

# Expression of a Ripening-Related Avocado (*Persea americana*) Cytochrome P450 in Yeast<sup>1</sup>

Kristin R. Bozak, Daniel P. O'Keefe, and Rolf E. Christoffersen\*

Department of Biological Sciences, University of California, Santa Barbara, California 93106 (K.R.B., R.E.C); and  
Central Research, The Dupont Company, Experimental Station, Wilmington, Delaware 19880-0402 (D.P.O.)

## ABSTRACT

One of the mRNAs that accumulates during the ripening of avocado (*Persea americana* Mill. cv Hass) has been previously identified as a cytochrome P450 (P450) monooxygenase and the corresponding gene designated *CYP71A1*. In this report we demonstrate that during ripening the accumulation of antigenically detected *CYP71A1* gene product (CYP71A1) correlates with increases in total P450 and two P450-dependent enzyme activities: *para*-chloro-*N*-methylaniline demethylase, and *trans*-cinnamic acid hydroxylase (tCAH). To determine whether both of these activities are derived from CYP71A1, we have expressed this protein in yeast (*Saccharomyces cerevisiae*) using a galactose-inducible yeast promoter. Following induction, the microsomal fraction of transformed yeast cells undergoes a large increase in P450 level, attributable almost exclusively to the plant CYP71A1 protein. These membranes exhibit NADPH-dependent *para*-chloro-*N*-methylaniline demethylase activity at a rate comparable to that in avocado microsomes but have no detectable tCAH. These results demonstrate both that the CYP71A1 protein is not a tCAH and that a plant P450 is fully functional upon heterologous expression in yeast. These findings also indicate that the heterologous P450 protein can interact with the yeast NADPH:P450 reductase to produce a functional complex.

P450<sup>2</sup>-dependent monooxygenases have been studied extensively in animals, bacteria, yeast, and fungi. Many individual members of the P450 gene superfamily, mostly from animals, have been characterized with regard to primary sequence and substrate specificity (10, 13, 17). In contrast, information concerning P450 from plant sources is still relatively limited (reviewed in refs. 8 and 19), and a published sequence is available for only one (3). In plants, these enzymes are thought to be essential in the biosynthesis of phenolics, membrane sterols, phytoalexins, and terpenoids, as well as in the metabolism of various xenobiotics (19, 26).

Microsomes from ripe avocado (*Persea americana* Mill.) fruit mesocarp have relatively high levels of P450-dependent enzyme activities including *p*CMA *N*-demethylase (5, 7, 15, 18) and tCAH (21). P450 protein isolated from this tissue has been shown to retain *p*CMA demethylase activity (18) and

under denaturing conditions can be resolved into two very similar polypeptides (ARP-1 and ARP-2). The N-terminal sequences of ARP-1 and ARP-2 are identical except that ARP-1 lacks an N-terminal Met, suggesting that these two polypeptides may arise from the same precursor through posttranslational modification. Although the P450 protein was only characterized in ripe fruit, several mRNAs that accumulate in ripening avocado mesocarp have been studied (4). Sequence analysis of a cDNA clone of one of these ripening-induced genes, *CYP71A1* (3), revealed a predicted protein sequence (CYP71A1) that exactly matches the N-terminal 40 amino acids of ARP-1 and ARP-2, suggesting that one or both are the mature *CYP71A1* gene product. What remained to be determined was whether the protein and associated activity also accumulated during ripening and whether the CYP71A1 substrate specificity matched that of the purified protein.

Because it has often been difficult to purify to homogeneity individual P450 isozymes, the ability to express heterologous P450 cDNAs in yeast has been an important tool in connecting gene family members with specific enzyme activities (24, 27), although this previously has not been accomplished with any plant P450. To determine the enzyme activity specifically associated with the CYP71A1 protein, we have expressed the *CYP71A1* cDNA in yeast cells and shown that the P450 gene product can be detected both antigenically and by its characteristic spectroscopic properties. In addition, we demonstrate that of the two P450-dependent enzyme activities that increase with ripening, *p*CMA demethylase and tCAH, yeast cells containing the CYP71A1 protein exhibit only *p*CMA demethylase activity.

