Planta (1985) 163:263–271

Planta

The appearance of polygalacturonase mRNA in tomatoes: one of a series of changes in gene expression during development and ripening

D. Grierson, A. Slater, J. Speirs* and G.A. Tucker
Department of Physiology and Environmental Science, University of Nottingham, School of Agriculture, Sutton Bonington, Loughborough LE12 5RD, UK

Abstract. Tomato mRNA was extracted from individual fruits at different stages of development and ripening, translated in a rabbit reticulocyte lysate and the protein products analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The results indicate that there are at least two classes of mRNA under separate developmental control. One group of approximately six mRNAs is present during fruit growth and then declines at the mature-green stage. Another group of between four and eight mRNAs increases substantially in amount at the onset of ripening, after the start of enhanced ethylene synthesis by the fruit, and continues to accumulate as ripening progresses. Studies of protein synthesis in vivo show that several new proteins are synthesised by ripening fruits including the fruit-softening enzyme polygalacturonase. One of the ripening-related mRNAs is shown to code for polygalacturonase, by immunoprecipitation with serum from rabbits immunised against the purified tomato enzyme. Polygalacturonase mRNA is not detectable in green fruit but accumulates during ripening. It is proposed that the ripening-related mRNAs are the products of a group of genes that code for enzymes important in the ripening process.

Key words: Fruit ripening – *Lycopersicon* (fruit ripening) – mRNA (tomato ripening) – Polygalacturonase.

Introduction

The cells of ripening fruit undergo a series of physiological and biochemical changes which render them attractive to eat. These changes affect several cell compartments and include degradation of starch, chlorophyll, alkoloids, cell walls, and altered metabolism of pigment, organic acids and sugars (Davies and Hobson 1981). Ripening used to be thought of essentially as a degradative process, caused by a breakdown of cellular organisation and the liberation of hydrolytic enzymes. However, there is increasing evidence that the expression of specific genes is required for normal ripening (Grierson et al. 1981) and biochemical evidence supports this conclusion. In ripening fruit there is synthesis of high-molecular-weight ribosomal RNA and polyadenylated RNA (Rattanapanone et al. 1977), proteins (Frenkel et al. 1968; De Swardt et al. 1973), increase in polyribosomes and the appearance of two new translatable mRNAs (Rattanapanone et al. 1978; Christoffersen et al. 1982). One interpretation of these observations is that macromolecular synthesis is required for the production of new enzymes essential for ripening. However, there is also evidence that ripening may involve the substantial turnover of existing proteins (Brady and O'Connell 1976).

Studies on tomato fruit polygalacturonase provide the clearest evidence for synthesis of enzymes of known function. Polygalacturonase activity is absent from unripe fruit and accumulates during ripening by de-novo synthesis (Tucker and Grierson 1982). There are three isoenzyme forms which are structurally and immunologically related (Tucker et al. 1980, 1981; Tucker and Grierson 1982; Ali and Brady 1982; Crookes and Grierson 1983). The role of the isoenzymes in cell-wall degratation during tomato ripening has been established. Purified isoenzymes have been shown to attack isolated tomato fruit cell walls (Themmen et al. 1982) and to solubilise cell walls when applied to segments of unripe tomato tissue (Crookes and Grierson 1983). The appearance of polygalact-

* *Present address:* CSIRO Division of Food Research, Plant Physiology Unit, School of Biological Sciences, Macquarie University, North Ryde, NSW 2113, Australia

Abbreviation: SDS = sodium dodecyl sulfate

uronase is related to ethylene, which in climacteric fruit such as tomato, functions as a ripening hormone. The natural synthesis of ethylene at the onset of ripening precedes polygalacturonase production (Grierson and Tucker 1983) and other ripening events.

We are interested in identifying ripening-related genes and studying how they are regulated by ethylene. In this paper we show that ripening-related changes in mRNA are more extensive than previously thought and involve the disappearance of several major mRNA species and the accumulation of new ones. Polygalacturonase mRNA has been identified by in-vitro translation and immunoprecipitation using an antibody raised against the purified enzyme. The mRNA is not detectable in unripe fruit but accumulates once ripening has been initiated.

Materials and methods

Plant growth and fruit production. Tomato seeds (*Lycopersicon esculentum* Mill. cv. Ailsa Craig) were surface-sterilised by washing for 10 min each in 50% (v/v) ethanol and 2% (v/v) domestic bleach (Domestos; Lever Brothers, Kingston upon Thames, Surrey, UK) 20 min in saturated trisodium orthophosphate, rinsed several times in sterile water and sown in autoclaved Levington compost (Fisons, Ipswich, UK) in 10-cm pots. Seedlings were grown in controlled-environment rooms in cycles of 16 h light, 22° C:8 h dark, 14° C. Illumination was by banks of warm-white fluorescent tubes which gave 33W m^{-2} at 1 m. Plants were transferred to 20-cm pots when the first flowers opened, after about six weeks. After fruit set, plants were fed twice a week with a balanced nutrient solution. Fruits began to ripen approximately eight weeks after fertilisation, when they weighed about 45 g. Where appropriate, the date of fertilisation and growth of individual fruits was monitored so that their stage of development was known accurately. Throughout the period of plant growth and fruit production, precautions were taken to exclude the possibility of virus contamination. Four different classes of fruit were used for RNA extraction, as follows. Immature-green fruit, which were about half the size of mature ones, were picked after approximately four weeks growth. Mature-green fruit were picked after approximately seven weeks and their ethylene production rate checked (Grierson and Tucker 1983). Only fruit with an ethylene diffusion rate through the calyx scar of less than 0.1 nl $g^{-1}h^{-1}$ were used as mature-green for RNA extraction. Upon cutting, these fruit showed no sign of colour change internally. Other fruits were picked at the mature-green stage and allowed to ripen naturally at 25° C. The ethylene production rate of these fruit was measured daily so that the onset of ripening could be pinpointed. This generally occurred 1–2 d before any visible change. The RNA was extracted from individual fruits at known times after the start of ripening. Alternatively, fruit allowed to ripen on the plants were picked 7–10 d after they showed the first signs of colour change and used immediately for RNA extraction.

