would best be realized by the retail or processing industries rather than the farmer or tomato grower. The development of the business vision based on this 'added value concept' has been described in detail (Best 1993). In summary, Zeneca Seeds provides technology to the specific sectors in the value chain of the fresh produce or processing industries. To realize this vision, a new business division of Zeneca Seeds has been established and partners who could provide strength in the provision of germplasm and in the processing industry have been identified.

7. IMPLEMENTATION OF THE BUSINESS STRATEGY

The Petoseed Company was identified as the most appropriate partner for germplasm, as they are a major international vegetable seed company with leading elite hybrid lines in all the major tomato markets. It cannot be underestimated how important the quality of germplasm is for any genetic modification work. The farming community requires confidence demonstrated by high consistent yield from trusted varieties. To maximize the potential of the effect genes, they must be combined with excellent agronomic performance and already high standards of fruit quality to achieve the step change benefits which the effect genes provide. To this end Zeneca Seeds has collaborated with Petoseed since 1989. Field trials

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have been carried out over the past two years in Chile and California to verify the effects determined from glasshouse research.

Hunt Wesson are the largest and most diversified processed tomato product company in the world. They have joined the three-way collaboration in which specific commercially known hybrids have been targeted, matching the effect genes with the appropriate quality in the Petoseed germplasm. The leading products, which will enter development, are derived from tomato hybrids in which viscosity parameters have been improved through the introduction of the PG effect gene. It is anticipated that commercial products resulting from this work will reach the US market by 1995.

The full range of benefits of enhanced viscosity tomatoes have not yet been fully evaluated. Detailed product evaluation on material from field trials is currently under way. There are clearly benefits to the processing company which lie in cost savings due to increased product yield. However, a broader range of benefits can be expected from the introduction of these modified plant varieties, affecting the grower, consumer and environment. For instance, improved handling and yield could reduce farm waste and the use of water and other inputs. For the consumer, the increased paste quality could provide an improved range of new products while keeping costs down.

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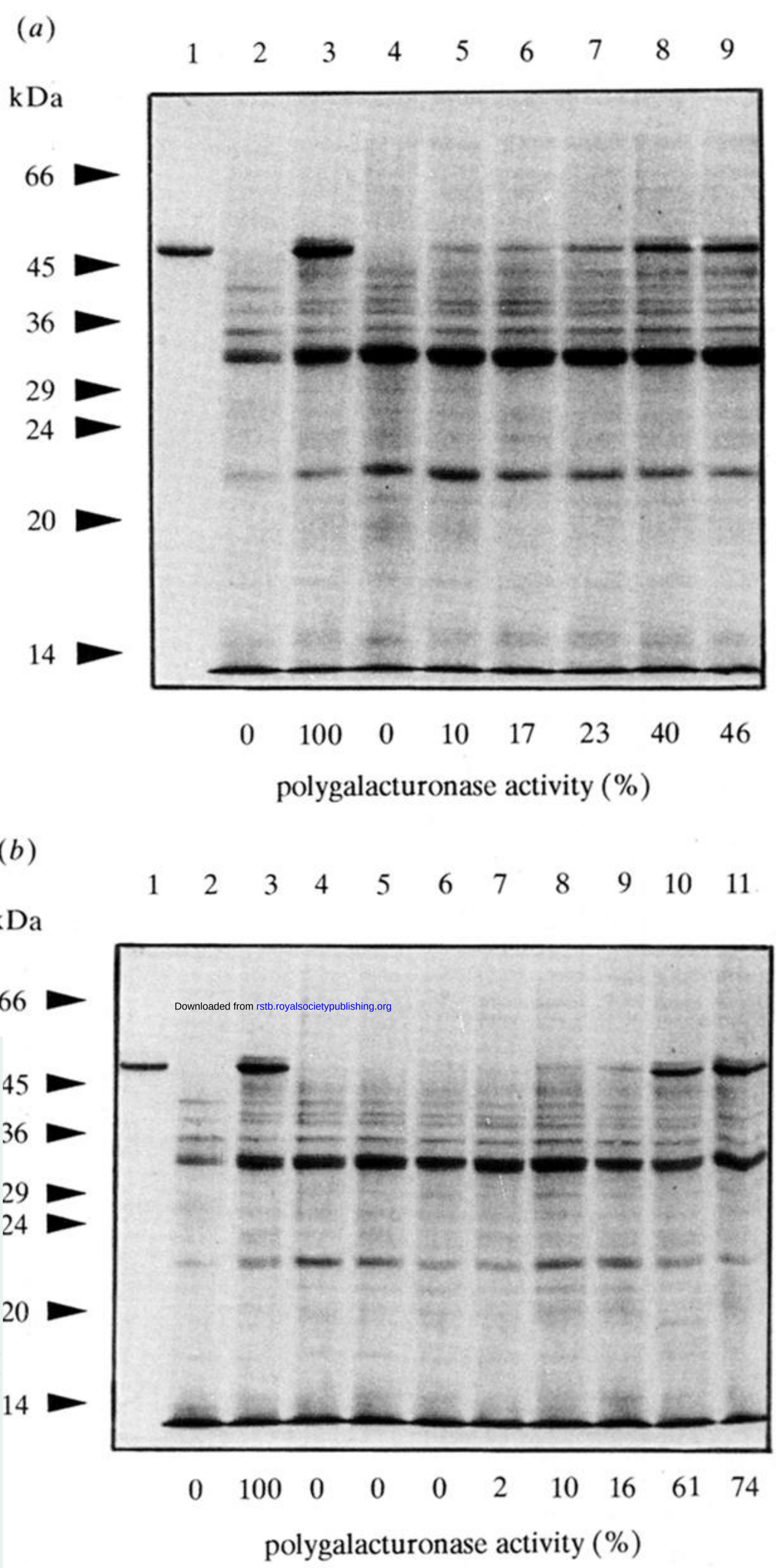

