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Gene expression during fruit ripening in avocado

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Abstract. The poly(A)$^+$RNA populations from avocado fruit (*Persea americana* Mill cv. Hass) at four stages of ripening were isolated by two cycles of oligo-dT-cellulose chromatography and examined by in-vitro translation, using the rabbit reticulocyte lysate system, followed by two-dimensional gel electrophoresis (isoelectric focusing followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis) of the resulting translation products. Three mRNAs increased dramatically with the climacteric rise in respiration and ethylene production. The molecular weights of the corresponding translation products from the ripening-related mRNAs are 80,000, 36,000, and 16,500. These results indicate that ripening may be linked to the expression of specific genes.

Key words: Fruit ripening – Gene expression – mRNA – *Persea*.

Introduction

The ripening of avocado fruit, initiated upon removal of the fruit from the tree, involves a series of complex biochemical and physiological changes, the climacteric, that lead to the development of a soft, edible fruit (for a recent review, see Biale and Young 1981). The means by which fruit ripening is coordinated and regulated is unknown, but two hypotheses have been proposed to explain the ripening behavior of climacteric fruit such as the avocado. According to one, the process is regulated at the level of transcription-translation, i.e., the de-novo synthesis of a specific set of mRNAs leads to the biochemical events that attend ripening. Alternatively, it has been suggested that all the essential enzymes necessary are present and need only be activated, possibly by changes in the permeability of cell membranes that allow access of ions, substrates, or cofactors to the inactive enzymes (for a complete discussion, see Rhodes 1980).

Evidence for the transcription-translation theory comes from three separate lines of research: 1) many workers have observed an increase in the labeling of protein and RNA by radioactive amino acids and nucleotides during the early stages of the climacteric, and Richmond and Biale (1967) found that ^{32}P incorporation into a presumed messenger-RNA fraction increased during this time; 2) inhibitors of protein synthesis have been shown to interfere with the ripening of bananas and pears (Frenkel et al. 1968; McGlasson et al. 1971); and 3) new mRNA species were found in the poly(A)$^+$RNA fraction of ripe tomato fruit as compared with the poly(A)$^+$RNA from green fruit (Rattanapanone et al. 1978).

We have examined the mRNA populations of avocado fruit at four successive stages of ripening with the goal of establishing how changes in the mRNA relate temporally to the climacteric rise in respiration and ethylene production.

Material and methods

Plant material. Avocado fruit (*Persea americana* Mill. cv. Hass) from a local tree were harvested and immediately placed in 4-l glass jars through which a continuous flow of water-saturated air was passed (100 ml/min). CO_2 production was monitored with an automatic sampling system connected to a nondispersive infrared gas analyzer (Anarad, Santa Barbara, Cal, USA; Model AR-500). This system measures the CO_2 production of each sample for 7.5 min every 1.5 h. Ethylene production was monitored with a gas chromatograph (F & M Scientific Corp., Avondale, Pa., USA; Model 810) fitted with a poropak N column (Waters Associates, Milford, Mass., USA). A cork borer was used to remove 0.6-cm plugs from avocado fruit at the following stages of ripening: shortly after harvest – stage I; preclimacteric – stage II; climacteric – stage III; postclimacteric – stage IV (Fig. 1). The resulting holes were covered over with lanolin. The tissue plugs were frozen in liquid nitrogen and stored at −70° C till extraction. To avoid variability among fruits between harvest and the climacteric stage

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– which is reached in 6–10 d – samples of the four stages were taken from a single fruit, and experiments were repeated a number of times.

