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Cloning of eggplant hypocotyl cDNAs encoding cytochromes P450 belonging to a novel family (CYP77)

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Abstract

From eggplant hypocotyl tissues we have cloned two closely related cDNAs encoding cytochromes P450 (P450s) by PCR amplification using a primer designed based on the highly conserved sequence among the known eggplant P450s One cDNA lacks the NH_2-terminal short sequence that is present in the other, full-length cDNA The two predicted protein sequences are 71% identical with each other and show less than 30% identity with any other known P450s It is concluded that these P450s, which are termed CYP77A1 and -A2, belong to a hitherto unknown P450 family

Key words Cytochrome P450, cDNA sequence, Hypocotyl, *Solanum melongena*

1. Introduction

Cytochromes P450 (P450s) constitute a superfamily of enzymes that are involved in the metabolism of both foreign chemicals and endogenous substrates [1]. In higher plants, P450s play a crucial role in oxidative detoxification of various xenobiotics including herbicides [2,3] Plant P450s also catalyze important oxidative steps leading to the biosynthesis of secondary metabolites such as phenolics, sterols, phytoalexins, and flavonoids [2,4,5]. Studies on higher plant P450s have lagged far behind those on animal and microbial counterparts, because of the difficulty in purification of P450s from higher plant sources. Recently, however, several cDNAs encoding higher plant P450s have been cloned and sequenced These include CYP73, CYP74, and CYP75, which encode cinnamate 4-hydroxylase [6,7], allene oxide synthase [8], and flavonoid B-ring hydroxylase [9–12], respectively Another plant P450 cDNA, called CYP71A1, from avocado is thought to encode a monoterpene hydroxylase [13–15]

We have previously cloned two CYP75 cDNA from eggplant and petunia and found that expression of these genes is controlled by ultraviolet light [11,12]. Using the eggplant CYP75 cDNA as a probe, we have also cloned cDNAs belonging to two other P450 families again from eggplant The cDNAs belonging to one family are highly homologous with avocado CYP71A1 and, therefore, designated as CYP71A2-4 [16], whereas the other cDNAs encode a P450 belonging to a novel family (CYP76) [17] Here we report the cloning and sequence analysis of two cDNAs from eggplant hypocotyl tissues and show that they belong to yet another family (CYP77)

2. Materials and methods

2 1 *Materials*

Eggplant seeds (*Solanum melongena* cv Sinsadoharanasu) were purchased from Tohoku Seeds Co Japan [α-^{32}P]dCTP ($1\ 1 \times 10^{14}$ Bq/mmol) was obtained from New England Nuclear Nylon membranes were obtained from Amersham The Ampli Taq PCR kit and the DNA Thermal Cycler (system 9600) were obtained from Perkin-Elmer

2 2 *Cloning and sequencing*

Poly(A)$^+$ RNA was isolated from eggplant hypocotyl tissues grown for two weeks under red light followed by white light irradiation for two days, and a cDNA library was constructed as described previously [11] The degenerate primer used in PCR amplification was designed (Fig 1a) based on a highly conserved sequence among eggplant CYP71A4, 75, and 76A1 [11,16,17], and as the counterpart primer the sequence corresponding to T7 promoter (5′-CGTAATACGACTCACTATAG) in λ ZAP II was used The primers were synthesized using an Applied Biosystems automatic DNA synthesizer model 394 The recombinant phage DNA used in PCR was prepared from a plate lysate of the cDNA library, and 0 1 μg of DNA was incorporated into the reaction The thermal cycle was set at 95°C (1 5 min), 45°C (2 min), and 72°C (2 5 min), and it was repeated 25 times The DNA products obtained were separated on a 2% low melting point agarose gel, and the region ranging from 310 to 420 bp was recovered The DNA fragments recovered from the gel were subcloned into the TA Cloning vector (Invitrogen Co), and double stranded DNAs were automatically sequenced using the Taq Dye Primer cycle sequence kit (the 370A sequencer from Applied Biosystems) Approximately 2×10^6 clones from the cDNA library were screened using the ^{32}P-labelled DNA fragment produced by PCR in a hybridization solution consisting of 5 × SSPE, 5 × Denhardt's solution,