Figure 1. Inhibition of tomato polygalacturonase accumulation during ripening of transgenic fruit containing an antisense or sense gene. Cell wall proteins were extracted from unripe or ripe wild-type tomatoes (lanes 2 and 3) and fractionated by SDS-polyacrylamide gel electrophoresis, together with extracts from a range of ripe transgenic fruit containing different levels of PG enzyme activity (lanes 3–11). The gels were stained with Coomassie Blue to reveal protein bands and compared to purified tomato PG (lane 1). (*a*) Transgenic fruit containing truncated antisense PG genes. (*b*) Transgenic fruit containing truncated sense PG genes. The level of PG activity measured by enzyme assay is shown beneath each lane. (After Smith *et al.* 1993.)

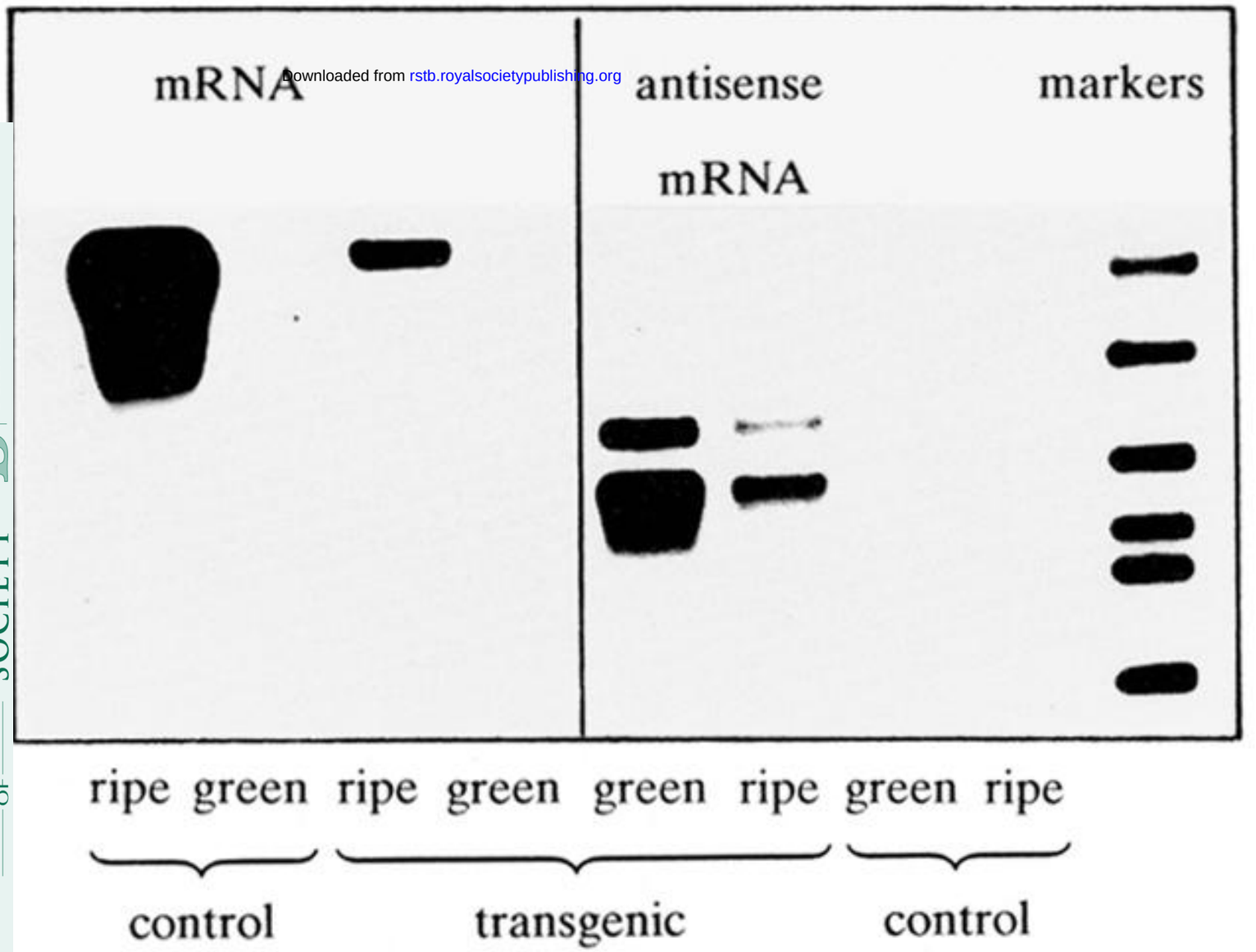

Figure 2. Effect of PG antisense genes on the accumulation of PG mRNA during ripening of tomato fruit. RNA was extracted from unripe and ripe wild-type tomatoes (control) and from transgenic fruit containing antisense genes in which there was a major reduction in PG enzyme, fractionated by agarose gel electrophoresis, blotted onto a nylon membrane and hybridized with either a sense-specific or antisense-specific single stranded RNA probe. The two PG antisense RNA bands are believed to arise by RNA processing at fortuitous poly A-addition signals in the antisense transcript. (After Smith *et al.* 1988.) Note the reduction in PG sense and antisense RNA during ripening of transgenic fruit.

