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Ripening-Related Gene from Avocado Fruit[1]

Ethylene-Inducible Expression of the mRNA and Polypeptide

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ABSTRACT

Fruit ripening involves a series of changes in gene expression regulated by the phytohormone ethylene. *AVOe3*, a ripening-related gene in avocado fruit (*Persea americana* Mill. cv Hass), was characterized with regard to its ethylene-regulated expression. The *AVOe3* mRNA and immunopositive protein were induced in mature fruit within 12 hours of propylene treatment. The *AVOe3* mRNA levels reached a maximum 1 to 2 days before the ethylene climacteric, whereas the immunopositive protein continued to accumulate. RNA selected by the pAVOe3 cDNA clone encoded a polypeptide with molecular mass of 34 kilodaltons, corresponding to the molecular mass of the AVOe3 protein determined by immunoblots. The protein was soluble, remaining in solution at 100,000 gravity and eluted as a monomer on gel filtration. Because of its pattern of induction and relationship to an ethylene-related gene of tomato, the possible involvement of *AVOe3* in ethylene biosynthesis is discussed.

Ripening of climacteric fruit is associated with a concurrent increase in ethylene production. Activities of ethylene biosynthetic enzymes increase, notably ACC[3] synthase and EFE (3, 24). ACC synthase converts *S*-adenosylmethionine to ACC, and EFE catalyzes the oxidation of ACC to ethylene (18). Ethylene plays a crucial regulatory role for both ethylene biosynthesis and ripening: application of exogenous ethylene accelerates the onset of ethylene biosynthesis and ripening, whereas inhibition of ethylene biosynthesis or action arrests these processes (3).

It is well documented that fruit ripening involves ethylene-related changes in gene expression (3, 7). Ethylene is required for the induction and maintenance of several ripening-related genes (9, 19). In avocado fruit (*Persea americana* Mill. cv Hass), a variety of genes are induced through ripening or application of ethylene. These include genes for cellulase (6), a cytochrome P-450 oxidase (2), and a small number of genes of unknown function (6, 21, 25). Given that the ripening process and increased ethylene biosynthesis proceed concomitantly, it is expected that this set of ripening- and ethylene-responsive genes includes genes for ethylene biosynthetic enzymes.

We have previously reported the sequence of pAVOe3, a ripening-related cDNA, and its relationship to homologous genes in tomato (21). The pAVOe3 cDNA bears extensive homology with pTOM13 of tomato, a cDNA implicated in the final step of ethylene biosynthesis (14). In this paper, we report the ethylene-related expression of the *AVOe3* gene. We have constructed a fusion protein of β-gal and AVOe3 to generate AVOe3-specific antisera. We present here the first characterization of a polypeptide in this family with regard to native structure and expression. The AVOe3 protein was characterized as a soluble monomer with molecular mass of 34 kD. The *AVOe3* mRNA and protein are induced in a manner consistent with its implied identity with EFE.

MATERIALS AND METHODS

Plant Material

Forty mature avocado fruit (*Persea americana* Mill. cv Hass) were harvested from a local tree and placed into glass jars at 20°C with a continuous flow of water-saturated air at approximately 50 mL/min. After 24 h, fruit were either maintained in air or switched to a constant flow of 500 μL/L propylene. Endogenous ethylene production was monitored at 12-h intervals by GC. Mesocarp tissue from fruit at differing stages of ripening was cut into pieces and frozen immediately in liquid nitrogen. Frozen tissue was then stored at −70°C for future use.