Extraction of RNA. All glassware, centrifuge tubes and solutions used for RNA extraction were autoclaved and disposable gloves were worn while handling samples. Fruits were wiped with moist tissue and all seeds and woody tissue were removed. The pericarp was sliced, immediately frozen in liquid N_2 and ground to a powder with liquid N_2 in a mortar and pestle. Approximately 40 g of frozen powder was added to 70 ml of the following medium and quickly homogenised with a mortar und pestle: 0.2 M 2-amino-2-(hydroxymethyl)-1.3-propanediol (Tris)-HCl, pH 8.5, 0.2 M sucrose, 60 mM KCl, 30 mM $MgCl_2$, 0.31% (v/v) β-mercaptoethanol (added immediately before use). The partially-frozen slurry was squeezed through four layers of moist muslin which had previously been autoclaved. The filtrate was centrifuged at 20000 *g* for 10 min at 3° C. To the resulting supernatant, 0.1 volume of 10% (w/v) sodium dodecyl sulphate (SDS) was added. The suspension was deproteinised by shaking with an equal volume of "phenol-chloroform" mixture (Grierson and Covey, 1976) and centrifuged at 2500 *g* for 15 min. The supernatant was again shaken with phenol-chloroform and centrifuged at 2500 *g* for 15 min. The supernatant was carefully removed, made 0.1 M in KCl, and nucleic acid precipitated with two volumes of ethanol in a refrigerator overnight. Nucleic acid was pelleted by centrifugation at 2500 *g* for 15 min, drained, dissolved in 0.5% SDS/0.15 M sodium acetate (adjusted to pH 6 with acetic acid) and precipitated with 2.5 volumes of ethanol at -20° C for at least 2 h. The nucleic acid was pelleted by centrifugation as before, drained and suspended with vigorous shaking in 5 ml sterile 3 M sodium acetate (adjusted to pH 6 with acetic acid). The suspension was centrifuged at 8000 *g* for 20 min at 3° C and the pellet drained, suspended in 80% ethanol containing 0.2 M KCl and centrifuged at 8000 *g* for 20 min at 3° C. The pellet was drained, again suspended in fresh 80% ethanol/0.2 M KCl and centrifuged at 8000 *g* for 20 min at 3° C, drained, dried in vacuum and dissolved in distilled water. Insoluble material was removed by centrifugation and the solution stored at -80° C at a concentration between 2.4 and 14 µg μl^{-1}.

In-vitro protein synthesis. Total high-molecular-weight RNA was translated in micrococcal nuclease-treated rabbit reticulocyte lysate in the presence of [^{35}S] methionine, obtained from Amersham International, Amersham, Bucks., UK. Reaction mixtures contained 8 µl lysate, 1 µl RNA (containing 2.5–3 µg) and 1 µl [^{35}S] methionine (approx. $3.7 \cdot 10^5$ Bq; $3.7 \cdot 10^{13}$ Bq $mmol^{-1}$) and were incubated for 40 min to 1 h at 30° C. At the end of the incubation the mixture was either diluted with two volumes of: 10% sucrose, 10% SDS, 5% (v/v) β-mercaptoethanol, boiled for 2 min and used for electrophoresis, or diluted with three volumes of: 10 mM NaH_2PO_4 adjusted to pH 7.4 with NaOH, 150 mM NaCl, 1% nonidet P-40 (Sigma, Poole, Dorset, UK) and used for immunoprecipitation. Aliquots of the reaction mixture were spotted onto a filter paper, dried and batch-washed sequentially in 10% trichloroacetic acid containing unlabelled methionine, boiling 5% trichloracetic acid, cold 5% trichloracetic acid then ethanol, dried and counted to measure total incorporation into protein.

Immunoprecipitation. Translation mixtures were diluted with three volumes of 10 mM NaH_2PO_4, pH 7.4, 150 mM NaCl, 1% nonidet P-40 and the following were added per 40 µl of diluted sample: 0.5 µl of 500 mM phenyl methyl sulphonyl fluoride (dissolved in hot ethanol), 5 µl serum from rabbits immunised against purified polygalacturonase according to the method of Foster and Crighton (1974), 4 µg unlabelled polygalacturonase purified as described by Tucker et al. 1980, 10 mg protein A-sepharose (Sigma) swollen in 50 µl dilution buffer, and the mixture was shaken overnight in a cold room. The mixture was then centrifuged at 8000 *g* for 4 min, the supernatant was removed and the pellet resuspended in 600 µl 10 mM NaH_2PO_4, pH 7.4, 150 mM NaCl, 1% nonidet P-40 and recentrifuged at 8000 *g* for 4 min. The cycle of resuspension and washing in fresh buffer was repeated seven to ten times. Finally the pellet was

drained, 20 µl of 10% (w/v) SDS, 10% (w/v) sucrose and 5% (v/v) β-mercaptoethanol were added and boiled for 2 min. The protein A-sepharose was then pelleted by centrifugation and the supernatant saved. The pellet was re-extracted with a further 20 µl of the same buffer and the supernatants pooled and used for scintillation counting and gel electrophoresis.

Fractionation and detection of mRNA translation products. Protein samples were separated by electrophoresis in 1.5 cm thick 12–17% or 10–15% gradient polyacrylamide-SDS slab gels, fixed in 30% methanol, 14% acetic acid, impregnated for fluorography (Bonner and Laskey 1974), dried and exposed to preflashed X-ray film for 5–10 d at -80° C. The developed film was then scanned with a laser densitometer (LKB, Bromona, Sweden), or photographed. Protein molecular weights were estimated by comparison with the mobility of ^{14}C-labelled marker proteins from Amersham International which were fractionated in the same gel.

