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# Cloning of Cytochrome P450 Gene involved in the Pathway of Capsidiol Biosynthesis in Red Pepper Cells

**Soon-Tae Kwon*, Jae-Sung Kim[1], Do-Cheul Jung[2], Jeong-Hag Jeong, Jae-Moon Hwang and Sei-Myoung Oh**

*School of Bioresources Science, Andong National University, Kyungpook 760-749, Korea*
*[1]Korean Atomic Energy Research Institute, Daejeon, 305-353, Korea*
*[2]Department of Plant Resources, Sangju National University, Kyungpook 642-130, Korea*

## Abstract

In order to measure the enzyme activity of 5-*epi*-aristolochene hydroxylase, one of cytochrome P450 (P450) enzymes in eicitor-treated pepper cell, we used *in vivo* assay method and demonstrated a dramatic suppression of the activity by P450-inhibitors, ancymidol and ketocornazole. Using RT-PCR method with degenerate primer of the well conserved domains found within most P450-enzymes, and using cDNA library screening method, one distinct cDNA, being designated *P450Hy01*, was successfully isolated from elicitor-treated pepper cells. *P450Hy01* mRNA was all induced in elicitor-treated cells whereas never induced in control cells. Moreover, levels of *P450Hy01* expression were highly correlated with the levels of extracellular capsidiol production by different elicitors in cell cultures. *P450Hy01* transcript was also induced by several other elicitors such as, cellulase, arachidonic acid, jasmonic acid, yeast extract as well as UV stress. *P450Hy01* sequence contained high probability amino acid matches to known plant P450 genes and ORF with a conserved FxxGxRxCxG heme-binding domain. *P450Hy01* cDNA showed 98% of homology in sequence of nucleotide as well as amino acid to 5-*epi*-aristolochene-1,3-hydroxylase (5EA1,3H) which has been isolated in tobacco cells, suggesting that *P450Hy01* is prominent candidate gene for P450-enzyme encoding 5EA1,3H in pepper cell.

**Key words** – red pepper, elicitor, heme-binding domain, cytochrome P450, 5-*epi*-aristolochene hydroxylase

## Introduction

Capsidiol is a bicyclic, dihydroxylated sesquiterpene phytoalexin produced by several solanaceous species in response to a variety of environmental stresses, including exposure to UV light and infection by pathogens or microoganisms [2,3,12]. It is derived from the isoprenoid pathway via its hydrocarbon precursor, 5-*epi*-aristolochene in tobacco or red pepper [2,7,12]. The biosynthestic enzymes leading up to 5-*epi*-aristolochene formation has been studied, especially 5-*epi*-aristolochene synthase (5EAS). 5EAS commits carbon to sesquiterpene metabolism by catalyzing the cyclization of farnesyl diphosphate (FPP) to 5-*epi*-aristolochene (Fig. 1).

Many reports have suggested that the oxidation of 5-*epi*-aristolochene to capsidiol occurs in two step process with one of the hydroxylation steps being constitutive and the other being mediated by an elicitor-inducible cytochrome P450 (P450) [2,7,12,16]. Whitehead *et al.* [16, 17] suggested that 3-hydroxylase, responsible for hydr oxylation of 5-*epi*-aristolochene at C3 to generate 1-

*To whom all correspondence should be addressed
*Tel*: 82-54-820-5623, Fax: 82-54-820-5785
E-mail: skwon@andong.ac.kr

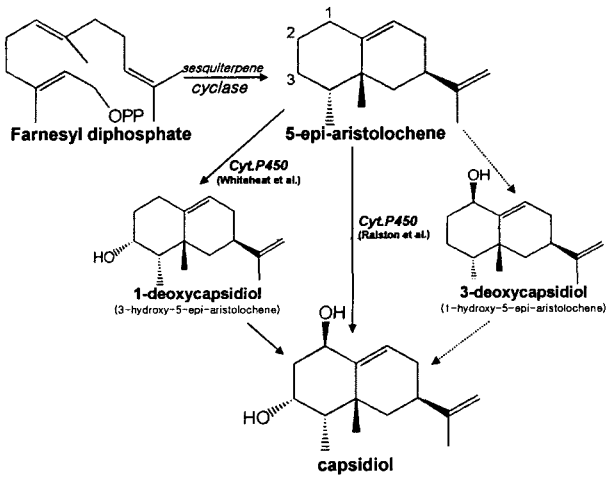


Fig. 1. Proposed pathways for the biosynthesis of capsidiol in elicitor-treated cells.
5-*epi*-Aristolochene is synthesized from FPP by the action of sesquiterpene cyclase, and is subsequently hydroxylated at C1 and C3 to form capsidiol (adapted from reference 12, 16, 17).

