Plant Physiol. (1993) 103: 227–234

Ethylene and Wound-Induced Gene Expression in the Preclimacteric Phase of Ripening Avocado Fruit and Mesocarp Discs[1]

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Whereas intact postharvest avocado (*Persea americana* Mill.) fruit may take 1 or more weeks to ripen, ripening is hastened by pulsing fruit for 24 h with ethylene or propylene and is initiated promptly by cutting slices, or discs, of mesocarp tissue. Because the preclimacteric lag period constitutes the extended and variable component of the ripening syndrome, we postulated that selective gene expression during the lag period leads to the triggering of the climacteric. Accordingly, we sought to identify genes that are expressed gradually in the course of the lag period in intact fruit, are turned on sooner in response to a pulse, and are induced promptly in response to wounding (i.e. slicing). To this end, a mixed cDNA library was constructed from mRNA from untreated fruit, pulsed fruit, and aged slices, and the library was screened for genes induced by wounding or by pulsing and/or wounding. The time course of induction of genes encoding selected clones was established by probing northern blots of mRNA from tissues variously treated over a period of time. Four previously identified ripening-associated genes encoding cellulase, polygalacturonase (PG), cytochrome P-450 oxidase (P-450), and ethylene-forming enzyme (EFE, or 1-aminocyclopropane-1-carboxylic acid synthase), respectively, were studied in the same way. Whereas cellulase, PG, and EFE were ruled out as having a role in the initiation of the climacteric, the time course of P-450 induction, as well as the response of same to pulsing and wounding met the criteria—together with several clones from the mixed library—for a gene potentially involved in preclimacteric events leading to the onset of the climacteric. Further, it was established that the continuous presence of ethylene is required for persisting induction, and it is suggested that in selected cases wounding may exert a synergistic effect on ethylene action.

Once harvested, avocado (*Persea americana* Mill.) fruit ripen in several days to weeks, depending on maturity. The ripening syndrome is biphasic, a variable and extended preclimacteric, or lag phase, preceding a fixed climacteric period of about 3 d. Endogenous ethylene, even at levels below ready detection, plays an important role in determining the duration of the lag period (Peacock, 1972; Lieberman, 1979; Yang, 1985, 1987). Upon dissipation of a putative inhibitory tree factor (Burg and Burg, 1964; Tingwa and Young, 1975), the low levels of preclimacteric ethylene are thought to mediate a gradual increase in tissue sensitivity, or responsiveness, to ethylene (see Trewavas, 1978) that culminates in the initiation of the climacteric with its attendant burst of autocatalytic ethylene production and ethylene-mediated changes in color, texture, aroma, and taste characteristic of the ripe fruit.

On the assumption that the selective expression (or suppression) of one or more genes during the lag period ultimately leads to the initiation of the climacteric, we sought evidence to that effect. To date, studies of selective gene expression associated with overt avocado ripening have been limited to the climacteric (Christoffersen et al., 1982, 1984), no attention having been paid to preclimacteric molecular events.

Because a 24-h pulse of ethylene or propylene following harvest shortens the lag period of mid-season fruit by several days (Starrett and Laties, 1991a), whereas wounding, i.e. slicing, causes mesocarp discs to ripen swiftly, we sought to identify genes that are activated gradually through the course of the lag phase in untreated fruit, are induced sooner by pulsing, and are turned on promptly by wounding. Such genes may conceivably be involved in initiating the climacteric phase while shortening the lag phase in pulsed and wounded tissue.

In what follows, we describe the preparation of a mixed cDNA library derived from poly(A)$^+$ mRNA from intact preclimacteric fruit, propylene-pulsed fruit, and aged discs and screening of the library for pulse and/or wound-induced genes. We subsequently probed northern blots of mRNAs from tissues treated variously over a period of time with isolates of choice to identify clones representing genes that met our criteria of gradual induction during the preclimacteric phase in untreated fruit and rapid or prompt induction in response to pulsing and/or wounding. Because wounding perforce leads to wound-ethylene production, the response to slicing could be attributed either to wounding per se or to wound ethylene. Accordingly, by aging discs in the presence of AVG (an inhibitor of ethylene synthesis) and NBD (an inhibitor of ethylene action) and probing northern blots of mRNA from such treated slices, we were able to distinguish

[1] Supported by a grant to G.G.L. from the National Science Foundation and by the University of California Los Angeles Academic Senate Committee on Research. D.A.S. was supported in part by a U.S. Department of Agriculture National Needs Fellowship.

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

specifically wound-induced genes from genes activated by wound ethylene. Although inhibition of disc ripening by the foregoing inhibitors suggests that it is wound-induced ethylene that initiates ripening and shortens or supplants the lag phase, the synergistic effect of wounding cannot be ruled out and is suggested herein.