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Abbreviations P450, cytochrome P450, PCR, polymerase chain reaction, SSC, 0 15 M NaCl/0 015 M Na_3-citrate pH 7 0, SSPE, 0 15 M NaCl/10 mM Na-phosphate/1 mM EDTA pH 7 4

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1% (w/v) SDS and 100 μg/ml of denatured salmon sperm DNA at 65°C The final wash was carried out in 2 × SSC at 65°C The positive clones were subcloned in vivo into pBluescript plasmid according to Stratagene's manual These clones were further characterized by restriction enzyme mapping Sequence comparison was carried out using DNASIS-Mac software (Hitachi Software Engineering Co)

2 3 Southern and Northern blot analyses

Genomic DNA and poly(A)$^+$ RNA was prepared from eggplant seedlings, and Southern and Northern blot analysis were performed as described previously [11] Briefly, DNA (10 μg) was digested with restriction enzymes, separated by 0 8% agarose gel electrophoresis, and transferred to Hybond N$^+$ membranes with 0 4 M NaOH The membranes were hybridized with ^{32}P-labelled DNA probes at 65°C, washed at 65°C in 0 1 × SSPE/0 1% SDS, and autoradiographed Poly(A)$^+$ RNA was size-fractionated electrophoretically on a 1% agarose gel containing 2 2 M formaldehyde [18], and transferred to Hybond N membrane with 10 × SSC The transferred RNA was hybridized with a ^{32}P-labelled CYP77A1 probe

3. Results and discussion

We are interested in P450s involved in the synthesis of secondary metabolites especially anthocyanins. We have so far cloned P450 cDNAs belonging to three families, i.e CYP71A2-4, CYP75, and CYP76A1-2. In an attempt to clone P450 cDNAs involved in anthocyanin biosynthesis, we screened an eggplant hypocotyl cDNA library using CYP75 cDNA as a probe under low stringency However, all the cDNAs thus isolated were found to encode P450s whose primary structures are more than 30% identical with that of CYP75 To isolate cDNAs encoding P450s possessing amino acid sequences that are less than 30% identical with all other P450s, we decided to employ PCR amplification For this purpose, a degenerate primer was constructed based on the sequences near the heme-binding cysteinyl residue of eggplant CYP71A4, 75 and 76A1 (Fig 1a). Along with this primer, a second primer corresponding to a vector sequence was used in PCR amplification with template DNA obtained from the cDNA library The DNAs that were produced migrated as diffuse bands, ranging between 310 and 420 bp (Fig 1b). These were cloned, and 40 randomly selected clones were sequenced Although inserts of most of the clones sequenced corresponded to fragments of CYP71, 75 and 76 cDNAs, two clones with an identical sequence were found to possess a hitherto unknown sequence. Since this sequence is characteristic of a P450, we used one of these clones as a probe to screen the cDNA library, and were able to isolate two positive clones. Restriction mapping showed that these cDNA are distinct from those encoding the known eggplant P450s. We, therefore, determined their nucleotide sequences

It was thus found that one clone, termed H1, has a 1,683-bp insert with an open reading frame encoding a polypeptide consisting of 499 amino acid residues and that it has a sequence identical with that of the probe DNA. Another clone, named H2, has a 1,673-bp insert