deoxycapsidiol, was pathogene or elicitor inducible, while the 1-hydroxylase, responsible for hydroxylating 1-deoxycapsidiol at the C1 to generate capsidiol, was constitutive. Recently, Ralston *et al.* [12] cloned one P450 hydroxylase, being designated CYP71D20 encoding 5-*epi*-aristolochene-1,3-dihydroxylase, from elicitor-treated tobacco cell, and interestingly, CYP71D20-encoded enzyme activity was capable of converting both 5-*epi*-aristolochene and 1-deoxycapsidiol to capsidiol *in vitro*.

Plant P450 hydroxylases are involved in mediating the formation of a wide range of plant metabolites, including phenylpropanoids, fatty acids, terpenoids, alkaloids, hormones, pigments and phytoalexins [5,14]. In addition, certain P450 enzymes have been reported to be involved in the detoxification of herbicides in plants and drug in mammalians [6,11]. Recently many investigators have succeeded in purifying some distinct P450 from plants [2, 5,6,12]. Isolation of P450 clones from plants was successfully performed by reverse transcription polymerase chain reaction (RT-PCR) using a set of degenerate primer corresponding to nucleotide sequence specifying a conserved region in P450 proteins. Sequence comparison of P450 protein from animals, microorganisms and plant have led to identification of several high conserved regions [5,14]. One of those domains, close to C-terminal end of the protein, is involved in binding of the P450 heme group. This domain contains the highly conserved amino acid sequence FxxGxRxCxG which has been regarded as fingerprint for P450 genes.

To understand the properties of P450 dependent hydroxylase responsible for the conversion of 5-*epi*-aristolochene to capsidiol in elicitor-treated pepper cells, gene families of P450 cDNAs were identified and characterized. In addition, effect of various elicitors on the production of capsidiol and expression of the P450 was investigated.

## Materials and Methods

### Cell culture and elicitation

Cell cultures of red pepper (*Capsicum annuum* cv. Subicho) were maintained in modified MS medium [10] as described previously [8]. Cultures in their rapid phase of growth (4 days old) were used for all experiments. Elicitation was initiated by addition of 5.0 μg cellulase per 100 ㎖ of cell cultures for the specific length of time before collecting cells and media. Control and elicitor-treated cells and media were harvested by filtration and stored at -70℃.

### *In vivo* assay of P450 hydroxylase activity

P450 hydroxylase, 5-*epi*-aristolochene hydroxylase (5EAH), activity was measured as the incorporation of [$^3$H]-labelled 5-*epi*-aristolochene into extracellular capsidiol by intact cell. [$^3$H]-5-*epi*-aristolochene was produced by incubating an excess of [$^3$H]-farnesyl diphosphate with 5-*epi*-aristolochene synthase (5EAS) [12]. At 6 h intervals after the addition of elicitor, 100,000 dpm of [$^3$H]-5-*epi*-aristolochene was carefully added to the surface of 10 ㎖ cell cultures. After 3 h further incubation, the medium was collected and extracted with chloroform and sub-

jected to TLC on silica plate with EtOAc-cyclohexane (1:1) as developing solvent. Capsidiol was visualized by spraying color reagents [12] on TLC plate, and the area corresponding to the capsidiol was scraped into scintillation vials and radioactivity was determined. Inhibition study was performed by addition of the P450-inhibitors, ancymidol or ketocornazole, directly into the cell cultures. Cell cultures were incubated in the presence of cellulase and 50 μM of ancymidol or ketocornazole for 6 h prior to the addition of [$^3$H]-5-*epi*-aristolochene as substrate for 5EAH. After a further 3 h incubation period, the cells and media were collected. The amount of radioactivity incorporated into extracellular capsidiol was determined as describe above.

