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Molecular Cloning and Characterization of Sesquiterpene Cyclase cDNAs from Pepper Plant Infected with *Phytophthora capsici*

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Pepper plants (Nogkwang, 60-day old) were inoculated with *Phytophthora capsici* to induce sesquiterpene cyclase associated with the biosynthesis of phytoalexin (capsidiol), a substance related to the defense against pathogens in plants. One day after inoculation, mRNA was isolated from the root, cDNA synthesized, and a library constructed in a ZAP express XR vector. The efficiency was 2×10^6 pfu/μg. Sesquiterpene cyclase cDNA from *Hyoscyamus muticus* was labeled with ^{32}P and used as a probe for screening the cDNA library. After the third screening, 25 positive clones were selected. Through restrictive digestion and DNA gel-blot analysis, six different cyclase gene expressions were identified. PSC1B sequences of the six clones were determined, which were 1966 base pairs encoded 556 amino acids with an expected molecular weight of 63.8 kDa. Response against the pathogen was different between the resistant and susceptible peppers. After the infection of the pathogen, the expression of PSC genes continued in the resistant peppers while the plants were alive. The expression in the susceptible peppers lasted for only 4 days.

Key words: *sesquiterpene cyclase, phytoalexin,* Capsicum annuum, Phytophthora capsici.

Phytoalexin has been known to be a low molecular-weight antimicrobial compound, synthesized by plants during the hypersensitive response to the invasion of pathogenic microorganisms.[1,2] Phytoalexin synthesis and accumulation are observed not only under biotic condition (microbial attack, oligosccharide,[3] protein[4] or lipid[5]) but also under abiotic stresses (heavy metal[6] or UV light[7]). Phytoalexins are classified into two groups, isoflavonoid and terpenoid compounds. Isoflavonoid phytoalexins are synthesized in roots of soybean, pea, alfalfa, chickpea, and green bean,[8] while terpenoid phytoalexins are the major phytoalexin class of Solanaceous species and several important Gramineous species.[9] Terpenoid phytoalexins are synthesized via mevalonate pathway. 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is the enzyme involved in the first rate-limiting step. Incorporation of exogenous ^3H-mevalonate, product of the HMGR-catalyzed reaction, into sesquiterpenoids was observed in tobacco cultures treated with fungal elicitor.[10] Sesquiterpenoids are derived from farnesyl pyrophosphate (FPP) through the condensation of three isopentenyl molecules.

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Abbreviations: PSC, pathogen-induced sesquiterpene cyclase; SC(UV), UV-induced sesquiterpene cyclase; HMGR, 3-Hydroxy-3-methylglutaryl coenzyme A reductase; FPP, farnesyl pyrophosphate; VS, Vetispiradien synthase; EAS, 5-*epi*-aristolochene synthase; PAL, phenylalanine ammonia lyase.

Farnesyl diphosphate is an intermediate of the isoprenoid biosynthetic pathway positioned at an important and potential regulatory branch point. Under normal conditions of cell multiplication, some portions of the FPP are diverted toward sterol biosynthesis, and the remaining are partitioned between the synthesis of other prenyl-lipid moieties including ubiquinones and dolichols.[10,11] Since both sesquiterpene cyclase and squalene synthetase compete for the same substrate, farnesyl diphosphate, it is assumed that the suppression of squalene synthetase increases the pool of available substrate for sesquiterpene cyclase and thus allows more efficient synthesis of sesquiterpenoid phytoalexins.[11-13]

Although sesquiterpenoids have been found in several plant families at various stages of their life cycles, a dramatic accumulation of antibiotic sesquiterpenoids has been observed in plants challenged with microbial and viral pathogens.[9] Tobacco produces at least eight different sesquiterpenoids in response to invasion of pathogens. One of these, casidiol accumulates at a high level in tobacco cell cultures that have been treated with elicitors.[14,15] Capsidiol is a monocyclic sesquiterpene hydrocarbon that serves as a phytoalexin in tobacco and pepper. Sesquiterpene cyclase catalyzes the cyclization of FPP to form monocyclic hydrocarbon. Eight bicyclic sesquiterpenoids, including rishintin, capsidiol, lubimin, phytuberin, and phytuberol, have been isolated from plants belonging to the Solanaceae and correlated with the defensive response of plants to the invading pathogens.[9] Typically, in potato, pepper or tobacco, accumulation of the specific sesquiterpenoids

occurs when the plant tissues or cell cultures are challenged with a microorganism or an elicitor.