In conjunction with the studies of selected clones from our mixed library, we investigated the expression of four ripening-associated genes screened from an early cDNA library (Christoffersen et al., 1984), viz. genes encoding cellulase, PG, P-450, and EFE.

Although there is no reason to believe that cellulase and PG influence ripening in the lag phase—since their enzyme activity is lacking in preclimacteric fruit (Awad and Young, 1979), and EFE induction has been ruled out as causative to the initiation of the climacteric (Starrett and Laties, 1991a)—nevertheless we wished to know how induction of the genes in question is influenced by pulsing and wounding and whether induction is simply triggered by ethylene or rather requires the continuous presence thereof. With respect to P-450, as will be seen, the time course and responsiveness of induction fit our criteria for lag phase involvement and resemble the behavior of several genes screened from the mixed library.

MATERIALS AND METHODS

Whole Fruit

Avocado fruits (*Persea americana* Mill. cv Hass) were harvested and treated as described previously (Starrett and Laties, 1991a). Fruit were harvested from the University of California South Coast Field Station between 10 and 12 AM, or from an orchard in Carpenteria, CA, between 11 AM and 1 PM. Within 2 to 3 h, single fruit were placed in 500-mL leakproof plastic canisters (Tupperware) fitted with input and output ports and kept at 20°C in a constant-temperature incubator. The fruit were treated with a continuous 30 mL/min flow of water-saturated air or propylene at 500 μL/L in air, propylene being an effective ethylene analog that permits the determination of endogenous ethylene production in response to treatment by exogenous propylene (Eaks, 1980; Starrett and Laties, 1991a). A system of control valves allowed easy switching of gas mixtures during the course of an experiment. Respiration was measured every hour by computer-controlled monitoring of CO_2 production with an IRGA (Anarad, model AR500) (Starrett and Laties, 1991a).

For T = 0 pulse treatments, fruit were pulsed for 48 h with propylene beginning within a few hours after harvest. For T = 24 pulses, fruit were pulsed with propylene for 24 h beginning 24 h after harvest. Preceding and following propylene pulsing, fruit were held in water-saturated air. Control fruit were held in water-saturated air throughout. At the time points indicated in the tables and figures, ethylene evolution was measured, after which fruit were cut into discs and 30 to 40 discs were frozen immediately in liquid nitrogen and stored at −70°C for subsequent RNA extraction (Starrett and Laties, 1991a).

Discs

Discs were prepared and treated as described by Starrett and Laties (1991b). Within 2 to 3 h of harvest, whole fruit were bathed for 5 min in bleach (5% NaOCl) diluted 1:20 in water, and rinsed in deionized water. Each fruit was subsequently halved longitudinally, the seed was removed, and each half was cut along its length into slices 3 mm thick with a slicer comprising an adjustable microtome knife fixed in an appropriate bed. Slices were punched with a #9 cork borer, yielding discs 14 mm in diameter. There being an inherent variability in the ripening behavior of different regions of the fruit, discs were punched only from the equatorial region of slices, from tissue abutting the seed. In this way, 75 to 150 discs were obtained per fruit, depending on its size. Discs from all fruit were combined, randomized, quickly rinsed five times with distilled water, and blotted dry. Subsequently, discs were placed on screens in canisters and respiration and ethylene evolution were measured.

Discs (also called slices herein) were variously treated with NBD (10,000 μL/L), AVG (25-μL droplets of 4 mM), or NBD and AVG together (Starrett and Laties, 1991b). At indicated time points, ethylene evolution was measured and 30 slices were assayed for ripeness (Starrett and Laties, 1991b), while a similar set of slices was frozen in liquid nitrogen and stored at −70°C for subsequent RNA extraction. Because NBD is applied as a gas, whereas AVG is applied as liquid droplets, both wet and dry controls were used; this explains the designation of dry or wet for untreated control slices.

RNA Isolation

Fifteen grams of frozen tissue were mixed with 20 g of dry ice and pulverized in an electric coffee grinder (Krups), then placed at −70°C for 48 h to allow the CO_2 to sublime. The tissue powder was homogenized in guanidinium buffer (Chirgwin et al., 1979) in a mortar and pestle and filtered through Miracloth (Chicopee Mills, Inc., Milltown, NJ) and the homogenate was centrifuged for 10 min at 12,000*g* in an SS-34 rotor (Beckman) to separate cellular debris and oil. The supernatant was made to 1.3 M CsCl, layered over a 10-mL pad of 5.7 M CsCl, and centrifuged for 5.5 h in a Ti-50.2 rotor (Beckman) at 48,000 rpm (average, 225,000*g*). The resulting pellets (total RNA) were resuspended in water and precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol at −20°C.