Chemicals

L-[^{35}S]methionine at >1200 Ci/mmol, γ-[^{32}P]ATP at >5000 Ci/mmol, and [α-^{32}P]dCTP at >3000 Ci/mmol were obtained from Amersham Corporation. Restriction enzymes and avian myeloblastosis virus reverse transcriptase were obtained from Promega or New England Biolabs. Guanidine hydrochloride was obtained from American Research Products Company; acrylamide, from Research Organics, Inc.; agarose, from Bethesda Research Laboratories; polyclonal antiserum to β-gal, from 5 Prime to 3 Prime, Inc.; and nitrocellulose from Schleicher and Scheull, Inc. All other

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[3] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; β-gal, β-galactosidase; bp, nucleotide base pairs; EFE, ethylene-forming enzyme; nt, nucleotide.

chemicals were obtained from Sigma Chemical Company or Fisher Scientific, except as otherwise stated.

RNA and Protein Extraction

For RNA extraction, frozen mesocarp tissue was ground to a fine powder in liquid nitrogen with a Waring blender and homogenized in lysis buffer (5 M guanidine hydrochloride, 10 mM Na_2EDTA, 100 mM Tris-HCl [pH 7.5], 100 mM β-mercaptoethanol) using an SDT Tissumizer (Tekmar Company). Following homogenization, samples were centrifuged at 30,000*g* for 40 min at 4°C and filtered through Miracloth (Calbiochem). The RNA was then precipitated with LiCl and isolated by the method of Cathala *et al.* (5).

Proteins were extracted using the Tris buffer and extraction method of Kanellis *et al.* (17). For sizing of the native protein, extraction was carried out following the method of Britsch and Grisebach (4).

Preparation of Antiserum

A fusion of the pAVOe3 cDNA and the *lac-z* gene of *Escherichia coli* was constructed as follows. The pAVOe3 clone, in pBR322 (6), was cut with restriction enzymes *Pst*I and *Bgl*II. The succeeding 750-bp fragment, encoding the 192 C-terminal amino acids of AVOe3 protein, was then ligated into *Bam*HI-*Pst*I-digested pWR590–0 (12) and transformed into *E. coli* strain JM101. The resulting 84-kD AVOe3/β-gal fusion protein was identified on SDS-PAGE and electroeluted from the gel. Polyclonal antiserum was then generated in rabbit by standard procedures (15). Antiserum was further purified by subjecting it to affinity chromatography using a column of soluble proteins from JM101/pWR590–0 linked to CNBr-activated Sepharose (Sigma) following the protocol of Harlow and Lane (15).

Blotting and Detection of RNA and Proteins

Proteins (20 μg/lane) were electrophoretically separated by SDS-PAGE. After they were electrotransferred to nitrocellulose, immunoblots were performed following the method of Young (28). Molecular mass was determined by reference to SDS Molecular Weight Standards-Low (Bio-Rad) stained with Coomassie brilliant blue R-250.

Total RNA (10 μg/lane) was electrophoretically separated on a 1% (w/v) agarose gel, containing 0.67 M formaldehyde, blotted to nitrocellulose, and hybridized with nick-translated pAVOe3 cDNA. The blot was washed at Tm −20°C and exposed to Kodak XAR-5 film at −70°C with an intensifying screen.

For dot blots, a series of dilutions of each RNA sample was blotted to nitrocellulose (8). After hybridization and autoradiography as above, the signals were quantified by densitometric scanning (LKB UltroScan XL) and comparison to a dilution series of pAVOe3 plasmid on the same blot.

Primer Extension

Primer extension was carried out as described by Dunsmuir *et al.* (11). All solutions were made RNase free by treatment with 0.05% (v/v) diethyl pyrocarbonate. E318p, an 18-mer oligodeoxynucleotide primer (5′-CAGTATTGTAGGTCAGGG-3′) derived from the untranslated region near the 5′-end of the pAVOe3 cDNA (21), was synthesized, gel purified, and 5′-end labeled to a specific activity of 2 × 107 dpm/pmol DNA. Primer annealing was carried out with 7 μg total RNA and 0.1 pmol ^{32}P-labeled E318p primer. The length of extended products was determined by comparison to a known sequencing ladder after PAGE.