### RT-PCR and cloning strategy for P450 cDNA

Total RNAs were isolated from control and 12 h elicitor-treated pepper cells, and poly(A)$^+$RNAs were prepared by oligo(dT) column (GIBCO, Life Technology). First strand cDNA was synthesized using an oligo(dT) primer and reverse transcriptase. P450-specific cDNAs were isolated using RT-PCR strategy based on sequence of already known P450s [14]. A forward primer [5′-GCGGATCCGA(G/A)GA(G/A)TT(C/T)(A/C)G(G/A/T/C)CC(G/A/T/C)GA(G/A)(A/C)G-3′) including *Bam*HI site (underlined) was based on amino acid sequence of EEFLP and reverse primer was 25 bp oligo(dT) (5′-CGGAATTCT TTTTTTTTTTTTTTT-3′) including *Eco*RI site (underlined). The RT-PCR products uniquely generated from elicitor-induced poly(A)$^+$RNA were isolated from an agarose gel and ligated into pBluescript-SK plasmid (Stratagene), and then transformed into *E. coli* strain TB1.

### cDNA library screening

To obtain the upstream sequence of RT-PCR product, pcDNAII-plasmid cDNA library, prepared from elicitor-treated pepper cells, in *E coli* strain DH1F was amplified on ampicillin/LB plate overnight. All bacterial colonies containing cDNA library were harvested by scraping into ampicillin/LB liquid medium and overnight cultured and purified plasmid DNA. Plasmids of pcDNAII, having cDNA inserts between *Bst*XI site, were directly amplified by PCR using gene specific reverse primer positioned in the termination codon of RT-PCR product, and T7 forward primer positioned in the plasmid DNA. The PCR products generated from the gene specific primer was recovered, subcloned into pBluescript SK, transformed and sequenced.

### Northern analysis

Northern analysis was carried out using 10 ㎍ of total RNA. RNA samples were size fractionated on 1.0% agarose formaldehyde gel, and transferred onto membrane filter in 10×SSC solution. Hybridization was performed at 42℃ in 5 ㎖ of buffer [13] probed with [$^{32}$P]-labelled RT-PCR product using commercial kit (Stratagene). Membrane was washed twice at room temperature for 20 min with 2×SSC, 0.1% SDS, and twice for 30 min with 0.1 x SSC, 0.1% SDS. Hybridization signal was visualized by autoradiography.

## Results

### Assay of 5-*epi*-aristolochene hydroxylase activity

*In vivo* feeding assay is indirect method for measuring 5-*epi*-aristolochene hydroxylase (5EAH) activity described by Ralston *et al.* [12]. This assay measures the incorporation rate of exogenously supplied [$^3$H]-5-*epi*-aristolochene into extracellular capsidiol by cell cultures. Using this assay method, a detailed induction time course of 5EAH activity in elicitor-induced cell cultures was able to determined relative to enzyme activity of P450 inhibitor-treated cells. Because the 5EAH assay is an indirect method, it was not possible to determine the enzyme activity quantitatively. Therefore, the enzyme activities between cells treated with cellulase only and cells treated with P450 inhibitors, ancymidol or keto-

cornazole were compared. As shown in Fig. 2, 5EAH activity is not detectable in control cell cultures, but is induced significantly after an apparent lag phase of 18 h, a rapid induction of 5EAH activity was observed 18 to 24 h after elicitor addition to the cell cultures, reaching its maximum level at 36 h of treatment. If the 5EAH activity measured in this assay is a P450-mediated reaction, the P450 inhibitor would be expected to hinder the incorporation of [$^3$H]-5-*epi*-aristolochene into capsidiol by the elicitor-treated pepper cell. Enzyme activities of 5EAH in cell cultures treated with cellulase plus either 50 μM of ancymidol or ketocornazole were completely inhibited. Ralston *et al.* [12] reported that 50% of 5EAH activity in elicitor-treated tobacco cells was inhibited by either 25 μM ancymidol or ketocornazole, and more than 80% by 75 μM ancymidol and 95% by 100 μM ketocornazole. However, in case of pepper cells, 5EAH activity was completely inhibited by either 50 μM ancymidol or ketocornazole suggesting that dose response of 5EAH activity to P450 inhibitors is different between tobacco and pepper cell.