Poly(A)$^+$ mRNA purification was accomplished essentially as described by Tucker and Laties (1984), including the additional step of passing the resuspended poly(A)$^+$ mRNA through a cellulose column equilibrated with binding buffer to remove contaminating polysaccharides. The polysaccharide-free effluent was passed twice by gravity flow through an oligo(dT)-cellulose column equilibrated with binding buffer. Poly(A)$^+$ mRNA was eluted from the column and precipitated in 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol at −20°C.

The poly(A)$^+$ mRNA pellet was resuspended in water, aliquoted into 0.5-mL Eppendorf tubes, and stored at −70°C. Poly(A)$^+$ mRNA used for cDNA library construction was resuspended in elution buffer and purified on oligo(dT)-

cellulose columns again, without the cellulose column step. The RNA was then ethanol precipitated and resuspended in water as above.

cDNA Library Construction and Screening

A mixed cDNA library was constructed from equal amounts of poly(A)$^+$ mRNA isolated separately from 24-h ethylene-pulsed fruit, 24-h aged slices, and 24-h untreated fruit. Eight micrograms of poly(A)$^+$ mRNA from each tissue type were mixed together and delivered to Stratagene (La Jolla, CA) for construction of the customized cDNA library. cDNA synthesis was primed using both oligo(dT) and random primers, and the cDNA was ligated into the *Eco*RI restriction site of the λZAPII vector (Stratagene). The cDNA insert size was greater than 400 bp. The library comprised 1.2×10^7 recombinant phage before amplification, which was done by Stratagene.

Two hundred fifty thousand recombinants were differentially screened using radiolabeled cDNA probes synthesized from the same poly(A)$^+$ mRNA used for library construction. For each of the three mRNA populations, cDNA was synthesized with dATP, dTTP, dGTP, and [α-^{32}P]dCTP (80 μCi of 3000 Ci/mmol, Amersham) using 1 μg of poly(A)$^+$ mRNA as a template. Unincorporated [^{32}P]dCTP was separated out with Nick columns (Pharmacia). cDNA synthesis and library screening were done according to Sambrook et al. (1989) and the Stratagene custom library protocols.

Recombinant phage of the mixed library were plated on the recommended bacterial host strain SURE (Stratagene), and three replicate filters were made of each plate according to the method of Benton and Davis (see Sambrook et al., 1989). Each of the three radiolabeled cDNA populations was hybridized to a different replicate filter with 10^6 cpm/mL of radiolabeled cDNA. Recombinants that showed dramatic differences in hybridization to the three different cDNA populations were identified and the corresponding plaques were isolated. Phage from each plaque were plated out in low numbers and rescreened.

cDNA clones were subcloned into the Bluescript SK plasmid (Stratagene) in the bacterial host strain SURE as per the in vivo excision protocol in the Stratagene literature. Bacteria containing the recombinant plasmids were streaked, and single colonies were isolated and grown up. Plasmid minipreparations were done according to Sambrook et al. (1989). Selected clones were nick translated (BRL Nick Translation Kit) using [α-^{32} P]dCTP (50 μCi of 3000 Ci/mmol, Amersham) and used to screen dot blots of poly(A)$^+$ mRNA from tissue of the three types to confirm the differential expression patterns.

Northern Blot Analysis

Selected clones isolated as described above, as well as four previously isolated ripening-related avocado clones, were used for northern blot analysis. Northern blots were prepared from poly(A)$^+$ mRNA isolated from fruit and slices at various time points under various conditions of fruit and slice ripening. Intact fruit comprised fruit endogenously ripened, fruit treated with propylene for 24 h beginning 24 h after harvest, and fruit treated with propylene for 48 h beginning immediately after harvest. Slices were either untreated, treated with AVG, with NBD, or with AVG plus NBD. Wet control slices were treated with 25 μL of deionized water. Dry control slices were untreated. Northern blot analysis was done according to Sambrook et al. (1989). After the gel was run, the size-marker lane was cut off and stained. The remaining gel was blotted onto a Nytran filter (Schleicher & Schuell) in 20× SSC overnight. Filters were baked and stored under vacuum at room temperature until use.

The northern blots were probed with selected clones isolated from the mixed library, as well as with four clones previously isolated: a cellulase clone (Cass et al., 1990); a PG clone (Kutsunai et al., 1993); a Cyt P-450 clone (Bozak et al., 1990); and a clone for EFE (McGarvey and Christoffersen, 1992). Plasmids were nick-translated as noted above, and hybridized blots were exposed to Kodak XAR-5 x-ray film at −70°C with a Cronex Lightning Plus enhancing screen (Dupont) for 18 h to 5 d.

Materials

AVG was purchased from Sigma. NBD was purchased from Aldrich Chemical Co. Ethylene and propylene (both 99.9% pure) were purchased from Scott Specialty Gases, and compressed air was from Liquid Air Corporation.

