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Cloning of Elicitor-Inducible 5-epi-Aristolochene Hydroxylase in Tobacco Cell Suspension Culture

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Abstract

The last enzyme of the sesquiterpen phytoalexin capsidiol synthesis in tobacco cell, 5-epi-aristolochene hydroxylase which convert 5-epi-aristolochene(EAS) to capsidiol, was cloned by a reverse transcription polymerase chain reaction strategy and cDNA library screening. Cloned *CYP-B3* contained high probability amino acid matches to known plant cytochrome P450 sequences and open reading frame with the conserved FxxGxRxCxG heme-binding region. Transcripts of *CYP-B3* were not detected in control cells, but induced in elicitor-treated cells. Furthermore, *CYP-B3* transcripts were induced by fungal extracts and cellulase but not by other stimuli(chilling, heat shock and 2,4-D). Induction of *CYP-B3* transcripts by elicitor treatment was not affected by ancymidol and ketoconazole treatments suggesting that an inhibition of hydroxylase activity by Cyt P450 inhibitors resulting from post translational processing event.

Key words : *Capsidiol, 5-epi-aristolochene hydroxylase, Cytochrome P450, Elicitor*

Introduction

One of the plant cell's responses to challenge by pathogen or pathogen-drived factors known as elicitors is the production of antimicrobial phytoalexins. The chemical class of phytoalexins produced by plants seem to be family-specific. The phytoalexins produced by Solanaceous are terpene derivatives. Many reports have demonstrated that addition of fungal elicitor to tobacco cell suspension cultures results in the induction of sesquiterpenoid, including capsidiol, biosynthesis with the induction of a sesquiterpene cyclase enzyme activity[1,2]. The sesquiterpene cyclase catalyzes the conversion of farnesyl diphosphate(FPP), a common precursor of both sesquiterpenoid and sterol biosynthesis, to 5-epi-aristolochene(EAS), a bicyclic sesquiterpene intermediate predicted for the ultimate production of capsidiol[2]. Capsidiol is a principal phytoalexin found in tobacco tissues challenged with pathogene or fungal elicitors[3-5]. Although much is known about the biosynthetic pathway for EAS in relation to the characterization of sesquiterpene cyclase enzyme in tobacco and other Solanaceae[4,6,7], little is known about the enzymatic regulation of the hydroxylation of EAS to form capsidiol. Hoshino et al.[8] suggested that EAS hydroxylase is a member of cytochrome(Cyt) P450s, in that microsomal enzyme activity required both NADPH and O2 for activity, and Cyt P 450 inhibitors, such as ancymidol, ketoconazole and CO

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gas, suppressed the enzyme activity in a dose dependent manner.

Plant Cyt P450 monooxygenases are involved in mediating the formation of a wide range of plant metabolites, including phenylpropanoids, fatty acids, terpenoids, alkaloids, hormones, pigment and phytoalexins[9,10]. In addition, certain Cyt P450 enzymes have been reported to be involved in the detoxification of herbicides in plants, and drugs in mammalians[11,12]. Wide range of reactions are mediated by specific forms of those proteins which might be expressed only under certain circumstances, for example, exposure to pathogens, herbicides, fungal elicitors, manganese and wounding[8,9,12]. Recently, hundreds of mammalian Cyt P450 cDNA clones have been isolated and the regulation of the expression of their corresponding genes is the subject of extensive research[11,13,14]. Despite of prevalency and physiological importance, relatively little is known about the number of Cyt P450 enzymes in plant tissues and their expression patterns. The limited knowledge of plant Cyt P450 enzyme due to difficulties encountered in isolating and purifying those enzymes. Especially, the low Cyt P450 contents of most plant tissues, instability of these enzymes after tissue homogenation and protein solublization represent major problems[13,15]. However, many investigators have succeeded in purifying some distinct Cyt P 450 from plants[10,16-18]. Isolation of Cyt P450 cDNA clones from plants was successfully performed by reverse transcription polymerase chain reaction (RT-PCR)-based method using a set of degenerate PCR primer corresponding to nucleotide sequence specifying a conserved region in Cyt P450 proteins[9,10]. Sequence comparisons of Cyt P450 proteins from animals, microorganisms and plants have led to identification of several high conserved region. One of these domains, close to C-terminal end of the protein, is involved in binding of the Cyt P450 heme group. This domain contains the highly conserved sequence FxxGxRxCxG which has been regarded as fingerprint for cloning Cyt P450 genes[9,10,18].