Hybrid-Select Translation

Hybrid selection of *AVOe3* RNA was carried out essentially as described by Parnes *et al.* (23) with the following modifications. For *AVOe3* mRNA selection, the cDNA insert alone was immobilized to filters, and total RNA (1 mg/300 μL hybridization mix) was used for the selection rather than polyadenylated RNA. The hybrid-selected RNAs were then translated with a wheat germ translation kit (Promega) according to the manufacturer's instructions, separated by SDS-PAGE, and exposed to Kodak XAR-5 film following drying of the gel.

Sizing of the Native Protein

Protein (760 μg), extracted under nondenaturing conditions, was loaded onto a Superose 12 column at 2°C. The column was eluted with buffer A (100 mM Tris [pH 7.4] and 10% [v/v] glycerol, deaerated and equilibrated with nitrogen) plus 33 mM Na-ascorbate, and 0.5-mL fractions were collected. Because ascorbate has a high A_{280}, protein was quantified using 20 μL of each fraction in a Bradford assay (Bio-Rad). The remainder of each fraction was then concentrated by TCA precipitation and subjected to SDS-PAGE and immunoblotting as described above. Gel permeability of molecular weight standards (Bio-Rad) was determined by A_{280} in a separate run on the same column, using buffer A without Na-ascorbate.

Subcellular Fractionation

Fractionation of avocado mesocarp tissue was accomplished by the method of Bennett and Christoffersen (1) using fresh tissue. Briefly, avocado mesocarp tissue was homogenized on ice in homogenization buffer (200 mM sucrose, 150 mM KCl, 25 mM Tris/Mes [pH 7.0], supplemented with 0.5 mM PMSF). The homogenate was then filtered through Miracloth and centrifuged at 20,000*g* for 20 min, yielding three fractions: a floating layer, supernatant, and pellet. The floating layer and pellet were resuspended in homogenization buffer and centrifuged a second time, yielding fractions "top" and "pellet 1," respectively. The initial supernatant was recentrifuged at 20,000*g* ("supernatant 1") and then at 100,000*g*, resulting in fractions "supernatant 2" and "microsomal pellet." Extraction with an homogenization buffer of lower ionic strength (100 mM Mops-NaOH [pH 7.0], 300 mM sorbitol, 5 mM EDTA, 0.1% [w/v] BSA, 0.05% cysteine [22]) gave identical results (not shown).

RESULTS

Characterization of the *AVOe3* mRNA

RNA blot analysis revealed the presence of pAVOe3 homologous mRNA in ripe avocado fruit. Comparison with RNA standards gave a size of 1450 nt for this message in ripe fruit (Fig. 1a) and throughout ripening (not shown). Primer extension analysis with E318p, an oligodeoxynucleotide primer derived from the 5′-untranslated region of the pAVOe3 cDNA (21), localized the major transcriptional start site 63 nt from the 5′-end of the E318p primer (Fig. 1b) and, hence, 30 bp upstream from the 5′-end of the pAVOe3 cDNA clone (21). (The doublet at 62/63 nt is likely due to pausing of reverse transcriptase at the 5′-cap of the mRNA.) This implies an untranslated sequence of 73 nt 5′ to the presumed translation start site of the *AVOe3* message (21). Minor primer-extended products might result from premature termination of the extension due to secondary structure in the mRNA; alternatively, these products may represent the presence of variable transcription start sites or additional gene family members.