RESULTS

cDNA Library Screen

After the first round screen, roughly 100 plaques representing differentially expressed mRNAs were isolated. Twenty-five of these clones were screened a second time, and 15 plaques exhibiting differential expression were picked and the inserts subcloned. These clones were hybridized to poly(A)$^+$ mRNA dot blots to verify their differential expression. Clones showing various patterns of differential expression in 24-h untreated fruit, 24-h propylene-treated fruit, and 24-h-old slices were then selected for further characterization (nine of these are presented in Table I), including northern blot analysis.

Table I. *Approximate insert size and expression patterns of isolated cDNA clones*

Clone designations as follows: pAPW, avocado pulse- and wound-induced; pAW, avocado wound only; pACO, avocado control; pACN, avocado constitutive.

	Clone	Insert Size	Expression Pattern
Group I	pAPW7C	800	Induced by pulsing and wounding;
	pAPW24C	1750	expression similar to P-450
Group II	pAW6B	2000	Induced by wounding; expressed
	pAW8E	1020	in slices at all time points and
	pAW23B	1350	unexpressed in intact fruit
Group III	pACO4C	1300	Expression reduced by wounding
	pACO8F	950	
Group IV	pACN3A	2490	Constitutive
	pACN8B	1900	

Characterization of Selected Differentially Expressed Genes

Nine selected clones reflecting various patterns of differential gene expression fall into four rough categories: clones pAW6B, pAW8E, and pAW23B represent wound-induced genes, whereas clones pAPW7C and pAPW24C represent genes induced by both pulsing and wounding. The expression of genes corresponding to clones pACO4C and pACO8F is reduced by wounding, whereas genes homologous to clones pACN3A and pACN8B appear to be constitutively expressed. Restriction maps show all clones to have insert sizes ranging from 800 to 2500 bp (Table I) and suggest that clones within a group are different from each other (data not shown).

Northern Blot Analysis of Differentially Expressed Genes

Northern blots were carried out with poly(A)$^+$ mRNA isolated from control fruit and pulsed fruit at time points encompassing the full span of the well-defined lag period and the climacteric, as well as with poly(A)$^+$ mRNA from ripening slices (Fig. 1; Tables II and III). Probing was then effected with clones of choice. Preclimacteric samples were taken well within the lag period to avoid any contribution by mRNA from incipient climacteric tissue.

Wound-Induced Genes

The wound-induced genes (clones pAW6B, pAW8E, and pAW23B) are expressed in slices at all time points after t_o,

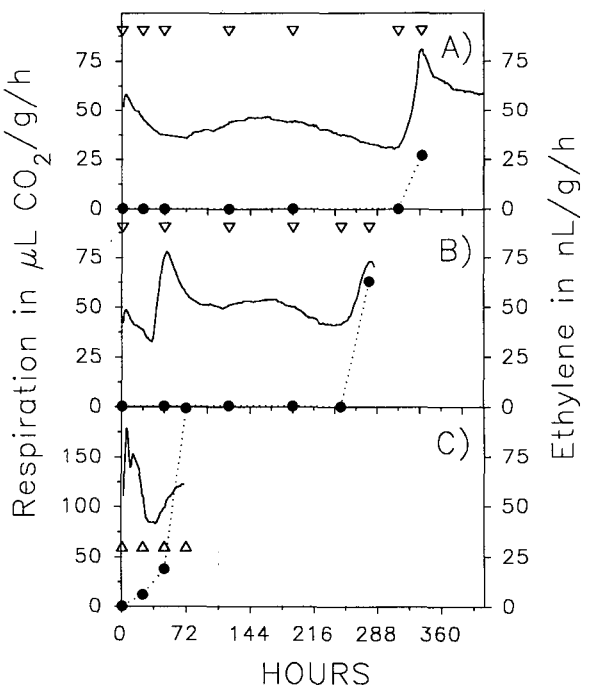

Figure 1. Respiration and ethylene profiles of whole fruit and slices used for RNA extractions. CO_2 production (—) and ethylene production (●—●) of control (i.e. endogenously ripened) fruit (A); 24-h propylene-pulsed fruit (B); and untreated slices (C). CO_2 production was monitored every hour. Ethylene production is shown for each time point at which tissue was frozen for subsequent RNA extraction (Δ). Profiles for inhibitor-treated slices are not shown.

Table II. *Time points for mRNA isolation, northern blot analysis, and measurement of ethylene production in intact, ripening fruit*

T = # represents age of fruit in h after harvest. For propylene pulses, fruit were treated with 500 μL/L of propylene in air for the indicated times, at which fruit were assayed for ethylene production. All fruit were sliced and frozen for subsequent RNA extraction.