## MATERIALS AND METHODS

### Plant Material and Membrane Preparation

Mature avocado fruit (*Persea americana* Mill. cv Hass) were used in all experiments. Fruit were harvested from a local tree and immediately placed in glass jars at 20°C with a continuous flow of water-saturated air at approximately 50 mL/min. After 24 h of air treatment, ripening was stimulated by continuous exposure to 500  $\mu$ L/L of propylene, an ethylene analog. In some experiments, fruit were allowed to ripen naturally without propylene treatment. Endogenous ethylene production was monitored at 12-h intervals by GC. Two fruit were removed each day, and the mesocarp tissue was cut into pieces, frozen immediately in liquid N<sub>2</sub>, and stored at -70°C. Microsomal fractions were prepared from frozen

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<sup>2</sup> Abbreviations: P450, Cyt P450; *p*CMA, *para*-chloro-*N*-methylaniline; tCAH, *trans*-cinnamic acid hydroxylase; SGLu, 2% (w/v) glucose (repressing conditions); SGal, 2% (w/v) galactose (inducing conditions).

tissue samples as previously described (19). The final microsomal fractions were resuspended in 0.1 M Mops (pH 7.0), 50% (v/v) glycerol, frozen in liquid N<sub>2</sub>, and stored at -70°C.

### Protein Analyses and Assays

Protein concentrations were determined by the Bio-Rad protein assay. P450 concentration was estimated from the carbon monoxide difference spectrum of dithionite-reduced microsomes on a Johnson Foundation SDB-3A scanning dual wavelength spectrophotometer, using a difference extinction coefficient of 91 cm<sup>-1</sup> mM<sup>-1</sup> (20).

Microsomal proteins from fruit or yeast were subjected to immunoblot analysis following separation by SDS-PAGE on a 10% acrylamide gel (14). After electrophoresis, proteins were transferred to nitrocellulose using transfer conditions as described by Towbin et al. (25). Blocking and antibody incubations were done in Tris-buffered saline containing 1.5% (w/v) BSA. The primary antiserum to avocado P450 (18) was used at a 1:2000 dilution for both the avocado and yeast immunoblots. Following incubation with peroxidase conjugated goat anti-rabbit immunoglobulin G (Bio-Rad), color was developed with the substrate 4-chloro-1-naphthol (Bio-Rad) for analysis of avocado microsomes. For analysis of the yeast microsomes, a color development system using both diaminobenzidine (Sigma) and 4-chloro-1-naphthol (28) was used, which increased the signal from the peroxidase-conjugated second antibody.

The demethylation of pCMA was assayed fluorimetrically as previously described (22) using a 15-min incubation at room temperature. Protein (300 µg) was used in each assay for the analysis of avocado microsomes, and 1.1 mg was utilized for yeast analysis. The amount of product formed was determined by comparison of the fluorescence to that of *para*-chloroaniline standards.

To measure tCAH activity, either 250 µg of microsomal protein (avocado membranes) or 1.1 mg (yeast membranes) was added to 1 mL of 0.1 M Mops (7.0) containing 200 µM *trans*-cinnamic acid and 500 µM NADPH and incubated at room temperature. After 15 min, 20 µL of concentrated hydrochloric acid was added to stop the reaction, *ortho*-coumaric acid was added as an internal standard, and the products were extracted into ethyl acetate. After excess solvent was evaporated, the extract was analyzed by HPLC on a Zorbax ODS column (0.45 × 8 cm) using a linear gradient of acetonitrile in water (20–75% over 10 min, 1.5 mL/min, both solvents containing 1% H<sub>3</sub>PO<sub>4</sub>). The retention times of *ortho*-coumaric acid, *para*-coumaric acid, and *trans*-cinnamic acid were determined to be 4.1, 5.2, and 6.9 min, respectively, from the chromatographic behavior of reference standards detected by their A<sub>280</sub>. As isolated here, avocado microsomes contain considerable *para*-coumaric acid, and rates of tCAH were determined after subtraction of this background amount.