RNA extraction and fractionation. The frozen plugs were ground in liquid nitrogen with a mortar and pestle. The RNA was extracted from this powder essentially by the method of Hall (1979). Thirteen ml of buffer [1% (w/v) SDS (sodium dodecyl sulfate); 30 mM EGTA (ethyleneglycol-bis[β-aminoethyl ether]-N,N′-tetraacetic acid); 5 mM dithiothreitol; 200 mM Na borate; pH adjusted to 9 with NaOH] heated to 100° C were added to 5 g of frozen, powdered tissue in a mortar and pestle; thereupon the mixture was homogenized at room temperature. 6.5 mg of proteinase K (Beckman Instruments, Palo Alto, Cal., USA) was added and the mixture incubated at 37° C for 1 h. Then, 1 ml of 2 M KCl was added and the extract centrifuged at 10,000 *g* for 10 min. To the supernatant, 0.25 volume of 10 M LiCl was added, the mixture quickly frozen, then stored at 4° C overnight. The RNA was pelleted by centrifugation at 10,000 *g* for 10 min and the resulting pellet washed twice with 2 M LiCl, then dissolved in 1 ml of H_2O and centrifuged at 10,000 *g* for 10 min to remove insoluble contaminants. The RNA was precipitated with 0.1 volume 3 M Na acetate, pH 6.0, and 2.5 volumes of cold (−20° C) ethanol, then stored at −20° C until further use. Subsequently, the RNA was suspended in buffer [0.5 M NaCl; 0.1% SDS; 1 mM EDTA (ethylenediaminetetraacetic acid); 10 mM Tris·HCl (tris[hydroxymethyl]aminomethane·HCl), pH 7.6] and passed over a cellulose column equilibrated with the same buffer to remove polysaccharide contaminants (Mozer 1980). Poly(A)$^+$RNA was separated by a modification of the method of Aviv and Leder (1972). To avoid adhesion of messenger and ribosomal RNA, the mixture was heated to 68° C for 3 min before loading onto an oligo dT-cellulose affinity column. The poly(A)$^+$RNA fraction isolated in the first cycle of affinity chromatography was reheated and rechromatographed on the affinity column. The final poly(A)$^+$RNA fraction at all stages of ripening contained at most 25% rRNA as a contaminant as shown by methylmercury agarose gel electrophoresis (Bailey and Davidson 1976).

In-vitro translation. The rabbit reticulocyte lysate system of Pelham and Jackson (1976) was used for in-vitro translation of the poly(A)$^+$RNA in the presence of $185 \cdot 10^4$ Bq [^{35}S]methionine ($3.7 \cdot 10^{13}$ Bq/mmol = 1,000 Ci/mmol; New England Nuclear, Boston, Mass., USA) in a total volume of 25 µl.

Two-dimensional electrophoresis. Aliquots of labelled translation products containing equal quantities of trichloroacetic-acid-precipitable [^{35}S]methionine were lyophilized and resuspended in 25 µl of O'Farrell's lysis buffer (O'Farrell 1975) with 0.2% SDS added to reduce streaking (Brandhorst 1976). After fixation and staining, the gels were infiltrated with 2,5-diphenyloxazole, dried, and exposed to Kodak XR-5 X-ray film (Eastman Kodak Co., Rochester, N.Y., USA) at −70° C (Laskey and Mills 1975).

Results and discussion

Figure 1 shows the time course of carbon dioxide and ethylene production after harvest of a single avocado fruit. The removal of small tissue plugs at stages I, II, III, and IV did not appreciably alter the climacteric behavior of the avocado fruit. Awad and Young (1979) showed that a similar sampling method did not affect the appearance of cellulase or polygalacturonase activity, the respiratory rise, or ethylene production during the climacteric. This sampling method allowed us to study changes in the mRNA populations of individual fruit without concern for variability in the rate of ripening between individual fruits.

The total amount of RNA per gram fresh weight was constant throughout ripening until stage IV,

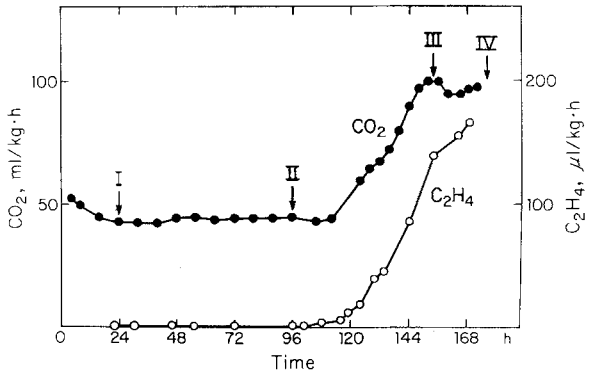


Fig. 1. The time course of carbon dioxide and ethylene production from a single avocado fruit. Tissue plugs were removed at the designated times (ripening stages I, II, III, and IV) following fruit harvest at 0 h

Table 1. The yield of total RNA and poly(A)$^+$RNA from avocado fruit at various stages of ripening

Stage of ripening	Total RNA (µg/5 g FW)	Poly(A)$^+$RNA (µg/5 g FW)	Poly(A)$^+$RNA as % of total RNA
I	280	5.7	2.0
II	300	7.8	2.6
III	300	15.0	5.0
IV	140	5.4	3.8