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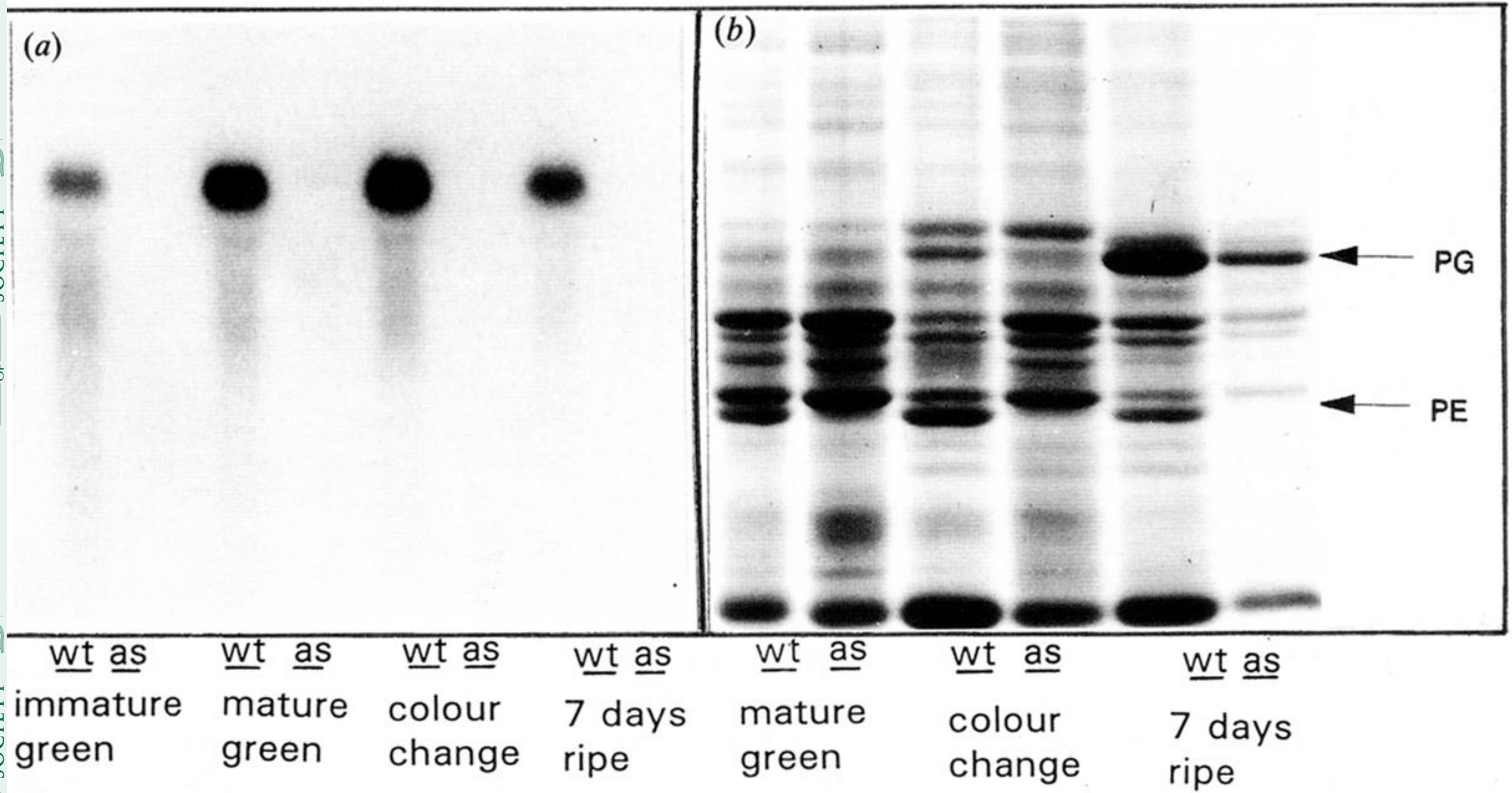

Figure 3. Inhibition by antisense genes of pectinesterase mRNA and enzyme synthesis in unripe and ripe tomatoes. Wild-type (control) and transgenic fruit containing PE antisense genes were assayed for the fruit-specific PE mRNA and protein by (*a*) northern blotting and (*b*) SDS-polyacrylamide gel electrophoresis. The positions of PG and PE proteins are shown by the arrows. (After Hall *et al.* 1993.)