### Bacterial and Yeast Strains, Vectors, and Media

Plasmids were maintained in the *Escherichia coli* strain DH-5α. The yeast expression vector pYES1.2 was purchased from Invitrogen (San Diego, CA). The protease-deficient yeast

strain BJ5465 (a; *ura3-52, trp1, leu2Δ1, his3Δ 200, pep4::HIS3, prblΔ1.6R, can1, GAL*) was from the Yeast Genetic Stock Center. Nontransformed yeast were grown in 1% (w/v) yeast extract, 2% (w/v) bacto-peptone, 2% (w/v) glucose. pYES-transformed yeast were selected by growth in synthetic media (0.67% [w/v] nitrogen base for yeast without amino acids, 0.5% [w/v] casamino acids, 0.003% [w/v] leucine, 0.002% [w/v] tryptophan, 0.002% [w/v] adenine). The carbon source was either SGal (inducing conditions) or SGlu (repressing conditions).

### Construction of pYEScyp71

A complete P450 cDNA was generated by ligation of two overlapping cDNAs of the avocado P450 gene *CYP71A1* (3) at their shared *SacI* site and insertion into the polylinker region of pBluescript SK+ to yield pSKcyp71A1. An *XbaI* site at the 5' end of the cDNA was filled in with Klenow enzyme to create a blunt end for ligation with the unique *SspI* site in the polylinker of pYES1.2. At the 3' end of the cDNA in pSKcyp71A1, digestion with *KpnI* yielded a compatible end for ligation into the unique *KpnI* site in pYES1.2. A 32-bp fragment carrying an AUG initiation codon located within the polylinker of pYES1.2 was removed by partial digestion with *SpeI*, selection of the correct size band from an agarose gel, and recircularization. The resulting plasmid contains a transcription fusion 94 bases from the +1 site of the *GAL1* promoter. Between the transcription fusion and the avocado P450 AUG initiation codon are also 6 bp of the avocado 5'-untranslated leader.

### Expression of *CYP71A1* in Yeast Cells

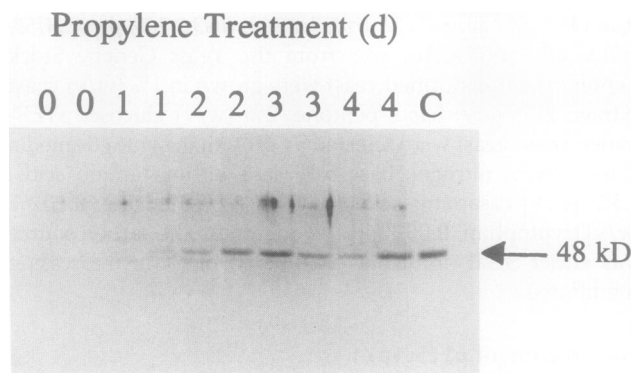
The transformation of yeast cells was accomplished by lithium chloride treatment. A culture of yeast was grown to an A<sub>660</sub> of 0.5 in 1% (w/v) yeast extract, 2% (w/v) bacto-peptone, and 2% (w/v) glucose. The cells were collected, resuspended in 0.1 M lithium chloride, and gently agitated at 30°C for 1 h. An aliquot of cells was then removed and incubated for 30 min with 2 µg of plasmid DNA and 50 µg of denatured calf thymus DNA in 0.1 M LiCl and 40% (w/v) PEG. Transformed cells were then spread onto SGlu plates for selection of URA+ transformants.

The large-scale isolation of yeast microsomal proteins began with inoculation of 1-L cultures of SGal or SGlu with transformed yeast cells (initial A<sub>660</sub> of approximately 0.2). After 40 h of growth at 30°C with vigorous shaking, cells were harvested, and microsomes were prepared according to the method of Yasumori et al. (27).

