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Sequence analysis of ripening-related cytochrome P-450 cDNAs from avocado fruit

(cytochrome P-450 oxidase/fruit ripening/*Persea americana*/ethylene/gene families)

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ABSTRACT **The ripening of avocado fruit is associated with the expression of a number of mRNAs concomitant with overt changes in texture and flavor. Two overlapping cDNAs for a mRNA that accumulates during ripening were identified. Sequence analysis of these two cDNAs revealed a polypeptide of 471 amino acids with characteristics of a typical P-450: an N-terminal hydrophobic membrane anchor, a conserved heme-binding domain in the C-terminal region, and patches of similarity to various P-450 family members. Further evidence that this polypeptide represents a cytochrome P-450 oxidase comes from the recent isolation and characterization of a cytochrome P-450 from ripe avocado mesocarp [O'Keefe, D. P. & Leto, K. J. (1989) *Plant Physiol.* 89, 1141–1149]. The N terminus of the predicted polypeptide in the cDNAs is identical to the N terminus of the purified avocado P-450. Gel blot analysis of RNA from fruit at various stages of ripening showed the accumulation of an 1800-nucleotide P-450 mRNA that hybridized to the P-450 cDNA. The P-450 protein predicted by the avocado cDNA sequence shares <40% positional identity with any known P-450 gene family. We propose therefore that it be placed in a separate family, P450LXXI, and that the corresponding gene from avocado be named *cyp71A1*.**

The ripening of climacteric fruit is accomplished through a series of biochemical and physiological changes that lead to the softening of the fleshy tissues and the synthesis of secondary metabolites associated with flavor and aroma. Ethylene plays a major regulatory role in this developmental transition (1, 2). The ripening of avocado fruit is associated with changes in the pattern of gene expression in the mesocarp tissues (3, 4). Two mRNAs of avocado that accumulate during ripening have been characterized through analysis of cDNA clones. One mRNA encodes a cellulase (5, 6), while another encodes a polypeptide of unknown function (45) but is homologous to an ethylene-related mRNA of tomato fruit (7).

The mesocarp of ripe avocado fruit has a relatively high level of cytochrome P-450 enzyme activity (8, 9). Recently, a cytochrome P-450 polypeptide from this tissue was purified to homogeneity and 40 residues of the N-terminal sequence were determined by Edman degradation (10). To our knowledge, this partial sequence of a cytochrome P-450 is the first derived from plants. Cytochrome P-450 oxidases are widespread in nature and have been characterized in vertebrates, yeast, and bacteria (11, 12). Amino acid sequences of at least 71 P-450 genes, grouped in 14 different gene families, are currently available (13). However, information on plant cytochromes P-450 is still limited. Various cytochrome P-450-dependent enzyme activities in plants, typically involving hydroxylations of secondary metabolites such as terpenes, aromatics, and fatty acids, have been demonstrated (14). Any of these compounds could play a role in the flavor of ripe fruit (15). In addition, the metabolism of exogenous substrates, such as herbicides or pesticides, has been shown in some cases to be dependent on cytochrome P-450 oxidases (16).

In this paper, we report the sequence analysis of two overlapping cDNAs from ripe avocado fruit.§ From the open reading frame within these cDNAs we predict a polypeptide with an N-terminal sequence identical to that of the purified avocado P-450 described above. Comparison of this plant P-450 sequence with all known P-450 families (13) indicates that the avocado gene belongs to a separate family, which we propose be designated the P450LXXI family. Because cytochromes P-450 are often associated with plant secondary metabolism, we also propose that this enzyme is involved in the metabolism of compounds associated with the development of flavor in the ripening avocado fruit.

MATERIALS AND METHODS

cDNA Libraries and Colony Screening. Construction of the cDNA library from which pAVOd8 was isolated has been described (5). This truncated clone was identified by differential colony hybridization using probes derived from mRNAs of ripe and unripe fruit. The pAVOd8 cDNA was then used to identify an overlapping clone from another cDNA library, which was also made from mRNA of ripe fruit (6).

