Short Communication

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PCR-amplification of Sequences Encoding Heme-binding Region of Plant Cytochrome P450

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Cytochrome P450 monooxygenases are membrane-bound heme-containing enzymes which have been found widely in prokaryotes and eukaryotes. Dozens of P450s are expressed simultaneously in eukaryotic tissue. In higher plants, they are involved in the oxidative detoxification of xenobiotics and the biosynthesis of secondary metabolites such as lignin phenolics [1], fatty acids [2], sterols, gibberellins, phytoalexins [3] and flavonoids [4]. Since plant cytochrome P450s mostly included in microsomal fraction are a small quantity and instable after tissue homogenization, it has been difficult to isolate and purify enough enzymes for further investigations.

Over 450 DNA fragments of cytochrome P450 from bacteria, mammalian and plants have been isolated and sequenced [5, 6]. Human cytochrome P450s are classified into 28 families/subfamilies from their general characteristics, functions and chromosomal distribution. Sequence comparisons of their proteins have revealed that the heme-binding domain of all characterized P450 proteins contains highly and evolutionally conserved sequence [7]. Ripening-related P450 cDNAs from the mesocarp tissue of avocado fruit were the first cloned in plants [8]. The amino acid sequence predicted from the cDNA sequence indicates that the ripening related P450 shares a homologous sequences with the heme-binding domain of animal proteins. cDNAs of flavonoid 3′-hydroxylase, flavonoid 3′, 5′-hydroxylase from petunia [9] and 16 different polypeptides from *Catharanthus roseus* [10] have been cloned by means of polymerase chain reaction (PCR) using degenerate primers, which anneal to the conserved region and poly(A) tail. Although wide varieties of enzymes are classified in the P450 family, the homology of their amino acid sequences are low except for the heme-binding region.

In this paper, we report the cloning strategies of a partial sequence of a wide variety of P450s from petunia flower petals by low-stringency PCR (LS-PCR [11-13]) at a low annealing temperature using a set of degenerate primers.

Petunia plants (*Petunia hybrida* var. falcon blue; Sakata Seed) had been grown in a green house. Ten grams of flower petals that pigmented slightly on the rim were cut off and ground in liquid nitrogen. Isolation of total RNA was carried out by the guanidin-hydrochloride method described by Logemann *et al.* [14]. Poly(A)$^+$ RNAs were purified by binding oligotex™-dT30 (Japan Roshe) and cDNAs were synthesized using cDNA Synthesize System Plus (Amersham) according to the instructions provided by the manufacturer.

We designed 8 sense PCR primers (S-primer) and 12 antisense PCR primers (R-primer) as listed in **Fig. 1.** All the degenerate PCR primers were synthesized using Cyclone Plus™ DNA synthesizer (Milligen/Biosearch).

PCRs were carried out with 0.1 $\mu g/ml$ cDNA as the template and 0.2 μM of each primer. The reaction mixture consisted of 67 mM Tris-HCl (pH 8.3), 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, 0.2 mM of each dNTPs and 2.5 U AmpliTaq (Takara Syuzo). The 25 μl of reaction mixture was incubated in a Thermal Cycler (Perkin Elmer) under the temperature program of 95°C for 3 min., 3 cycles of 24°C for 10 sec. and 93°C for 30 sec., 40 cycles of 55°C for 10 sec., 93°C for 30 sec., followed by 55°C for 10 min. Amplifying fragments were so short that extension step at 72°C was omitted. PCR products were separated on a 10% polyacrylamide gel. Approximate 52 bp bands were then excised and purified by the crash and soak method [15].

The 52bp-PCR products purified from polyacrylamide gel were double digested with *Bam*HI and *Eco*RI, and cloned into pBS$^{(+)}$ (STRATAGENE) cleaved with *Bam*HI and *Eco*RI. Recombinant plasmids were transformed into *E. coli* strain JM109, purified and sequenced by Taq DyeDeoxy™ Terminator Cycle Sequencing kit and the Model 373A DNA Sequencing Systems (Applied Bio Systems).

We have searched the protein data base PIR (R25.0) and some references for amino acid sequences of