## RESULTS

### Increases in P450 and Associated Enzyme Activities with Avocado Ripening

To establish whether the amount of P450 protein increases with ripening, we examined microsomal fractions from avocado fruit at various stages of ripening for P450 antigen. The microsomal fraction from unripe fruit contains no detectable 48-kD P450 antigen (Fig. 1, 0 day propylene treatment), although some other weakly cross-reacting antigens are pres-



**Figure 1.** Immunoblot analysis of ripening avocado P450 antigen. Avocado fruit were ripened by exposure to 500  $\mu\text{L/L}$  of propylene for the times indicated. Thirty milligrams of microsomal protein from each fruit was loaded in each lane and resolved by SDS-PAGE. Lane C contains 320 ng of purified avocado P450 (18) as a positive control. Molecular mass markers (Sigma, high mol wt markers) were used to estimate the molecular mass of antigen.

ent. Because the microsomes from unripe fruit have no spectrally detectable P450 (see below), it is unlikely that these antigens are P450 proteins. A 48-kD antigen comigrating with purified native avocado P450 begins to accumulate within 24 h of propylene (an ethylene analog) treatment and steadily increases throughout the 4 d of propylene-induced ripening (Fig. 1). Upon longer electrophoretic separation, this single band resolves into two bands (data not shown) that represent the two avocado P450 polypeptides (ARP-1 and ARP-2) previously reported (18).

Microsomal fractions derived from unripe and ripe avocado tissue were also analyzed spectrophotometrically for total P450 (Table I). The level of P450 increases from undetectable in the unripe fruit to a value in ripe fruit that is comparable to that observed in tulip bulbs (21) and wounded Jerusalem artichoke tubers (2). This increase in total P450 during ripening correlates with the accumulation of 48-kD antigen shown in Figure 1. These data support the hypothesis that

**Table I.** P450 Content and Associated Enzyme Activities in Unripe and Ripe Avocado

P450, pCMA demethylation, and tCAH were determined as described in "Materials and Methods." The rates shown for unripe tissue are from a fruit frozen 24 h after detachment from the tree and not treated with propylene (no detectable ethylene production). The ripe avocado was allowed to ripen without propylene treatment for 20 d and was 2 d past the peak in ethylene production.

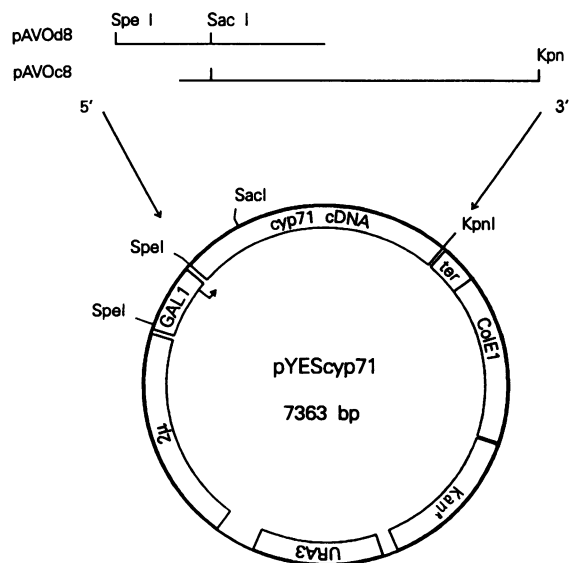
Assays	Microsomes from Avocado Fruit at Two Stages of Ripening	
	Unripe	Ripe
P450 (pmol/mg of protein)	<3	210
<i>para</i> -Chloro- <i>N</i> -methylaniline demethylase (pmol min <sup>-1</sup> mg <sup>-1</sup> )	55	603
tCAH (pmol min <sup>-1</sup> mg <sup>-1</sup> )	<25	173

the ARP-1 and ARP-2 polypeptides are the major P450 polypeptides present in ripe avocado microsomes (18). Furthermore, these increases in P450 antigen and chromophore parallel the previously reported increase in *CYP71A1* mRNA during ripening in avocado (3).

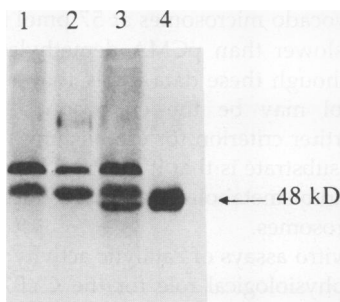
To determine what P450-dependent enzyme activities increase concomitantly with the increase in P450 antigen and chromophore, pCMA demethylase and tCAH were measured in microsomes from both unripe and ripe fruit (Table I). Both of these enzyme activities have been previously reported in ripe avocado fruit (18, 21). The pCMA demethylase increased 11-fold, whereas the tCAH activity increased at least 6-fold (Table I). Thus, the level of P450-dependent activity detected in a particular sample can vary by more than an order of magnitude depending on the precise stage of ripening of the fruit from which it is derived. Analysis of pCMA and tCAH rates in fruit throughout propylene-induced ripening confirmed this (data not shown), by the steady increase of activities throughout ripening. Previous studies in which avocado tissue was used have not correlated P450 levels or activities with the precise stage of ripening. The increase in total P450 and associated enzyme activities during ripening may explain the differing absolute values for P450-dependent activities previously reported.