Timepoint	Description	Ethylene Production
		nL C_2H_4 g^{-1} h^{-1}
Air control fruit		
T = 0	Freshly harvested	0.6
T = 18	18 h	0.1
T = 24	24 h	0.1
T = 48	48 h	0.1
T = 120	Early valley (5 d)	0.2
T = 192	Mid valley (8 d)	0.3
BC	Bottom of climacteric rise	0.3
TC	Top of climacteric rise	54.1
Propylene pulse: 24 h at T = 24		
T = 48	48 h	0.6
T = 120	Early valley (3 d after pulse)	1.1
T = 192	Mid valley (6 d after pulse)	1.0
BC	Bottom of climacteric rise	0.5
TC	Top of climacteric rise	126.0
Propylene pulse: 48 h at T = 0		
T = 48	48 h	0.4

Table III. *Time points for mRNA isolation, northern blot analysis, and ethylene production in ripening slices*

$t_{no.}$ represents age of slices in h after cutting from freshly harvested fruit. Eight fruit were sliced and the slices randomized. For AVG treatments, 4 mM AVG was applied as a 25-μL droplet to each slice at t_0 and t_{24}. Twenty-five-microliter droplets of water were applied to wet control slices at t_0 and t_{24}. NBD was applied at a gas concentration of 10,000 μL/L and replenished every 8 h. At the indicated times, slices were assayed for ethylene production and then frozen for subsequent RNA extraction. For illustration of time points, see Figure 1.

Timepoint	Description	Ethylene Production
		nL C_2H_4 g^{-1} h^{-1}
t_0	Fresh	0.1
t_{24} W[a]	24-h wet control	9.8
t_{48} W	48-h wet control	46.8
t_{72} W	72-h wet control	234.0
t_{24} AN[b]	24-h AVG + NBD	0.6
t_{48} AN	48-h AVG + NBD	0.6
t_{72} AN	72-h AVG + NBD	0.4
t_{72} A[c]	72-h AVG	0.9
t_{72} N[d]	72-h NBD	82.4
t_{72} D[e]	72-h dry control	165.6

[a] W, Wet. [b] AN, AVG + NBD. [c] A, AVG. [d] N, NBD. [e] D, Dry.

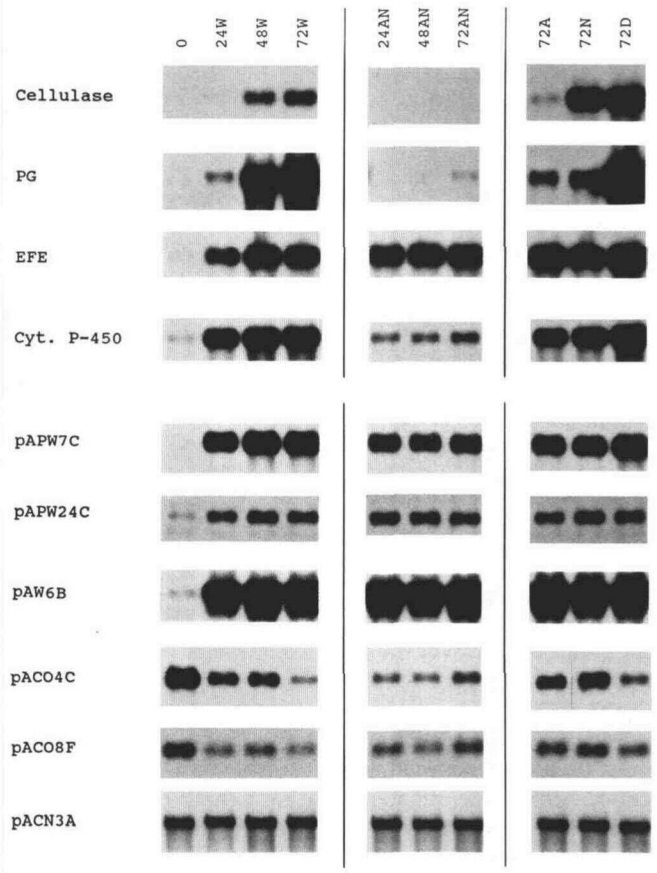

Figure 2. Northern blot analysis of wound-, pulse-, and ripening-induced cDNA clones, hybridized to slice mRNA extracted at designated times. One microgram of poly(A)$^+$ mRNA was run in each lane of a formaldehyde-denaturing gel and probed with nick-translated, radiolabeled cDNA probes. For lane designation descriptions, see Table III.

roughly to the same degree (Fig. 2; all three clones exhibit similar expression patterns; only pAW6B is shown for clarity). Inhibitors of ethylene synthesis and action, such as AVG and NBD, respectively, do not alter the mRNA levels. However, in intact fruit, the wound-related mRNA levels are very low and remain so throughout the lag period and climacteric, as well as during a propylene pulse (Fig. 3). Although the accelerated lag period witnessed in avocado slices may be due to wound ethylene, some true wound-induced genes are expressed.