To understand the properties of elicitor-inducible Cyt P450 dependent hydroxylase responsible for the conversion of EAS to capsidiol in elicitor-treated tobacco cells, EAS hydroxylase was cloned. In addition, effects of Cyt P450 inhibitors, ancymidol and ketoconazole, on the enzyme activity and expression of hydroxylase was investigated.

Materials and Methods

Cell Cultures and Elicitor Treatment

Cell cultures of *Nicotiana tabacum* cv. KY14 were maintained in modified MS medium as described previously[2]. Cells under the rapid growing, corresponding to 3 days after subculturing, were used for all the experiments presented. Elicitor treatment was initiated by the addition of 0.5 μg of cellulase(*Trichoderma viride*, type RS, Onozuka) per mℓ of cell suspension cultures for the indicated length of time before collecting cells and media. Control and elicitor and/or ancymidol or ketoconazole-treated cells and media were harvested by gentle vacuum filtration through a single sheet of miracloth and used for enzyme activity and expression study. Assay for cyclase activity was performed as previous report[2].

RNA Extraction, RT-PCR and Cloning

Total RNAs were isolated from control and 12 h elicitor-treated tobacco suspension cells using phenol/chloroform extraction method[19], and poly(A)$^+$RNA was prepared by oligo(dT) cellulose chromatography column (GIBCO, Life Technology). First strand cDNA was synthesized using an oligo(dT) primer and reverse transcriptase(cDNA Cycle Kit, Invitrogen). A cDNA clone of mRNA was isolated using reverse transcription polymerase chain reaction(RT-PCR) strategy based on sequence comparison of Cyt P450 monooxygenase genes from avocado[20], pea[9] and Jerusalem artichoke[18]. A degenerate 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 restriction site (underlined) was synthesized based on amino acid sequence of EEF(R/L)PER and reverse primer was 25-bp oligo(dT) primer (CGGAATTCTTTTTTTTTTTTTTTT) including *Eco*RI restriction site (underlined). The 413-bp RT-PCR products uniquely generated from elicitor-induced poly(A)$^+$RNA were isolated from an agarose gel using DE-81 ion exchange paper (Whatman, Maidston, England). The RT-PCR product was restriction enzyme digested with *Bam*HI and *Eco*RI at 37℃ for 1 h, and ligated into corresponding restriction site of pBluescript SK(Stratagene), and then transformed into E. coli strain TB1 according to $CaCl_2$ transformation procedure[19]. The transformants were selected on X-gal/ampicillin LB plate and subjected to northern hybridization and sequence analysis.

Screening of the cDNA library using PCR method

To obtain the upstream sequence of 413-bp RT-PCR product, pcDNAII-plasmid cDNA library, prepared from elicitor-treated tobacco suspension 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 subjected to purifying plasmid DNA. Plasmids of pcDNAII, having cDNA library inserts between *Bst*XI restriction enzyme sites, were directly amplified by PCR using gene specific reverse primer (5'-CGGGATCCCTTAAGCATATTATGCAGCAATAGG-3', *Bam*HI site underlined) positioned in the translation termination codon(bold) of 413-bp 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 as described above.

Northern Analysis

Total RNAs were electrophoresised on 1.0% agarose-formaldehyde gel[19]. The RNA was transferred overnight onto a nylon membrane(Zetta probe, BioRad, Hercules, CA) in 10x SSC(1x SSC=0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) solution. Following transfer, the membrane was air dried and UV cross-linked using Stratalinker(Stratagene). RNA blot hybridization was performed at 42℃ in 5 mℓ of hybridization buffer(5x SSPE, 2x denhardt's solution, 0.2% SDS, 20 μg $mℓ^{-1}$ tRNA, 50 % foramid) probed with ^{32}P-labeled 413-bp RT-PCR product or sesquiterpene cyclase gene using a commercial kit (Prime-It Kit, Stratagene). RNA blot was washed twice at room temperature for 10 min with 2x SSC, 0.1% SDS, and twice for 30 min with 0.1x SSC, 0.1% SDS. Hybridization signal was visualized by autoradiography.