In this paper, the characteristics of PSC gene are described. This research also revealed that six different transcripts of PSC genes were induced by the pathogenic *P. capsici*, and that they were expressed differentially by the progressing invasion of the pathogen. It was also postulated that varieties resistant to *P. capsici* continue to transcribe the PSC genes, and that a gene of PSCs may be related to the pathogen-resistant property in pepper.

Materials and Methods

Plant materials. Seeds of *Capsicum annuum* var. Nogkwang, resistant varieties (CM331 and PI201234), and susceptible varieties (Longfruit and Taean) were grown in pots for two months under greenhouse conditions (RH, 65%; Temp, 25°C).

Preparation of microspores. A piece of *Phytophthora capsici* mycelium was placed on V-8 juice media (V-8 juice 183 m*l*, $CaCO_3$ 2 g, agar 20 g per liter distilled water), and cultured at 26-28°C for 7 days in the dark. The mycelia were removed with tea spoon, then placed under fluorescent lamp for 3 days without a cover. Inocula were harvested in water, counted with haemacytometer, and adjusted to 10^4 sporangia per ml before inoculation.

PSC mRNA induction and cDNA library construction. *P. capsici* (5×10^5 microspores/plant) were inoculated on the soil surface planted with *C. annuum* var. Nogkwang. After 24 h, total RNAs were prepared using the guanidine isothiocyanate/LiCl method,[16] and then poly(A^+) RNA was isolated through oligo(dT)-cellulose chromatography. cDNA was synthesized from the poly(A^+) RNA, and a cDNA library was constructed into ZAP Express XR vector (Stratagene, La Jolla, CA).

Screening of PSC cDNA clones. Vetispiradien synthase (VS) cDNA of *Hyosciamus muticus* (the clone obtained from Dr. Chapell's Lab, Department of Biochemistry, Kentucky University) was used for the screening of the cDNA library. VS cDNA was labeled with [α-^{32}P] dCTP using the random primed DNA labeling kit(Boehringer Mannheim, Mannheim, Germany). Approximately 5×10^6 recombinant plaques were screened through plaque hybridization. After 4 h prehybridization in a solution containing 0.5 M Na_2PO_4 (pH 7.2), 1% bovine serum albumin fraction V (Sigma, St. Louis, MO), 7% SDS, 100 $\mu g \cdot ml^{-1}$ denatured salmon sperm DNA, the nylon membranes blotted with the plaques were incubated with the probe at 65°C overnight. The membranes were then washed in 2× SSC, 0.5% SDS at 60°C, 1× SSC, 0.5% SDS and 0.5× SSC, 0.5% SDS each for 20 min. The blots were exposed overnight to Kodak XAR-5 X-ray film (Kodak, Rochester, NY) at -70°C.

DNA sequencing. A series of overlapping deletion subclones were generated using exonuclease III and Mung Bean nuclease. Dideoxynucleotide sequencing was performed with Sequenase II (United States Biochemical, Cleveland, OH) using synthetic oligonucleotide primer and ExoIII/Mung Bean nuclease-deleted mutant. The DNA was also sequenced through the dideoxy chain termination method using a Taq Dye primer cycle sequencing kit (Perkin-Elmer, Foster city, CA) in conjunction with an Applied Biosystem 373A automatic DNA sequencer (Perkin-Elmer, Foster city, CA). Nucleotide sequence analysis was carried out with the Lasergene (DNASTAR. Madison, WI) and BioEdit, and searches for sequence similarities were done using the BLAST algorithm as implemented by the National Center for Biotechnology Information server.