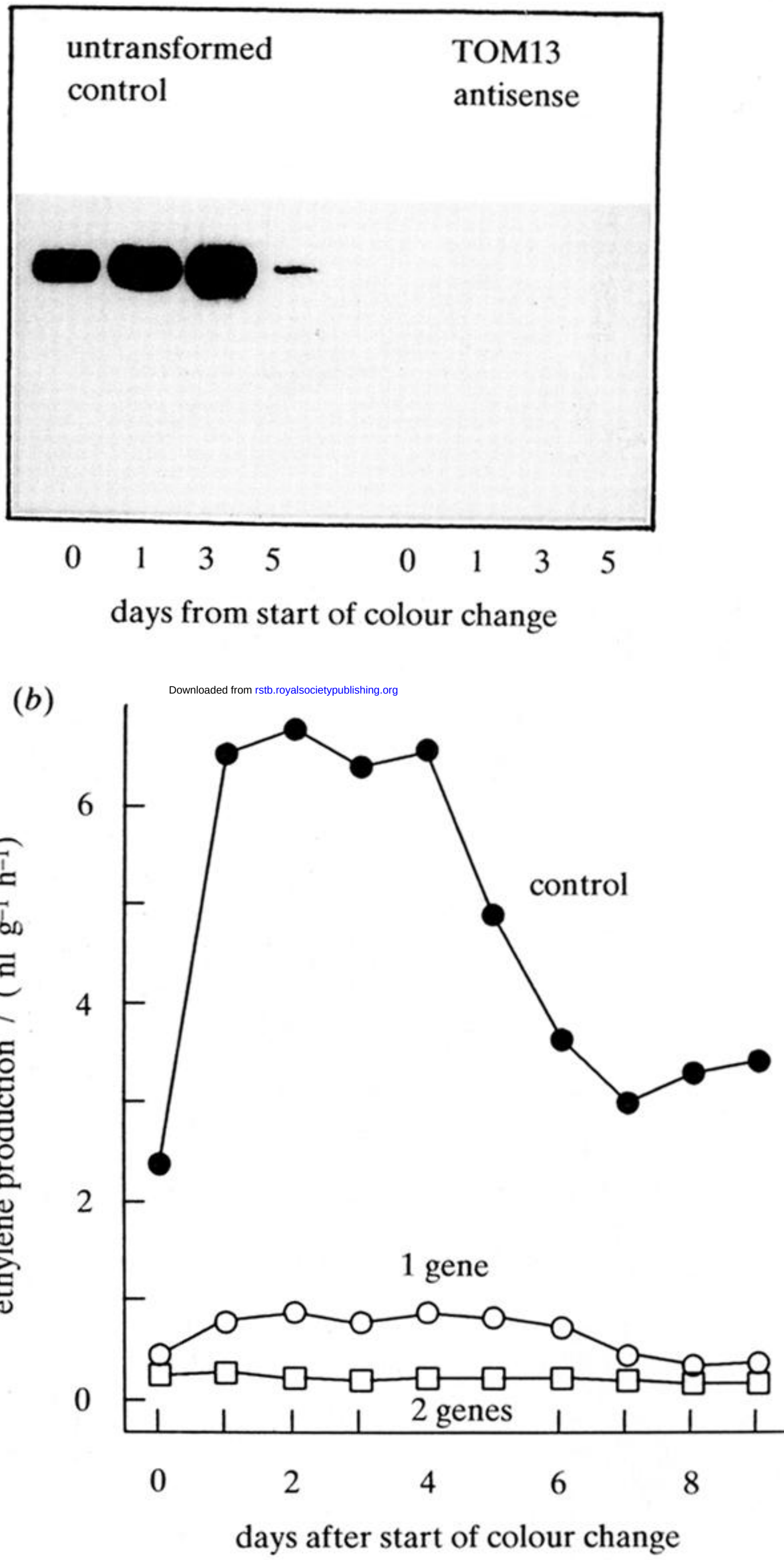

Figure 4. Inhibition by antisense genes of TOM13 (ACC oxidase) mRNA and ethylene synthesis during tomato ripening. Wild-type (control) and transgenic tomatoes containing TOM13 antisense genes were analysed for mRNA expression during ripening by (*a*) northern blotting and (*b*) ethylene synthesis. (After Hamilton *et al.* 1990.)