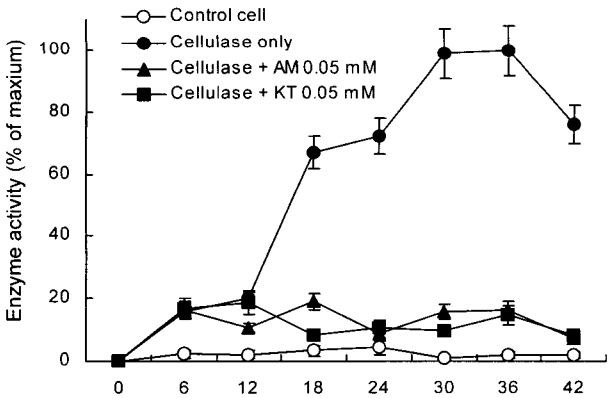


Fig. 2. Induction time course for 5-*epi*-aristolochene hydroxylase (5EAH) activity and inhibition of enzyme activity by Cyt.
P450 inhibitors, ancymidol and ketocornazole, in the cellulase-treated pepper cell cultures. Cell cultures were incubated in the presence of cellulase (0.05 μg/mℓ) and $10^5$ DPM of [$^3$H]-labelled 5-*epi*-aristolochene with or without Cyt. P450 inhibitors, and measured DPM of capsidiol which was converted by hydroxylation of 5-*epi*-aristolochene with the action of 5EAH.

## Cloning of P450 cDNA

In order to isolate elicitor-inducible hydroxylase cDNA out of extensive variations of P450 gene families, we used a two-step approach. First step was RT-PCR strategy using degenerate primer based on recently reported P450 genes [14], and second step was screening of cDNA library prepared against mRNA isolated from elicitor-treated cells. Based on *in vivo* assay, we confirmed that 5EAH activity was dramatically increased at 18 h after elicitor addition. cDNAs prepared from control cells and elicited cells were PCR amplified by degenerate forward primer encoding a conserved amino acid sequences, EEFLP, located in approximately 30 amino acid upstream from the heme-binding domain in P450 gene families. The forward primer was based on sequence conservation in the region of the *Helianthus tuberosis* CYP73A1 encoding a trans-cinnamic acid hydroxylase [15], the *Persea americana* CYP71A1 encoding a p-chloro-N- methylaniline activity [1]. From RT-PCR reaction, 98 PCR products which were uniquely generated by elicitor-induced mRNA were subcloned into plasmid and transformed into *E. coli*. Of the 98 transformants that were sized, 26 independent clones had cDNA insert in the size approximately 400 to 600 bp. Out of 26 independent cDNA clones, twelve clones showed high specificity to elicitor-induced mRNA by Northern analysis (Data not shown). Twelve northern positive clones were designated from *P450Hy01* to *P450Hy12*, meaning 'cytochrome P450 hydroxylase' (*P450Hy$_n$*). In this report, we are going to consider just one clone, *P450Hy01* showing high specificity to elicitor-treated cells (Fig. 3). From cDNA library screening using *P450Hy01* specific internal sequence, we obtained full length *P450Hy01* with the length of 1,684 bp containing open reading frame (OFR) of 1,419 bp nucleotides and 473 amino acids, and contained high probability amino acid matches to already known P450 sequences, and ORF with the conserved FxxGxRxCxG

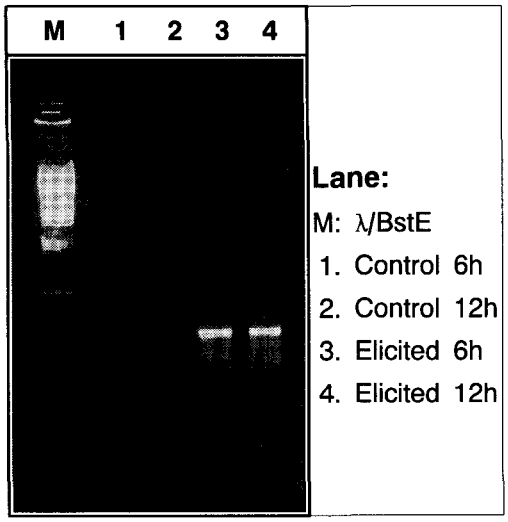


Fig. 3. Identification of elicitor-inducible Cyt. P450 cDNA detected by RT-PCR strategy.
Forward primer is degenerated from amino acid sequence, EEFLP, and reverse primer is Olig (dT).

heme-binding domain. An analysis of sequence for *P450Hy01* and other species showed 98% of homology in nucleotide sequence as well as amino acid sequence to CYP71D20 (GeneBank Accession No. AF368376) encoding 5-*epi*-aristolochene-1,3-hydroxylase (5EA1,3H), and 92% homology to CYP71D21 which have been isolated in elicitor-treated tobacco cells [12].