Incorporation of radioactivity into proteins by tomato discs. Discs of tomato pericarp were cut with a 1-cm-diameter cork borer. Each disc was sliced transversely and the resulting pair of discs were placed cut-side upwards in a small Petri dish. 5 µl of [^{35}S] methionine plus 5 µl of H_2O was spread on the cut surface of each disc. The discs were incubated for 16 h at 25° C in a water-saturated atmosphere.

"Total" protein was extracted by homogenising each pair of discs on ice with an equal volume of 4% (w/v) SDS, 100 mM Tris-HCl, pH 6.8, 10% (w/v) sucrose, 10% (v/v) β-mercaptoethanol. The homogenate was heated to 100gC for 2 min and centrifuged for 5 min at 8000 *g*. The supernatant fraction was loaded directly onto SDS-polyacrylamide gels.

Cell-wall-bound proteins were extracted by first homogenising the labelled discs in an equal volume of H_2O on ice and centrifuging at 8000 *g* for 5 min. The water-soluble proteins in the supernatant fraction were removed and the pellet was then resuspended in the same volume of 0.9 M NaCl, 0.1 M NaH_2PO_4, pH 6.0 and extracted on ice for 3 h before centrifugation at 8000 *g* for 5 min. The high-salt-extracted proteins ("cell-wall-bound" proteins) were precipitated by addition of trichloroacetic acid to a final concentration of 10% (w/v) and collected by centrifugation after 2 h at 4° C. The precipitate was washed twice with 80% (v/v) acetone, dried under vacuum, and redissolved in SDS-polyacrylamide gel sample buffer (2% (w/v) SDS, 50 mM Tris-HCl, pH 6.8, 5% (w/v) sucrose, 5% (v/v) β-mercaptoethanol) before loading onto gels.

Results

Changes in mRNA during development and ripening. The nucleic-acid content of tomato fruit was relatively low, as shown previously (Rattanapanone et al. 1977) and the yield of purified high-molecular-weight RNA varied from 15–60 µg g^{-1}, depending on fruit ripening stage, and age and nutritional status of the plants. Total RNA stimulated the incorporation of [^{35}S] methionine into protein in the rabbit reticulocyte lysate between three and ten fold above background. In general, RNA from ripe fruit was more active in directing protein synthesis than that from green fruit. The mRNA translation products varied in MW from 200000 to about 8000 and there were obvious

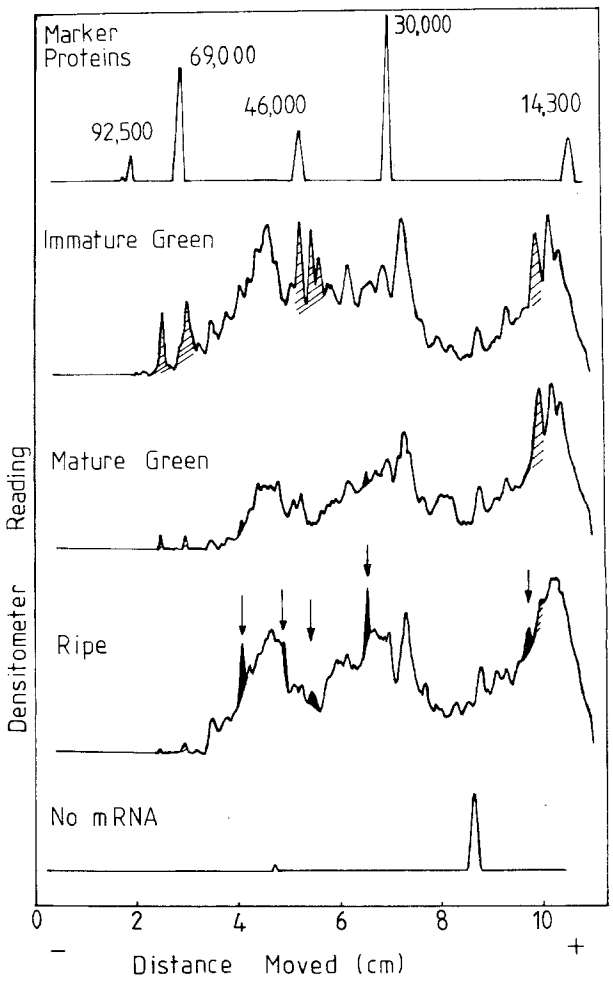

Fig. 1. Changes in translatable mRNA during fruit maturation and ripening. Samples of RNA from immature-green, mature-green and ripe fruit were translated in reticulocyte lysate. Samples of the translation products were treated with mercaptoethanol and SDS and similar quantities of radioactive proteins fractionated by polyacrylamide gel electrophoresis in parallel tracks. Radioactive marker proteins and a sample of reticulocyte lysate incubated without added RNA were inluded in the same gel. Radioactive proteins were detected by fluorography and the developed film was scanned with a densitometer to locate and quantify individual protein bands. Prominent mRNA translation products that disappear during fruit maturation and ripening are *hatched.* Additional translation products that appear or accumulate during ripening are shown black and are indicated by *arrows*

changes in translatable mRNA at different stages of fruit development (Fig. 1). One class of mRNAs coding for at least six major proteins (hatched in Fig. 1), was present in immature-green fruit but declined in amount at the mature-green stage and during ripening. A second group of mRNAs, coding for between four and eight proteins, either appeared or increased greatly in amount at the onset of ripening. Some of these are shown black in Fig. 1. An additional high-molecular-weight