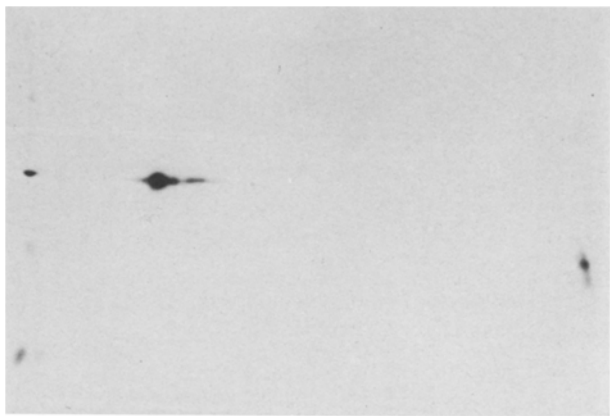


Fig. 2. Two-dimensional electrophoresis fluorograph of the polypeptides synthesized by the reticulocyte lysate in the absence of added avocado poly(A)$^+$RNA

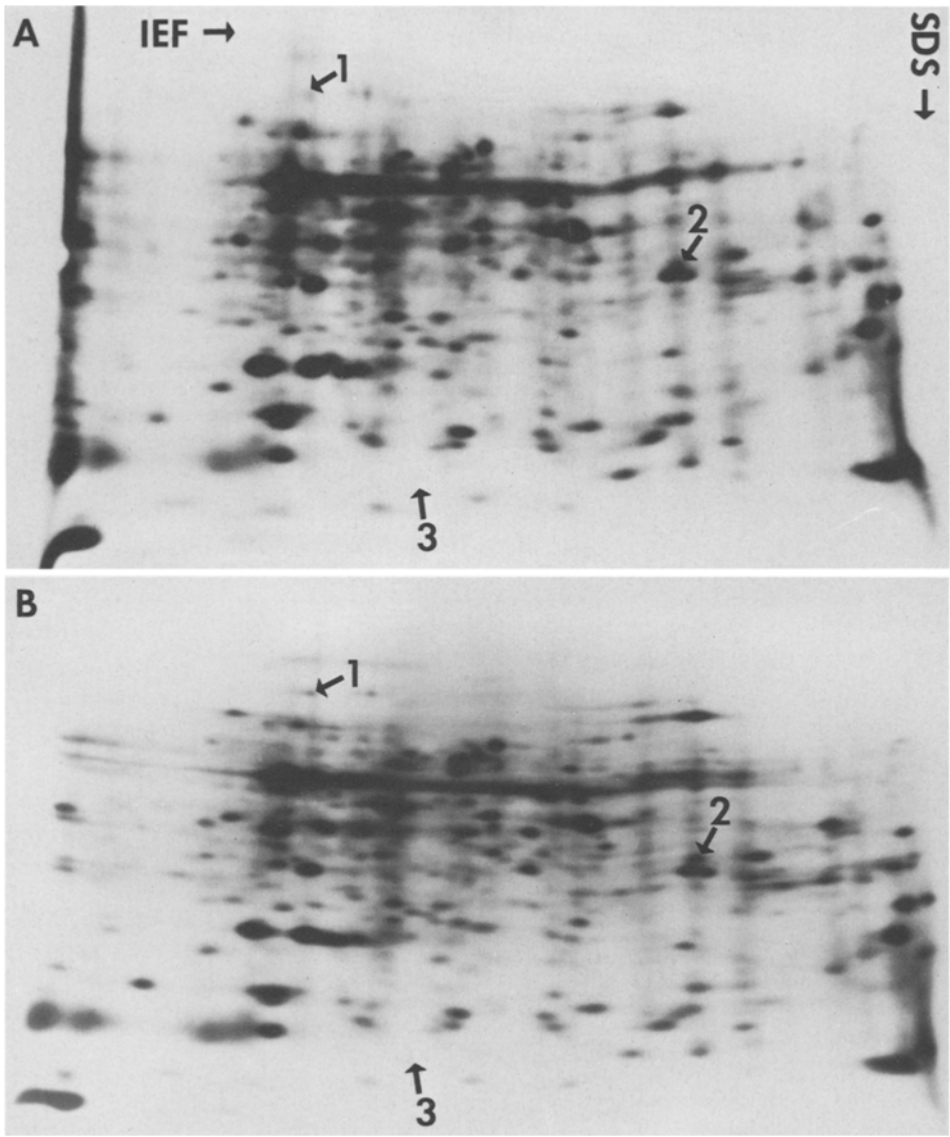
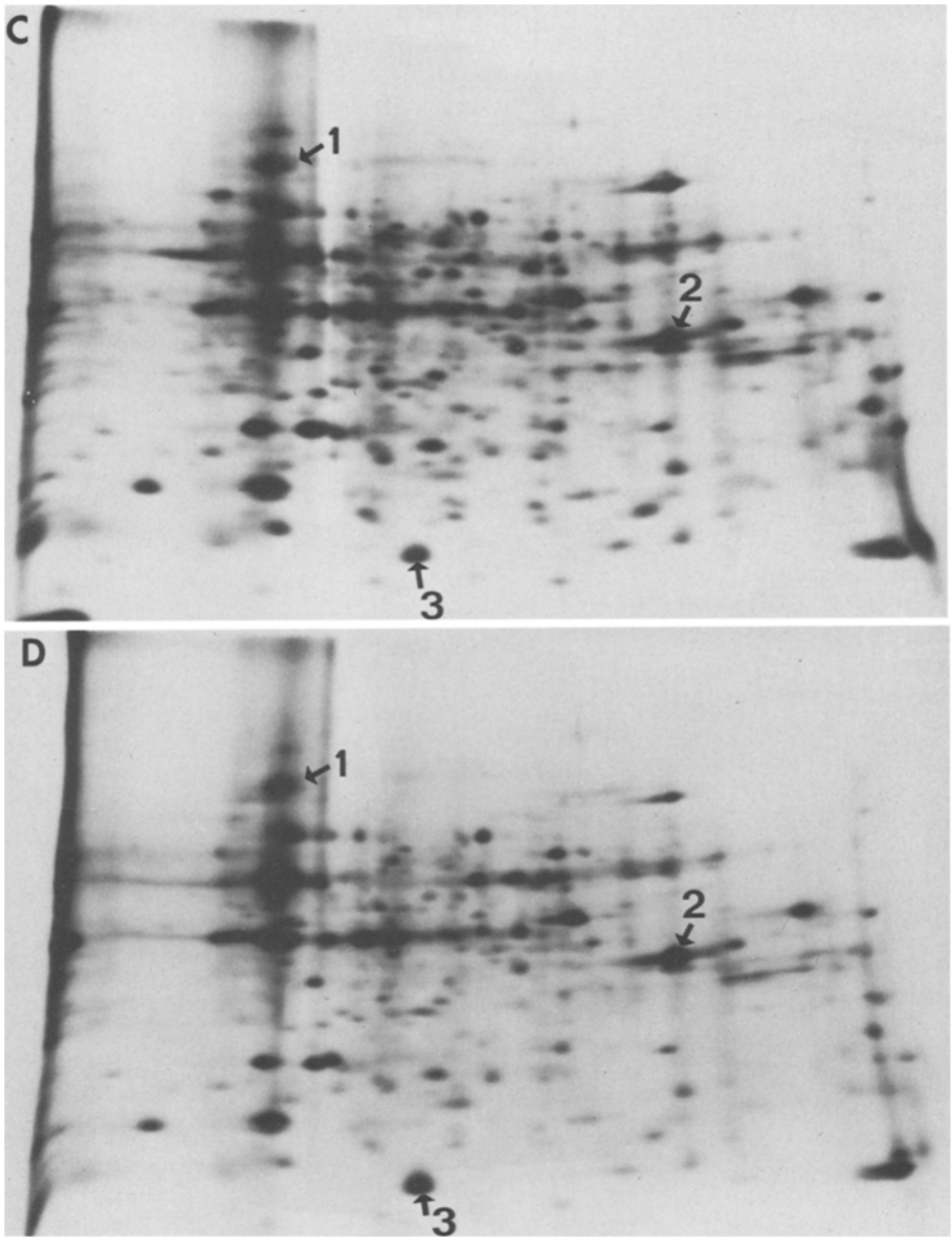


Fig. 3a–d. Two-dimensional electrophoresis fluorograph of [^{35}S]methionine-labelled polypeptides synthesized in vitro in response to poly(A)$^+$RNA isolated from stage I (**a**), II (**b**), III (**c**) and IV (**d**) avocado fruit. The polypeptides identified by arrows correspond to the translation products characterized in Table 2

when we observed a 50% reduction (Table 1). Whether this decrease in recoverable RNA was due to degradation or loss during extraction, or reflected a true decrease in the cellular concentration is not known. It is unlikely, however, that degradation during extraction accounts for the observed difference, since we were able to translate stage-IV poly(A)$^+$RNA, and extensive degradation is normally accompanied by a deterioration of translation efficiency. Furthermore, with respect to extraction effectiveness, stage IV tissue is soft and extraction is facilitated accordingly.

