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

Soon-Tae Kwon*, Jae-Sung Kim¹, Do-Cheul Jung², Jeong-Hag Jeong, Jae-Moon Hwang and Sei-Myoung Oh

School of Bioresources Science, Andong National University, Kyungpook 760-749, Korea
¹Korean Atomic Energy Research Institute, Daejeon, 305-353, Korea
²Department 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 elicitor-treated pepper cell, we used in vivo assay method and demonstrated a dramatic suppression of the activity by P450-inhibitors, ancyimidol and ketocornazone. 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 FxxGxRxGxG 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 microorganisms [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 biosynthetic 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 hydroxylation of 5-epi-aristolochene at C3 to generate 1-
deoxycapsidiol, was pathogene or elicitor inducible, while the 1-hydroxylase, responsible for hydroxylating 1-deoxy-
capsidiol 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
testingly, 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 suc-
cessfully performed by reverse transcription polymerase
chain reaction (RT-PCR) using a set of degenerate primer
 corresponding to nucleotide sequence specifying a con-
served 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 hy-
droxylase responsible for the conversion of 5-epi-aristoloch-
ene to capsidiol in elicitor-treated pepper cells, gene
families of P450 cDNAs were identified and charac-
terized. 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 annum 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 ml 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°C.

In vivo assay of P450 hydroxylase activity

P450 hydroxylase, 5-epi-aristolochene hydroxylase (5EAH),
activity was measured as the incorporation of [3H]-
labelled 5-epi-aristolochene into extracellular capsidiol by
intact cell. [3H]-5-epi-aristolochene was produced by
incubating an excess of [3H]-farnesyl diphosphate with
5-epi-aristolochene synthase (5EAS) [12]. At 6 h intervals
after the addition of elicitor, 100,000 dpm of [3H]-5-epi-
aristolochene was carefully added to the surface of 10 ml
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, ancamidol or ketocornazole, directly into the cell cultures. Cell cultures were incubated in the presence of cellulase and 50 μM of ancamidol or ketocornazole for 6 h prior to the addition of [3H]-5-epi-aristolochene as substrate for 5EH. 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 BamHI site (underlined) was based on amino acid sequence of EELP and reverse primer was 25 bp oligo(dT) (5'GGGAATCT TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3') including EcoRI 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, pcDNA11-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 pcDNA11, having cDNA inserts between BstXI 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 μg 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°C in 5 ml of buffer [13] probed with [32P]-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 (5EH) activity described by Ralston et al. [12]. This assay measures the incorporation rate of exogenously supplied [3H]-5-epi-aristolochene into extracellular capsidiol by cell cultures. Using this assay method, a detailed induction time course of 5EH activity in elicitor-induced cell cultures was able to determined relative to enzyme activity of P450 inhibitor-treated cells. Because the 5EH 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, ancamidol 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 [3H]-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 ancyimidol 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 ancyimidol or ketocornazole, and more than 80% by 75 μM ancyimidol and 95% by 100 μM ketocornazole. However, in case of pepper cells, 5EAH activity was completely inhibited by either 50 μM ancyimidol or ketocornazole suggesting that dose response of 5EAH activity to P450 inhibitors is different between tobacco and pepper cell.

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 tuberosus 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). 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 (ORF) 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 FxxGxRxGxG.
Cloning of Cytochrome P450 Gene involved in the Pathway of Capsidiol Biosynthesis in Red Pepper Cells

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 steadystate 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, AgNO3, chitosan, yeast extract and NaF in order. But the treatment of pectinase, agar, v-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, Phytophthora 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, AgNO3 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
Soon-Tae Kwon, Jae-Sung Kim, Do-Cheul Jung, Jeong-Hag Jeong, Jae-Moon Hwang and Sei-Myoungh Oh

CaP450Hy01 1 ---MQFFSLVSIFLFLAFLLFLRLRKWKSNSQSKKLHSGPWKIPILGSLIHMGEPHHLHVI
NtCYP71D20 1 ---MQFFSLVSIFLFLALFLRRLRKWKSNSQSKKLHSGPWKIPILGSLIHMGEPHHLHVI
NtCYP71D21 1 ---MBFFSLVSIFLFLALFLRRLRKWKSNSQSKKLHSGPWKIPILGSLIHMGEPHHLHVI
MpCYP71D15 1 MBILGLALTFLVIYWTVGQSLINQRKPKFKCQFLFPCGFLPLCHLICRLEKCHL
MsCYP71D18 1 --MBEGLSATFLVIYTIVSLINQRKKSQQLPLFPCGFLPLCICRLEKCHL