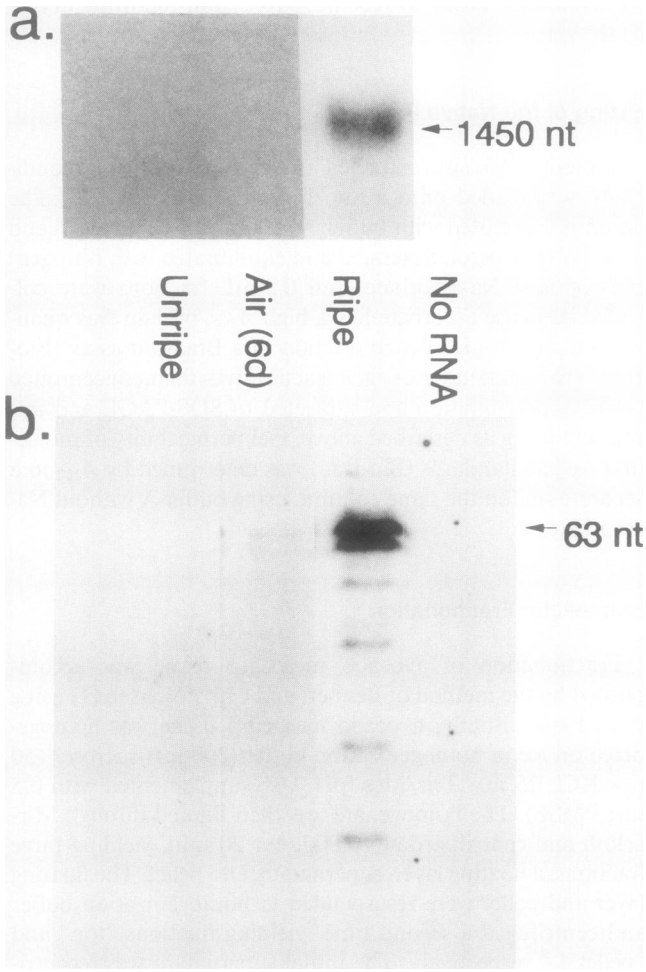

Figure 1. Characterization of the *AVOe3* mRNA. a, RNA blot analysis of total RNA (10 μg/lane) from freshly harvested fruit (Unripe), precli-macteric fruit kept for 6 d in air (Air [6d]), and fruit ripened in 500 μL/L propylene (Ripe). b, Primer extension of primer E318p using total RNA (7 μg/lane) from the same fruit as in a. As a control, one reaction was carried out with no RNA added to the hybridization (No RNA).

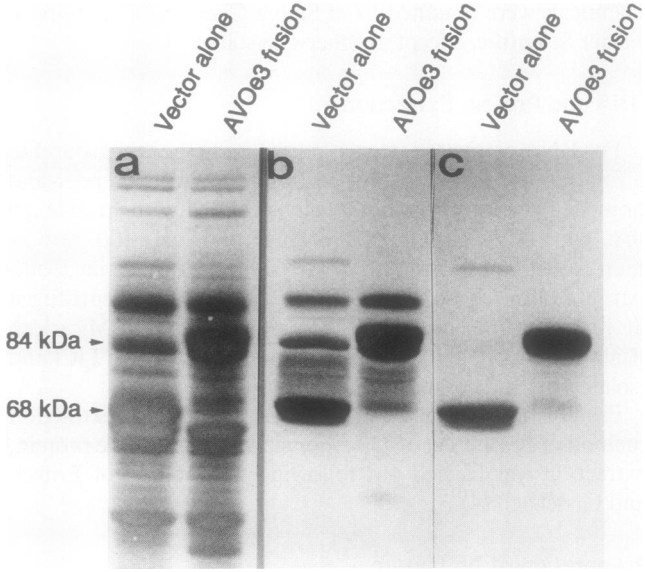

Figure 2. Characterization of antisera to AVOe3 polypeptide. a, Coomassie-stained gel of total protein from *E. coli* JM101 cells transformed with pWR590–0 (Vector alone) or the fusion construct (AVOe3 fusion). b, Immunoblot of the same samples, using the crude antiserum. c, Immunoblot of the same samples, using the affinity-purified antiserum.