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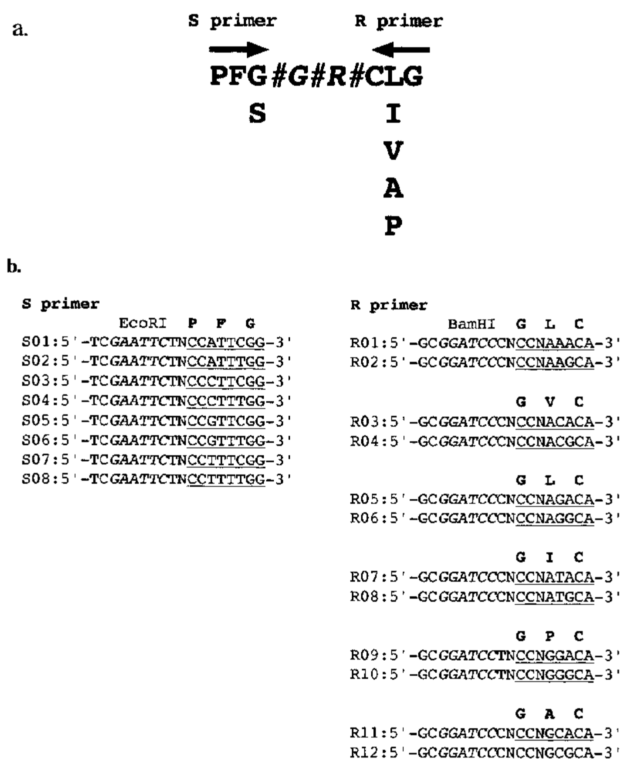

Fig. 1 Amino acid sequence of the heme-binding region of cytochrome P450 and the list of S- and R-primers used in this study.
A: Consensus amino acid sequence of cytochrome P450. Arrow indicates the position and the orientation of the primers. # is not designated amino acid.
B: Nucleotide sequence of S- and R-primers. Regions corresponding to the conserved sequence were underlined. A restriction site for *Eco*RI or *Bam*HI is added to the 5′ end of the primers (*italic*). N means a mixture of A, C, G and T.

P450 and found 193 independent P450s. By comparing the heme-binding domain in the carboxyl-terminal portion of these P450s, it has been shown that 11 amino acid sequences in the region flanking cysteine residue were relatively well conserved. The amino acid sequence PFG#G#R#CXG, in which # is any kind of amino acids and X is either L, I, V, A or P, appeared in approximately 80 % of the eukaryotic P450s (**Fig. 1-A**). For the purpose of amplifying and cloning the DNA fragments encoding the conserved region by PCR, 8 degenerate sense primers (S01 to S16) and 12 degenerate antisense primers (R01 to R12) corresponding to nucleotide sequences specifying consensus sequences were designed with addition of *Eco*RI/*Bam*HI cutting sites in the 5′ end (**Fig. 1-B**). PolyT sequence, which anneals 3′-terminal region of cDNAs, was not used as an antisense primer to enhance fidelity of the LS-PCR. Ninety-six PCRs were carried out, using all the possible combinations of a S- and a R-primer. Annealing temperature for the first three cycles was reduced to 24°C to allow for mismatches. The expected 52bp-products were

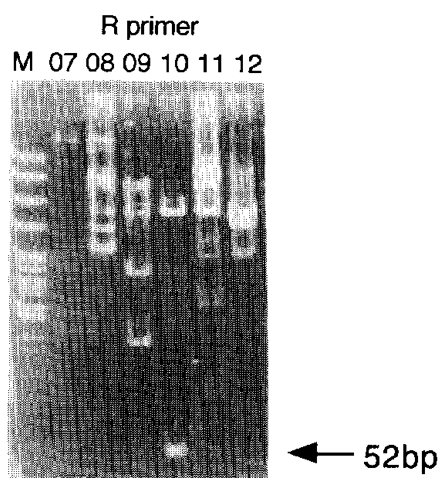

Fig. 2 Amplification of a heme-binding region of cytochrome P450 by LS-PCR. In the case that S04 and one of R07 - R12 primers were used in the reaction, 52 bp band (arrow) appeared only in the combination of S04 and R10 primers. M means pBR322/*Msp*I molecular size markers.

synthesized in 14 out of 96 reaction products (**Fig. 2**) and their libraries were constructed. Ninety-six clones were picked up from the libraries and their inserts were sequenced.

Sequence analysis represented that 62 clones have encoded the expected P450 heme binding sequence, PFG#G#R#CXG (**Table 1**). These clones were classified into 16 groups (Group 1 to 16) according to the predicted amino acid sequences. Some of the clones were amplified from one reaction. For example, four different DNAs were amplified in the reaction combined by No. 06 and 09 primers (**Table 1**). We suppose a pair of primers hybridized to the different cDNAs under the low-stringency condition. From the sequence analysis, P450s coding "CPG" sequence in the latter half of the conserved region was predominant, about 70 %, in the petunia petal. This sequence was found in avocado and other plants, but few in bacteria and animals. The conserved region of two flavonoid 3′, 5′-hydroxylase genes cloned by Holton *et al.* [9], which exist on different loci of a petunia genome, were identical to each other. We suppose that partial fragments displaying some differences within the nucleotide sequence cloned by this study have quite distinct P450.