### Expression of *P450Hy01* in pepper cells

RNA blot analysis were used to determine the steady-state levels of the mRNA coding for *P450Hy01* clone and 5EAS in control and elicitor-treated cells (Fig. 5) As we expected, the mRNA for P450 clone and 5EAS were rapidly and transiently induced with slightly different time courses relative to one another. *P450Hy01* mRNA displayed an induction pattern similar to that of 5EAS except that the maximum level of mRNA occurred at 6 h after elicitation for 5EAS and 12 h for *P450Hy01*. However, *P450Hy01* mRNA remained high until 18 h after elicitation and negligible at 24 h, 5EAS mRNA dramatically decreased from 6 to 12 h and almost disappeared at 24 h. Base on Northern analysis, 5EAS mRNA was induced and declined more rapidly than that of *P450Hy01* mRNA.

It is well known that a member of P450 enzymes are induced by a variety of environmental stresses and xenobiotics. Thus to investigate if *P450Hy01* clone was specifically induced during the hydroxylation of 5-*epi*-aristolochene and ultimately produce capsidiol, suspension cells and intact plants were subjected to various environmental stresses and elicitors. Figure 7 shows that extracellular accumulation of capsidiol in the media of pepper cell cultures treated with various elicitors and environmental stresses. In pepper cell cultures, cellulase treatment showed highest level of capsidiol production followed by arachidonic acid, jasmonic acid, UV, $AgNO_3$, chitosan, yeast extract and NaF in order. But the treatment of pectinase, agar, γ-ray did not induce the pepper cell. Further to investigate if *P450Hy01* mRNA is able to be expressed by the treatment of other elicitors and stresses which induced capsidiol production, total RNAs extracted from control and elicitor-treated cells and hybridized with *P450Hy01* probe (Fig. 8). Interestingly, levels of *P450Hy01* mRNA accumulation in pepper cells were higher than that in leaves regardless of elicitors used. However, *P450Hy01* mRNA in leaves is specifically induced, even very low level, by various elicitors, such as UV, *Phytopthora* infection, cellulase injection, paraquat, bialaphose and glyphosate treatment whereas no signal was seen in control and 50 Gy of gamma ray-treated leaf. *P450Hy01* mRNA expression in suspension cells showed different levels depended on elicitors. Highest expression was obtained in cellulase-treated cell and followed by arachidonic acid, jasmonic acid and yeast extract in order. Treatment of pectinase, agar, NaF, $AgNO_3$ also showed *P450Hy01* mRNA accumulation even though very low level as compared to cellulase and arachidonic acid. Levels of *P450Hy01* transcripts in suspension cells induced by various elicitors were very similar to amount of capsidiol production by the treatment. For example, cellulase, arachidonic acid and jasmonic acid showed