RNA Blot and Primer-Extension Analysis. Total RNA was isolated from mesocarp of avocado (*Persea americana* Mill. cv. Hass) fruits as described (17). Samples of total RNA (10 μg) were subjected to denaturing agarose gel electrophoresis followed by blot hybridization to radiolabeled pAVOd8 insert (18).

Total RNA from unripe and ripe fruit was analyzed by primer extension (19). A 17-base synthetic oligodeoxynucleotide complementary to the 5′ end of pAVOd8 (5′-GAGACACTAAGATAGCC-3′) was ^{32}P-labeled. For each reaction, 0.1 pmol of oligodeoxynucleotide was annealed at the indicated temperature to 5 μg of RNA, followed by extension with 10 units of reverse transcriptase.

DNA Sequencing. The cDNA inserts were sequenced as either M13mp18 subclones with Klenow enzyme (20) or pUC18 and pBluescript SK subclones by double-strand sequencing with the Sequenase enzyme (21) according to the manufacturer's instructions (United States Biochemical), with deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate (Amer-

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M32885).

sham). Deletions were made with *Exo* III and mung bean nucleases.

Protein Comparisons and Alignment. Pairwise comparisons of the avocado P-450 and representative members of 10 P-450 families were performed with GAP in the Genetics Computer Group (GCG) software package (22). A profile of representative members from 6 eukaryotic cytochrome P-450 families was generated by the GCG program PROFILE based on the alignment of Nelson and Strobel (23), with modifications in the C-terminal region (24). The predicted protein sequence of the avocado P-450 was then aligned with the profile of the eukaryotic cytochrome P-450 families with the GCG program PROFILEGAP. The locations of the four domains A, B, C, and D in the C-terminal region of the alignment are taken from Kalb and Loper (24).

RESULTS

cDNA Cloning and Characterization. A ripening-related cDNA clone, pAVOd8, was identified by differential colony hybridization (5). Preliminary characterization of the pAVOd8 cDNA indicated that it represented less than half the length of its corresponding mRNA. Accordingly, an overlapping cDNA, pAVOc8, was identified from an alternative cDNA library. These two cDNAs represent almost the entire length of the mRNA from which they were derived (Fig. 1).

The pAVOd8 and pAVOc8 cDNAs contain inserts of 844 and 1451 base pairs (bp), respectively, excluding the poly(A) tract of the pAVOc8 cDNA. Sequence analysis of pAVOd8 and pAVOc8 showed an overlap of 626 bp with no mismatches within the overlapping region of the two clones. In addition, the two cDNA clones hybridize to a contiguous segment of the avocado genome (K.R.B. and R.E.C., unpublished data). These data strongly suggest that the two cDNAs represent the same mRNA species. The orientation of the pAVOd8 insert with respect to the mRNA was confirmed by hybridizing cDNA probes derived from RNA of ripe fruit to both sense and antisense strands of pAVOd8 (data not shown). Thus, these two overlapping cDNAs encompass 1669 bp excluding the poly(A) tract at the 3′ end of the mRNA. The combined

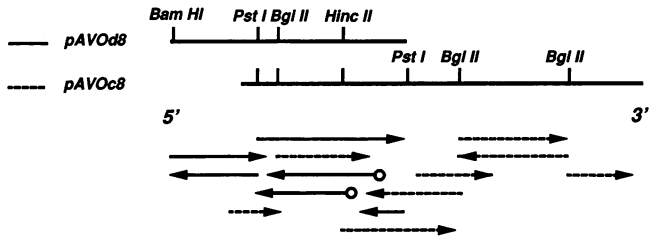

FIG. 1. Restriction maps and sequencing strategy of cDNA clones pAVOd8 and pAVOc8. Arrows indicate both the extent and direction of each sequencing reaction. Subclones generated by exonuclease III deletion are indicated by open circles.

sequence of the pAVOd8 and pAVOc8 clones (Fig. 2) contains an open reading frame that spans 471 amino acids and from which a protein of molecular weight 53,073 is predicted. The N-terminal residues of the predicted polypeptide are identical to the 40 amino acids previously determined by Edman degradation of a cytochrome P-450 protein (ARP-2) isolated from ripe avocado fruit (10).

