Plant Physiol. (1993) 102: 417–423

Ethylene-Mediated Posttranscriptional Regulation in Ripening Avocado (*Persea americana*) Mesocarp Discs[1]

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Discs of avocado (*Persea americana*) fruit (15 × 3 mm thick) kept in a stream of moist air ripen within 72 h. Following cutting, a modest evolution of wound ethylene that dissipates in 24 h is followed by a burst of autocatalytic ethylene production associated with a respiratory climacteric, much as in the intact fruit. Aminoethoxyvinylglycine (AVG), an inhibitor of ethylene synthesis, and 2,5-norbornadiene (NBD) and Ag^+, inhibitors of ethylene action, inhibit disc ripening, as does 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxin. On the other hand, none of the foregoing agents except Ag^+, at concentrations that delay or prevent ripening, suppress the induction of four ripening-related genes encoding cellulase, polygalacturonase (PG), cytochrome P-450 oxidase (P-450), and ethylene-forming enzyme (EFE, or 1-aminopropane-1-carboxylic acid oxidase), respectively. Whereas Ag^+ fully inhibits the production of cellulase and PG mRNAs, it has little effect on the induction of EFE and P-450 mRNAs. Cellulase and PG enzyme activities are absent in extracts of discs treated with AVG, NBD, or 2,4-D, as are antigenically detectable cellulase and PG proteins. The strong appearance of ripening-related mRNAs in discs inhibited from softening by ethylene antagonists suggests posttranscriptional control by ethylene. Similarly, inhibition of ripening by 2,4-D without suppression of mRNA induction suggests translational control. Whether ethylene inhibits transcription or postttranscriptional events or both depends on its concentration.

Avocado (*Persea americana*) fruit offer particularly desirable benefits for ripening studies inasmuch as the mature fruit remain on the tree, and ripening is not initiated until the fruit is picked (Biale, 1960). Whereas early or midseason fruit ripen within 1 to several weeks after harvest, depending on fruit maturity (Eaks, 1980), thin slices or discs of mesocarp tissue (3 mm thick) ripen in 3 to 4 d (Starrett and Laties, 1991b). Although discs submerged in aerated solution fail to ripen, discs held in a humid flow-through system behave much like their whole-fruit counterparts in which ripening is accompanied by a respiratory climacteric and autocatalytic ethylene production along with changes in texture, flavor, and enzymic activity (Ben-Yehoshua, 1964; Imasaki et al., 1968; Rhodes and Wooltorton, 1971).

In fruit, the ripening syndrome is biphasic, an extended and variable lag period preceding a fixed climacteric phase of about 3 d (Eaks, 1980). In discs, it is the lag period that is curtailed, and the duration of the climacteric remains much the same as in intact fruit (Starrett and Laties, 1991a). During the lag period, the buildup of endogenous low levels of ethylene is thought to trigger the climacteric as sensitivity or responsiveness to ethylene increases (McMurchie et al., 1972; Peacock, 1972; see Trewavas, 1983). Because slicing causes the production of wound ethylene, it is an open question whether the acceleration of ripening in discs is caused by wound ethylene, wounding per se, or a combination thereof.

In this paper, we examine in ripening avocado discs the induction of four genes known to be induced in ripening fruit, namely cellulase, PG, P-450, and ACC oxidase (formerly EFE). To discriminate between the effect of wound ethylene and wounding per se, the behavior of untreated discs was compared with that of discs treated with an inhibitor of ethylene synthesis (AVG) or inhibitors of ethylene action (NBD and Ag^+). Because, historically, a so-called tree factor has been suggested to be responsible for the failure of avocado fruit to ripen on the tree (Tingwa and Young, 1975a), and the putative tree factor has been proposed to be an auxin (Frenkel and Dyck, 1973), we have also examined the effect of 2,4-D, an auxin, on disc ripening and mRNA induction.

We discovered that levels of AVG and NBD at concentrations that fail to inhibit transcription caused a marked reduction of activity and antigenically detectable cellulase and PG protein. The evidence suggests that ethylene exerts posttranscriptional regulatory properties as well as causes induction, and that the nature of its action depends on its concentration.

MATERIALS AND METHODS

Plant Material

Avocado fruits (*Persea americana* Mill. cv Hass) were obtained from the University of California South Coast Field Station (Irvine, CA) or from an orchard in Santa Paula, CA. Individual whole fruits were sorted by weight before discs were prepared as described by Starrett and Laties (1991b). The freshly cut discs (roughly 80–100 per fruit; 3 mm thick × 14 mm diameter) were washed together in several changes of aerated distilled water and blotted dry.

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Abbreviations: AVG, aminoethoxyvinylglycine; EFE, ethylene-forming enzyme; NBD, 2,5-norbornadiene; P-450, Cyt P-450 oxidase; PG, polygalacturonase.