Purification and Characterization of Antiserum

A fusion of the *lac-z* gene of *E. coli* and the 3′ 750 bp of the pAVOe3 cDNA, encoding 192 C-terminal amino acid residues, was expressed in *E. coli*. Expression of this AVOe3/β-gal fusion protein resulted in the appearance of a new polypeptide with a molecular mass of 84 kD, whereas the pWR590–0 vector alone expressed the truncated β-gal protein with a molecular mass of 68 kD (Fig. 2a). The 84-kD fusion protein was partially purified by SDS-PAGE and used to generate crude antiserum. This crude antiserum recognized the fusion protein and β-gal as well as a number of other polypeptides of similar molecular mass (Fig. 2b). After partial purification of the antiserum by affinity chromatography (see "Materials and Methods"), β-gal and the fusion protein were the major polypeptides of *E. coli* recognized (Fig. 2c). Immunoblots with commercially prepared polyclonal antisera to β-gal detected no cross-reacting antigen in avocado fruit (not shown). Hence, the partially purified antiserum should detect only AVOe3-related antigen in avocado fruit extracts.

Characterization of the AVOe3 Polypeptide

Immunoblots of avocado proteins were probed with the partially purified antiserum described above. An immunopositive polypeptide with a molecular mass of 34 kD was detected in extracts from ripe avocado fruit (Fig. 3). *In vitro* translation of total RNA selected by the pAVOe3 insert

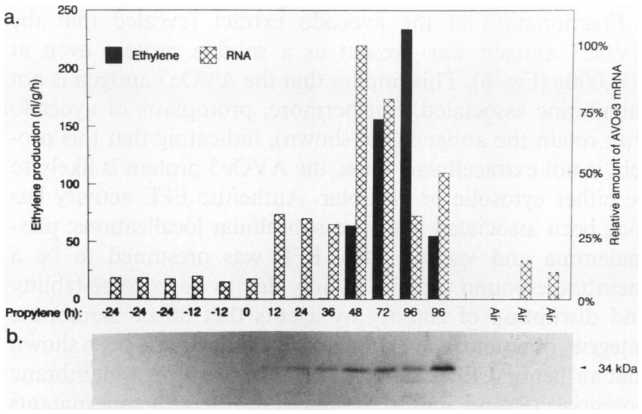

Figure 3. Characterization and expression of the *AVOe3* mRNA and antigen. a, Ethylene production and relative *AVOe3* mRNA levels in avocado fruit treated with 500 μL/L propylene. Propylene treatment was initiated 24 h after harvest. Each pair of bars represent data from a single fruit. Sixteen representative fruit were chosen from 40 initially assayed. b, Immunoblot analysis of total soluble protein (20 μg/lane) from the same fruit as in a.

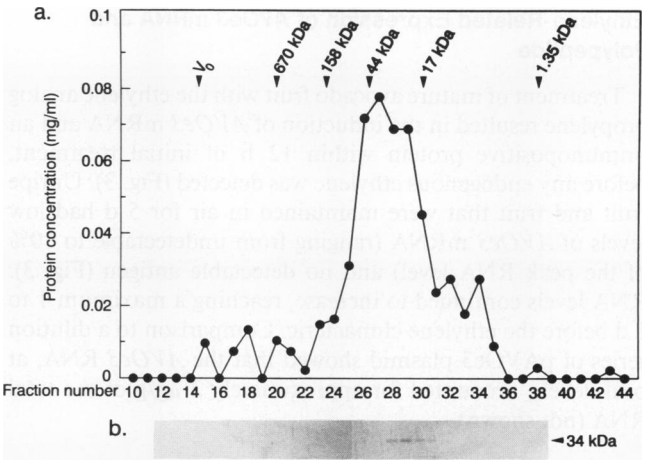

Figure 5. Superose 12 gel chromatography of the AVOe3 antigen. a, Protein elution profile for the Superose 12 column. Fractions of 0.5 mL each were collected, beginning immediately after injection of soluble protein extract from ripe avocado fruit. Elution profile of molecular weight standards (Bio-Rad) was determined from a separate run on the identical column. b, Immunoblot analysis of the corresponding fractions from the Superose 12 column.

yielded also a polypeptide with a molecular mass of 34 kD, which was present in ripe fruit but not in unripe (Fig. 4). These results are consistent with the molecular mass of 36,230 D predicted by the pAVOe3 cDNA-derived protein sequence (21). Attempts to immunoprecipitate the *in vitro* or *in vivo* translated polypeptide were unsuccessful (not shown). The antiserum, generated from the denatured AVOe3/β-gal fusion protein, presumably does not recognize the native AVOe3 protein.