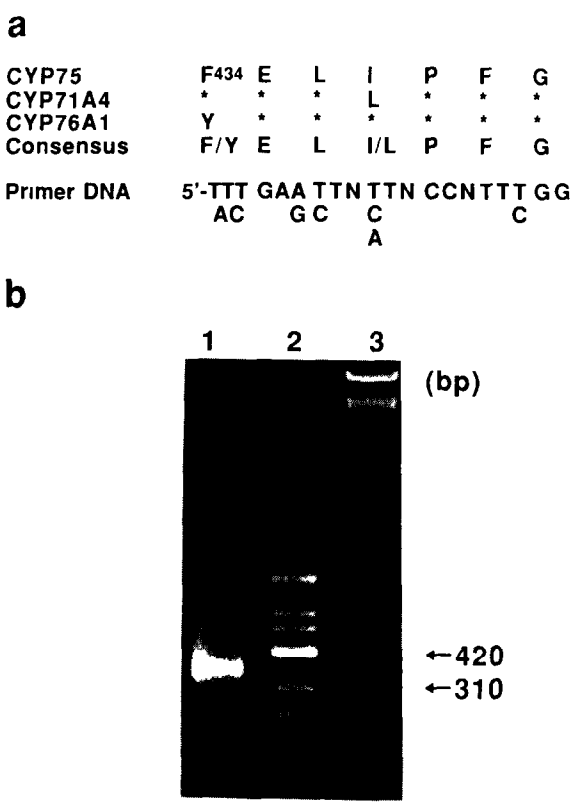

Fig 1 PCR amplification of DNA fragments (a) The DNA sequences of a degenerate primer, aligned with the peptide sequences conserved in eggplant CYP71A4, 75 and 76A1 (b) Ethidium bromide-stained agarose gel showing the products of PCR amplification from library DNA (lane 1) The size markers used were φX174 *Hinf* I (lane 2), and λ *Eco*RI/*Hin*dIII (lane 3)

encoding a protein of 511 amino acid residues (M_r and isoelectric point are 58,112 and 9 3, respectively) (Fig. 2). A comparison of the two deduced primary structures indicates that they are 71% identical with each other and that H1 is truncated in the NH_2-terminal 15 residues A computer search using the SWISS PROT database (release 23) shows that the deduced amino acid sequences of H1 and H2 contain segments that are significantly similar to the sequences highly conserved in many P450 families [19]. However, no P450s including those from eggplant exhibit less than 30% primary structural identity with H1 and H2. As P450s in the same family share more than 40% amino acid similarity with one another [20], it is considered that H1 and H2 belong to a novel P450 gene family, this is the fourth family to be found in the eggplant. Recently, Nebert and Nelson have officially named H1 and H2 as CYP77A1 and A2, respectively (personal communication).

Southern hybridization was carried out to examine the genomic organization of these cDNAs. There were one or two bands in each restriction digestion, indicating the copy number is low, as is usual with other eggplant P450 genes [11,16,17] (Fig. 3a,b). To examine the CYP77A1 transcript, mRNAs from eggplant hypocotyl tissues