Pulse and Wound-Induced Genes

In this study, genes expressed in fruit in response to a propylene pulse (e.g. genes represented by clones pAPW7C and pAPW24C) were also induced by slicing (Figs. 2 and 3). Because induction in slices was unaffected by AVG plus NBD, the genes in question are deemed to be induced by either pulsing or wounding. By contrast, strictly wound-induced genes, such as that represented by clone pAW6B, for example, are not expressed significantly in intact fruit. Parenthetically, the cellulase and PG genes would seem to be true ethylene-induced genes, since their induction in slices is repressed by AVG plus NBD (Figs. 2 and 3).

Expression of the pulse- and wound-induced clones pAPW7C and pAPW24C rises gradually through the lag period of endogenously ripened intact fruit, and more dramatically in the climacteric (Fig. 3). mRNA levels are increased by a propylene pulse, only to drop back to control levels following the pulse. The expression of these two clones in whole fruit resembles the expression pattern of P-450 (see below).

In slices, mRNA levels of pAPW7C and pAPW24C are already at near climacteric levels by 24 h, rising little more by the climacteric (Fig. 2). AVG and NBD combined or applied alone reduce mRNA levels only slightly, if at all. The expression patterns of these two clones are similar under most conditions and at all time points.

Wound-Reduced Genes

The levels of mRNAs that hybridize with clones pACO4C and pACO8F, respectively, are significantly reduced by slicing (Fig. 2), reduction being evident as early as 18 h (data not shown). Because reduction remains marked in the presence of AVG and NBD together, wounding per se is presumably the causative agent. Consistent with this deduction, a propylene pulse has no effect on the level of the mRNAs in question in intact fruit. In both control and pulsed fruit,

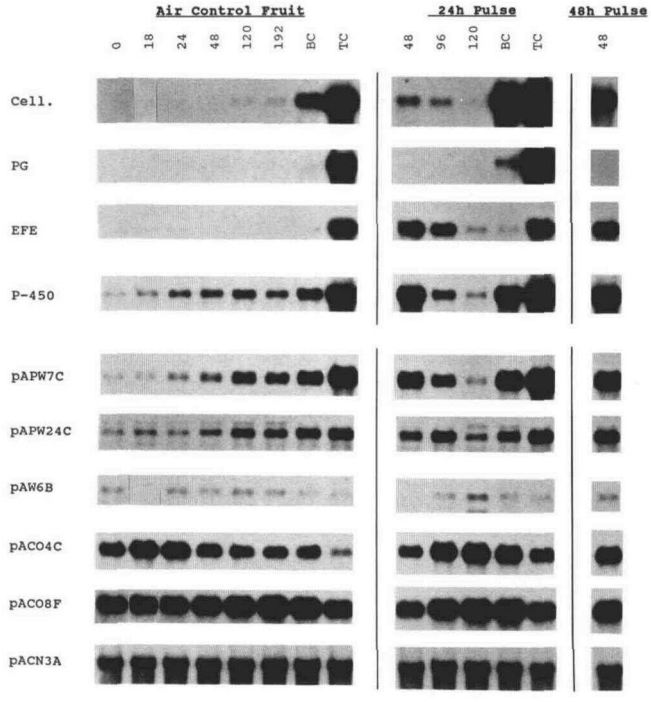

Figure 3. Northern blot analysis of wound-, pulse-, and ripening-induced cDNA clones hybridized to whole fruit mRNA at designated times. One microgram of poly(A)$^+$ mRNA was run in each lane of a formaldehyde-denaturing gel and probed with nick-translated, radiolabeled cDNA probes as described in "Materials and Methods." For lane designation descriptions, see Table II.

however, there appears to be a modest drop in the pertinent mRNA levels at the climacteric peak (Fig. 3).

Constitutive Genes

Clones pACN3A and pACN8B represent genes that appear to be constitutively expressed, directing the production of roughly equal amounts of poly(A)$^+$ mRNA in whole fruit and slices under all conditions, including freshly harvested fruit (Figs. 2 and 3; only pACN3A is shown for clarity). pACN3A is also expressed in flower buds, at slightly higher levels (data not shown).

Northern Blot Analysis of Previously Isolated Clones

Cellulase

Northern blot analysis of the cellulase clone shows this gene to be expressed during the climacteric of intact fruit as expected (Fig. 3; cf. Christoffersen et al. [1984] and De Francesco et al. [1989]), as well as in response to a 24- or 48-h propylene pulse, although to a lesser degree than in the climacteric. Following the pulse, induced mRNA levels drop to near those in untreated fruit levels.

In slices, mRNA appears by 48 h (perceptible by 24 h at longer exposures) and continues to rise through 72 h, nearly reaching levels found in whole climacteric fruit (Fig. 2, cf. Fig. 3). AVG and NBD applied together reduce the mRNA level to below the vanishingly low levels in untreated, intact preclimacteric fruit, whereas AVG alone reduces mRNA to levels near those in untreated fruit, and NBD alone reduces the mRNA level to a lesser degree. It should be noted that water-treated slices (Fig. 2, 72W) serve as the control for AVG-treated slices, and untreated slices (Fig. 2, 72D) serve as the control for NBD-treated slices. Because the mRNA levels in wet and dry slices differ, the differences in mRNA level due solely to AVG or NBD may not be as disparate as they appear.