Proteins were also extracted from avocado fruit under conditions that retain dimerization and activity of flavanone 3-hydroxylase (4), a protein with sequence similarity to the AVOe3 polypeptide. Gel filtration chromatography of this native protein extract resulted in elution of the immunopositive polypeptide with a molecular mass of approximately 30 kD (Fig. 5); thus, the AVOe3 protein behaved as a monomer under these extraction conditions.

Subcellular fractionation of the AVOe3 polypeptide was carried out by means of differential centrifugation of a protein extract. Extraction was done under nondenaturing conditions and a low ionic strength buffer to maintain possible protein-membrane interactions (1, 22). The AVOe3 polypeptide was soluble, remaining in the supernatant fraction even at 100,000*g* (Fig. 6). These results suggest that the AVOe3 polypeptide is not associated with cellular membranes or organelles.

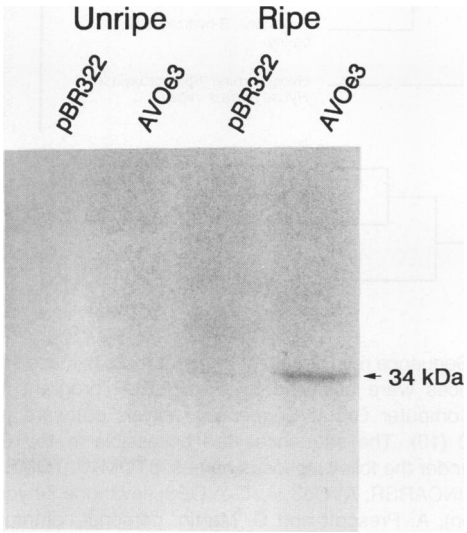

Figure 4. *In vitro* translation products of hybrid-selected RNAs. The pAVOe3 insert or linearized pBR322 was used to select from total RNA of ripe or unripe avocado fruit.

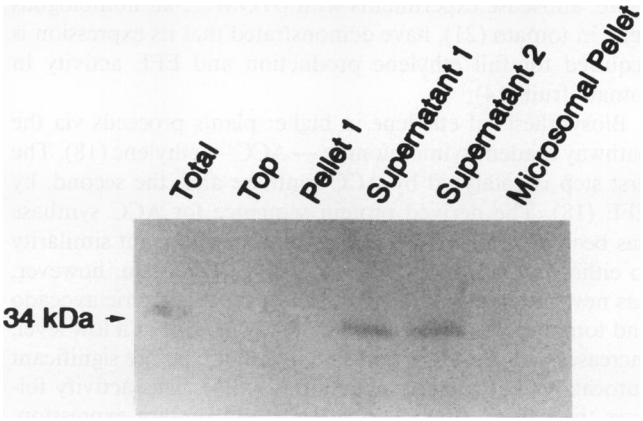

Figure 6. Immunoblot analysis of proteins from avocado fruit, fractionated by differential centrifugation. Protein fractions (20 μg/lane) of crude homogenate (Total); 20,000*g* floating layer (Top), pellet (Pellet 1), and supernatant (Supernatant 1); and 100,000*g* supernatant (Supernatant 2) and pellet (Microsomal Pellet).