Primer-extension analysis indicated that the P-450 mRNA extends 16 bases upstream from the 5′ end of pAVOd8 (Fig. 3). The sequence of these 16 bases was derived from a genomic clone of this gene (K.R.B. and R.E.C., unpublished data). We suspect that this 16-bp sequence is part of the mRNA sequence because of the presence of a TATA box 30 bases upstream from the tentative +1 position in the genomic DNA.

Ripening-Induced Accumulation of a Cytochrome P-450 mRNA. To correlate levels of P-450 mRNA expression with extent of ripening, total RNA was extracted from avocado fruit at different stages of ripening and was analyzed by RNA gel blot assay with pAVOd8 used as a probe. Endogenous ethylene synthesis was used as a physiological marker to indicate the response of individual fruit to propylene treatment and the precise ripening stage of each fruit (17). Fig. 4 shows that P-450 mRNA is undetectable in unripe fruit (0-day propylene treatment). As propylene-induced ripening progressed, P-450 mRNA began to accumulate within 24 hr and reached a maximum level in postclimacteric, fully ripe fruit

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                                                                                                                    120
TCACTCTAGAGTAATAATCCATGGCTATCTTAGTGTCTCTGCTCTTCTTAGCCATTGCTCTCACCTTCTTCCTCCTAAAACTCAACGAAAAACGAGAGAAGAAACCGAACCTACCCCCTT
                  M  A  I  L  V  S  L  L  F  L  A  I  A  L  T  F  F  L  L  K  L  N  E  K  R  E  K  K  P  N  L  P  P  S
                                                                                                                    240
CTCCTCCCAACCTTCCCATCATCGGAAACCTCCATCAGCTCGGTAATCTCCCACACCGCTCTCTTCGCTCCCTTGCAAACGAACTCGGACCCTTGATACTCCTCCATCTGGGTCACATCC
  P  P  N  L  P  I  I  G  N  L  H  Q  L  G  N  L  P  H  R  S  L  R  S  L  A  N  E  L  G  P  L  I  L  L  H  L  G  H  I  P
                                                                                                                    360
CCACTCTCATAGTCTCCACCGCTGAGATTGCCGAAGAGATCTTGAAAACCCATGATCTCATCTTTGCTAGCCGACCATCCACAACTGCAGCTCGACGAATCTTCTATGACTGCACCGACG
  T  L  I  V  S  T  A  E  I  A  E  E  I  L  K  T  H  D  L  I  F  A  S  R  P  S  T  T  A  A  R  R  I  F  Y  D  C  T  D  V
                                                                                                                    480
TGGCGTTCTCTCCCTACGGCGAATATTGGAGGCAAGTAAGGAAGATCTGTGTACTCGAGCTCCTTAGCATAAAGAGAGTCAACTCCTACCGTTCGATCAGGGAAGAAGAGGTGGGCCTCA
  A  F  S  P  Y  G  E  Y  W  R  Q  V  R  K  I  C  V  L  E  L  L  S  I  K  R  V  N  S  Y  R  S  I  R  E  E  E  V  G  L  M
                                                                                                                    600
TGATGGAGAGGATCTCTCAATCTTGCTCGACAGGTGAAGCTGTTAATCTATCAGAGCTGTTGCTATTGCTATCAAGCGGCACAATAACAAGGGTTGCTTTTGGGAAGAAGTACGAAGGAG
  M  E  R  I  S  Q  S  C  S  T  G  E  A  V  N  L  S  E  L  L  L  L  L  S  S  G  T  I  T  R  V  A  F  G  K  K  Y  E  G  E
                                                                                                                    720