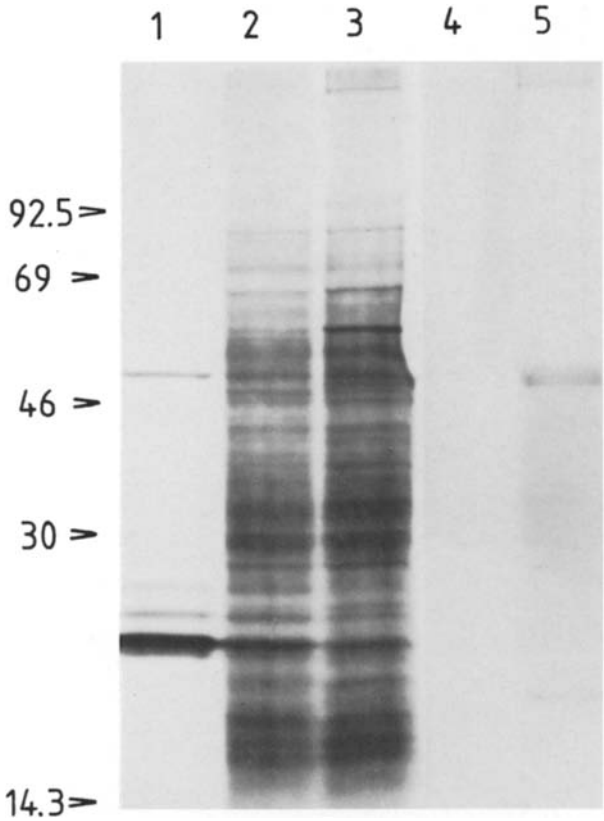

Fig. 2. Immunoprecipitation of mRNA translation products in vitro with polygalacturonase antibody. Samples of RNA from mature-green and ripe tomatoes were translated in a reticulocyte lysate and the major part of each sample was treated with antibody to polygalacturonase and the immunoprecipitate recovered as described in Material and methods. Approximately 1% of the total translation products from red RNA were precipitated with the antibody. Samples of the complete translation mixtures and the immunoprecipitates were fractionated in parallel tracks of a polyacrylamide gel together with radioactive marker proteins and a sample of reticulocyte lysate incubated without added mRNA. Radioactive proteins were detected by fluorography. *Track 1*, reticulocyte lysate without added RNA; *track 2*, translation products from RNA from green fruit; *track 3*, translation products from RNA from red fruit; *track 4*, immunoprecipitate from greem RNA translation products; *track 5*, immunoprecipitate from red RNA translation products. The positions of marker proteins (MWsx 10^{-3}) are indicated by *arrows*

ripening-specific translation product is shown in Fig. 2. A third class of mRNAs appeared to be present at all stages of fruit development and ripening. The molecular weights of the proteins coded for by mRNAs which increased in amount during ripening, calculated from a number of different gel profiles using ^{14}C-labelled proteins as molecular-weight markers, are shown in Table 1. These are divided into two groups: major translation products and less prominent ones observed frequently. At least one of the ripening mRNAs in the former group, coding for a protein with a MW of 55000 was present in small quantities in green tissue, whereas the mRNAs coding for proteins with MWs of 190000 and 48000 were only detected in ripening fruit (Fig. 2). Similar mRNA translation profiles were obtained using total RNA, poly(A)$^+$ RNA and polysomal RNA.

Table 1. Molecular weights of proteins coded by ripening-specific mRNAs

Major translation products found consistently	Other translation products observed frequently
190000	80000
55000	57000
48000 (polygalacturonase)	44000
35000	20000

Appearance and accumulation of polygalacturonase mRNA during ripening. The physiological significance of the ripening-specific mRNAs was investigated by identifying and studying the mRNA coding for polygalacturonase, an enzyme which is known to be synthesised de novo during ripening (Tucker and Grierson 1982).

Polygalacturonase antiserum was raised against isoenzyme 2 which was shown to be pure judged by electrophoresis in SDS-polyacrylamide gels (Fig. 3). The antiserum has been shown by radioimmunossay to react with polygalacturonase isoenzymes 1 and 2 which are structurally related (Tucker et al. 1980). Immunodiffusion experiments showed no reaction between the antiserum and proteins from mature-green tomatoes and a single precipitin line with proteins from ripe fruit (Fig. 3). There was no reaction with pre-immune serum. Incubation of polygalacturonase with antiserum in the presence and absence of protein A-sepharose resulted in the inactivation of the enzyme (Table 2).

Polygalacturonase mRNA was identified by immunoprecipitation of its in-vitro translation product with antiserum from rabbits immunised against purified tomato polygalacturonase. No precipitate was obtained with pre-immune serum. In SDS-polyacrylamide gels the immunoprecipitated protein migrated slightly faster than an endogenous radioactive band produced by rabbit reticulocyte lysate without added RNA (Fig. 2). This endogenous band has been shown to arise from post-translational addition of [^{35}S]methionine to a protein already present in the lysate (Morch and Benicourt 1980).

The polygalacturonase in-vitro translation product had an apparent MW of 48000 which is

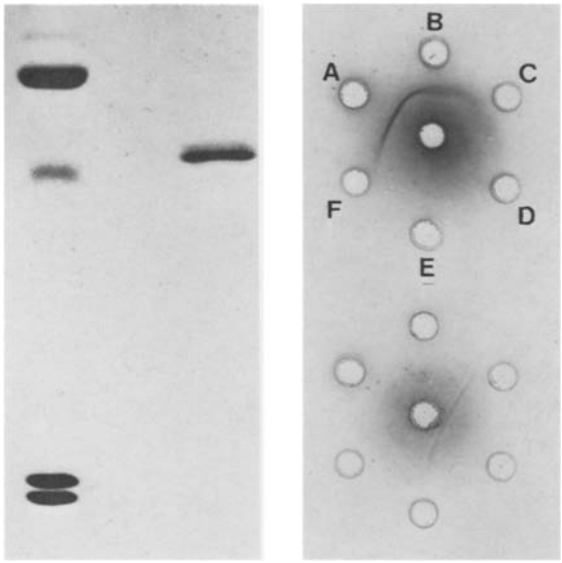

Fig. 3. Characterisation of polygalacturonase protein and antibody. *Left* Protein samples were fractionated by electrophoresis in a 10–15% gradient polyacrylamide-SDS gel and stained with Coomassie blue: *track 1*, marker proteins with MWs of 66000, 45000, 16950, 14300; *track 2*, purified polygalacturonase 2 (MW 46000). *Right* Ouchterlony double-diffusion in 1.5% agarose, 0.15 M NaCl, 0.01 M Tris-HCl pH 8. Cell-wall-bound proteins from ripe fruit at *A*, 1 mg ml^{-1}; *B*, 0.5 mg ml^{-1}; *C*, 0.25 mg ml^{-1} or mature-green fruit at *D*, 1 mg ml^{-1}; *E*, 0.5 mg ml^{-1}; *F*, 0.25 mg ml^{-1} were tested against purified polygalacturonase anti-serum (*top*) or pre-immune serum (*bottom*)