Northern blot analysis. Total RNAs were prepared from resistant varieties (CM331 and PI201234) and susceptible varieties (Longfruit and Taean) following the predescribed method.[17] Total RNAs (20 μg) were fractionated on 1.0% agarose gels containing formaldehyde, blotted onto Hybond N^+ membranes (Amersham, Buckinghamshire, England), and incubated at 80°C for 2 h. The membrane was prehybridized, hybridized, and washed following the same procedures to those of the cDNA clone screening protocol.

DNA gel blot analysis. Plasmid DNAs were prepared using the alkaline method[18] and microspin plasmid prep kit (Qiagene, Hilden, Germany). They were digested by *Eco*RI and *Xho*I and electrophoresed in 1.0% agarose gel, transferred onto a positively charged Hybond nylon membrane (Amersham, Buckinghamshire, England) by capillary blotting in a 10× SSC, and immobilized by baking at 80°C for 2 h. The membrane was prehybridized, hybridized, and washed following the same procedures to those of the cDNA clone screening protocol.

Results

PSC mRNA induction. For the induction of sesquiterpene cyclase mRNA, *P. capsici* zoospores (5×10^5) were inoculated to the roots of *C. annuum* var. Nogkwang. Twenty-four hours after inoculation, mRNA was isolated from the root and stem. Northern blot analysis was carried out to confirm the induction of sesquiterpene cyclase transcripts. The PSC mRNA was strongly induced in roots, but weak in stems, and was undetectable in the control plant roots (data not shown).

Screening of library for PSC cDNA clones. The cDNA was synthesized on the basis of the mRNA from roots of infected pepper plant, and cDNA library was constructed into ZAP express XR vector. The efficiency was 2×10^6 pfu/μg of ZAP vector. The first screening of cDNA library was carried out on 2.5×10^5 plaques with ^{32}P-labeled vetispiradiene cyclase cDNA. Finally, 25 PSC cDNA clones were selected after the third screening (Fig. 1A) and confirmed through Southern blot analysis (Fig. 1B).

The clones, considered as full-length in size, were classified into six groups (Figs. 1C and 1D) on the basis of the restriction fragments produced with the enzymes *Eco*RI and *Xho*I. Groups I and II had two insert fragments of 0.4 and 1.6 kb,