AAGAAGAAAGGAAGAACAAGTTTGCGGATCTTGCAACTGAGTTGACAACTCTTATGGGAGCTTTCTTCGTGGGAGACTACTTTCCTTCGTTTGCATGGGTTGATGTTCTAACAGGGATGG
  E  E  R  K  N  K  F  A  D  L  A  T  E  L  T  T  L  M  G  A  F  F  V  G  D  Y  F  P  S  F  A  W  V  D  V  L  T  G  M  D
                                                                                                                    840
ATGCGAGGTTGAAGAGAAATCATGGTGAATTGGATGCTTTTGTGGATCACGTAATTGATGACCATCTCCTTAGTAGAAAAGCGAACGGCTCAGATGGAGTGGAGCAGAAAGATTTAGTGG
  A  R  L  K  R  N  H  G  E  L  D  A  F  V  D  H  V  I  D  D  H  L  L  S  R  K  A  N  G  S  D  G  V  E  Q  K  D  L  V  D
                                                                                                                    960
ATGTGCTGCTACATCTGCAGAAGGATTCCTCACTCGGCGTCCATCTCAACAGAAATAACCTTAAAGCCGTCATCTTGGACATGTTCTCTGGTGGAACTGATACGACGGCTGTGACCTTAG
  V  L  L  H  L  Q  K  D  S  S  L  G  V  H  L  N  R  N  N  L  K  A  V  I  L  D  M  F  S  G  G  T  D  T  T  A  V  T  L  E
                                                                                                                   1080
AATGGGCTATGGCAGAGCTTATAAAGCATCCCGATGTGATGGAGAAAGCCCAACAAGAGGTAAGAAGAGTTGTGGGGAAAAAAGCAAAGGTGGAAGAAGAAGATCTTCATCAGTTGCACT
  W  A  M  A  E  L  I  K  H  P  D  V  M  E  K  A  Q  Q  E  V  R  R  V  V  G  K  K  A  K  V  E  E  E  D  L  H  Q  L  H  Y
                                                                                                                   1200
ACTTAAAACTAATCATCAAAGAGACTCTGCGCTTGCATCCTGTTGCTCCATTATTAGTTCCACGCGAGTCCACAAGGGATGTAGTTATAAGGGGCTATCATATTCCTGCAAAGACAAGAG
  L  K  L  I  I  K  E  T  L  R  L  H  P  V  A  P  L  L  V  P  R  E  S  T  R  D  V  V  I  R  G  Y  H  I  P  A  K  T  R  V
                                                                                                                   1320
TCTTTATTAATGCATGGGCAATAGGAAGAGACCCCAAGTCATGGGAGAATGCTGAGGAATTTCTCCCAGAGAGATTTGTCAATAATTCTGTTGATTTCAAGGGGCAAGATTTTCAACTTA
  F  I  N  A  W  A  I  G  R  D  P  K  S  W  E  N  A  E  E  F  L  P  E  R  F  V  N  N  S  V  D  F  K  G  Q  D  F  Q  L  I
                                                                                                                   1440
TTCCTTTTGGAGCAGGGAGGAGGGGCTGTCCGGGGATTGCATTTGGCATCAGTTCAGTTGAGATTTCTCTTGCCAATCTCTTGTACTGGTTCAACTGGGAATTACCTGGGATCTAACCAA
  P  F  G  A  G  R  R  G  C  P  G  I  A  F  G  I  S  S  V  E  I  S  L  A  N  L  L  Y  W  F  N  W  E  L  P  G  I  *
                                                                                                                   1500
AGAAGATCTGGACACGCTCGAAGCCGTTGGGATAACCGTTCACATGAAGTTTCCTCTGCA.....
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FIG. 2. Nucleotide and deduced amino acid (single-letter code) sequence of the avocado cytochrome P-450 cDNAs. Sixteen nucleotides at the 5′ end were derived from a genomic DNA clone using a primer that was the complement of nucleotides 23–40. The underlined amino acids are identical to the N-terminal sequence of ARP-2, a cytochrome P-450 polypeptide isolated from avocado mesocarp.