Disc Aging System and Ethylene Measurements

Discs were aged for various times in a stream of water-saturated air or air + 10 μL/L of ethylene at 20°C in a constant temperature incubator. For a complete description of the disc-aging system and respiration measurements, see Starrett and Laties (1991b). With the exception of NBD, all compounds were administered in solution as 25-μL droplets to the surface of each disc as indicated in the figure legends. A computer-controlled sample changer linked to an IRGA provided continuous respiration measurements of control and treated discs in order to follow the course of ripening. Because of the caustic nature of NBD, respiration measurements were forgone and liquid NBD (yielding 5000 μL/L of gaseous NBD upon evaporation) was added directly to a piece of filter paper in a 4-L jar containing 100 g of discs on screens. Ethylene evolution, except in the case of NBD-treated discs, was quantified by periodically withdrawing a 10-mL gas sample from the system effluent and injecting it into a gas chromatograph (Hach Carle, Loveland, CO) equipped with a proprietary column designed to enhance ethylene resolution (Hach Carle application 254-C).

Ripeness Assay

Ripeness assays were conducted 72 h after slicing, with scoring assessed by evaluating softness, texture, and taste. For simplicity, discs were assigned to one of three categories: hard, intermediate, or soft (Starrett and Laties, 1991b).

Poly(A)$^+$ mRNA Isolation and mRNA Blot Analysis

Total mRNA was isolated from treated discs by the guanidinium isothiocyanate method (Chirgwin et al., 1979). The total RNA pellet was purified and subjected to oligo(dT)-cellulose column chromatography essentially as described by Tucker and Laties (1984). Isolated poly(A)$^+$ RNA (3 μg/lane) was fractionated on denaturing 1.2% agarose gels containing 0.67 M formaldehyde followed by capillary blotting according to Maniatis et al. (1982) to nitrocellulose membranes (Schleicher & Schuell). Each membrane was subjected to four successive hybridizations with cDNAs (i.e. pUC-18 plasmids with the pertinent inserts) of cellulase, PG, EFE, and P-450, respectively. Cellulase and PG cDNAs were amplified in pUC-18, and small preparations were isolated by the alkaline lysis method (Maniatis et al., 1982). Gel-purified (Sepharose CL-4B) plasmids with insert were radiolabeled with [α-^{32}P]-CTP using a nick translation kit (BRL) and separated from unincorporated nucleotides using Nick columns (Pharmacia). Blots were hybridized with 1×10^6 cpm/mL (specific activity $>10^8$ cpm/μg) nick-translated probe. The cDNA probes for P-450 (Bozak et al., 1990) and EFE (McGarvey et al., 1992) were kindly provided by Rolf Christoffersen; the cellulase clone (Tucker et al., 1987) was the gift of Mark Tucker (U.S. Department of Agriculture, Beltsville, MD); the PG clone (Kutsunai et al., 1993) was isolated in our laboratory.

PG and Cellulase Activity Assay

PG activity was assayed at 25°C by measuring the formation of reducing groups (Bernfeld, 1955) released from polygalacturonic acid. The reaction mixture consisted of 50 μL of cell-free extract (1:3 tissue:buffer ratio; 50 mM sodium acetate, pH 5.0, 50 mM sodium chloride, 5 mM β-mercaptoethanol), 0.77% polygalacturonic acid, and 80 mM sodium chloride. Reducing groups were measured with DNS reagent (0.1% [w/v] 3,5-dinitrosalicylic acid, 30% [w/v] sodium potassium tartrate, 1.6% [w/v] sodium hydroxide) with α-D-galacturonic acid as a standard. One unit of PG activity was defined as producing 1 μmol of reducing groups per hour.

Relative cellulase activity was assayed viscometrically at 25°C by a method described by Awad and Lewis (1980). Cell-free extracts (1:3 tissue:buffer ratio) were diluted 50-fold with extraction buffer (20 mM Tris-HCl, pH 8.1, 0.1 M sodium chloride, and 3 mM EDTA) containing 1.5% (w/v) carboxymethylcellulose (medium viscosity, Sigma). Relative activity was defined as the difference in intrinsic viscosity per hour between extracts of aged, treated discs and unripe (T = 0 h) discs at 25°C (Almin et al., 1967). Protein concentration was determined as described by Bearden (1978).

Immunoblots

Cell-free extracts (1:3 tissue:buffer ratio; 0.4 M Tris-HCl, pH 8.5, 0.5% [v/v] β-mercaptoethanol, 10 mg/mL of EDTA, 1.0% [v/v] SDS, 1.0 mM PMSF, 10 mM *N*-acetyl-Cys) were run on duplicate 12.5% SDS-PAGE gels (2.3 μg of protein/lane) for both cellulase and PG determinations using the buffer system of Laemmli (1970). One duplicate gel for cellulase and one for PG were fixed and stained to confirm equal sample loading. The remaining unstained duplicate gels were transferred by electroblotting to nitrocellulose membranes (Schleicher & Schuell) in transfer buffer consisting of 25 mM Tris, 192 mM Gly, 20% (v/v) methanol, and 0.01% SDS. Preincubation and incubation overnight with the appropriate antisera were performed at 4°C in Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 500 mM sodium chloride) and 10% (w/v) nonfat dry milk. Blots were washed in 1× Tris-buffered saline, 0.1% (v/v) Triton X-100. Visualization was carried out using an anti-rabbit immunoglobulin G horseradish peroxidase conjugate and color development reagents supplied with an Immuno-Blot Assay Kit (Bio-Rad).