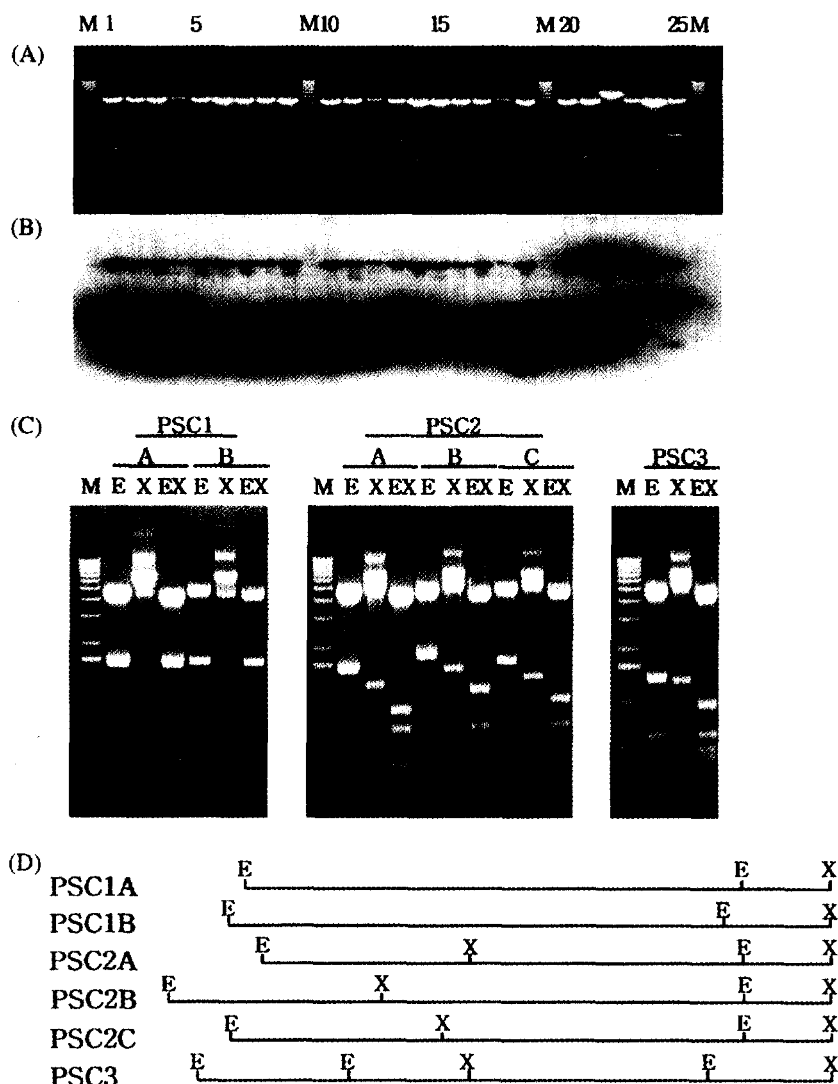

Fig. 1. Confirmation and size-mapping of PSC cDNA clones. Plasmid DNA (1 μg) was digested with *EcoR*I (E) and *Xho*I (X), and loaded on 1% agarose gel. (A) restriction patterns in 1% agarose gel. (B) radiophotogram after hybridization with ^{32}P-labeled probe. (C) restriction patterns of six groups classified on the basis of the restriction fragments. (D) restriction size maps of PSC clones.

and 0.34 and 1.6 kb, respectively. Groups III, IV, and V had three fragments of 0.3, 0.7, and 0.9 kb, 0.3, 0.7, and 1.2 kb, and 0.3, 0.7, and 1.0 kb, respectively. Group VI had four fragments of 0.3, 0.4, 0.6, and 0.9 kb. Groups I-VI were designated as PSC1A and 1B for the group I and II, PSC2A, 2B, and 2C for the group III, IV, and V, and PSC3 for the group VI, respectively. PSC1B clone, longer in size among PSC1 clones, was sequenced and used in the expression analysis as a probe.

DNA sequencing and gene analysis. The sequence of the PSC1B clone was registered in GenBank with accession number of AF326118. PSC1B sequence comprises 1966 bp, including 83 bp of 5'-untranslation sequences, 215 bp of 3'-untranslation sequences, and 1668 bp of coding sequences. Nucleotide sequence of the coding region of PSC1B showed above 85% identity with those of other plants (*C. annuum*, *H. muticus*, *L. esculantum*, and *N. tabacum*), and the 5'- and 3'-untranslated regions were highly divergent. The potent polyadenylation signal TAAATAAT was located at a position, 64 bp downstream of the putative termination sequence. Open reading frame of PSC1B encoded 556 amino acids. The predicted translation product initiated at the first in-frame ATG following the transcription start site has a molecular mass of 63.8 kDa, close to the value of 60 kDa determined for the 5-*epi*-aristolochene synthase(EAS) protein via SDS/PAGE.[19] The identity of this gene also could be verified by comparing the deduced amino acid sequences with the sequences of other known sesquiterpene cyclase (Fig. 2). At the amino acid level, the PSC1B has similarity above 70% compared to the sesquiterpene cyclase from other plants. Asp-Asp-Xaa-Xaa-Asp, the specific nature of terpenoid cyclases,[20] is located at 308-312 deduced amino acid of PSC1B.

Expression of the PSC gene in the pathogen-infected pepper plant. The expression patterns of PSC in response to *P. capsici* were examined in resistant varieties (CM331 and PI201234) and susceptible varieties (Longfruit and Taean).