CaP450Hy01 58 RDLAKKYGLMLGBIGASAVVTVSDMMAKEKLTEDIVVAFSPKIVAMDIGNOSDIAS
NtCYP71D20 58 RDLAKKYGLMLGBIGASAVVTVSDMMAKEKLTEDIVVAFSPKIVAMDIGNOSDIAS
NtCYP71D21 58 RDLAKKYGLMLGBIGASAVVTVSDMMAKEKLTEDIVVAFSPKIVAMDIGNOSDIAS
MpCYP71D15 61 ASVABYGFPAHVLQGESHVSLSSRATKFMKLVPAPCLNFSSICTRMWMYNEDHII
MsCYP71D18 60 RSPACKFYPFAHVLQGESHVSLSSRPAACFQKVLDPNPCDIMFSSTRTMYKQDDII

CaP450Hy01 118 FSIPYGDHRWQMRKICKVMEEHKANAKVRSFSSISRDEVVRLIDSIRSDSSGELVNFTORII
NtCYP71D20 118 FSIPYGDCHRWQMRKICKVMEEHKANAKVRSFSSISRDEVVRLIDSIRSDSSGELVNFTORII
NtCYP71D21 118 FSIPYGDCHRWQMRKICKVMEEHKANAKVRSFSSISRDEVVRLIDSIRSDSSGELVNFTORII
MpCYP71D15 121 FSIPYGRWQMRKICKVMEEHLKCNVRSFGTIFDRESSLIRLREG--SACAAVDYTERIE
MsCYP71D18 120 FSIPYGDHRWQMRKICKVMEEHLKCNVRSFGTIFDRESSLIRLREG--SCCAAVDVBVGS

CaP450Hy01 178 WFASSMSMTSACSGQVQVKGQIDFAPKAKERIVIGLAEQJLDVIWDDFTYFKLHLVSCMRKLLN
NtCYP71D20 178 WFASSMSMTSACSGQVQVKGQIDFAPKAKERIVIGLAEQJLDVIWDDFTYFKLHLVSCMRKLLN
NtCYP71D21 178 WFASSMSMTSACSGQVQVKGQIDFAPKAKERIVIGLAEQJLDVIWDDFTYFKLHLVSCMRKLLN
MpCYP71D15 179 TLTCGTICRACFGVLDNMRVGLKDLNMAEGLALMDPPSGLNMLCMRKSQKWR
MsCYP71D18 178 KWSVATCCRACFGVLDNMRVGLKDLNMAEGLALMDPPSGLNMLCMRKSQKWR

CaP450Hy01 238 AHLEKDAIVEDVINBHKN--LARGKSNAGELDMAFA
NtCYP71D20 238 AHLEKDAIVEDVINBHKN--LARGKSNAGELDMAFA
NtCYP71D21 238 AHLEKDAIVEDVINBHKN--LARGKSNAGELDMAFA
MpCYP71D15 239 MRRLVHILALVBDHFKKSCBPGFEGIDIDVLFRMQKDIQKVTNSIKFFIFRTES
MsCYP71D18 238 MRRLVHILALVBDHFKKSCBPGFEGIDIDVLFRMQKDIQKVTNSIKFFIFRTES

CaP450Hy01 272 AGTETSSSTTVVAMAENMKQESVEKTAQAEVREAFDKVSFDENDBEKLKLYKLVIKBT
NtCYP71D20 272 AGTETSSSTTVVAMAENMKQESVEKTAQAEVREAFDKVSFDENDBEKLKLYKLVIKBT
NtCYP71D21 272 AGTETSSSTTVVAMAENMKQESVEKTAQAEVREAFDKVSFDENDBEKLKLYKLVIKBT
MpCYP71D15 299 AGTETSSTTYELAMLRENEAIQAKQAEVAGAFKRERTDQEKIFREIFH
MsCYP71D18 298 AGTETSSSTVWALSLEMNPLAKAPMAAEQAVREALGKFAFGLEFLVEFQIKLVE