Ethylene-Related Expression of *AVOe3* mRNA and Polypeptide

Treatment of mature avocado fruit with the ethylene analog propylene resulted in the induction of *AVOe3* mRNA and an immunopositive protein within 12 h of initial treatment, before any endogenous ethylene was detected (Fig. 3). Unripe fruit and fruit that were maintained in air for 5 d had low levels of *AVOe3* mRNA (ranging from undetectable to 10% of the peak RNA level) and no detectable antigen (Fig. 3). RNA levels continued to increase, reaching a maximum 1 to 2 d before the ethylene climacteric. Comparison to a dilution series of pAVOe3 plasmid showed that the *AVOe3* RNA, at peak levels, accounted for approximately 2 μg/g of the total RNA (not shown).

DISCUSSION

A number of specific mRNAs increase with ripening (6) or ethylene treatment (25) of mature avocado fruit. We have analyzed the expression of *AVOe3*, a ripening-related gene from avocado fruit, as part of our overall goal toward understanding function and regulation of ripening-related genes.

The *AVOe3* mRNA was present at varying low levels in freshly harvested fruit, although no antigen was detected (Fig. 3). This may be a reflection of the relative sensitivity of RNA dot blots and immunoblots or an indication of posttranscriptional regulation. When propylene was applied, mRNA and antigen levels increased substantially (Fig. 3). At its peak, the *AVOe3* mRNA accounted for approximately 2 μg/g of total RNA, a moderately abundant message. This level is also comparable to the mRNA levels of pTOM13, an homologous gene of ripe tomato fruit (16). Comparison of propylene- and air-treated fruit showed that induction of the *AVOe3* message and immunopositive protein was dependent upon propylene. The protein and mRNA, however, appeared before endogenous ethylene production (Fig. 3). This expression is consistent with the expected expression of an ethylene biosynthetic gene, *i.e.* induction by ethylene and expression preceding or concurrent with endogenous ethylene production. Furthermore, antisense experiments with pTOM13, an homologous gene in tomato (21), have demonstrated that its expression is required for full ethylene production and EFE activity in tomato fruit (14).

Biosynthesis of ethylene in higher plants proceeds via the pathway *S*-adenosylmethionine → ACC → ethylene (18). The first step is catalyzed by ACC synthase and, the second, by EFE (18). The derived protein sequence for ACC synthase has been determined (26) and bears no significant similarity to either pTOM13 or pAVOe3. The EFE protein, however, has never been characterized (18). In preclimacteric avocado and tomato fruit, EFE activity, already present at a low level, increases within 24 h of ethylene treatment, before significant autocatalytic ethylene production (20, 24). This activity follows the pattern of *AVOe3* mRNA and protein expression. EFE activity *in vivo*, however, decreases sharply after the climacteric ethylene peak (24); whereas the AVOe3 protein continues to accumulate (Fig. 3). EFE activity may be limited by physiological factors of the cellular environment in postclimacteric fruit.

Fractionation of the avocado extract revealed that the AVOe3 antigen was present as a soluble protein even at 100,000*g* (Fig. 6). This implies that the AVOe3 antigen is not membrane associated. Furthermore, protoplasts of avocado fruit retain the antigen (not shown), indicating that this protein is not extracellular. Thus, the AVOe3 protein is likely to be either cytosolic or vacuolar. Authentic EFE activity has long been associated with two subcellular localizations: plasmalemma and vacuole (18). EFE was presumed to be a membrane-bound protein, mainly due to its extreme lability and disruption of activity by agents that affect membrane integrity or potential (18). Recently, though, it has been shown that authentic EFE activity is not dependent on a membrane potential (13) and, indeed, is present in 100,000*g* supernatants (27; D.J. McGarvey and R.E. Christoffersen, manuscript in preparation). It is likely that EFE is indeed a soluble protein such as the AVOe3 antigen. Further localization of the AVOe3 antigen and its relationship to ethylene biosynthesis is at present under investigation.