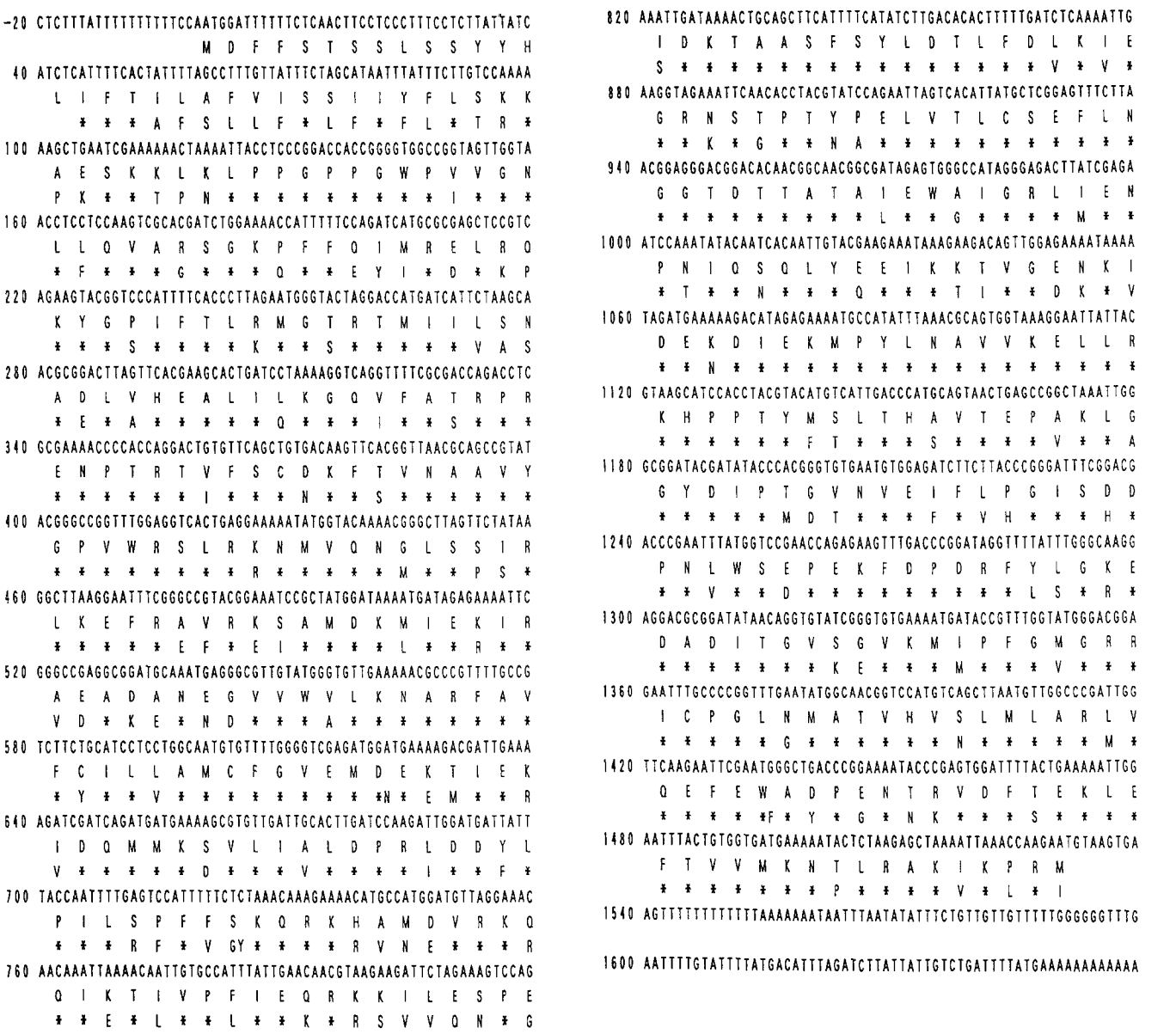

Fig 2 The nucleotide and the deduced amino acid sequences of the CYP77A1 and 77A2 cDNA clones The cDNA inserts were subcloned and sequenced on both strands by the dideoxynucleotide chain termination method, using double stranded DNA as a template The amino acid sequences are shown below the nucleotide sequence of 77A2 DNA, and the nucleotides are numbered on the left hand side The star (*) indicates residues of 77A1 identical with the 77A2 sequence These sequences have been submitted to the EMBL Data Library under accession numbers X71655 (CYP77A1, only the deduced amino acid sequence is shown) and X71656 (CYP77A2), respectively

grown under red light, and those grown under white light following red light irradiation were Northern blotted and hybridized with the cDNA There was a weak signal of 1 8 kb in the mRNA from tissues grown under white light (Fig. 3c). It is reasonable that a low signal intensity is observed due to the low frequency of clones in the cDNA library (2×10^{-6}), or it may be that this gene is transcribed transiently We isolated a P450 cDNA, named CYP75, which was accumulated under white light and is considered to encode a flavonoid B-ring hydroxylase [11]. When the source of irradiation was changed, the accumulation of mRNAs from other genes involved in anthocyanin synthesis was observed This fact suggests that CYP77A1 may also be associated with the metabolism of anthocyanins As CYP77A1 shows less than 30% homology with cinnamate 4-hydroxylase (CYP73) [6,7] and CYP75, we consider that this cDNA may encode a protein catalyzing another type of P450 dependent hydroxylation, such as flavone synthase [21,22], although there is no direct evidence supporting this speculation

We further conclude that the present PCR method using primers based on the consensus sequence found in P450s is a powerful means of isolating unknown P450

Table 1
Alignment of the amino acid sequences of the heme-binding region of P450s