Estimation of Avocado Tissue Auxin Levels

Frozen 24- to 48-h-aged avocado discs, as well as frozen tissue from untreated whole fruit, and 48-h ethylene-pulsed whole fruit were sent to Dr. Jerry D. Cohen (U.S. Department of Agriculture/Agricultural Research Service, Beltsville, MD) for analysis. Analyses described in Table II were performed by Dr. Lech Michalczuk in Dr. Cohen's laboratory. Samples were extracted and purified using the mini-column method (Chen et al., 1988). Auxin content was measured as described by Cohen et al. (1987). The content of free IAA and its esters was determined in triplicate by quantitative GC-MS.

RESULTS

Ripening Inhibition by Ethylene Antagonists and 2,4-D

In the course of avocado disc ripening, early concomitant peaks of wound-respiration and wound-ethylene production

are followed by a respiratory and ethylene climacteric, with attendant changes in flesh texture and flavor much as in the whole fruit (Starrett and Laties, 1991b). Discs from mid- and late-season fruit usually ripen within 72 h. Table I sets out the effects of inhibitors of ethylene synthesis (AVG) and ethylene action (Ag^+ and NBD), as well as of the auxins IAA and 2,4-D, and the anti-auxin 2,4,5-trichlorophenoxyisobutyric acid. Ethylene production rates at the height of the climacteric are also noted. The range of values reflects seasonal variation due to fruit maturity. Control discs treated with water behaved much like untreated "dry" discs, although some delay in softening was apparent. Ag^+, an inhibitor of ethylene action (Tucker and Brady, 1987), is notably the most effective ethylene antagonist in terms of ripening (i.e. softening) prevention. The lesser effect of AVG and NBD, reflected by the percentage of discs in the "intermediate" category in Table I, denotes incomplete inhibition at the concentrations employed. This is particularly true of NBD, since the latter is a competitive inhibitor and avocado is a prodigious ethylene producer. Although the level of auxin used seems inordinately high, auxin was administered as a single 25-μL droplet to each disc. Because the actual concentration within the tissue was uncertain following dilution by free-space fluid and absorption, the effective concentration was determined empirically. Nevertheless, the action of 2,4-D is specific and not simply a toxic response, since the anti-auxin, 2,4,5-trichlorophenoxyisobutyric acid, is without effect (Table I).

Effect of Ripening Inhibitors on Specific mRNA Levels

Measurement of the relative accumulation of cellulase, PG, P-450, and EFE poly(A)$^+$ mRNAs in aged discs from 0 to 72 h after cutting was accomplished by successive hybridization of single nitrocellulose blots with homologous ripening-related cDNA probes (Fig. 1, A–D). The time course of mRNA appearance was essentially the same in dry and wet discs (Fig. 1, A–D). Of the ethylene antagonists, Ag^+ totally suppressed cellulase and PG mRNA appearance, whereas AVG and NBD did not. The disparity between the effect of Ag^+ and that of the latter inhibitors was greater than was the case for ripening inhibition, for reasons explained in "Discussion." Most striking is the lack of inhibition of the buildup of cellulase and PG mRNA by 2,4-D, as well as by AVG and NBD (Fig. 1, A and B), in the absence of overt softening at