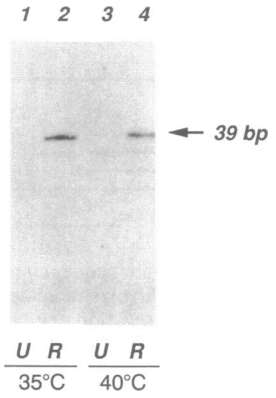

FIG. 3. Primer-extension analysis of a cytochrome P-450 mRNA from ripe fruit. The synthetic oligodeoxynucleotide primer was complementary to nucleotides 23–40 in Fig. 2. RNA from unripe fruit (U) (day 0 from Fig. 4) was used in lanes 1 and 3, whereas RNA from ripe fruit (R) (day 5 from Fig. 4) was used in lanes 2 and 4. The primer/RNA annealing temperatures were 35°C (t_m − 15°C) for lanes 1 and 2 or 40°C (t_m − 10°C) for lanes 3 and 4.

(5-day propylene treatment). At maximum, the P-450 mRNA was ≈10-fold less abundant (data not shown) than the mRNA for cellulase (5), which is estimated to be 0.013% of the total RNA mass in ripe fruit (46). The pAVOd8 probe detects a single RNA size class of 1800 bases, which agrees with the 1669-bp size of the two overlapping cDNA clones when the presence of a poly(A) tail is included.

Comparison of Avocado Cytochrome P-450 with Other P-450 Families. The avocado P-450 protein sequence was compared to representative members of 10 different P-450 families from outside the plant kingdom. After optimal pairwise alignment, the avocado P-450 sequence showed from 19.7% (CIA1, *P. putida* $P450_{cam}$) to 28.5% (XXIA1, bovine c21) positional identity to the different representative family members. Because the avocado P-450 shares <40% positional identity with any characterized member of the cytochrome P-450 superfamily, it belongs to another P-450 family. We propose that this family be named P450LXXI and this particular gene from avocado be designated *cyp71A1* following the nomenclature system of Nebert *et al.* (13).

The avocado P-450 protein sequence was aligned to six representative members of different cytochrome P-450 gene families (Fig. 5) using a method that was developed by Gribskov and Burgess (25) to detect similarities between distantly related proteins. Initially, a profile of six P-450 gene families was generated by using previously published alignments (23, 24). The avocado P-450 sequence was then aligned to this profile and a consensus sequence was generated. Of a total of 126 highly conserved positions among the different P-450 families, the avocado sequence was included in forming the consensus line at 103 positions (asterisks in Fig. 5). The majority of these positions are located in the C-terminal region of the proteins, beginning 275–310 amino acids from the N termini, and continuing for ≈170 residues.

The C-terminal region of cytochromes P-450 has been divided into four conserved domains designated A, B, C, and D (24), as shown in Fig. 5. Within the conserved domains of the C-terminal region, the avocado P-450 sequence was included in forming the consensus strand in 18/20, 8/9, 17/21, and 9/13 positions within domains A, B, C, and D, respectively. Included in domain D of the avocado sequence is a highly conserved peptide (FXXGXXXCXG), which serves as the thiolate ligand to the heme of all cytochromes P-450 (27).