Approximately 80% of the conserved region of eukaryotic P450s that were researched shared the amino acid sequence PFG#G#R#CXG in their protein database. We found that the conserved sequences of cinnamate 4-hydroxylase and a maize P450 whose function is not elucidated belong to Group 13 and 3, respectively. This suggests that a number of P450 conserved regions could be cloned by the LS-PCR using the set of degenerate primers constructed

Table 1.
A list of nucleotide sequences cloned in this study.

<AMINO ACID>		<DNA>		
type	sequence	No. of primer (S/R)	sequence	No. of clones
1	PFG AGRRI CAG	06/11	CCG TTT GGt gct gga cga aga att TGT GCA GGG	1
2	PFG AGRRI CPG	02/10	CCA TTT GGt gct ggt cga aga ata TGC CCT GGT	1
		02/10	CCA TTT GG- --- --a --- --- --- TGC CCG GGG	1
		06/09	CCG TTT GG- --- --a a-- c-- --- TGT CCG GGA	2
		06/09	CCG TTT GGc --- --a a-- c-- --- TGT CCT GGT	1
3	PFG AGRRV CPG	02/09	CCA TTT GGt gct ggt aga aga gtg TGT CCT GGT	4
4	PFG FGPRK CVG	05/03	CCG TTC GGc ttt ggt cct cga aaa TGT GTG GGG	1
		05/04	CCG TTC GGc --- --- --- --- --- TGC GTG GGG	2
		07/03	CCT TTC GGg --- --g --- --- --- TGT GTT GGG	5
5	PFG GGPRK CVG	01/04	CCA TTC GGt gga gga cca aga aaa TGC GTC GGG	4
6	PFG GGPRR CPG	06/09	CCG TTT GGt gga gga cca cgg cga TGT CCC GGG	3
7	PFG GGRRI CPG	06/09	CCG TTT GGt ggt gga aga agg ata TGT CCT GGG	1
8	PFG SGPRI CLG	05/06	CCG TTC GGt agt gga agg agg att TGC CTG GGA	1
9	PFG SGRRI CPG	02/10	CCA TTT GGt agt gga agg agg att TGC CCA GGG	1
		03/10	CCC TTC GG- --- --- --- --- --- TGG CCA GGA	2
		04/10	CCC TTT GG- --- --- --- --- --- TGC CCA GGT	1
		06/10	CCG TTT GG- --- --- --- --- --- TGC CCT GGT	1
10	PFG SGRRS CPG	02/09	CCA TTT GGc tcg gga aga cga tct TGT CCA GGG	1
11	PFG TGRRI CPG	06/09	CCG CTT GGc act ggt cga cga att TGT CCG GGG	3
12	PFG VGLRM CPG	03/10	CCC TTC GGa gta ggc cta aga atg TGC CCT GGG	1
		04/10	CCC TTT GG- --- --- --- --- --- TGC CCA GGA	3
13	PFG VGRRS CPG	02/10	CCA TTT GGt gtt ggt agg agg agt TGC CCT GGA	2
		06/10	CCG TTT GG- --- --- --- --- --- TGC CCC GGA	3
		06/10	CCG TTT GG- --- --- --- --- --c TGC CCT GGT	1
14	PFG VGPKM CPG	02/10	CCA TTT GGa gtc ggc ccc aaa atg TGC CCG GGC	1
		03/10	CCC TTC GG- --- --- --- --- --- TGC CCG GGA	3
		04/10	CCC TTC GG- --- --- --- --- --- TGC CCG GGT	1
		06/10	CCG TTT GG- --- --- --- --- --- TGC CCA GGT	1
		04/10	CCC TAT GG- --- --t --- --- --- TGC CCG GGT	1
15	PFG SGFCS CPG	06/09	CCG TTT GGc agt ggt ttc tgc tca TGT CCT GGG	1
16	PFG VGPNM CPG	02/10	CCA TTT GGa gtc ggc ccc aac atg TGC CCT GGT	1

Nucleotide sequences of the cloned PCR products which code for the putative heme-binding region of cytochrome P450. They are classified into 16 types by the amino acid sequences deduced from their nucleotide sequences (left). S/R indicates the combination of the S- and R-primer used for PCR. DNA sequences corresponding to the PCR primer are indicated by capital letters, and internal sequences are indicated by small letters. The same nucleotide as above is indicated by "-".

in this study. A full-length cDNA of specific P450 is easy to clone by using amplified sequences as a probe for screening cDNA libraries. In this way, we have cloned full-length cDNAs of several flower specific P450s (manuscript in preparation), and the analysis of those genetic functions is in progress.

Estimates of the copy number of plant individual P450 genes range from 60 to 250 per vertebrate genome. There has been little investigation into how the expression of these P450 genes are regulated tissue-specifically, and how the ancestral genes had obtained different substrate specificities and diversified in the process of evolution. The method presented in this report may be widely applicable not only for the cloning of a cytochrome P450 families/subfamilies from various sources but also for analyzing expression patterns of P450 genes.

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