Results

Cloning by RT-PCR and cDNA Screening

To isolate elicitor-inducible hydroxylase gene(s) out of extensive variations of Cyt P450 gene families in tobacco cells, we used an RT-PCR strategy using degenerate primer based on recently reported genes. To maximize opportunities for cloning hydroxylase gene(s) which might be specifically involved in the hydroxylation of EAS, PCR products generated by poly(A)$^+$RNA from control cells were compared to those generated by 12 h-induced cells on 1.0% agarose electrophoresis gel. In preliminary experiment, we confirmed that hydroxylase activity was rapidly increased at 12 h after elicitor addition. This would be expected to produce abundant mRNA encoding for the enzyme at this time. The mRNA was reverse transcribed using oligo(dT) reverse primer complementary to the 3' polyadenine tail of mRNA, and PCR amplified by degenerate forward primer encoding a conserved amino acid sequences, EEF(R/L)PER, located in approximately 30 amino acids upstream from the highly conserved heme-binding domain in Cyt P450 gene families. The forward degenerate primer was based on sequence conservation in the region of the Jerusalem artichoke *CYP73A1*[18], the avocado *CYP71A1*[20], and the pea *CYP73A9* sequence encoding trans-cinnamic acid 4-

hydroxylase(t-CAH)[9]. From the RT-PCR reactions, PCR products which were uniquely generated by elicitor-induced mRNA were cloned using *Bam*HI and *Eco*RI sites included in 5' and 3' PCR primers, respectively. Of the 60 transfromants that were sized, 12 independent clones had inserts in the size approximately 400 to 550 bp, and only one(413-bp) of those clones was shown high specificity to elicitor-induced mRNA by northern hybridization(Fig. 1). To obtain the upstream sequence

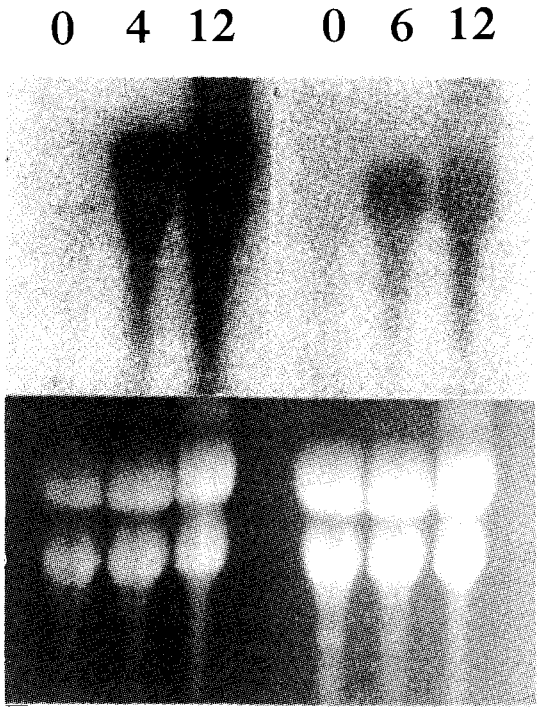

Fig. 1. Northern analysis of sesquiterpene cyclase and *CYP-B3* in elicitor-treated tobacco suspension cells. Total RNAs isolated from indicated times(0, 6, and 12 h) of elicited-cells were electrophoresised on a 1.0% agarose formaldehyde gel, blotted onto nylon membrane, and probed with ^{32}P labeled 413-bp RT-PCR product of *CYP-B3* and full length of tobacco sesquiterpene cyclase cDNA probe.

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GGA GAA AAG CAT AAG ATA AGC TGT GCT ATT GAT CAC ATT ATA GAT GCC GAA ATG AAA GGA      60
 G  E  K  H  K  I  S  C  A  I  D  H  I  I  D  A  E  M  K  G

GAA ATA AAT GAG CAA AAT GTA CTC CAT ATT GTG GAG AAT ATC AAT GTT GCA GCA ATT GAA     120
 E  I  N  E  Q  N  V  L  H  I  V  E  N  I  N  V  A  A  I  E

ACA ACT CTA TGG TCC ATG GAA TGG GCC ATA GCT GAA CTT GTA AAT CAT CCC ATT GTT CAA     180
 T  T  L  W  S  M  E  W  A  I  A  E  L  V  N  H  P  I  V  Q