#### Expression of *CYP71A1* in Yeast Cells

To separate *CYP71A1* from other potential P450 species in avocado tissue, the P450 cDNA was expressed in yeast using a nonintegrating plasmid that carries a galactose-inducible promoter and yeast terminator sequence (Fig. 2). A previous report indicated that heterologous expression of P450 protein in yeast is optimal when the AUG initiation codon is as close as possible to the +1 site of transcription



**Figure 2.** The construction of pYEScyp71A1. The avocado P450 cDNA was placed under control of the *GAL1* promoter in the yeast expression vector pYES1.2. Details of the construction are described in "Materials and Methods."



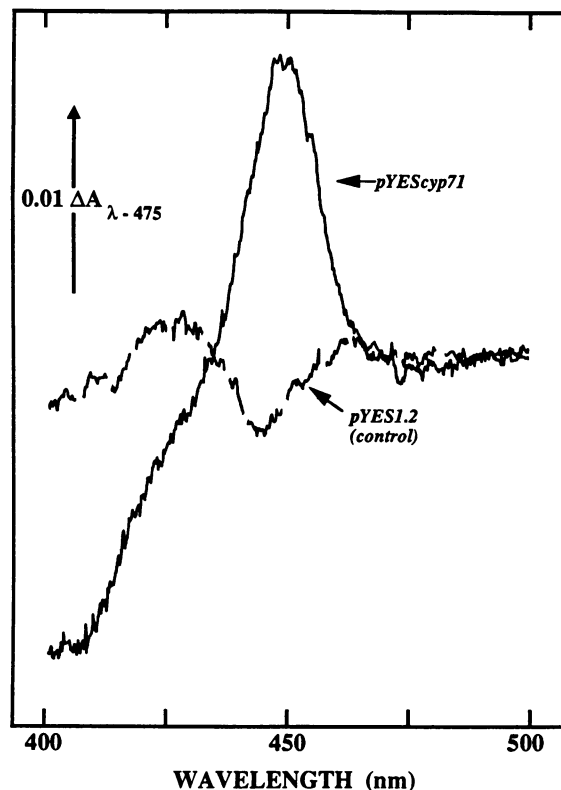
**Figure 3.** Immunoblot analysis of yeast microsomal proteins. In each lane 60 mg of yeast microsomal protein was electrophoretically separated using SDS-PAGE, transferred to nitrocellulose, and incubated with antibodies to the purified avocado P450. Lane 1 shows microsomal proteins from SGal-grown yeast carrying vector pYES1.2. Lanes 2 and 3 are microsomal proteins from yeast carrying pYEScyp71 grown in SGlu and SGal, respectively. Purified native avocado P450 (240 ng) (lane 4) was used as a positive control.

(6). Therefore, we engineered pYEScyp71 so that the cDNA AUG is immediately downstream of the *GAL1* untranslated leader, removing all pYES1.2 and pBluescriptSK+ polylinker DNA, as well as a portion of the 5'-untranslated *GAL1* leader.

Placing the *CYP71A1* cDNA under control of the *GAL1* promoter in yeast allowed for galactose-inducible expression of a 48-kD P450 antigen that comigrates with purified avocado P450 (Fig. 3). Induction of this antigen was repressed by glucose, indicating that P450 is under control of the *GAL1* promoter and, therefore, derived from *CYP71A1*. Two other polypeptides that cross-react with the antisera are seen in all lanes (Fig. 3), although there is no spectrally detectable P450 in the yeast strain without P450 cDNA insert (Fig. 4), and these antigens do not respond to galactose. Microsomal fractions from yeast transformed with pYEScyp71 exhibited a characteristic P450 spectrum (Fig. 4). The specific P450 content (0.05 nmol of P450/mg of protein) of microsomes from these protease-deficient cells carrying pYEScyp71 was nearly 5-fold higher than the highest levels observed in a yeast strain that was not protease deficient. The observation of native P450 absorption spectra in pYEScyp71A1-transformed yeast suggests that at least some of the *CYP71A1* is assembled in its native conformation. The microsomal difference spectrum of the galactose-grown yeast culture carrying only the pYES1.2 vector demonstrates that the endogenous P450 level in this strain of yeast grown under these conditions is very low, which implies that the predominant P450 in the transformed cells is *CYP71A1*.