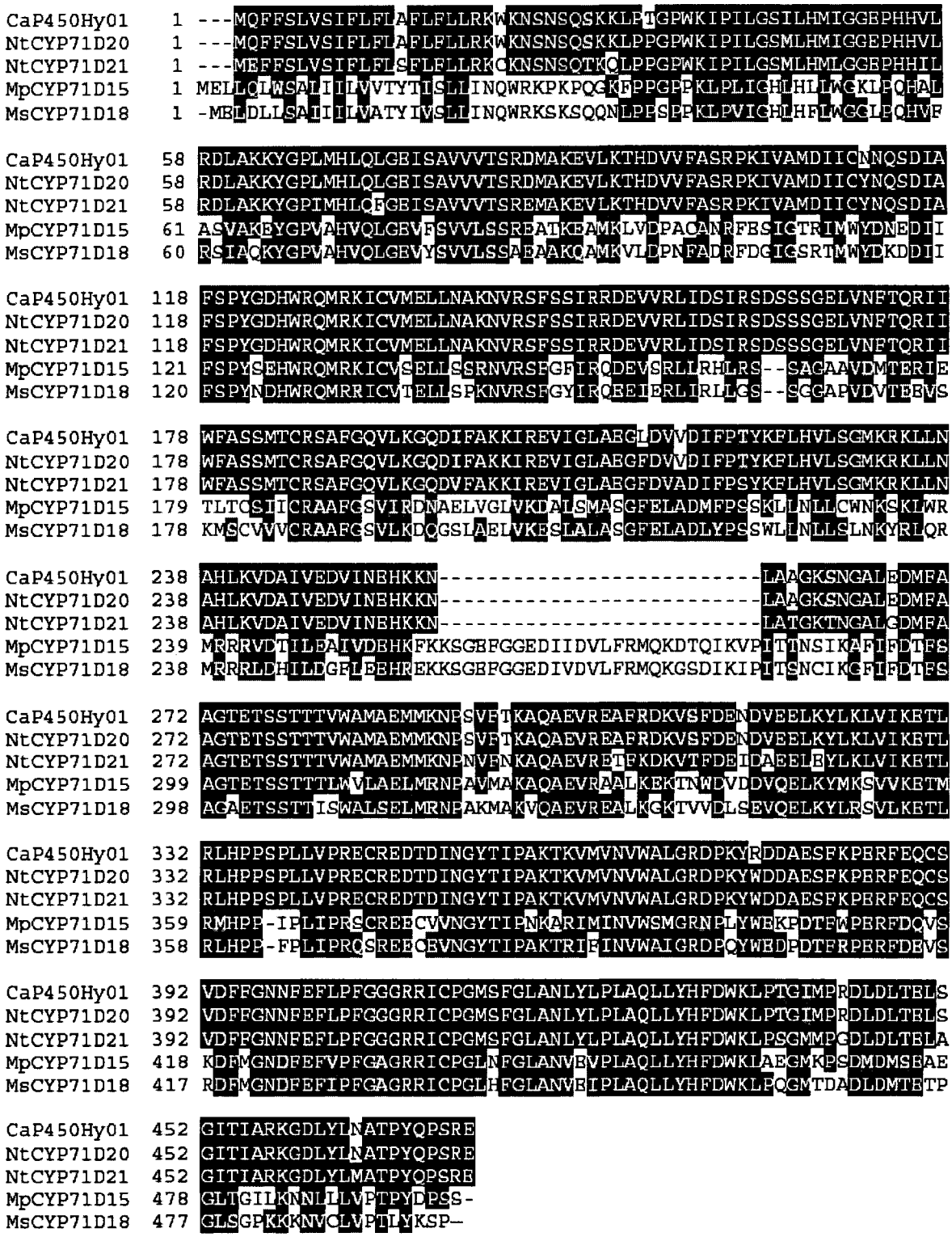


Fig. 4. Multiple amino acid sequence alignment of *P450Hy01* with other plant terpene hydroxylase.
NtCYP71D20 (Accession No. AF368376), NtCYP71D21 (AF368377), MsCYP71D18 (AAD44150), MpCYP71D15 (AAD44152).

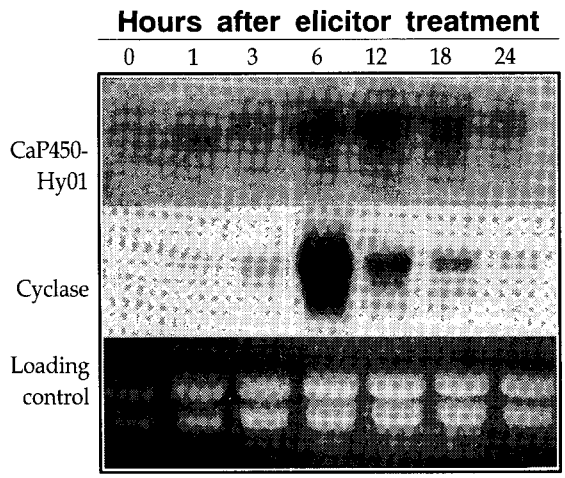


Fig. 5. Induction time course for *P450Hy01* and sesquiterpene cyclase transcripts accumulation in elicitor-treated cells.
Total RNA was extracted from pepper suspension cell treated with the 0.05 ㎍/㎖ cellulase elicitor for the indicated time.