CAG AAG ATT AGG GAT GAA ATC TCA ACA GTC CTC AAA GGC AGA TCA GTC ACA GAA TCA AAC     240
 Q  K  I  R  D  E  I  S  T  V  L  K  G  R  S  V  T  E  S  N

CTC CAT GAG CTG CCT TAC TTG CTA GCA ACA GTA AAT GAA ACA TTA AGA CTC CAC ACA CCA     300
 L  H  E  L  P  Y  L  L  A  T  V  N  E  T  L  R  L  H  T  P

ATA CCT TTA CTT GTA CCA CAT ATG AAC CTT GAA GAA GCA AAG CTA GGT GGT TAC ACT ATT     360
 I  P  L  L  V  P  H  M  N  L  E  E  A  K  L  G  G  Y  T  I

CCT AAA GAA ACT AAG GTG GTT GTG AAT GCA TGG TGG CTG GCT AAC AAC CCT GCC TGG TGG     420
 P  K  E  T  K  V  V  V  N  A  W  W  L  A  N  N  P  A  W  W

AAA AAC CAG AAC GAA TTC CGG CCC GAG CGG TTT CTC GAG GAG GAT AGT AGC ACA GAG GCA     480
 K  N  Q  N  E  F  R  P  E  R  F  L  E  E  D  S  S  T  E  A

GCT GTT GCT GGT GGC AAG GTT GAT TTC AGG TAC TTG CCC TTC GGT ATG GGG AGG CGG AGC     540
 A  V  A  G  G  K  V  D  F  R  Y  L  P  F  G  M  G  R  R  S
                                          *           *

TGC CCC GGA ATC ATC CTT GCA CTG CCA ATT CTG GGG CTT GTC ATA GCC AAA CTA GTG TCA     600
 C  P  G  I  I  L  A  L  P  I  L  G  L  V  I  A  K  L  V  S
 *     *

AAT TTT GAA ATG CAG GGT CCA CCA GGT GTG GAA AAG GTT CAT ACA AGT GAA AGA GGA GGG     660
 N  F  E  M  Q  G  P  P  G  V  E  K  V  H  T  S  E  R  G  G

CAG TTT AGC TTG CAC ATT GCA AAA CAT TCC ACG GTT GTC TTC AAG CCT ATT GCT GCA TAA     720
 Q  F  S  L  H  I  A  K  H  S  T  V  V  F  K  P  I  A  A

TATGCTTAAGCTATCCTTGTTTTAATTATATTTGTCTTACCACAAAGCAAAACTACTAAGTTACTCGATAAAGATTTCA     799

ATGAATATTACAGTTTTTGTTACCNAAAAAAAAAAAAAAAAA                                      842
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Fig. 2. Nucleotide and predicted amino acid sequence in single letter code of the cDNA *CYP-B3*. The translation termination codon TAA is underlined. Amino acids conserved in the heme-binding region are designated with asterisks.

of 413-bp RT-PCR product, cDNA library in pcDNAII-plasmid was directly amplified by PCR using internal reverse primer specific to 413-bp RT-PCR product and T7 forward primer positioned in the plasmid. From PCR amplification, we obtained specific clone, namely *CYP-B3* that had 842-bp cDNA sequences containing open reading frame of 239 amino acids, and contained high probability amino acid matches to known eukaryotic Cyt P450 sequences (GenBank data base), an open reading frame with the conserved FxxGxRxCxG heme-binding region and intact forward and reverse PCR primers used(Fig. 2). An analysis of the deduced amino acid sequence for *CYP-B3* and other species show 70% of identities to t-CAH cloned from *Phaseolus aureus*[21], *Glycin max*(GenBank accession No. X92437) and *Zinnia eleganse*(GenBank No. U199[22], and 69% identities to that from *Pisum sativum*[9] and *Helianthus tuberosus*[18]. Comparison of amino acid sequence indicated that *CYP-B3* polypeptide was significantly homologous to Cyt P450-dependent gene families in other species(Fig. 3).