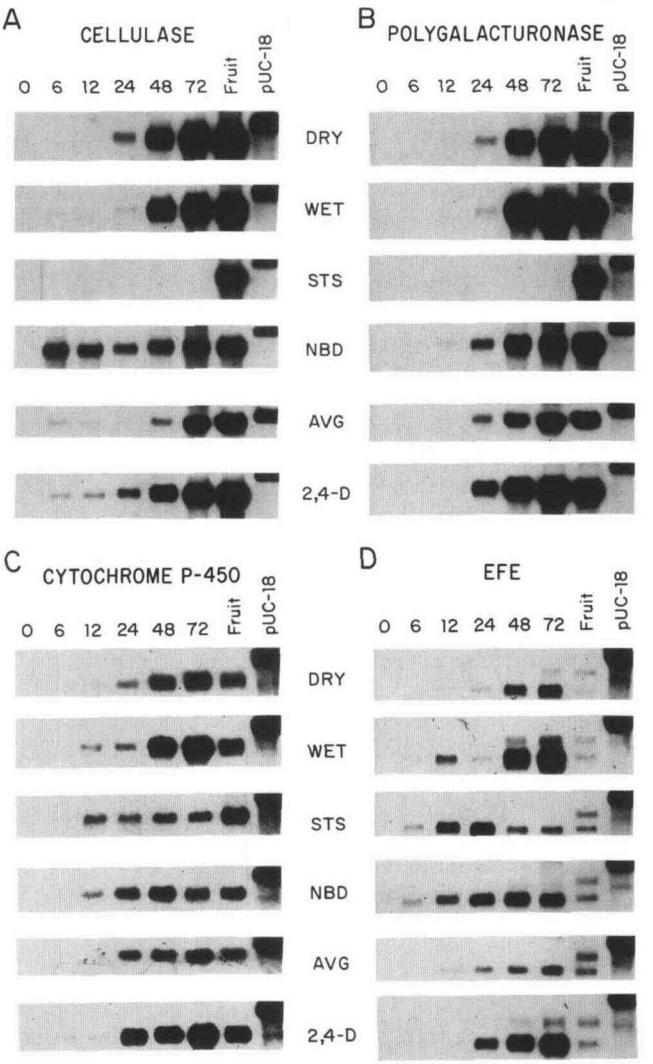

Figure 1. Accumulation of four ripening-related mRNAs in aged avocado discs under various treatment conditions. Each lane contains poly(A)$^+$ RNA (3 μg) from discs treated, respectively, with 25 μL/disc of water (WET), 10 mM Ag^+ (STS), 4 mM AVG, or 10 mM 2,4-D, or with 5000 μL/L NBD in the gas phase, or untreated (DRY). Disc age was from the time of slicing. At the end of each treatment period, discs were frozen in liquid nitrogen and stored at −70°C. Single RNA gel blots representing each treatment assayed sequentially using cDNA probes of cellulase (A); PG (B); Cyt P-450 (C); and EFE (or ACC oxidase) (D). Lanes marked "Fruit" contain 3 μg of poly(A)$^+$ RNA extracted from ripe, whole fruit treated with ethylene (10 μL/L) for 3 d. pUC-18 (0.5 μg) was used in each blot as positive control and to ensure uniform autoradiography between blots.

Table I. *Effect of ethylene antagonists and auxin on ripening*

All discs were sliced from fruit within a few hours of picking. Discs were scored for ripeness at 72 h. Values represent the percentage of discs scored within each category (see "Materials and Methods"). Approximately 100 discs were scored per experiment. Ethylene production was measured every 12 to 24 h and presented as the range of peak values among all experiments for a given treatment.

Treatment	Hard	Intermediate	Soft	No. Exps.	C_2H_4 Peak
	% *of discs*				*nL* g^{-1} h^{-1}
Dry	6	1	93	8	187–295
Wet	4	14	82	11	118–298
NBD[a]	40	60	0	4	ND[b]
AVG (4.0 mM)	43	35	22	3	<45
2,4-D (10 mM)	74	15	11	5	113–418
IAA (10 mM)	57	16	27	3	200–235
TCPA[c] (10 mM)	0	0	100	1	126–142
2,4-D + C_2H_4[d]	79	21	0	2	ND
Ag^+ (10 mM)	100	0	0	2	<50

[a] 5000 μL/L. [b] Not determined. [c] 2,4,5-Trichlorophenoxyisobutyric acid. [d] 10 μL/L of C_2H_4.

72 h (Table I). The early stimulation of cellulase mRNA seen at 6 h with NBD is specific and real, since a similar pattern of expression was not observed with other cDNA probes hybridized to the identical membrane (i.e. PG message over the same time course does not appear until 24 to 48 h, and rises progressively). Perhaps wound ethylene inhibits cellulase induction much as it inhibits the induction of ACC synthase, with the result that suppression of wound-ethylene action by NBD overcomes the inhibition (Starrett and Laties, 1991b). In contrast, the appearance of P-450 and ACC oxidase (EFE) mRNAs is not inhibited by Ag^+, let alone by AVG and NBD. This fact, together with the rapid course of induction, suggests an independent wound response. 2,4-D fails to inhibit mRNA appearance in all cases (Fig. 1, A–D) while fully preventing softening (Table I).

EFE mRNA is detected within 6 h after slicing. The appearance of two EFE mRNA bands in ripe discs and in whole fruit suggests two transcripts, although the possibility of cross-reactivity with homologous but extraneous mRNAs cannot be ruled out. In whole fruit the two mRNAs are of equal intensity, whereas in 72-h discs the larger message is either absent or undetectable.

The inhibitory effect of Ag^+ where noted may be due to its known binding of sulfhydryl groups. However, *N*-ethylmaleimide, a sulfhydryl binding reagent, proved totally without effect on the accumulation of PG, P-450, and EFE mRNAs under similar conditions (data not shown). Thus, inhibition of mRNA appearance by Ag^+ is attributed to its blocking of ethylene action. In summary, at the concentrations used, ethylene antagonists (except for Ag^+) and 2,4-D have little effect on induction of the four ripening-related genes in this study, whereas they do fully inhibit ripening.

Cellulase and PG Enzyme Activity

Unripe, whole fruit produced detectable but low levels of cellulase and PG activity (Figs. 2 and 3A), whereas dry and wet aged discs exhibited increasing levels of activity concomitant with a rise in ethylene and CO_2 production. The suppressive effect of AVG, NBD, and 2,4-D on activity is most notable with 2,4-D-treated discs, as an example, yielding nearly 50-fold less cellulase activity than comparable wet controls (Fig. 2). The diminished level of both cellulase and PG activity is consistent with the general inhibition of disc softening by AVG, NBD, and 2,4-D (Table I). However, the lack of significant cellulase and PG activity is in stark contrast to the abundant levels of cellulase and PG mRNA at 72 h in inhibitor-treated discs (Fig. 1, A and B). The small burst in PG activity in treated discs between 6 and 12 h is unexplained (Fig. 3A). The incremental increase in PG activity above 0-h levels at 48 and 72 h is totally suppressed by the inhibitors in question, suggesting that a constitutive level of PG activity is under a different set of controls.