Table 2. Effect of anti-polygalacturonase serum and pre-immune serum on enzyme activity. Total cell-wall-bound protein (20 µg) was incubated ± serum (25 µl) for 24 h at 4° C in a total volume of 35 µl 14 mM phosphate, 0.15 M NaCl pH 7.2. Protein A-sepharose (50 µl of a 100 mg ml^{-1} suspension) was added where shown and samples left for a further 24 h at 4° C. Samples were then centrifuged (5 min, 8000 *g*) and the supernatant and pellet fractions assayed for polygalacturonase (PG) activity

Incubation conditions	Enzyme activity (µmol galacturonic acid min^{-1} mg^{-1}	
	Supernatant	Pellet
Enzyme only	0.245	0.013
Enzyme + PG antibody	0.002	0.023
Enzyme + preimmune serum	0.246	0.004
Enzyme + PG antibody + protein A-sepharose	0.017	0.083
Enzyme + preimmune serum + protein A-sepharose	0.238	0.027

slightly larger than the 46000 MW subunit polypeptide of polygalacturonase isoenzymes (Fig. 4). Traces of a protein of MW 190000 were sometimes also found in the polygalacturonase immunoprecipitate (Fig. 2). It is not clear whether this protein

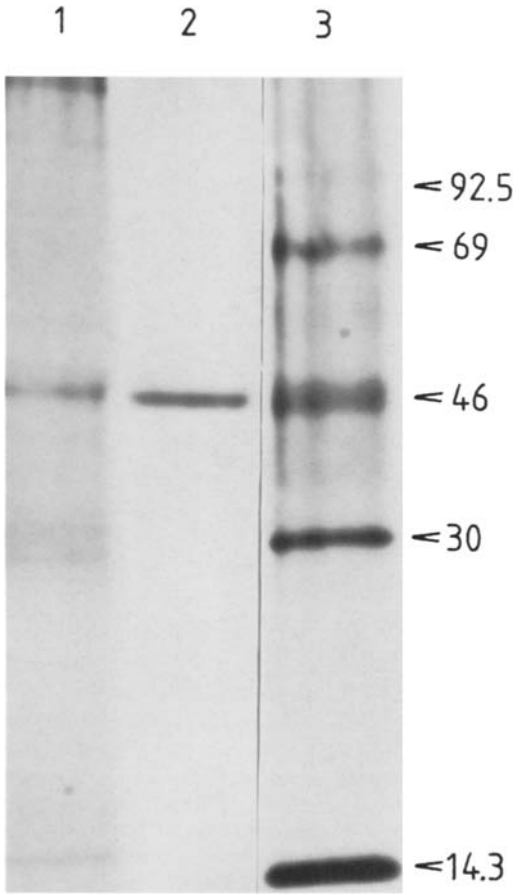

Fig. 4. Comparison of immunoprecipitates abtained by treatment of tomato in-vitro and in-vivo translation products with polygalacturonase antibody. Proteins from ripening tomatoes were labelled in vivo with [^{35}S]methionine, purified as described in Material and methods, treated with polygalacturonase antibody and the immunoprecipitate recovered. The immunoprecipitate from [^{35}S]methionine-labelled in-vitro translation of mRNA from ripe fruit was also prepared (see Fig. 2) and the two samples were fractionated in a polyacrylamide gel, together with marker proteins. The radioactive protein bands were detected by fluorography. *Track 1*, in-vitro polygalacturonase immunoprecipitate; *track 2*, in-vivo polygalacturonase immunoprecipitate; *track 3*, radioactive marker proteins (MWs $X10^{-3}$ are shown)

is immunologically related to polygalacturonase or is a non-specific contaminant.

Translatable mRNA coding for polygalacturonase was not detectable in immature-green or mature-green tomatoes and only appeared in fruit after they initiated the increase in ethylene synthesis normally associated with the onset of ripening (Fig. 2). When detached fruit were allowed to ripen naturally, without added ethylene, several ripening-specific mRNAs, including that coding for polygalacturonase, appeared in a coordinated manner after endogenous ethylene production began (Fig. 5). These mRNAs continued to increase in quantity