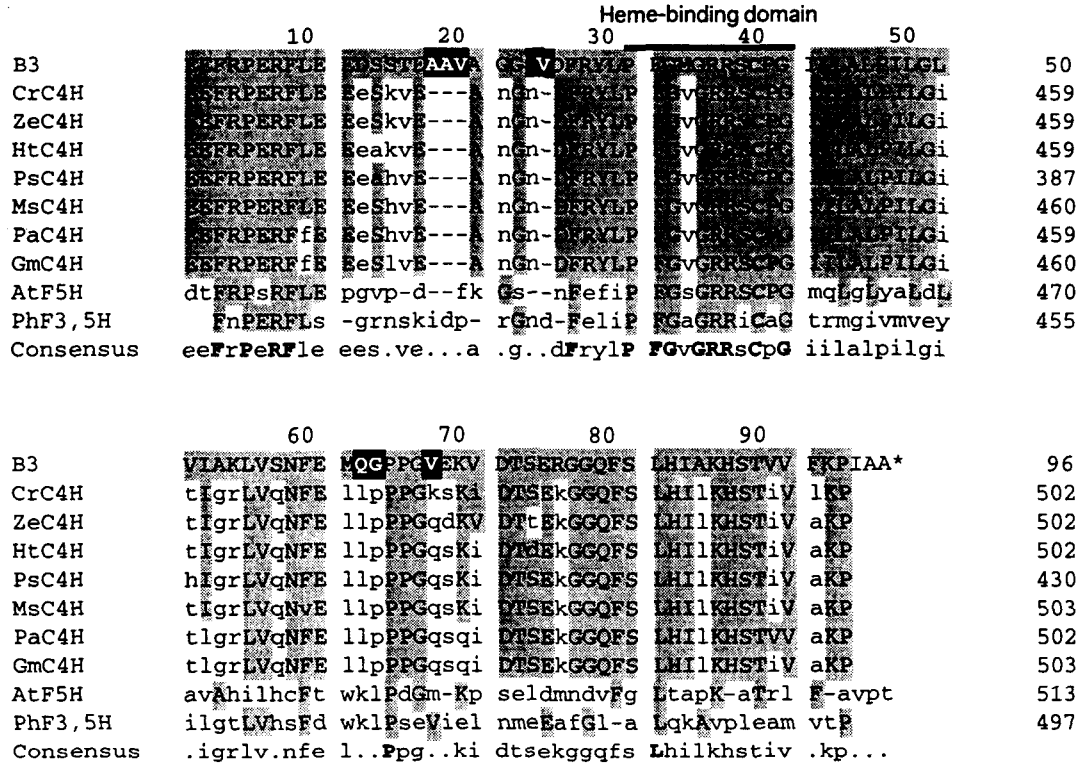

Fig. 3. Alignment of *CYP-B3* with other Cyt P450 sequences.
Amino acid comparison of Cyt P450 genes, cinnamic acid 4-hydroxylase from *Phaseolus aureus*(Mizutani et al., 1993), *Glycin max*(GenBank accession No. X92437), *Zennia eleganse*(GenBank No. U19922), *Pisum sativum*(Frank et al., 1996), *Helianthus tuberosus*(Teutsch et al., 1990) and *Catharanthus roseus*, flavonoid 5-hydroxylase from *Arabidopsis thaliana*, and flavonoid 3,5-hydroxylase from *Petunia hybrida*. Numbers indicate the amino acid residues in the sequences, gaps in the alignment are designated by dashes. Amino acid which are common to all five sequences are denoted as the concensus sequences, large capital. Overlines numbered with roman numerals indicates highly conserved regions.

Enzyme Activity and Northern Analysis in Respond to Elicitor and/or Cyt P450 Inhibitors

Cyt P450-dependent enzymes inhibitors ancymidol and ketoconazole would be expected to inhibit the incorporation of [^{3}H] labeled EAS into capsidiol by inhibiting hydroxylase enzyme activity. Extracellular accumulation of [^{3}H] labeled capsidiol which might be synthesized by hydroxylation reaction of [^{3}H] labeled EAS, was determined in tobacco cell suspension cultures, treated with cellulase plus different concentrations of ancymidol or ketoconazole for 12 h, followed by 3 h incubation with [^{3}H] labeled EAS. In addition to the hydroxlyase activities, effects of those chemicals on sesquiterpene cyclase activity in tobacco suspension cells (*in vivo*) as well as cyclase purified from bacterial extracts were examined(*in vitro*)(Fig. 4). Enzyme activity of hydroxylase was inhibited in a dose dependent manner, greater than 30% by 5 μM ancymidol and 25% by as little as 1 μM ketoconazole, and subsequently suppressed more than 80% and 95 % by 75 μM ancymidol and 100 μM ketoconazole, respectively. However, activities of both types of sesquiterpene cyclases, one