The inhibition of the development of cellulase and PG

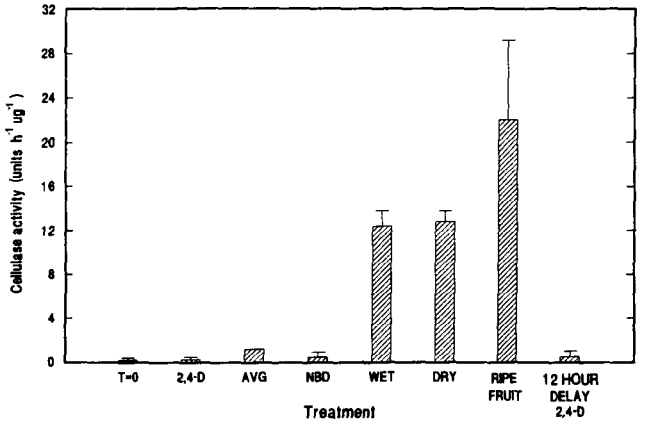

Figure 2. Cellulase activity in 72-h variously treated aged discs. Inhibitor concentrations are identical to those in Figure 1. Discs were treated at 0 and 24 h with water, AVG, or 2,4-D. NBD was given continuously at 5000 μL/L. The last bar represents discs treated once with 2,4-D at 12 h only. Each bar represents the midrange of two replicate experiments.

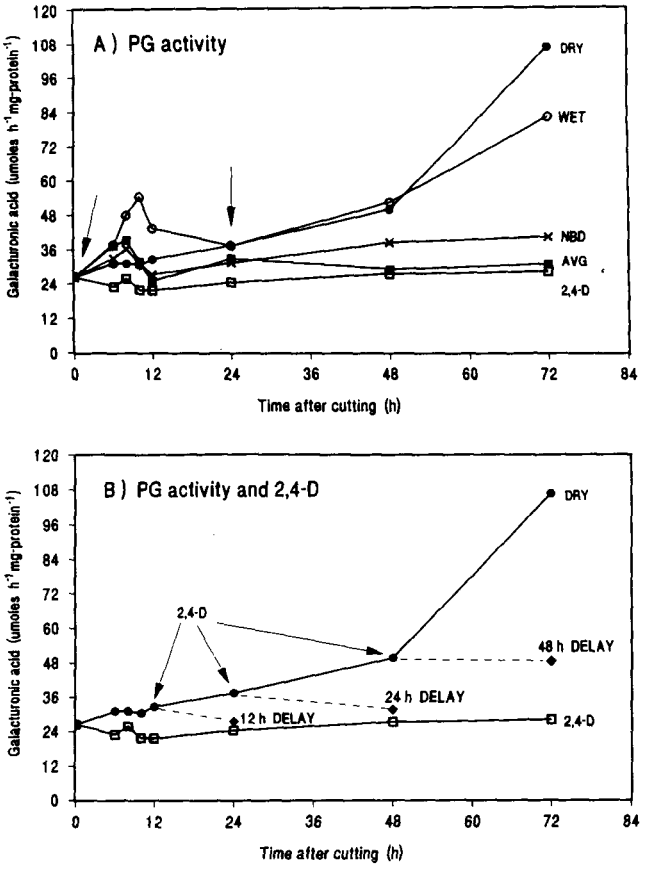

Figure 3. PG activity of variously treated aged discs. Each data point is the midrange of two replicate experiments. A, Discs were treated twice (arrows) with the specified inhibitor (concentrations identical to those in Fig. 1). NBD was given continuously at 5000 μL/L. Discs were frozen in liquid nitrogen at 0, 6, 8, 10, 12, 24, 48, and 72 h after cutting. B, Discs treated with 2,4-D in the usual manner (0 and 24 h) compared with discs treated once with 2,4-D after the specified delay of 12, 24, or 48 h. Arrows indicate the point at which a sampling of discs was removed from the "dry" container to a separate container and treated with 2,4-D as indicated. PG determinations were made 12 or 24 h later.