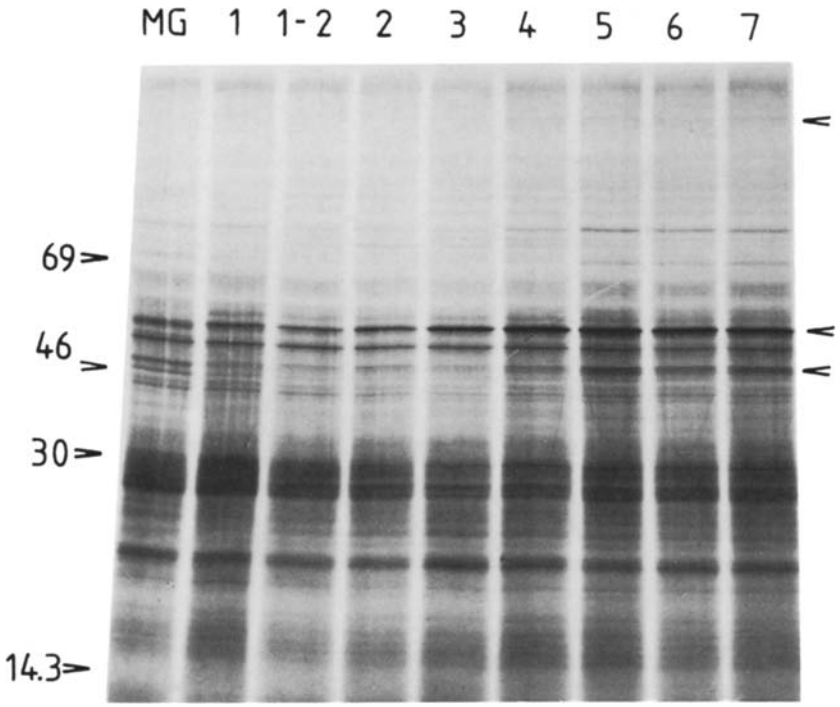

Fig. 5. Changes in mRNA translation products with respect to endogenous ethylene production. Detached mature-green fruit were allowed to ripen under normal laboratory conditions. Ethylene production by each fruit was monitored daily. RNA was extracted from individual fruit on different days after the onset of ethylene production. The RNA was translated in a reticulocyte lysate and the [^{35}S]methione-labelled translation products were separated by SDS-polyacrylamide gel electrophoresis and detected by fluorography. Translation products of RNA from mature-green fruit (*MG*) with no detectable ethylene production were compared with those from individual ripening tomatoes sampled from *1* to *7* d after the onset of ethylene production. The positions of marker proteins (MW X10^{-3}) run in a parallel track on the same gel are indicated. The *arrows* on the *right hand side* indicate translation products which increase during ripening

throughout the ripening period and were extracted in an undegraded form even from soft red fruit (Fig. 5). This is consistent with earlier studies on the incorporation of [^{3}H]uridine into RNA, which indicated that synthesis of polyadenylated RNA continues during ripening (Rattanapanone et al. 1977).

Effect of virus infection. In experiments using fruit from plants grown in a general glasshouse, quite different mRNA translation profiles were occasionally observed. These were found to be due to the infection of plants by a strain of tobacco mosaic virus (TMV) which showed no visible symptoms on tomato plants. The presence of the virus in such plants was confirmed by electron microscopy. Translation products of virus RNA were identified by comparison with those obtained with RNA purified from TMV particles isolated from infected tobacco plants. Infected tomato plants contained large amounts of virus RNA which were efficiently translated in vitro and tended to obscure the translation products of the fruit mRNA (Fig. 6). The majority of the RNA molecules directing the synthesis of virus-related products was present in the non-polyadenylated fraction of infected fruit. Translation of the polyadenylated mRNAs revealed those normally found in fruit (data not shown). Investigations with the tomato cultivar 'sonatine' which carries TMV-resistance genes, showed that translatable virus RNA could also be present in these fruit.

Protein synthesis in vivo during ripening. When discs cut from fruit pericarp tissue were labelled with [^{35}S]methionine, radioactivity was rapidly incorporated into a large number of proteins. Many of the proteins sythesised by mature-green and ripening tissue were similar but a number of new proteins appeared in ripening tissue (shown by arrows in Fig. 7a). A protein of MW 46000 was found to be a major component of the cell-wall-bound proteins synthesised by discs of ripening fruit (shown by arrows in Fig. 7b) but was absent in green fruit. This protein was identified as polygalacturonase by immunoprecipitation (Fig. 4).

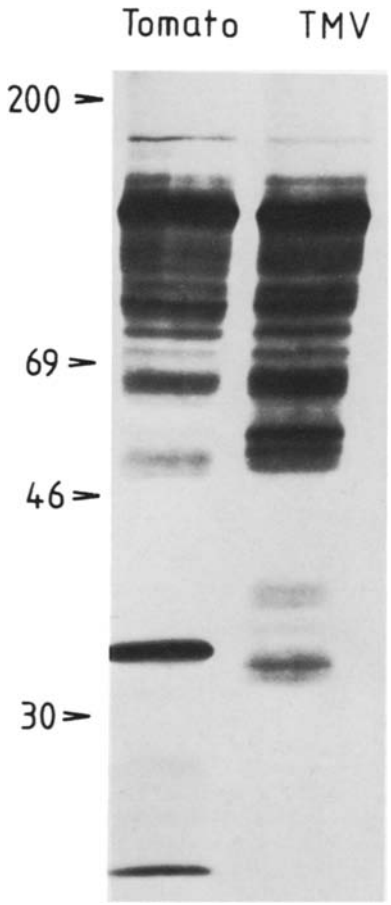

Fig. 6. Effect of tobacco-mosaic-virus infection on fruit mRNA translation products in vitro. Total cytoplasmic RNA was extracted from tomatoes grown without special precautions in a general glasshouse (*Tomato*) and viral RNA was isolated from tobacco-mosaic-virus-infect tobacco leaves (*TMV*). The RNA was translated in a reticulocyte lysate and the translation products were compared by separation in parallel tracks in an SDS-polyacrylamide gel. The positions of protein markers (MW X10^{-3}) run on the same gel are indicated

Discussion

There have been two previous reports of changes in mRNA associated with fruit ripening, in tomato (Rattanapanone et al. 1978) and avocado (Christoffersen et al. 1982). In both studies, evidence was presented for the appearance of two new mRNAs during ripening but the identity of the proteins they coded for was not established. Our present results indicate that the changes in mRNAs during fruit development and ripening are much more extensive than was thought previously. Furthermore, one of the ripening-specific mRNAs in tomato has been shown to code for polygalacturonase, an important softening enzyme (Themmen et al. 1982; Crookes and Grierson 1983) which is synthesised de novo during ripening (Tucker and Grierson 1982).