#### Resolution of pCMA Demethylase and tCAH Activities

To determine whether the pCMA demethylase or tCAH activity in ripe avocado microsomes is derived from *CYP71A1*, the yeast microsomes were assayed for both of these activities (Table II). Microsomes derived from cells carrying pYES1.2 showed neither of these enzyme activities. However, the membranes from yeast cells carrying pYEScyp71 had a significant level of pCMA demethylase activity but no detectable tCAH (Table II). This demonstrates that the *CYP71A1* protein is the previously characterized



**Figure 4.** Dithionite-reduced carbon monoxide difference spectra of yeast microsomes.

pCMA demethylase P450 from avocado fruit membranes (5, 7, 15, 18). Previous results with other P450s have demonstrated that when these proteins are expressed in yeast they retain their substrate specificity (13, 24, 27); therefore, it is unlikely that the yeast-expressed *CYP71A1* would still metabolize pCMA and yet be specifically inactivated for *trans*-cinnamic acid hydroxylation.

Additional experimental evidence with the P450 from ripe avocado suggests that the tCAH activity is not a function of the major P450. Enzymic turnover of nearly 10 times that supported by NADPH can be obtained using pCMA and cumene hydroperoxide as an oxygen donor in avocado microsomes, and this activity is retained in the purified protein (18); however, no detectable tCAH activity can be observed with cumene hydroperoxide either in microsomes or with the purified P450. Although pCMA exhibits type I-binding spectra with both the microsomal and purified P450 (18), none has been observed with *trans*-cinnamate at concentrations of

**Table II.** Enzyme Activities of Yeast Microsomal Fractions

Rates of pCMA demethylase and tCAH were measured as described in "Materials and Methods."

Enzyme Assay	pYES1.2	pYEScyp71
	<i>pmol min<sup>-1</sup> mg<sup>-1</sup></i>	
<i>para</i> -Chloro- <i>N</i> -demethylase	10	275
tCAH	<1	<1

up to 1 mM. In contrast, a pronounced type I spectrum is observed upon addition of *trans*-cinnamate to microsomes from wounded Jerusalem artichoke tubers, in which tCAH is the predominant P450 and is antigenically related to the same enzyme in avocado (9). These results suggest that in microsomes from ripe avocado mesocarp tCAH is being carried out by a different P450 than that which binds and metabolizes pCMA. Taken together with the yeast expression of CYP71A1, we conclude that the CYP71A1 is not capable of *trans*-cinnamate hydroxylation and the ripening-induced tCAH activity is due to another P450 present in much lower abundance than CYP71A1. Because the polypeptides ARP-1 and ARP-2 have always been observed in nearly equal abundance, it is unlikely that either of these proteins is responsible for the tCAH activity.

### DISCUSSION

Avocado fruit microsomes display a variety of P450-dependent enzyme activities (5, 7, 11, 15, 18). We have shown that two of these activities, pCMA demethylase and tCAH, increase with ripening along with spectrally and antigenically detected P450 levels. Utilizing a cDNA clone of the ripening-related gene *CYP71A1*, we have shown that CYP71A1 protein expressed in a heterologous yeast system displays only one of these activities, pCMA demethylase.