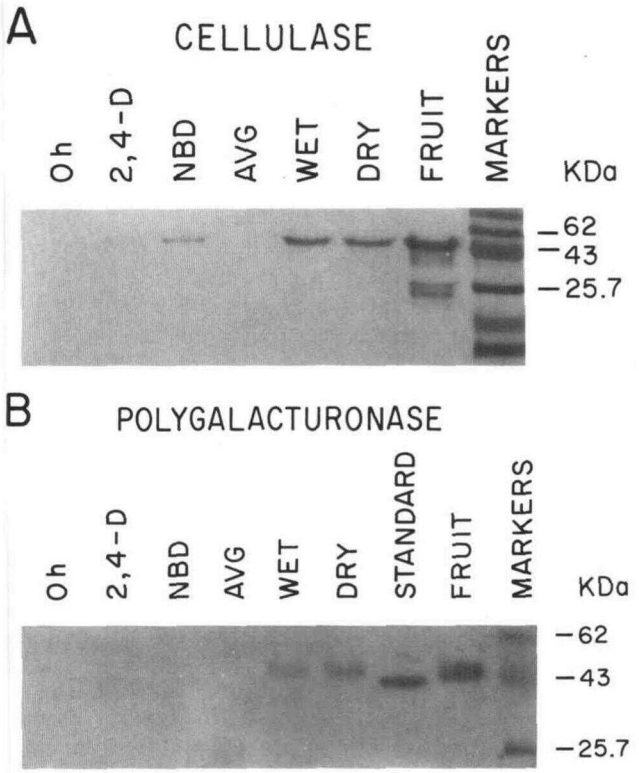

Figure 4. Cellulase and PG immunoblots of 72-h-aged discs. Protein extracts (2.3 μg of total protein per lane) were separated on SDS-PAGE, electroblotted onto nitrocellulose, and challenged with either A, antiserum against cellulase, or B, antiserum against PG. A duplicate gel for each set stained with Coomassie blue (data not shown). Visualization of the antigen was accomplished with a horseradish peroxidase-conjugated second antibody. In B, a purified isoform of PG was run as a control. Protein extracts used in both immunoblots were obtained from discs of the same experiment.

activity by 2,4-D as measured at 72 h was preceded by nearly 24 h by a peak in ethylene production (data not shown). By contrast, AVG (Table I) and NBD sharply reduce ethylene production (Starrett and Laties, 1991b). Furthermore, when presentation of 2,4-D is delayed from 12 to 48 h, the rise in PG activity is inhibited from the time of 2,4-D addition (Fig. 3B). Existing PG activity is unaffected by inhibitor addition, thus emphasizing that it is the buildup of enzyme activity and not activity per se that is impaired. Since PG mRNA accumulation in 2,4-D-treated discs reaches its highest level within 48 to 72 h, the foregoing observations suggest ethylene involvement in translational control.

Immunoblots

As seen in Figure 4, A and B, fresh tissue (T = 0 h) and discs treated with either NBD, 2,4-D, or AVG contained little or no detectable levels of cellulase or PG antigen, whereas ripe, whole fruit extracts, as well as extracts of wet and dry aged control discs, contained considerable levels of immunoreactive protein. Overall, the pattern of cellulase and PG antigen expression is consistent with the diminished softening observed in 72-h-aged discs treated with NBD, AVG, or 2,4-D (Table I) and supports our findings on the absence of enzyme activity. Although the purified PG enzyme (Fig. 4B) migrates with an apparent molecular mass lower than that of PG in the extracts, the difference is most likely due to the choice of but one among several PG isozymes for use as the standard in Figure 4B. A number of isozymes have been purified from avocado, and numerous PG bands can be demonstrated in western blots; in fact, PG isozymes are encoded by a single gene, isozymes being the consequence of posttranslational modification (personal communication, R. Christoffersen, University of California, Santa Barbara).

A small trace of cellulase antigen is detectable in NBD-treated discs (Fig. 4A). Although inhibition by NBD is not total, the relative level of protein and enzyme activity compared with that in controls is negligible, notwithstanding the abundance of PG and cellulase mRNAs (Fig. 1, A and B).

IAA Content of Aged Discs and Whole Fruit

Although the influence of exogenous IAA on ripening in avocado and other plant tissues has been studied (Vendrell, 1969; Frenkel and Dyck, 1973; Tingwa and Young, 1975b), little has been done to investigate ripening-related IAA changes in vivo. Frenkel (1972) proposed that endogenous IAA may be selectively destroyed upon detachment of the fruit from the tree, thus rendering the fruit more sensitive to subsequent exposure to ethylene. Because IAA as well as 2,4-D inhibits ripening of avocado discs (Table I), the level of IAA and its esters in freshly picked preclimacteric fruit was compared with levels in aged discs and ripe, whole fruit.

The content of free IAA and IAA esters was analyzed in aged discs 24 and 48 h after cutting and in preclimacteric, ethylene-treated, ripe, whole fruit. IAA content ranged from a low of 86 μg/g fresh weight to a high of 122 μg/g fresh weight. The lesser value for total IAA (compared with free IAA plus IAA ester) in Table II constitutes an unexplained experimental anomaly but bespeaks the absence of any significant quantity of amide conjugates. No significant evidence of IAA destruction was observed in the samples relative to the control tissue, suggesting that wounding and/or ethylene have little impact on in vivo IAA levels in avocado fruit.

Table II. *Content of IAA and its derivatives in avocado tissue*

The content of IAA and its derivatives in avocado tissue following cutting or ethylene treatment is shown. Two subsets of a single sample were analyzed for each treatment. Each analysis was replicated three times, and the values reported are the average.