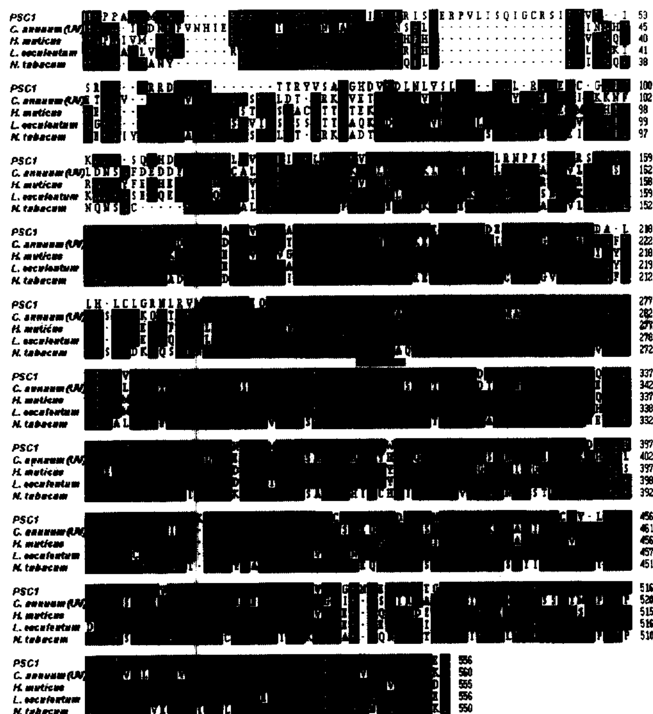

Fig. 2. Comparison of the amino acid sequences of PSC1B with those of other plants. Multiple sequence alignment was performed using the BioEdit program. Identical residues in the sequences are indicated in white letters. Predicted substrate binding site is marked with thick black line. The amino acid sequences employed are as follows: GenBank accession number AF061285 (*C. annuum*), U20187 (*H. muticus*), AF171216 (*L. esculantum*), and L04680 (*N. tabacum*).

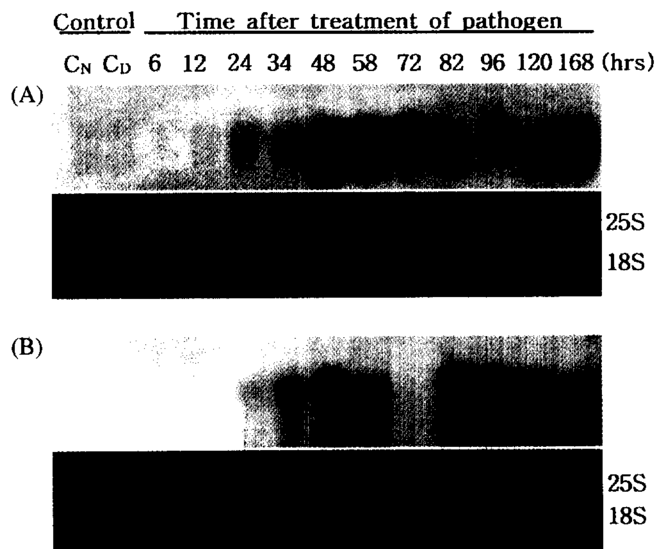

Fig. 3. Expression profiles of PSC genes via infection with pathogen in resistant varieties. The induction and accumulation of PSC mRNAs in resistant varieties (A, CM331; B, PI201234) in response to *P. capsici* were monitered with ^{32}P-labeled PSC cDNA. Total RNAs were isolated from roots of the control and pathogen-treated pepper plants at various times after inoculation of *P. capsici*. Twenty microgram of total RNAs were loaded on 1.5% formaldehyde gel. C_N and C_D: controls sampled at night and day time, respectively.