PG

The PG gene is not induced by a pulse in intact fruit; rather, it is expressed solely at the peak of the respiratory climacteric (Fig. 3). The PG gene is expressed within 1 d in slices, however, and expression is fully suppressed by AVG and NBD together and reduced substantially by either inhibitor alone (Fig. 2). The suppressible induction of the PG gene by ethylene inhibitors in slices in the absence of induction by an ethylene-analog (propylene) pulse in fruit suggests a synergistic effect of wounding on ethylene-mediated PG induction in slices (see "Discussion").

P-450

Expression of P-450 mRNA in intact fruit exhibits a pattern different from that of the two genes described above (Figs. 2 and 3). mRNA levels rise gradually through the lag period of endogenously ripened intact fruit, increasing sharply in the climacteric. mRNA levels increase to near climacteric levels in 24 or 48 h in propylene-pulsed fruit. As with cellulase, mRNA drops to control levels upon discontinuation of the pulse.

In slices, mRNA levels have already increased dramatically by 24 h, and rise only gradually thereafter, reaching levels near those in climacteric intact fruit by 72 h. AVG and NBD applied together reduce mRNA levels to those in untreated fruit, whereas AVG or NBD alone has a lesser effect.

EFE (ACC Oxidase)

EFE transcript is evident in untreated intact fruit solely at the climacteric peak, whereas mRNA is strongly induced in whole fruit following a 24-h propylene pulse only to fall away with time after the propylene is withdrawn (Fig. 3). Slicing elicits EFE mRNA within 24 h in a manner unaffected by AVG and NBD together (Fig. 2). Because both pulsing and wounding induce EFE and AVG and NBD fail to inhibit EFE induction in slices, it follows that either pulsing or wounding per se causes induction.

DISCUSSION

The foregoing study sought to identify genes that might be involved in preclimacteric events leading to the initiation of the climacteric in avocado fruit. To this end, we searched for ripening-related genes expressed increasingly with time over the lag period in fruit, whose induction was accelerated by pulsing fruit with propylene and by slicing—procedures that individually advanced the onset of the climacteric and ripening. Although emphasis is placed on induction, lag-phase transitions could just as well entail down-regulation, with the dissipation of a ripening inhibitor, perhaps the putative tree factor.

As noted previously, in evaluating the basis of induction in response to slicing we must distinguish the effect of wounding per se from that of wound ethylene. The criteria are as follows: (a) Where induction is achieved in intact fruit by a pulse and induction is inhibited in slices by AVG plus NBD, we consider induction to be ethylene mediated. (b) When induction in slices is inhibited by AVG plus NBD and a propylene (ethylene) pulse fails to cause induction in intact fruit, it is believed that wounding exerts a synergistic effect on induction by ethylene. (c) When induction is effectuated as readily by a pulse as by slicing in the presence of AVG plus NBD, it is deduced that induction is achieved either by ethylene or by wounding per se.

Of the previously identified genes shown to be ripening associated, neither cellulase, PG, nor EFE is a candidate for involvement in the initiation of the climacteric. Although all three are induced in slices, and cellulase and EFE are induced by pulsing, none are induced during the lag period in intact fruit (Fig. 3). Cellulase and PG are involved in cell wall degradation and ostensibly in softening, but there is no apparent softening of fruit tissue during or after a pulse, softening being associated solely with the climacteric. Nevertheless, it is noteworthy that ethylene must be present continuously to sustain the pulse response.

The induction of cellulase and PG in slices is inhibited by AVG and NBD together (Fig. 2), bespeaking the involvement of wound ethylene in the process. In seeming contradiction, however, PG is not induced in fruit by a propylene pulse (Fig. 3). Accordingly, the prompt induction of PG in slices in

the absence of inhibitors suggests a synergistic response wherein wounding makes the tissue responsive to ethylene. The reported indifference to ethylene of PG induction in tomato pericarp (Oeller et al., 1991) may be due to the absence of wounding in the latter instance.

EFE, being induced by an ethylene pulse in fruit (Fig. 3) or by slicing even in the presence of AVG and NBD, represents a gene induced by either ethylene or wounding per se (cf. Oeller et al., 1991). Thus, there appear to be at least two different mechanisms controlling the expression of the EFE gene, or possibly two different EFE genes. Previous work on EFE activity has shown that the EFE gene is probably not involved in determining lag period length (Starrett and Laties, 1991a).