Plant Material	Treatment	Free	+Ester	Total
		μg g^{-1} fresh weight		
Control (0 h)	None	49	114	122
		42	114	107
Aged discs	24 h	50	136	86
		52	136	104
Aged discs	48 h	38	132	109
		56	102	92
Whole ripe fruit	48 h C_2H_4	53	142	94
	(10 μL/L)	43	144	99

DISCUSSION

Whereas endogenous ripening of postharvest fruit may take from 1 to several weeks, exogenous ethylene at concentrations of 1 to 10 μL/L initiates ripening promptly (Biale, 1960). Because discs ripen in 3 to 4 d and discs produce wound ethylene on cutting, we examined whether wound ethylene shortens the lag period in discs, and to what extent wounding, apart from induction of wound ethylene, supplants preclimacteric developmental changes that take place during the lag period in intact fruit. Accordingly, ripening behavior was studied in discs treated with inhibitors of ethylene synthesis (AVG) and/or ethylene action (NBD, Ag^+), with particular regard to the induction of four genes known to be induced in the course of fruit ripening, namely cellulase, PG, P-450, and EFE (ACC oxidase). In addition, we examined the effect of the auxin 2,4-D on gene induction and ripening, inasmuch as auxin has been hypothesized to be a ripening inhibitor, the so-called tree factor (Frenkel and Dyck, 1973).

In every case, AVG, NBD, and 2,4-D were found to inhibit ripening with little effect on mRNA accumulation of the genes in question. In contrast, Ag^+ fully suppressed appearance of cellulase and PG mRNA. The fact that AVG and NBD individually were less effective than Ag^+ suggests incomplete inhibition by the former, a prospect borne out by the fact that AVG and NBD given together suppress transcription completely (personal communication, D. Starrett, University of California, Los Angeles). Because exogenous ethylene overcomes ripening inhibition caused by AVG and NBD but not by 2,4-D, the effect of 2,4-D is considered to be independent of direct ethylene action.

If mRNA transcription were the sole determinant of the presence and activity of cellulase and PG protein, the mRNA levels in treated discs at 72 h should have been sufficient, since they matched or exceeded the levels in ripe, intact fruit. Because, however, disc ripening was retarded or prevented at inhibitor levels permissive to transcription, softening is controlled by more than transcription alone. In this connection, we report that AVG, NBD, and 2,4-D suppress both the antigenicity and activity of cellulase and PG protein. Accordingly, we propose that ethylene mediates posttranscriptional events at concentrations higher than those that control transcription alone. Posttranscriptional influence by 2,4-D is further indicated by the prompt inhibition of developed PG activity by 2,4-D whenever added, with no effect on the endogenous or basal level of PG activity (Fig. 3B). In this case, translational control is suggested.

Ag^+, a particularly effective inhibitor of ethylene action (Tucker and Brady, 1982), as well as AVG and NBD, fail to suppress the induction of P-450 and EFE (ACC oxidase) mRNA (Fig. 1, C and D). Together with the rapid appearance of these mRNAs, this suggests a direct-wound response. Furthermore, it should be noted that Oeller et al. (1991) reported that induction of PG mRNA in tomatoes is independent of ethylene, and Theologis (1992) demonstrated an ethylene requirement for the expression of PG protein, with the suggestion that ethylene controls either translation or protein stability. In our view, wound/ethylene synergism may explain the ethylene dependence of PG mRNA induction in avocado discs (manifested by the inhibition by Ag^+; Fig. 1B) compared with its purported ethylene independence in tomato fruit. Parenthetically, when Van der Straeten et al. (1989) studied ACC synthase induction in halves of tomato fruit, pericarp tissue was sliced after the elicitation of ethylene production with LiCl, with induction estimated 2 to 3 h after wounding.

The strong appearance of ripening-related mRNAs in discs inhibited from softening by ethylene antagonists offers the prospect of multiple ethylene thresholds for different regulatory targets. Specifically, the concentration of ethylene required for transcription induction is lower than that for posttranscriptional events (e.g. translation; cf. Lincoln and Fischer, 1988). Whereas ethylene is involved in both transcriptional and posttranscriptional control, wounding may provide a signal that synergizes ethylene action and is susceptible to inhibition by 2,4-D.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Jerry Cohen and Dr. Lech Michalczuk (U.S. Department of Agriculture, Beltsville, MD) for their extensive analysis of IAA and IAA conjugates in avocado tissue. We also owe our gratitude to Dr. Rolf E. Christoffersen at University of California, Santa Barbara for providing us with cDNA clones of EFE and P-450 and for the purified PG protein and antiserum, and to Dr. Mark Tucker of U.S. Department of Agriculture/Agricultural Research Service, (Beltsville, MD) for his kind gift of the cellulase cDNA clone. Avocado fruit were graciously provided by Professor Howard Ohr at University of California, Riverside from the South Coast Field Station, and by Bill Catlin from his orchard in Carpenteria, CA.

Received November 19, 1992; accepted March 1, 1993.