Characterisation of fruit mRNA translation products provides further support for the "gene expression" hypothesis for the control of fruit ripening. There appear to be two separate classes of mRNA which are under developmental control. One class is associated with fruit growth and development: these mRNAs decline in amount before and during ripening. The second class of mRNAs accumulate during ripening: they are either present in very small amounts in mature-green fruit or are absent altogether. Between four and eight ripening-related mRNAs have been detected by in-vitro translation but it is possible that other less abundant mRNAs were not detected. Some ripening-specific tomato mRNAs have been shown elsewhere (Speirs et al. 1984) to be associated with polyribosomes and to direct the synthesis of a number of proteins in a wheat-germ protein-synthesis system. However, no translation products were identified in these experiments and not all the high-molecular-weight products described here (Figs. 2, 5) were detected previously, probably because of the lack of fidelity shown by wheat-germ extracts in translating large mRNAs.

The striking effect virus infection has on mRNA translation profiles highlights the importance of maintaining virus-free plants in this type of investigation (Fig. 6). The use of a virus-resistant cultivar ('sonatine') was not satisfactory and stringent precautions against physical contamination of plants by TMV had to be taken. This is especially important with tomatoes, since visible symptoms of virus are not always apparent. The virus RNA in fruit of infected plants tends to dominate the in-vitro translation profiles and obscure the fruit mRNAs (Fig. 6).

It is obviously important to demonstrate that developmental changes in mRNA have genuine importance for the process being studied. There are several reasons for concluding that the ripening-specific mRNAs are not artifacts arising from the degradation of pre-existing mRNAs. Firstly, several of the mRNAs code for high-molecular-weight proteins which do not decrease in amount even when mRNA samples are extracted from soft red fruit (Fig. 5). Secondly, poly(A)$^+$ mRNA has been shown to be synthesised in ripening tomato tissue (Rattanapanone et al. 1977). Thirdly, one of the ripening-specific mRNAs codes for a protein which is immunologically related to polygalacturonase (Figs. 2, 4). This mRNA is not detectable in mature-green fruit but accumulates after the initiation of ripening, when polygalacturonase is

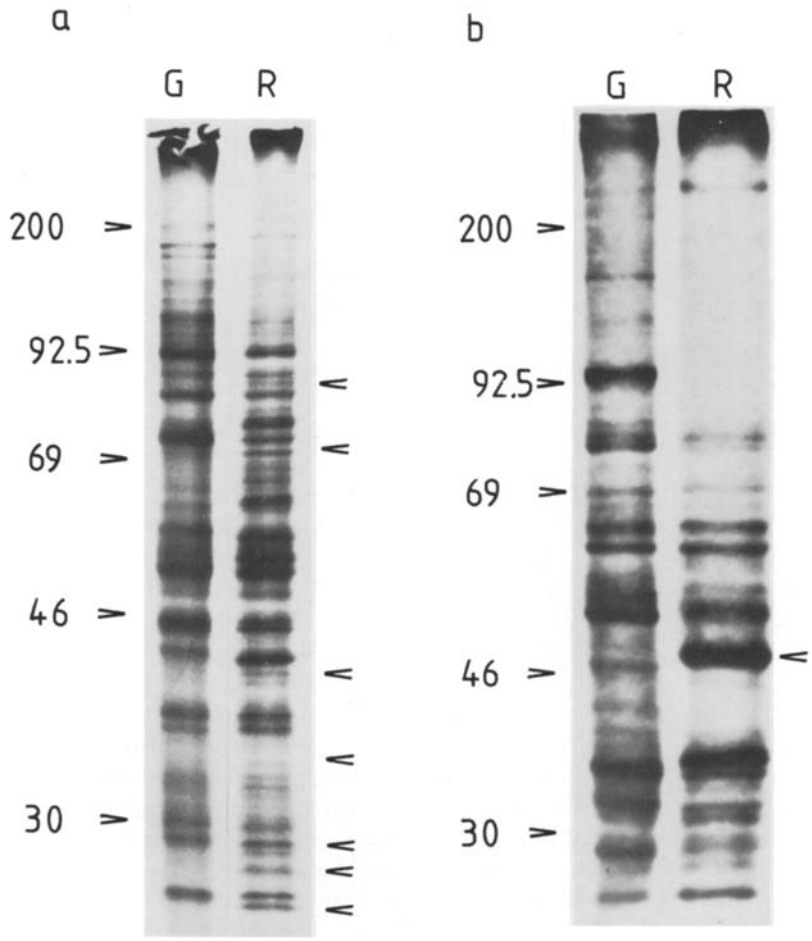

Fig. 7. Comparison of labelled proteins sythesised in green and ripening tomato discs. Discs of pericarp cut from green (*G*) or ripening (*R*) fruit were labelled with [^{35}S]methione for 16 h. Labelled proteins were extracted as described in Material and methods and were separated by SDS-polyacrylamide gel electrophoresis and detected by fluorography. The positions of protein markers (MW X10^{-3}) are indicated. **a** Total SDS-soluble proteins. Proteins labelled in discs from ripening fruit but not in discs from green fruit are indicated by *arrows*. **b** Cell-wall-bound protein. The position of a major cell-wall protein labelled in ripening tomato discs but not in green tomato discs, corresponding in size to polygalacturonase, is indicated by the *arrow*

synthesised (Tucker and Grierson 1982; Grierson and Tucker 1983).

The molecular weight of the polygalacturonase in-vitro tanslation product is slightly greater than that of the native enzyme (Figs. 3, 4) and it may be an unmodified precursor protein. Since mature polygalacturonase is glycosylated (Moshrefi and Luh 1983) and secreted into the cell wall, it is probable that the initial translation product undergoes processing and glycosylation reactions in-vivo.