The poly(A)$^+$RNA fraction increases during the ripening up till the climacteric peak (stage III), then sharply declines at the postclimacteric stage (stage IV; Table 1). The increase is not attributable to contaminating rRNA since the latter comprises no more than 25% of the poly(A)$^+$RNA fraction and remains relatively constant throughout ripening. Furthermore, stage I and stage III poly(A)$^+$RNAs translate in vitro equally well on a microgram basis. These results are consistent with the finding of Richmond and Biale (1967) in the Fuerte avocado, that ^{32}P labelling of a putative mRNA fraction increased during the climacteric rise and ceased at the climacteric peak. Presumably their observations correspond to our observed increase in poly(A)$^+$RNA during the climacteric rise.



The poly(A)$^+$RNA from various stages of ripening was translated in vitro by a rabbit reticulocyte lysate system, and the resulting incorporation of [^{35}S]methionine into protein was assayed as acid-precipitable radioactivity. As thus measured, poly(A)$^+$RNA from fruit at all stages stimulated the reticulocyte in-vitro translation system at least five-fold and in some cases up to 14-fold over background incorporation. However, an amount of non-protein acid-precipitable label is found in the lysate reaction mixture immediately following the addition of [^{35}S]methionine. This label constitutes an appreciable fraction of the total radioactivity in the control at the end of an incubation period, while contributing little on a fractional basis to the radioactivity of the sample. Upon gel electrophoresis the non-protein label is lost. Accordingly the ratio of labelled protein in the experimental gels (Fig. 3) to protein label in the control gels (Fig. 2) is considerably greater than the ratio of their total acid precipitates. The endogenous mRNA present in the reticulocyte lysate coded for one major translation products as shown by two-dimensional electrophoresis (Fig. 2).

When the resulting labelled translation products were electrophoretically separated by one-dimensional SDS-PAGE (sodium dodecyl sulfate-polyacryl-

Table 2. Characterization of translation products from three ripening-related RNAs of avocado fruit

	Molecular weight (dalton · 10^{-3})	Isoelectric point
R1	80	6.3
R2	36	5.1
R3	16.5	5.7

amide gel electrophoresis) and visualized by fluorography, the various stages displayed no apparent differences (data not shown). Accordingly, the translation products were separated by a two-dimensional system which utilized isoelectric focusing in the first dimension and SDS-PAGE in the second. This resulted in the resolution of approx. 330 individual polypeptides, whereas the one-dimensional SDS-PAGE system resolved but 35 bands at most. Comparison of the various stages of ripening by the two-dimensional system showed three species of mRNA to increase dramatically as ripening proceeded (Fig. 3C, D). These mRNAs were characterized by the molecular weight and isoelectric point of their corresponding translation products (Table 2). Other, less dramatic quantitative changes were also noted. In each individual fruit examined the three aforementioned mRNAs always increased in the same manner and at the same developmental stage. The major part of the increase in concentration of these three RNAs always occurred between stages II and III, a period which coincides with the rise in respiration and ethylene production (Fig. 1). Similar results were obtained using [^{3}H]leucine as label (data not shown).

Rattanapanone et al. (1978) showed that three mRNAs appear during ripening of tomato fruit in the absence of an increase in poly(A)$^+$RNA (Rattanapanone et al. 1977) as judged by the one-dimensional separation of translation products. On the basis of molecular weights, the ripening-related polypeptides of tomato do not appear to bear a close relationship to the ripening-induced translation products in avocado. This may not be surprising as tomato and avocado display different patterns of ripening from the biochemical standpoint. Although both show an increase in the level of polygalacturonase activity upon ripening, only in the avocado does an increase in cellulase activity correlate closely with softening (Hobson 1964, 1968; Awad and Young 1979).

Further studies are being planned using recombinant cDNA probes to ascertain whether the observed increase in the levels of ripening-specific mRNAs reflects increased rates of mRNA synthesis or lower rates of cytoplasmic mRNA turnover. An affirmative hybridization assay for these specific messenger sequences would also rule out the possibility that the mRNAs are being unmasked during ripening, thus becoming detectable in our translation system.

Conclusion

The appearance of three predominant mRNAs was observed to be closely correlated with the climacteric of avocado fruit. While the functional significance of these mRNAs is unknown, we are examining the prospect that they are involved in the regulation of biochemical changes that occur during ripening. The results reported here, while not ruling out the activation hypothesis referred to in the Introduction, appear to favor the alternative hypothesis, according to which ripening is under the control of specific "ripening genes".

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