AVOe3, pTOM13, and Dincarsr, a carnation senescence-related gene of unknown function, all show homology to one another (Fig. 7). As previously noted by Hamilton *et al.* (14), pTOM13 bears similarity to incolorata, encoding flavanone 3-hydroxylase of snapdragon (A. Prescott and C. Martin, personal communication). The AVOe3/pTOM13/Dincarsr family, in turn, shows sequence similarity to several plant 2-oxoglutarate-dependent dioxygenases, including hyoscyamine

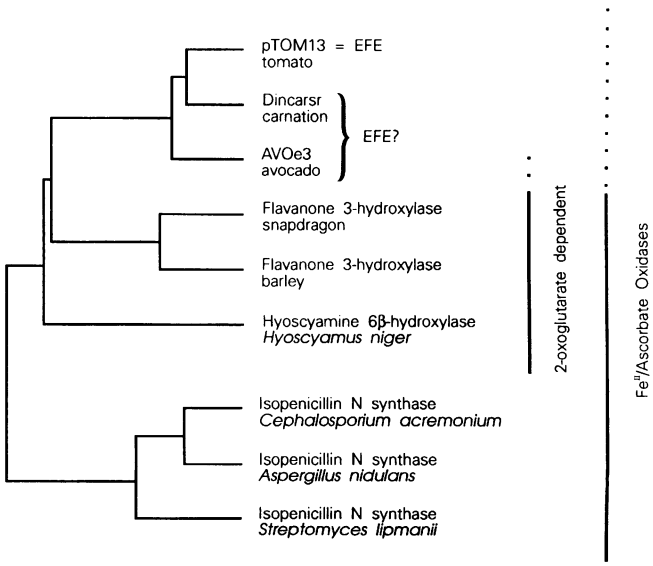

Figure 7. Sequence relationship of Fe^{II}/ascorbate oxidase superfamily. Sequences were analyzed by the PILEUP program from the Genetics Computer Group Sequence Analysis software package, version 7.0 (10). The sequences are accessible in the GenBank Database under the following locus names: pTOM13, TOMETHYBR; Dincarsr, DINCARSR; AVOe3, AVOAVOE3; flavanone 3-hydroxylase (snapdragon), A. Prescott and C. Martin, personal communication; flavanone 3-hydroxylase (barley), BLYFL3DOX; hyoscyamine 6β-hydroxylase, HYSH6H; isopenicillin *N*-synthase (*C. acremonium*), YSSIPSG; isopenicillin *N*-synthase (*A. nidulans*), ASNIPNS; isopenicillin *N*-synthase (*S. lipmanii*), STMIPNSA.

6β-hydroxylase of *Hyoscyamus niger* and flavanone 3-hydroxylase of snapdragon and barley (Fig. 7). These 2-oxoglutarate-dependent dioxygenases are part of a larger class of oxidases, all requiring Fe^{II} and a reductant, usually ascorbate, for catalytic activity. This larger class includes enzymes such as isopenicillin *N*-synthase that do not require 2-oxoglutarate. Isopenicillin *N*-synthase also bears sequence similarity to the AVOe3/pTOM13/Dincarsr family (Fig. 7). Aside from these Fe^{II}/ascorbate oxidases, no other sequences of known function in the GenBank database bear significant similarity to AVOe3. Figure 7 illustrates the sequence relationships of this Fe^{II}/ascorbate oxidase superfamily. Recent investigations (27; D. J McGarvey and R. E. Christoffersen, manuscript in preparation) have shown that EFE also belongs to the Fe^{II}/ascorbate class of oxidases. Thus, from its pattern of expression as well as sequence similarity with EFE-related enzymes, it appears likely that the AVOe3 polypeptide is indeed authentic EFE or a closely related enzyme.

Following submission of this paper, expression of pTOM13-related genes in *Xenopus* oocytes (P. Spanu, D. Reinhardt, T. Boller [1991] EMBO J **10:** 2007–2013) and in yeast (A. J. Hamilton, M. Bouzayen, and D. Grierson [1991] Proc Natl Acad Sci USA **88:** 7434–7437) was reported and has served to confirm the identity of these genes as EFE.

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