In yeast microsomes containing CYP71A1, the maximum NADPH-dependent enzymic turnover number for pCMA is  $5.5 \text{ min}^{-1}$ . This compares with turnover numbers of  $3 \text{ min}^{-1}$  (Table I) and  $8 \text{ min}^{-1}$  (18) measured previously with microsomes from tissue that was several days past climacteric. In avocado microsomes, the pCMA turnover number appears to be limited by electron transfer from NADPH (18), and therefore, the turnover number in transformed yeast probably reflects a similar limitation in the rate of P450 reduction by a yeast NADPH:P450 oxidoreductase. Because there is no detectable endogenous yeast P450 in these membranes (Fig. 4), it is likely that the level of oxidoreductase is not very high, and therefore, it is possible that increasing levels of expression of this protein in the transformed yeast could lead to higher P450-dependent activities. Indeed, yeast cells containing a translational fusion of rat P450c and NADPH-P450 reductase show 4-fold higher levels of monooxygenase activity than yeast cells expressing only rat P450c (16).

Because pCMA is an artificial substrate of P450-related activity, the *in vivo* substrate of CYP71A1 remains to be determined. A simple way of screening potential substrates of a P450 is to identify compounds that produce a type I substrate-binding spectrum (12). Various compounds have been assayed for type I binding to avocado microsomal fractions, with lauric acid, *N*-methyl-substituted anilines, xylenes, and alkylbenzenes giving a positive result (5), although the rate of substituted aniline (including pCMA) demethylation exceeds that of any other substrate. Based on binding studies of monoterpenes to avocado P450, Hallahan et al. (11) recently proposed that the monoterpene nerol, or a closely related compound, is the physiological substrate for the major avocado P450. This was based partly on the low apparent dissociation constant for nerol of  $7.2 \mu\text{M}$ , compared to  $180 \mu\text{M}$  for pCMA (18). However, the hydroxylation of

nerol by ripe avocado microsomes at  $57 \text{ pmol mg}^{-1} \text{ min}^{-1}$  is about 10-fold slower than pCMA demethylation (Table I, ripe tissue). Although these data imply that a monoterpene related to nerol may be the endogenous substrate for CYP71A1, a further criterion for establishing the identity of a physiological substrate is that it must be found in ripening avocado fruit and be metabolized at least as rapidly as pCMA in avocado microsomes.

Although *in vitro* assays of catalytic activity are indicative of a potential physiological role for the CYP71A1 protein, these must be correlated with the actual metabolic changes that occur *in vivo* during avocado fruit ripening. The precise metabolite changes that occur have not been well characterized, although the essential oil from ripe avocado was found to contain six volatiles of unknown chemical composition (1). In general, this fraction is an important component of flavor and aroma in plants and contains various terpenes, alcohols, and esters. In an effort to determine the *in vivo* substrate of CYP71A1, we are currently examining ripening-related changes in the avocado essential oil fraction to identify potential CYP71A1 substrates.

Because the catalytic activity of two P450s that are 97% homologous can differ markedly (23), yeast expression of CYP71A1 does not specifically address the relation between CYP71A1 and ARP-1/ARP-2. The CYP71A1 expressed in yeast is approximately the same mol wt and should have the same N-terminal sequence as ARP-2, but the overall similarity of ARP-1 and ARP-2 and the potential for difference in posttranslational processing in avocado and yeast make the interpretation of results equivocal, at best. Nonetheless, two groups of clones from an avocado genomic library have been characterized (K.R. Bozak, A. Lin, F.W. Percival, R.E. Christoffersen, unpublished data) that hybridize to the P450 cDNA (3). Sequence analysis has shown that one group contains the *CYP71A1* gene, whereas the second group contains a highly homologous gene designated *CYP71A2*. We have tentatively placed it within the *CYP71A* subfamily (17) because the predicted protein sequence is greater than 80% identical with the CYP71A1 protein. RNA transcripts from *CYP71A2* are not detected in ripe fruit (K.R. Bozak, A. Lin, F.W. Percival, R.E. Christoffersen, unpublished data), and thus a *CYP71A2* mRNA is not likely to encode either the ARP-1 or ARP-2 polypeptides. This strengthens the likelihood that both of these P450 polypeptides are derived from the *CYP71A1* gene and both are forms of the CYP71A1 protein. These findings, along with the work presented here, further suggest that avocado contains at least three distinct P450 genes: *CYP71A1*, encoding for the ripening-associated pCMA demethylase, a ripening-associated tCAH gene, and the putative *CYP71A2* gene with a currently undetermined function.

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