Total RNAs were isolated from the pepper plants at various times after inoculation of *P. capsici*. The expression of the PSC genes was monitored through Northern blot analysis using radiolabeled PSC1B cDNA as a probe. No PSC transcripts were detected in the control (no inoculation) RNA. The expressions of PSC genes in the roots of both resistant varieties CM331 and PI201234 were detected within 24 h of infection and reached maximum level after 48 h (Fig. 3). In CM331, the expression gradually decreased until 72 h then decreased steeply until 82 h, after which, the expression started to increase. For PI201234, the level of expression steeply decreased after 58 h, and was hardly detected after 72 h of infection, after which, the expression went on. On the other hand, the transcripts of PSC in susceptible varieties Longfruit and Taean were detected within 34 h, reached the maximum level after 48 to 58 h, then decreased gradually, and could not be detected after 120 h (Fig. 4).

Discussion

Genes related to the defense mechanism and phytoalexin biosynthesis were observed to exist in multigene families such as phenylalanine ammonia lyase (PAL),[21] chalcone synthase,[22] and sesquiterpene cyclase.[23] Several PAL and PSC are transiently expressed through the elicitor treatments,[4,24] abiotic stresses (heavy metal,[6] and exposure to UV light[7]).

Six different sized transcripts of PSC genes have been isolated from pepper plants infected with *P. capsici*, and one complete sesquiterpene cyclase cDNA (PSC1B) has been characterized. In PSC1, PSC1B is longer in size than PSC1A, and the small fragments produced only by *Eco*RI and *Xho*I are different in sizes. Sizes of middle fragments of PSC2 genes produced by *EcoR*I and *Xho*I are different from each other. It is, therefore, speculated that PSC1A and PSC1B could have been formed by splicing from the same primary transcript derived from one gene, and that PSC2A, 2B, and 2C may have resulted from alternative splicing of the same transcript from another gene. PSC3 may have been transcribed from another independent gene as well. These results indicate that the multiple PSC isozymes exist in pepper infected with *P. capsici*, suggesting that the enzymatic isoforms should be synthesized to respond effectively to the progression of pathogen invasion, and regulated at the post-transcriptional level. Meanwhile, the expression of sesquiterpene cyclase gene was also induced in *C. annuum* by UV exposure.[7] Amino acid sequences 11-15 and 58-64 in SC(UV) are not in PSC, and 35-48 in PSC is not in SC(UV). And the first 100 amino acid sequence of PSC and SC(UV) is very variable (Fig. 2). It suggests that the two different elicitors should induce the transcripts from different templates, and multigenes of sesquiterpene cyclase in genome should be transcribed by equivalent triggers.

Aspartate-rich consensus sequence, which is found in the monoterpene cyclase (limonene synthase), triterpene synthase (casbene synthase), and squalene synthetase,[20] has been pro-

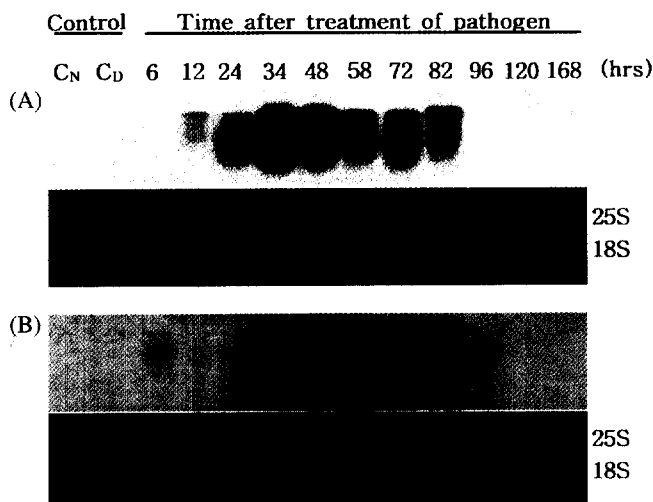

Fig. 4. Expression profiles of PSC genes via infection with pathogen in susceptible varieties. The induction and accumulation of PSC mRNAs in susceptible varieties (A, Longfruit; B, Taean) in response to *P. capsici* were monitered with ^{32}P-labeled PSC cDNA. Total RNAs were isolated from roots of the control and pathogen-treated pepper plants at various times after inoculation of *P. capsici*. Twenty microgram of total RNAs were loaded on 1.5% formaldehyde gel. C_N and C_D: controls sampled at night and day time, respectively.