Of the clones isolated from the mixed library, pAW6B, pAW8E, and pAW23B are considered wound induced because they remain unexpressed in intact and pulsed fruit while rising strongly in slices. Although their lack of expression in whole fruit makes it unlikely that these genes are involved directly in control of the lag period, it cannot be ruled out that they play a synergistic role in the accelerated lag period exhibited by slices. pACO4C and pACO8F are believed to be down-regulated by wounding. These may represent genes whose expression normally leads to inhibition of lag period events and thus to the extended lag period. The substantial reduction in their expression by wounding is consistent with the pronounced shortening of the lag period by wounding. pACN3A and pACN3B are viewed as constitutive and unaffected by wounding or pulsing.

Clones pAPW7C and pAPW24C represent genes of the type we have been seeking inasmuch as expression rises with time through the lag period in intact fruit, is induced by a propylene pulse, and responds sharply to wounding, much as is the case with P-450. Although such behavior suggests that the genes in question, as well as P-450, may be involved in lag period events that lead to the triggering of the climacteric, it does not prove that this is the case. It remains to be established whether interference with their induction or expression impairs ripening. Although there is a strong similarity in the time courses of induction and wound and pulse responses between P-450, APW7C, and APW24C, induction of P-450 in slices is inhibited by AVG plus NBD, whereas induction of the latter two genes is not (Fig. 2). Thus, wounding alone seemingly supplants ethylene in the latter instance, but not with P-450 induction. Accordingly, P-450 and the genes APW7C and APW24C are not identical. Furthermore, a more pronounced inhibition by NBD of APW24C induction in the early stages of slice treatment distinguishes the latter from APW7C (data not shown), as does the suggestive, albeit inconclusive, difference in restriction enzyme digestion patterns.

It bears remembering that continuous exposure of postharvest fruit to ethylene shortens the lag period (Biale, 1960) to the length of that in ripening slices. Thus, there is little question that ethylene mediates events of the lag period in intact fruit (even at levels below ready detection; Peacock, 1972). Our experiments suggest that in the absence of inhibitors, ethylene plays the same role in slices, although ethylene action may be synergistic with wounding (Starrett and Laties, 1991b). As suggested above, genes induced by slicing per se may play a role in the mediation of the wound-shortened lag period. In this view, the AVG/NBD-inhibitable appearance of PG mRNA in as little as 24 h in slices compared with its lack of induction even by a 48-h pulse suggests that in slices, wounding, in a synergistic interaction with ethylene, circumvents some of the lag period events that occur in intact fruit, leading directly to a climacteric-like state.

Whereas there is no doubt that EFE mRNA is induced by propylene (ethylene) in intact avocado fruit (Fig. 3; McGarvey et al., 1992), EFE mRNA is induced in avocado slices by wounding alone under conditions where ethylene synthesis and action are suppressed (Fig. 2). Although avocado EFE has been shown to be homologous with the tomato cDNA clone pTOM13 (McGarvey et al., 1990) that hybridizes with a small family of three tomato genes (Holdsworth et al., 1988), no evidence has been adduced for the expression of more than one EFE gene in avocado, although two distinct EFE proteins have been demonstrated (McGarvey and Christoffersen, 1992). Consistent with the EFE mRNA profiles in Figures 2 and 3, the in situ activity of EFE in intact avocado fruit is quite low during the lag period and rises sharply at the climacteric (Yang, 1985; Starrett and Laties, 1991a), whereas in slices EFE activity rises continuously from the time of cutting (Starrett and Laties, 1991b).

We have proposed one way to select genes expressed during the preclimacteric lag phase of postharvest fruit that may be involved in the initiation of the climacteric. Herein we have chosen but a few of the many genes that fit our postulated criteria. It is clear that genes heretofore most often associated with ripening—viz. cellulase and PG—are activated late in the game and are a consequence rather than the cause of the climacteric. It remains to sequence the potentially numerous genes that share the characteristics of APW7C and APW24C with respect to time course of induction and response to wounding and pulsing, and to deduce or determine their gene products.

We have shown previously that with early-season to midseason fruit there is approximately 1 d postharvest in which fruit is unresponsive to a propylene pulse with respect to the consequent shortening of the lag period (Starrett and Laties, 1991a). Unresponsiveness has been attributed to a postulated inhibitory tree factor. It will be of particular interest in the future to probe northern blots of mRNA from freshly harvested and 1-d-postharvest fruit with genes of the type identified herein that we suspect to be involved in lag-phase events leading to the climacteric.

ACKNOWLEDGMENTS

The authors wish to thank Professor Howard Ohr at the University of California, Riverside, for his kindness in providing us with avocado fruit from the South Coast Station orchard, and Bill Catlin for graciously providing us access to his orchard in Carpenteria, CA. We are indebted to Dr. John Lighton for the custom computer software programs developed for our use.

Received March 17, 1993; accepted May 24, 1993.

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