DISCUSSION

We have determined the sequences of overlapping cDNA clones from avocado that encode a ripening-related cytochrome P-450 (P450LXXIA1). From the open reading frame, we predict a protein of molecular weight 53,073, a size typical of cytochrome P-450 oxidases. Recently, two related cytochrome P-450 polypeptides, ARP-1 and ARP-2, have been isolated from ripe avocado fruit (10). The first 40 residues of the avocado P450XXIA1 protein are identical to the N-terminal sequence of ARP-2. In addition, the ARP-2 polypeptide migrates on lithium dodecyl sulfate/PAGE at 48 kDa (10). Further indication that the P450LXXIA1 protein may be identical to ARP-2 comes from the similar amino acid compositions of the deduced P450LXXIA1 sequence (data not shown) and the ARP-2 polypeptide (10).

While the ripe avocado has high levels of cytochrome P-450 protein and activity (8–10), it has not been demonstrated that these levels change during ripening. We show here that the *cyp71A1* mRNA accumulates during ripening, increasing from undetectable levels in the preclimacteric fruit to become a moderately abundant mRNA in the ripe fruit. It remains to be determined whether the protein accumulates in a similar manner during ripening.

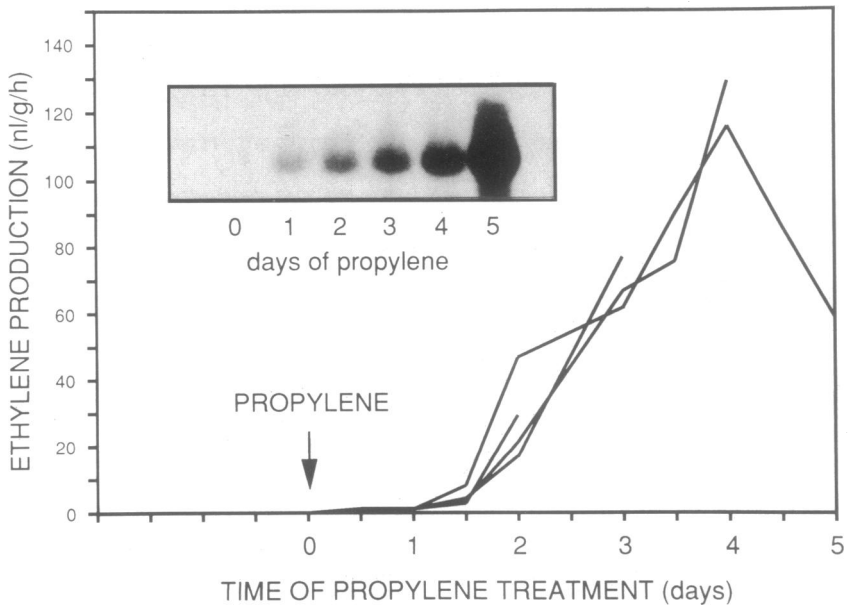

FIG. 4. Accumulation of P-450 mRNA and synthesis of endogenous ethylene during propylene-induced ripening. Freshly harvested avocado fruit were treated with propylene in air (500 μl/liter). During treatment, endogenous ethylene synthesis was monitored by gas chromatography. At the end of each treatment period, fruit were frozen in liquid nitrogen and stored at −70°C. (*Inset*) RNA was extracted from representative samples and analyzed by agarose RNA gel blot assay using pAVOd8 as a probe.