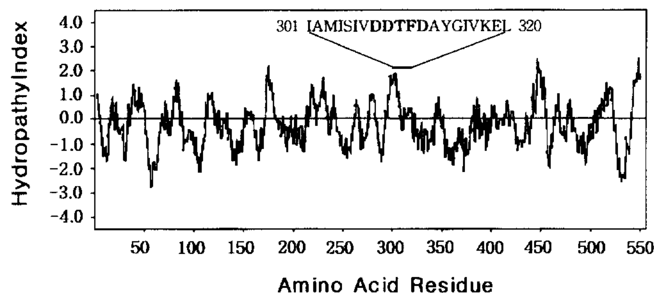

Fig. 5. Hydropathy indices. The hydropathy plot was calculated according to the Kyte-Doolittle parameters with an amino acid range of 11. The isoprene binding domain is indicated in bold letters. In the amino acid sequences (301 through 320) of PSC1B, the isoprene binding domain was positioned between the hydrophobic and weak hydrophilic regions.

posed as a binding domain for the substrate farnesyl pyrophosphate.[20] Deduced amino acids sequence of PSC1B also contain the putative consensus motif Asp-Asp-Xaa-Xaa-Asp, which is highly conserved in other plants including *H. muticus*, *L. esculentum*, and *N. tabacum*. Hydropathy plot shows that 20 amino acids (amino acid 301-320) around the FPP binding domain are actually located between hydrophobic and weak hydrophilic regions (Fig. 5). This suggests that the spatial environment of the enzymes facilitate the FPP access to the binding domain, because the molecule of farnesyl pyrophosphate has both the hydrophobic isoprene and the hydrophilic pyrophosphate groups.

The expréssions of PSC in the roots of both resistant varieties (CM331 and PI201234) were detected 10 h earlier compared to those of the susceptible varieties (Longfruit and Taean) (Figs. 3 and 4). After reaching a maximum level at 48 h after inoculation, the expressions decreased gradually, then the transcript level of PSC increased in both varieties. In susceptible varieties, the expression patterns were completely different. The expression was detected 10 h later compared to those of the resistant varieties. Once the transcript levels decreased after the maximum level, expression level did not increase again. In other words, while the expression of PSC genes was going on in the resistant pepper plant, it was declining in the susceptible ones after 96 h and was not detected after 120 h. These data suggest that the PSC gene expressed later could be related to the resistant property against pathogen *P. capsici*, showing that at least two different genes may be associated with the resistant properties in both CM331 and PI201234. One of the genes is expressed early, and the other is transcribed later. It is speculated that the expression of PSC may be temporally and time-differentially regulated.

It is suspected that the resistant pepper may continue to synthesize phytoalexin (capsidiol) and be able to resist against pathogens. The susceptible pepper, however, cannot overcome the decrease after the maximum level of phytoalexin, and may lose resistance to the pathogens. Martin *et al.* (1993) introduced *P. syringae* resistant gene (*Pto*) to tomatoes and showed that the tomato plant gained pathogen-resistance.[25] Use of genetic manipulation technique that transforms a plant with the genes related to pathogen-resistance is an important potential target to yield a more effective antimicrobial defense.[26] Therefore, it can be speculated that if the PSC genes are introduced into a plant, the sesquiterpenoid phytoalexins are synthesized effectively in the transgenic plant, and the resistant capability of the plant to the pathogen *P. capsici* will be reinforced. PSC cDNA can be used for the transformation of a susceptible plant into a resistant plant against pathogens suppressed by sesquiterpenoid phytoalexins.

Characteristics of each genes (PSC1, 2, and 3) for the expression and sequence structure remain to be elucidated. The differential and post-transcriptional regulations by alternative splicing of the PSC genes will be investigated as well.

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