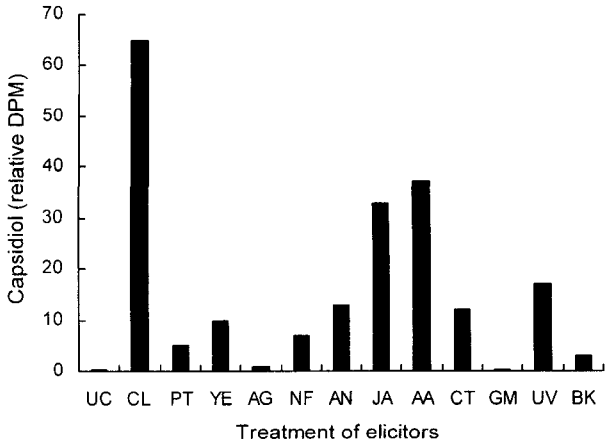


Fig. 6. Extracellular accumulation of capsidiol in the medium of pepper cell cultures treated with various elicitors and environmental stresses.
Cellulase 0.05 ㎍/㎖ (CL), pectinase 0.05 ㎍/㎖ (PT), yeast extract 5.0 ㎍/㎖ (YE), agar 5.0 ㎍/㎖ (AG), NaF 0.5 ㎍/㎖ (NF), $AgNO_3$ 0.5 ㎍/㎖ (AN), jasmonic acid 0.5 ㎍/㎖ (JA), arachidonic acid 0.5 ㎍/㎖ (AA), chitosan 0.5 ㎍/㎖ (CT), gamma ray 50 Gy (GM), ultraviolet-B for 24 hrs (UV), and back ground DPM (BK).

highest capsidiol production in cell cultures also showed highest *P450Hy01* transcript level. This result suggests that *P450Hy01* cDNA cloned in this experiment is closely

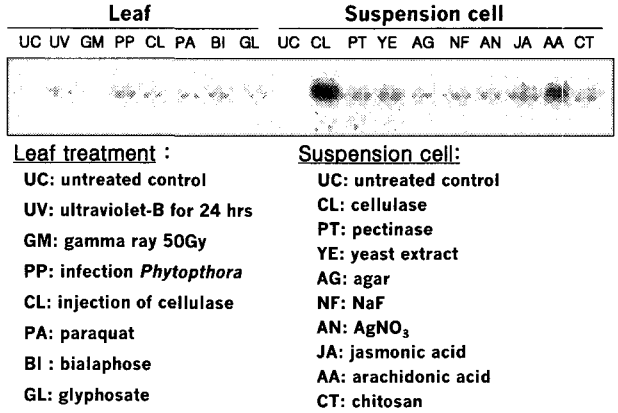


Fig. 7. Expression of *P450Hy01* by various elicitors and environmental stresses in pepper suspension cells and leaves.
Total RNA was extracted from cultured cells and leaves and then Northern hybridized with *P450Hy01* cDNA probe.

related to the production of capsidiol by hydroxylation of 5-*epi*-aristolochene.

## Discussion

The current results confirm that pepper cells respond to pathogen infection, elicitors and a variety of environmental stresses by inducing specific genes encoding for enzymes catalyzing capsidiol production. The *in vivo* assay results shown in Fig. 2 also demonstrate that the conversion of 5-*epi*-aristolochene to capsidiol is catalyzed by at least one elicitor-inducible P450-mediated reaction. However, this *in vivo* assay could not determine whether hydroxylation was catalyzed by one P450 enzyme or two, and whether one enzyme activity might be regulated by elicitation of the cells or two enzymes.

We have successfully cloned full sequence of the cDNA, corresponding to P450 gene families, uniquely expressed in elicitor-treated pepper cells, using RT-PCR and cDNA library screening. Even though we recognized elicitor- inducible gene or mRNA in pepper cell, it is still unclear that the gene is specifically responsible for synthesis of the enzyme catalyzing the hydroxylation of