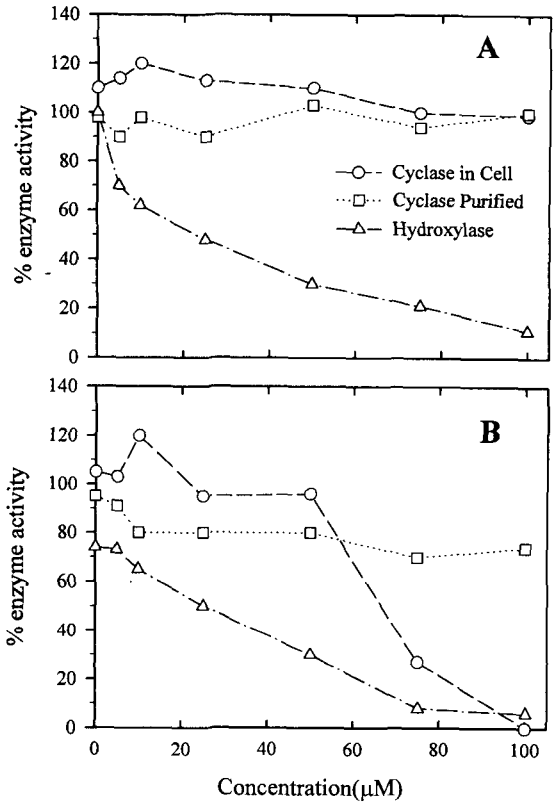

Fig. 4. Effect of ancymidol(A) and ketoconazol(B) on the activity of cyclase and 5-EAS hydroxylase.

from tobacco cells and the other from bacterial extracts, were not significantly affected by even 100 μM ancymidol. These results were almost identical with ketoconazole treated cyclase activities with the exception that enzyme activity of cyclase in tobacco cells, but not of bacterial extracts, was dramatically inhibited, greater than 78 % by 75 μM and up to 100 % by 100 μM ketoconazole, which might be caused by cell death by lethal concentration of the chemical, resulting in interception of cell induction as well as blocking all biochemical processes in the cells. It is well known that a member of Cyt P450 enzymes are induced by a variety of environmental stresses and xenobiotics. Thus, to demonstrate cloned *CYP-B3* was specifically induced by elicitor only, suspension cells were subjected to cold, heat and herbicide 2,4-D treatments. *CYP-B3* transcripts were detected only in the fungal extracts and elicitor cellulase (Fig. 5). Because *CYP-B3* transcripts were induced by fungal extracts and cellulase but not by cold, heat and herbicide, we could concluded that this *CYP-B3* clone was elicitor inducible plant defence related hydroxylase rather than other Cyt-P450 genes.

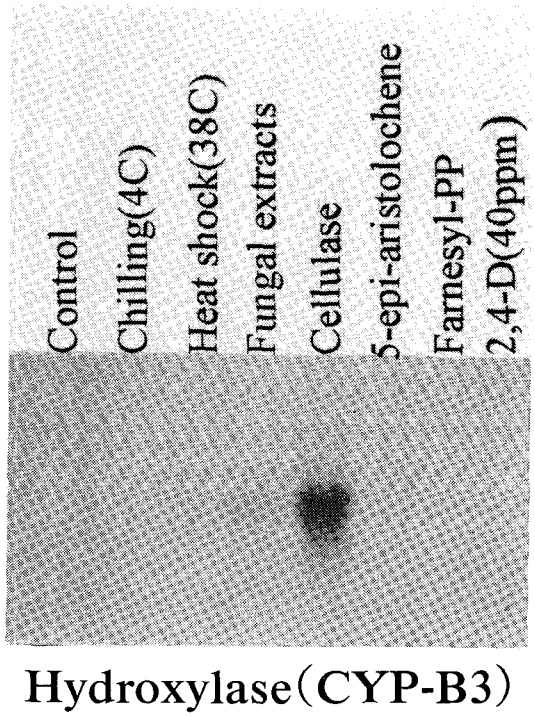

Fig. 5. Nothern analysis of *CYP-B3* in respond to chilling, heat shock, 2,4-D and elicitors.