Family	Amino acid sequence															Source	Reference
71A1	F	Q	L	I	P	F	G	A	G	R	R	G	C	P	G	avocado	[13]
A2	*	K	*	L	*	*	*	*	*	*	*	*	*	*	*	eggplant	[16]
A3	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	eggplant	[16]
A4	*	E	*	L	*	*	*	S	*	*	*	*	*	*	*	eggplant	[16]
72	A	T	Y	L	*	*	S	W	*	P	*	V	*	L	*	periwinkle	[24]
73	*	R	Y	L	*	*	*	V	*	*	*	S	*	*	*	mung bean	[7]
74	P	E	T	E	T	P	S	V	A	N	K	Q	*	A	*	flax	[8]
75	*	E	*	*	*	*	*	*	*	*	*	I	*	A	*	eggplant	[11]
76A1	Y	E	*	*	*	*	*	*	*	*	*	M	*	V	*	eggplant	[17]
A2	Y	G	*	*	*	*	*	*	*	*	*	M	*	V	*	eggplant	[17]
77A1	V	K	M	M	*	*	*	V	*	*	*	I	*	*	*	eggplant	(this work)
A2	V	K	M	*	*	*	*	M	*	*	*	I	*	*	*	eggplant	(this work)
1A1	E	K	V	L	V	*	*	M	D	K	*	R	*	I	*	trout	[25]
2A1	S	E	V	*	L	*	*	L	*	K	*	K	*	I	*	rat	[26]

*Indicates the same amino acid residue as CYP71A1

sequences. Similar strategies have been used to isolate a flavonoid 3′,5′-hydroxylase cDNA from petunia [9,10] and CYP72 from periwinkle [23], based on the use of the consensus sequence of avocado CYP71 and animal P450s Table 1 shows the amino acid alignment of the highly conserved cysteinyl-peptide involved in heme-binding in 12 P450s from higher plants, in addition to two P450s from other eukaryotic species. In this region, the P-F-G-X-G-R-R-X-C-X-G sequence is found to be highly conserved in higher plant P450s, with the exception of CYP72 and 74 Some of these residues are known to be highly conserved in eukaryotic P450s [14,19,20. These results agree with the phylogenetic observation that CYP72 diverged earlier than CYP71 and CYP73 [6], and support the hypothesis that CYP74 is a type I P450 localized in the chloroplasts [8]. If we attempt to clone more diverse sequences, such as CYP72, we must design other primers based on this sequence, or must carry out the PCR amplification under conditions of lower stringency [10] However, it is clear that CYP74 is too unrelated to be cloned by this strategy

The above results show that cDNAs encoding a novel P450 family were isolated by PCR amplification, and that the transcripts were accumulated by white light irradiation. Also, the degree of homology between plant P450s is listed, which may provide us with clues to the cloning of other P450s

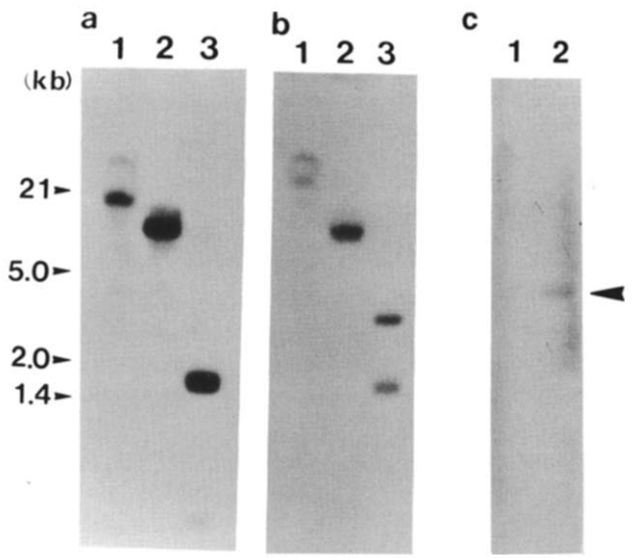

Fig 3 Gel blot analysis of CYP77A1 and A2 Genomic DNA from the eggplant (10 μg) was digested with *Bam*HI (lane 1), *Eco*RV (lane 2) or *Hin*dIII (lane 3). The DNA was transferred onto membranes, and was hybridized with CYP77A1 (a) or A2 (b) Northern analysis of CYP77A1 using eggplant hypocotyl tissues (c) Two μg of poly(A)$^+$ RNAs from hypocotyl tissues grown under red light (lane 1), or white light following red light irradiation (lane 2), were electrophoresed on a agarose gel, and were hybridized with ^{32}P-labelled CYP77A1 cDNA

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