5-*epi*-aristolochene giving rise to capsidiol production in pepper cell. However, Northern analysis demonstrated that the transcripts corresponding to *P450Hy01* were not detected in control cells, but detected at 6 and 12 h after elicitor addition. The patterns of *P450Hy01* mRNA accumulation were highly correlated with the time course changes of enzyme activities during cell induction. Moreover, levels of *P450Hy01* transcripts being induced by various elicitors were very similar to amount of capsidiol production by the treatments, suggesting that *P450Hy01* is closely related to the production of enzyme catalyzing the hydroxylation of 5-*epi*-aristolochene. There is about 12 to 24 h differences between maximal accumulation of *P450Hy01* mRNA and maximal 5EAH enzyme activity in elicitor-treated pepper cells. This result suggests that transcriptional induction of *P450Hy01* mRNA precedes by approximately 12 h the peak in hydroxylase activity, as might be expected for a transcriptional regulated gene product. Frank *et al.* [5] suggested that the long lag between mRNA accumulation and maximal enzymatic activity corresponds to the time during which P450 is post translationally attached to its heme ligand, inserted in microsomal membranes, and coupled which NADPH-P450 reductase, its electron transfer partner. Although we have not accurately monitored the proportions of two transcript between *P450Hy01* and sesquiterpene cyclase, 5EAS, visual intensity of autoradiography on Northern blot suggested that *P450Hy01* transcripts are relatively lower abundant than the 5EAS transcripts at maximal levels of each genes in elicitor-treated pepper cell. Furthermore, accumulation of *P450Hy01* and 5EAS transcripts is highly associated with activities of corresponding enzyme, at least in response to elicitor treatment. It is likely that induction of hydroxylase gene previously require for the induction of 5EAS, as a proceeding enzyme for the biosynthetic pathway of capsidiol.

A sequence homology search *P450Hy01* against GeneBank showed high homology to a number of P450 gene families, indicating that *P450Hy01* is a member of group of these genes responsible for translation into protein which may catalyze the hydroxylation of 5-*epi*-aristolochene in elicitor-treated pepper cell. Especially, *P450Hy01* cDNA showed 98% of homology in nucleotide as well as amino acid sequence to 5-*epi*-aristolochene-1,3-hydroxylase (5EA1, 3H) which has been isolated in elicitor-treated tobacco cells, indicating that *P450Hy01* is prominent candidate for P450 enzyme coding for 5EA1,3H in pepper cell. Comparison of amino acid sequence for *P450Hy01* with other known gene families in other plant species also showed high identity, and an ORF of the *P450Hy01* also contains heme-binding domain for P450 gene sequence.

Further study on the characterization of gene expression in relation to the hydroxylation activity of 5-*epi*-aristolochene and function of encoded protein to be expressed by cDNA in elicitor treated pepper cells, as well as genomic structure and its promoter, remains to be elucidated.

## Acknowledgments

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## 초록 : 고추세포에서 Capsidiol 생합성을 유도하는 Cytochrome P450 유전자의 탐색

권순태* · 김재성[1] · 정도철[2] · 정정학 · 황재문 · 오세명
(*안동대학교 생명자원과학부, [1]한국원자력연구소, [2]상주대학교 자원식물학과)

고추에서 항균 phytoalexin으로 알려진 capsidiol의 생합성을 촉매하는 5-*epi*-aristolochene hydroxylase는 cytochrome P450 (P450) 억제제인 ancymidol과 ketocornazol에 의해 그 활성이 특이적으로 억제되어, 이 효소가 P450계 효소임을 알 수 있었다. P450 효소가 공통으로 보유하는 염기서열을 지닌 primer를 이용하여 RT-PCR과 cDNA screening을 실시한 결과 고추배양세포에서 elicitor 처리에 의해 강하게 유도되는 cDNA (*P450Hy01*)를 cloning하였다. 배양세포에 cellulase, arachidonic acid, jasmonic acid, 자외선 등을 처리하여 capsidiol을 생합성하는 량과 *P450Hy01* mRNA의 발현정도는 밀접한 유사성이 있었다. *P450Hy01*의 염기서열은 담배에서 밝혀진 5-*epi*-aristolochene-1,3-hydroxylase와 98%의 유사성이 있었으며, P450 효소가 공통으로 지니는 heme-binding domain인 FxxGxRxCxG를 보유하고 있었다. 이러한 결과는 본 연구에서 cloning된 *P450Hy01*이 고추의 세포에서 capsidiol의 생합성을 촉매하는 5-*epi*-aristolochene hydroxylase 효소를 coding하고 있음을 시사한다.