Further to investigate if the inhibition of hydroxylase activity by Cyt P450 inhibitors is due to transcriptional or post transcriptional regulation, total RNAs extracted from control and induced cells, and elicitor+ancymidol or ketoconazole-treated cells were hybridized with ^{32}P labeled *CYP-B3*(Fig. 6). Specific accumulation of the transcripts corresponding to *CYP-B3* mRNA was detected at about 1.5 kb, whereas that of sesquiterpene cyclase was 1.8 kb as previously reported[22]. Regardless

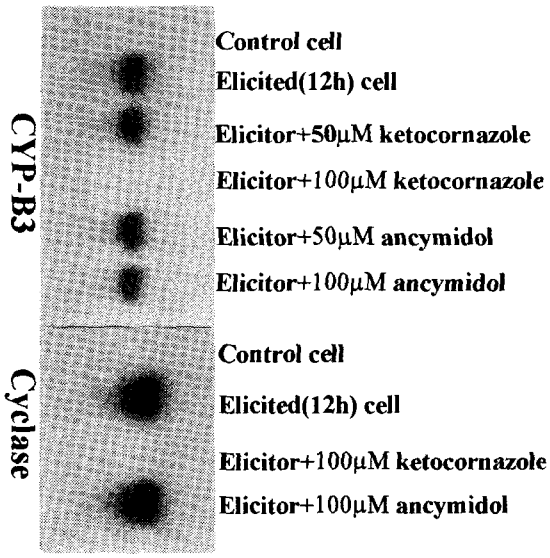

Fig. 6. Nothern analysis of *CYP-B3* and cyclase in respond to Cyt P450 inhibitors, ketoconazole and ancymidol. Cell cultures were incubated in the absence or presence of cellulase plus indicated concentrations of ancymidol and ketoconazole for 12 h prior to isolating total RNAs.

of concentrations applied, induction of transcripts corresponding *CYP-B3* and sesquiterpene cyclase genes by elicitor treatment were not affected by ancymidol treatment. However, 100 μM ketoconazole completely inhibited the expression of *CYP-B3* and sesquiterpene cyclase transcripts, whereas as 50 μM did not inhibit elicitor-induced gene expression. As previously explained in the biochemical analysis of enzyme activity, treatment of 100 μM ketoconazole completely suppressed hydroxylase and sesquiterpene cyclase activity due to cell death by lethal concentration. This suggests that absence of transcripts in 100 μM ketoconazole treated cells is not due to the inhibition of gene expression by this chemical but due to cell death resulting in blocking all biochemical processes in the cells. We concluded from this result that inhibition of enzyme activity of hydroxylase, as one of Cyt P450 dependent enzymes, is not involved in transcriptional level. In addition, sesquiterpene cyclase was insensitive to exogenously applied ancymidol or ketoconazole in the lower concentrations than lethal dose. Furthermore, the inhibition of extracellular capsidiol production in elicitor-treated tobacco cell suspension cultures by these chemicals is attributed to a post translational inhibition of hydroxylase(s), the enzyme(s) which hydroxylates the sesquiterpene cyclase reaction product giving rise to capsidiol.

Discussion

We have successfully cloned partial sequence of the cDNA, corresponding to Cyt P450 gene families, uniquely expressed in elicitor-treated tobacco suspension cells using RT-PCR strategy and cDNA library screening. Even though we recognized elicitor-inducible gene or mRNA in tobacco cell, it is still unclear that the gene is specifically responsible for the synthesis of enzyme catalyzing the hydroxylation of EAS giving rise to capsidiol production in tobacco cells. However, northern analysis demonstrated that the transcripts corresponding to *CYP-B3* were not detected in control cells, but detected at 6 and 12 h after elicitor addition. The patterns of *CYP-B3* mRNA accumulation were highly correlated with the time course changes of enzyme activities during cell induction, suggesting that *CYP-B3* is related to the production of enzyme catalyzing the hydroxylation of EAS(unpublished data). There is about 6 h differences between maximal accumulation of *CYP-B3* mRNA and maximal enzyme activity in elicitor-treated tobacco cells. This result suggests that transcriptional induction of *CYP-B3* precedes by approximately 6 h the peak in hydroxylase activity, as might be expected for a transcriptionally regulated gene product. Frank et al.[9] suggested that the long lag between mRNA accumulation and maximal enzymatic activity corresponds to the time during which Cyt P450 is post translationally attached to its heme ligand, inserted in microsomal membranes, and coupled with NADPH-Cyt P450 reductase, its electron