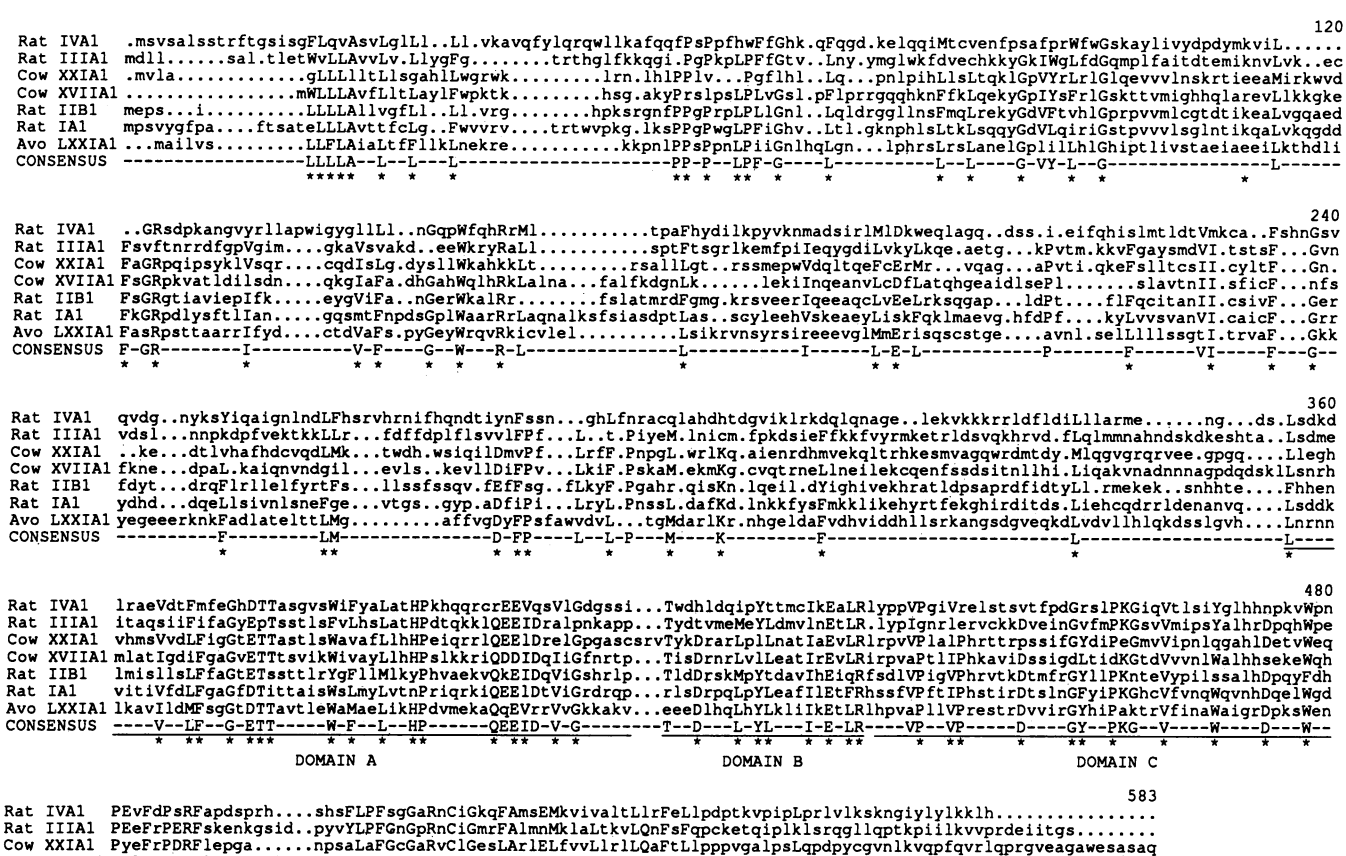

FIG. 5. Alignment of the avocado cytochrome P-450 with six vertebrate P-450 gene families. The consensus line was calculated including the avocado sequence with the six other families. Where a group of at least five of seven amino acids at any position had a threshold value of at least 1.0 in the normalized mutational difference matrix (25, 26), these highly conserved residues are shown in capital letters. The consensus residue was determined either by the most frequent conserved residue or, when no one residue was a majority, was chosen arbitrarily. Positions where the avocado P-450 sequence was included in calculating the consensus line are indicated by an asterisk. The domains A, B, C, and D are spanned by a horizontal line beneath the consensus sequence. Amino acids are designated by the single-letter code.

Examination of the sequence of the avocado cytochrome P-450 points to the many similarities between this plant P-450 and those previously characterized in animals. This includes a hydrophobic "anchor" at the N terminus and four conserved domains in the C-terminal region of the polypeptide, including a short sequence (FXXGXXXCXG), which is unique to P-450 proteins (28) and provides the thiolate ligand to the heme (27).