LITERATURE CITED

Almin KE, Ericksson KE, Jansson C (1967) Enzymatic degradation of polymers. II. Viscometric determinations of cellulase in absolute terms. Biochim Biophys Acta **139:** 248–251

Awad M, Lewis LN (1980) Avocado cellulase: extraction and purification. J Food Sci **45:** 1625–1628

Bearden JC (1978) Quantitation of sub-microgram quantities of protein by an improved protein-dye binding assay. Biochim Biophys Acta **533:** 525–529

Ben-Yehoshua S (1964) Respiration and ripening of discs of the avocado fruit. Physiol Plant **17:** 71–80

Bernfeld P (1955) Amylases a and b. Methods Enzymol **1:** 149–158

Biale JB (1960) The postharvest biochemistry of tropical and subtropical fruits. Adv Food Res **10:** 293–354

Bozak KR, Yu H, Sirevag R, Christoffersen RE (1990) Cloning and sequence analysis of ripening-related cytochrome P-450 cDNAs from avocado fruit. Proc Natl Acad Sci USA **87:** 3904–3908

Chen KH, Miller AN, Patterson GW, Cohen JD (1988) A rapid and simple procedure for purification of indole-3-acetic acid prior to GC-SIM-MS analysis. Plant Physiol **86:** 822–825

Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18:** 5284–5299

Cohen JD, Bausher MG, Bialek K, Buta JG, Gocal GFW, Janzen LM, Pharis RP, Reed AN, Sloven JP (1987) Comparison of a commercial ELISA assay for indole-3-acetic acid at several stages of purification and analysis by gas chromatography-selected ion monitoring-mass spectrometry using a $^{13}C_6$-labeled internal standard. Plant Physiol **84:** 982–986

Eaks IL (1980) Respiratory rate, ethylene production, and ripening response of avocado fruit to ethylene or propylene following harvest at different maturities. J Am Soc Hortic Sci **105:** 744–747

Frenkel C (1972) Involvement of peroxidase and indole-3-acetic acid oxidase isozymes from pear, tomato, and blueberry fruit in ripening. Plant Physiol **49:** 757–763

Frenkel C, Dyck R (1973) Auxin inhibition of ripening. Plant Physiol **51:** 6–9

Imaseki H, Uchiyama M, Uritani I (1968) Effect of ethylene on the inductive increase in metabolic activities in sliced sweet potato roots. Agric Biol Chem **32:** 387–389

Kutsunai S, Lin AC, Percival FW, Laties GG, Christoffersen RE (1993) Ripening-related polygalacturonase cDNA from avocado. Plant Physiol (in press)

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Lincoln JE, Fischer RL (1988) Diverse mechanisms for the regulation of ethylene-inducible gene expression. Mol Gen Genet **212:** 71–75

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular Cloning: A Laboratory Manual, Ed 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

McGarvey DG, Sirevag R, Christoffersen RE (1992) Ripening-related gene from avocado fruit. Plant Physiol **98:** 554–559

McMurchie EJ, McGlasson WB, Eaks IL (1972) Treatment of fruit with propylene gives information about the biogenesis of ethylene. Nature **237:** 235–236

Oeller PW, Min-Wong L, Taylor LP, Pike DA, Theologis A (1991) Reversible inhibition of tomato fruit senescence by antisense RNA. Science **254:** 437–439

Peacock BC (1972) Role of ethylene in the initiation of fruit ripening. Q J Agric Anim Sci **29:** 687–690

Rhodes MJC, Wooltorton LSC (1971) The effect of ethylene on the respiration and on the activity of phenylalanine ammonia lyase in swede and parsnip root tissue. Phytochemistry **10:** 1989–1997

Starrett DA, Laties GG (1991a) The effect of ethylene and propylene pulses on respiration, ripening advancement, ethylene forming enzyme, and 1-aminocyclopropane-1-carboxylic acid synthase activity in avocado fruit. Plant Physiol **95:** 921–927

Starrett DA, Laties GG (1991b) Involvement of wound and climacteric ethylene in ripening avocado discs. Plant Physiol **97:** 720–729

Theologis A (1992) One rotten apple spoils the whole bushel: the role of ethylene in fruit ripening. Cell **70:** 181–184

Tingwa PO, Young RE (1975a) Studies on the inhibition of ripening in attached avocado (*Persea americana* Mill.) fruits. J Am Soc Hortic Sci **100:** 447–449

Tingwa PO, Young RE (1975b) The effect of indole-3-acetic acid and other growth regulators on the ripening of avocado fruits. Plant Physiol **55:** 937–940

Trewavas AJ (1983) Growth substance sensitivity: the limiting factor in plant development. Physiol Plant **55:** 60–72

Tucker GA, Brady CJ (1987) Silver ions interrupt tomato fruit ripening. J Plant Physiol **127:** 165–169

Tucker ML, Durbin ML, Clegg MT, Lewis LN (1987) Avocado cellulase: nucleotide sequence of a putative full length cDNA clone and evidence for a small gene family. Plant Mol Biol **9:** 197–203

Tucker ML, Laties GG (1984) Interrelationship of gene expression, polysome prevalence, and respiration during ripening of ethylene and/or cyanide-treated avocado fruit. Plant Physiol **74:** 307–315

Van der Straeten D, Van Wiemeercsh L, Goodman HH, Van Montague H (1989) Purification and characterization of 1-aminocyclopropane-1-carboxylate synthase from tomato pericarp. Eur J Biochem **182:** 639–647

Vendrell M (1969) Reversion of senescence: effect of 2,4-dichlorophenoxyacetic acid and indoleacetic acid on respiration, ethylene production, and ripening of banana fruit slices. Aust J Biol Sci **22:** 601–610