CaP450Hy01 332 RLHPSLPLVPRECREDDITINGYITYPAKTIVMKVMOQVLNPSLALRDPOKIRDESPFEQCS
NtCYP71D20 332 RLHPSLPLVPRECREDDITINGYITYPAKTIVMKVMOQVLNPSLALRDPOKIRDESPFEQCS
NtCYP71D21 332 RLHPSLPLVPRECREDDITINGYITYPAKTIVMKVMOQVLNPSLALRDPOKIRDESPFEQCS
MpCYP71D15 359 MHPPEI-PPTSPSCRFPPCNGQITTPKARIMINWMSGRCNPWYFKDFTETFRRDQV
MsCYP71D18 358 RLHPSLPLVPRECREDDITINGYITYPAKTIVMKVMOQVLNPSLALRDPOKIRDESPFEQCS

CaP450Hy01 392 VDFGNNEIEFLFGGRRICPGMSEGLANLYLPLAQOLLYHPDKLPTCGIMRDPDLDLTLBS
NtCYP71D20 392 VDFGNNEIEFLFGGRRICPGMSEGLANLYLPLAQOLLYHPDKLPTCGIMRDPDLDLTLBS
NtCYP71D21 392 VDFGNNEIEFLFGGRRICPGMSEGLANLYLPLAQOLLYHPDKLPTCGIMRDPDLDLTLBS
MpCYP71D15 418 RKMGFDFEPFPAFCAGRICTPGLMFGLANLYLPLAQOLLYHPDKLIAKRESGQMSRBAE
MsCYP71D18 417 RKMGFDFEPFPAFCAGRICTPGLMFGLANLYLPLAQOLLYHPDKLIAKRESGQMSRBAE

CaP450Hy01 452 GITTARCGDLYATYPQYPSRE
NtCYP71D20 452 GITTARCGDLYATYPQYPSRE
NtCYP71D21 452 GITTARCGDLYATYPQYPSRE
MpCYP71D15 478 GITLQGNNILLVQITPDESS-
MsCYP71D18 477 CLSGFKKKVNLTVLPKSP-

Fig. 4. Multiple amino acid sequence alignment of P450Hy01 with other plant terpene hydroxylase.
NtCYP71D20 (Accession No. AF368376), NtCYP71D21 (AF368377), MsCYP71D18 (AAD4150), MpCYP71D15 (AAD4152).
Cloning of Cytochrome P450 Gene involved in the Pathway of Capsidiol Biosynthesis in Red Pepper Cells

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 μg/ml 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 μg/ml (CL), pectinase 0.05 μg/ml (PT), yeast extract 5.0 μg/ml (YE), agar 5.0 μg/ml (AG), NaF 0.5 μg/ml (NF), AgNO3 0.5 μg/ml (AN), jasmonic acid 0.5 μg/ml (JA), arachidonic acid 0.5 μg/ml (AA), chitosan 0.5 μg/ml (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 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

This research was supported by Basic Science Research Program in Korean Research Foundation. Gamma ray (60Co) was irradiated at Korean Atomic Energy Research Institute, Daejeon, Korea.

References

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


(Received August 26, 2003; Accepted November 17, 2003)
초록: 고추세포에서 Capsidiol 생성성을 유도하는 Cytochrome P450 유전자 탐색

권순태* · 김재성1 · 정도철2 · 정정학 · 황재문 · 오세명
(*안동대학교 생명자원과학부, 1한국원자력연구소, 2상주대학교 자원식물학과)

고추에서 항균 phytoalexin으로 알려진 capsidiol의 생성성을 촉매하는 5-epi-aristolochene hydroxylase는 cytochrome P450 (P450) 억제제인 ancymidol과 ketocornazol에 의해 그 활성을 특이적으로 억제되어, 이 효소가 P450계 효소임을 알 수 있었다. P450 효소가 공통으로 보유하는 염기서열을 지난 primer를 이용하여 RT-PCR과 cDNA screening을 실시한 결과 고추배양세포에서 elicitor 처리에 의해 강하게 유도되는 cDNA (P450Hy01)을 cloning하였다. 배양세포에 cellulase, arachidonic acid, jasmonic acid, 자외선 등을 처리하여 capsidiol을 생성성하는 량과 P450Hy01 mRNA의 발현정도는 밀접한 유사성이 있었다. P450Hy01의 염기서열은 항균 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 P450Hy01이 고추의 세포에서 capsidiol의 생성성을 촉매하는 5-epi-aristolochene hydroxylase 효소를 coding하고 있을음을 시사한다.