transfer partner. Although we have not accurately monitored the proportions of two transcripts between *CYP-B3* and sesquiterpene cyclase, relative to one another, visual intensity of autoradiography on northern blot suggested that *CYP-B3* transcripts are significantly lower abundant than the sesquiterpene cyclase transcripts in elicitor-treated tobacco suspension cells. Moreover, accumulation of *CYP-B3* and sesquiterpene cyclase transcripts is highly associated with activities of corresponding enzymes, at least in respond to elicitor treatment. It is likely that induction of hydroxylase gene previously require for the induction of sesquiterpene cyclase, as a proceeding enzyme for the biosynthetic pathway of capsidiol production. Several studies demonstrated that the transcripts corresponding to Cyt P450-dependent genes are specifically accumulated in the plants such as alfalfa [23,24] and pea[25,26] in respond to fungal elicitors.

The levels of sesquiterpene cyclase and *CYP-B3* transcripts in elicitor-treated cells were not affected by ancymidol or ketoconazole treatment at lower concentration than lethal dose. In the enzyme assay experiment, sesquiterpene cyclase enzyme activity was insensitive to those chemicals, whereas hydroxylase activity was greatly inhibited in a dose dependent manner. However, in northern analysis, induction of *CYP-B3* and sesquiterpene cyclase transcripts by elicitor treatment was not affected by ancymidol and ketoconazole treatment. This result suggests that the inhibition of extracellular capsidiol production in elicitor-treated tobacco cell suspension cultures by ancymidol and ketoconazole is due to the post translational inhibition of hydroxylase.

A sequence homology search of *CYP-B3* against GenBank data base showed significant homology to a number of Cyt P450 gene families, indicating that *CYP-B3* is a member of group of these genes responsible for translation into protein which may catalyze the hydroxylation of EAS in elicitor-treated tobacco cells. Comparison of deduced amino acid sequence for *CYP-B3* with known Cyt P450 gene families in other plant species also showed high identity, and an open reading frame of the *CYP-B3* contains the conserved FxxGxRxCxG heme-binding region for Cyt P450 gene. However, amino acid sequence of *CYP-B3* is only corresponded to about 239 amino acid upstream from C-terminal polypeptides of about 500 amino acid residues as compared to Cyt P450 genes from *Phaseolus aureus*[21], *Glycin max* (GenBank accession No. X92437), *Zinnia eleganse*(GenBank No. U199[22], *Pisum sativum*[9] and *Helianthus tuberosus*[18]. This results indicate that full length of *CYP-B3* may be about 1.5 kb gene encoding about 500 amino acid for the polypeptide of about 50−60 kDa.

Further studies on the characterization of gene expression in relation to the hydroxylation activity of EAS and function of encoded protein to be expressed by full length cDNA in the elicitor-treated tobacco cells, as well as genomic gene and its promoter, remains to be elucidated.

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Cloning of Elicitor-Inducible 5-epi-Aristolochene Hydroxylase in Tobacco Cell Suspension Culture

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초록 : 담배 현탁배양 세포의 Elicitor 유도성 5-epi-Aristolochene Hydroxylase 유전자의 클로닝

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담배의 phytoalexin으로 알려진 capsidiol 생합성의 마지막 단계에 관여하는 5-epi-aristolochene hydroxylase 유전자의 일부를 RT-PCR 방법으로 클로닝하였다. 클로닝한 *CYP-B3*는 콩, 완두 등의 cytochrome P450 계의 유전자와 높은 동일성을 보였으며 heme 결합부위로 알려진 FxxGxRxCxG을 포함하고 있는 것으로 나타났다. 또한 *CYP-B3*는 저온, 고온 또는 제초제 등에 의해서는 유도되지 않고 Elicitor에 의해서만 특이하게 유도되는 것으로 나타나 phytoalexin 생합성에 관여하는 유전자임을 확인하였다. Cyt P450 억제제인 ancymidol과 ketoconazol에 의해 *CYP-B3*의 전사는 억제되지 않는 반면 5-epi-aristolochene hydroxylase의 효소활성은 현저히 억제되는 것으로 나타나 이들 억제제는 전사후의 효소의 합성 또는 활성을 억제하는 것으로 나타났다.