The identification of polygalacturonase mRNA and the demonstration that it appears during ripening is important since it lends credibility to the suggestion that the other ripening-specific mRNAs detected in this investigation represent the transcription products of a range of genes which are coordinately expressed in ripening fruit. The finding that several new proteins are synthesised in vivo during ripening (Fig. 7) is consistent with this suggestion. With the exception of polygalacturonase, the identity of the products of these ripening genes is unkown. They may be involved in catalysing other aspects of ripening such as chlorophyll loss, starch metabolism and the synthesis of lycopene and ethylene.

The absence of polygalacturonase mRNA from green tomatoes contrasts with the situation for that coding for the 55000 MW protein, which is present in small amounts in green fruit and increases greatly during ripening (Figs. 2, 5). We speculate that the protein coded for by this mRNA functions at a low level in green tissue and is required in much greater quantities during ripening. In addition to accumulating new mRNAs, ripening fruit retain some of those which are present in mature-green tissue (Fig. 1). This is consistent with the finding that during ripening, fruit continue to synthesise many proteins made at the mature-green stage, plus a number of new ones (Fig. 7).

Similar mRNA translation profiles were obtained with RNA prepared as described in the Methods section and with total cell RNA preparations including the 20000 *g* fraction. This indicates that the major fruit mRNAs are not located in cell organelles such as plastids and

mitochondria. Since the detection of mRNA translation products by one-dimensional electrophoresis is a relatively insensitive procedure the eight mRNAs found to accumulate must be regarded as the minimum number of changes occurring during ripening. It is possible that more sensitive procedures will reveal others.

Information about the regulatory signals governing the expression of the ripening genes is of critical importance for our understanding of ripening control. Ethylene appears to be important in this process and its effects on mRNA production are being investigated.

This work was supported by a grant from the Agriculture and Food Research Council to D.G. During part of the work, D.G. was a Nuffield Foundation Science Research Fellow. J.S. gratefully acknowledges the support of ICI.

References

Ali, Z.M., Brady, C.J. (1982) Purification and characterisation of polygalacturonases of tomato fruits. Aust. J. Plant Physiol. **9**, 171–178

Bonner, W.M., Laskey, R.A. (1974) A film-detection method for 3H-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**, 83–88

Brady, C.J., O'Connell, P.B.H. (1976) On the significance of increased protein synthesis in ripening banana fruits. Aust. J. Plant Physiol. **3**, 301–310

Christoffersen, R.E., Warm, E., Laties, G.G. (1982) Gene expression during fruit ripening in avocado. Planta **155**, 52–57

Crookes, P.R., Grierson, D. (1983) Ultrastructure of tomato fruit ripening and the role of polygalacturonase isoenzymes in cell wall degradation. Plant Physiol. **72**, 1088–1093

Davies, J.N., Hobson, G.E. (1981) The constituents of tomato fruit – the influence of environment, nutrition and genotype. CRC Crit. Rev. Food Sci. Nutrit. **15**, 11205–11280

De Swardt, G.H., Swanepoel, J.H., Duvenage, A.J. (1973) Relation between changes in ribosomal RNA and total protein synthesis and the respiration climacteric in pericarp tissues of tomato. Z. Pflanzenphysiol. **70**, 358–363

Foster, J.P., Crighton, D.B. (1974) Luteinizing hormone (LH) release after single injections of a synthetic LH-releasing hormone (LH-RH) in the ewe at three different reproductive stages and comparison with natural release at oestrus. Theriogenology **2**, 87–100

Frenkel, C., Klein, I., Dilley, D.R. (1968) Protein synthesis in relation to ripening of pome fruits. Plant Physiol. **43**, 1146–1153

Grierson, D., Covey, S.N. (1976) The properties and function of rapidly-labelled nuclear RNA. Planta **130**, 317–321

Grierson, D., Tucker, G.A. (1983) Timing of ethylene and polygalacturonase synthesis in relation to the control of tomato fruit ripening. Planta **157**, 174–179

Grierson, D., Tucker, G.A., Robertson, N.G. (1981) The regulation of gene expression during the ripening of tomato fruits In: Quality in stored and processed vegetables and fruit, pp. 179–191, Goodenough, P.W., Atkin, R.K., eds. Academic Press, London New York

Morch, M.-D., Benicourt, C. (1980) Post-translational proteolytic cleavage of an in vitro-synthesised turnip yellow mosaic virus RNA-coded high-molecular-weight proteins. J. Virol. **34**, 85–94

Moshrefi, M., Luh, B.S. (1983) Carbohydrate composition and electrophoretic properties of tomato polygalacturonase isoenzymes. Eur. J. Biochem. **135**, 511–514

Rattanapanone, N., Grierson, D., Stein, M. (1977) Ribonucleic acid metabolism during the development and ripening of tomato fruits. Phytochemistry **16**, 629–633

Rattanapanone, N., Speirs, J., Grierson, D. (1978) Evidence for changes in messenger RNA content related to tomato fruit ripening. Phytochemistry **17**, 1485–1486

Speirs, J., Brady, C.J., Grierson, D., Lee, E. (1984) Changes in ribosome organisation and messenger RNA abundance in ripening tomato fruits. Aust. J. Plant Phys. **11**, 225–233

Tucker, G.A., Grierson, D. (1982) Synthesis of polygalacturonase during tomato fruit ripening. Planta **155**, 64–67

Tucker, G.A., Robertson, N.G., Grierson, D. (1980) Changes in polygalacturonase isoenzymes during the 'ripening' of normal and mutant tomato fruit. Eur. J. Biochem. **112**, 119–124

Tucker, G.A., Robertson, N.G., Grierson, D. (1981) The conversion of tomato fruit polygalacturonase isoenzyme 2 into isoenzyme 1 in-vitro. Eur. J. Biochem. **115**, 87–90

Themmen, A.P.N., Tucker, G.A., Grierson, D. (1982) Degradation of isolated tomato cell walls by purified polygalacturonase in vitro. Plant Physiol. **69**, 122-124

Received 7 June; accepted 23 August 1984