Evidence of structural similarity between putative plant P-450 polypeptides and both animal (P450IA1) and bacterial (P450CIA1) P-450 proteins has been obtained by using cross-reacting antisera (29, 30). Three of the four domains showing high conservation among eukaryotic P-450 families (Fig. 5) have been detected in the $P\text{-}450_{cam}$ (P450CLA1) sequence: domains A, C, and D (24). X-ray crystallographic studies of the $P\text{-}450_{cam}$ protein indicate that domains A and D are involved in forming the heme-binding pocket (31). The conservation of these C-terminal domains between plant and bacterial or animal P-450 proteins, as shown here, is likely to be the source of the cross-reacting epitopes.

Many members of the cytochrome P-450 superfamily are under strict developmental and hormonal regulation (12). In addition, exposure to various xenobiotic inducers can regulate P-450 gene expression at the transcriptional level (32). In higher plants, P-450 levels can be modulated by a wide variety of factors. Wounding (33), various chemical agents (34), and elicitor treatment (35, 36) will increase the levels of *trans*-cinnamic acid 4-hydroxylase, a P-450 enzyme of the phenylpropanoid biosynthetic pathway that converts *trans*-cinnamic acid to *p*-coumaric acid (37). We have shown that *cyp71A1* mRNA accumulates during avocado fruit ripening, implicating the phytohormone ethylene in the regulation of the *cyp71A1* gene.

The accumulation of P-450 mRNA during ripening points to a specific role that this gene may play in the ripening process. Unfortunately, the endogenous substrate of the avocado P450LXXIA1 protein has not been determined. The activity of the avocado P-450 was characterized as a *N*-demethylase using an artificial substrate, *p*-chloro-*N*-methylaniline (9, 10). One possible function of this P-450 may be related to the metabolism of compounds that contribute to the characteristic flavor of ripe avocado fruit. The specific hydroxylation pattern of metabolites can dramatically alter their taste (14, 38) and during ripening, avocado mesocarp undergoes a change in flavor and texture (39).

The ripe avocado fruit is known to contain a large number of different phenolic compounds such as epicatechin, *p*-coumaric acid, and isoflavone, which are the most prominent species, as well as other phenolic derivatives of the phenylpropanoid pathway (40, 41). These contribute to the browning of overripe fruit when acted upon by polyphenol oxidase. The avocado P-450 described here could be involved in phenolic metabolism during ripening, possibly by acting as *trans*-cinnamic acid 4-hydroxylase. This hypothesis is sup-

ported by the observation that *trans*-cinnamic acid 4-hydroxylase activity has been detected in microsomal preparations of ripe avocado mesocarp (42). Alternatively, because the total phenolic content of avocado fruit does not appreciably change during ripening (41), the primary role of this P-450 may be interconversion of preexisting phenolics rather than *de novo* synthesis through the phenylpropanoid pathway.

Ripening of climacteric fruit is usually associated with an increase in oxygen consumption (43). The majority of this increase in oxygen utilization has been assumed to be mitochondrial in origin even though precise measurements are difficult in bulky tissues such as fruit. It is possible that a portion of the respiratory climacteric results from an increase in microsomal oxygen consumption and that the ripening-induced cytochrome P-450 oxidase described here may participate in this respiratory burst. The observation that nonmitochondrial oxygen consumption (residual respiration) increases from 22% in slices of unripe avocado fruit to 47% in slices of ripe fruit (44) supports this hypothesis.

In summary, we have characterized cDNA clones for a ripening-induced cytochrome P-450 mRNA from avocado fruit. The polypeptide predicted within these cDNAs has features that are typical of P-450 proteins from the animal kingdom. Further study of this plant P-450 should lead to a better understanding of its physiological role in ripening and how ethylene may regulate its expression.

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