Korean Journal of Life Science
Vol. 12. No. 3. 264~273, 2002

Cloning and Expression of Antifungal Protein (PR5) Genes from Hot Pepper (*Capsicum annuum* L.)

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Abstract

We have isolated and artificially expressed three cDNA clones of *Capsicum annuum* PR5 genes for elucidating the antifungal activity against *Phytophthora capsici* which contracted a hot pepper root rot in field condition. Three divergent PR5 proteins from hot pepper were designated as CAPR5-1 and CAPR5-2 from susceptible cultivar (Subicho) as well as CAPR5-3 from resistant cultivar (CM331) in response to *P. capsici*. The cDNA similarity was found over 80% of identity among the three CAPR5s, and deduced amino acid sequence was characterized that all of CAPR5s contained 16 cysteine residues which possibly had a significant role in the structural formation. The result of genomic DNA blot showed that CAPR5-1 and CAPR5-2 existed as single copy in the Subicho genome. Three recombinant CPARs in *E. coli* were identified by SDS-PAGE, and each expressed protein was treated on the PDA medium which contained cultured pathogens. Although three CAPR5 proteins did not affected the hyphal growth of *Glomerella glycines* and *Colletotrichum lagenarium*, CAPR5-1, CAPR5-2, and CAPR5-3 showed a specific antifungal activities against *P. capsici*.

Key words – Hot pepper, Osmotin-like protein, Pathogenesis-related protein

Introduction

The term pathogenesis-related (PR) proteins was introduced in 1980 to designate it as proteins codes for by the host plant but induced only in pathological or related situations. PR proteins were firstly identified as new groups of proteins induced by TMV in tobacco which hypersensitively responded against its virulent invader [7]. On plants suffering a pathogen inducing hypersensitive necrosis, they enhanced systemic acquired resistance (SAR) to subsequent infection by various types of pathogens, and the SAR was related to the introduction of PR proteins in distant tissues or organs from the original inoculated site. These observation suggested that PR proteins were a crucial member of defense components in protecting pathogen colonization and propagation [3].

Based on their serological, enzymological, functional, and structural properties, PR proteins have been classified into fourteen families that were related to the plant responses interacting with virus, bacteria, and fungi [5,9]. PR proteins also acted against fungal parasite in vivo, in vitro, or both [14,21]. One group of fourteen PR protein families is sometimes called by thaumatin-like proteins (TLP) because of their high amino acid homology with thaumatin, a sweet tasting protein of *Thaumatococcus*

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danielli fruit [7]. PR5 proteins as antifungal activities inhibits hypha growth, reduces spore germination, and/or demolish spores in such fungi as *Candida albicans*, *Neurospora crassa*, *Trichoderma reesi*, *Fusarium oxysporum*, *Phytophthora infestans*, and *Asternaria solani* [1,8,11,12,19,20]. Furthermore, the transgenic rice over-expressed with rice PR5 gene strengthened an environmental affinity, by which the transgenic plant resisted *Rhizoctonia solani* causing sheath blight disease. Although thaumatin had the highly homologous amino acid with other PR5 proteins, its weak antifungal activity was represented in *C. albicans* [9,19].

PR5 proteins are classified into 3 subclasses; basic (osmotin), neutral (osmotin-like protein; OLP), and acidic (PR-S). A high accumulation of PR5 proteins was detected only in tobacco root tissues, which almost all of PR5 proteins was generally the osmotin-like protein as well as a small amount of basic osmotin. However, large amounts of the neutral PR5 protein (OLP) were mainly accumulated in the cultured tobacco cell, in contrast to the cultured medium contained large amounts of acidic form (PR-S). Another data showed that adaptation of salt stress was associated with a higher accumulation of basic PR5 (osmotin) and less neutral PR5 (osmotin-like protein) [9,11].

The synthesis of PR5 proteins inducible by biotic and abiotic stimuli in leaf tissue was also induced by various hormone such as ethylene, salicylic acid, and jasmonic acid [13,21,23,24]. Ethylene was found to induce a high accumulation of basic and neutral PR5 protein in the stimulated leaf. Salicylic acid, reactive oxygen species, ethylene, and methyl jasmonate which were recognized as putative signal molecules in SAR also elevated the expression of acidic PR genes. In the tobacco mosaic virus (TMV)-infected tobacco plant, the alternation of induced PR5 protein was differed from isoforms which was analyzed to higher accumulation of PR-5 acidic and neutral isoforms [10,11].

The biological function of PR5 proteins have been elucidated in some plants. Thaumatin-like proteins were induced in both mono- and dicotyledonous plants in response to both fungal and viral infection [4,6,16]. The constitutive antifungal TLP of low molecular mass (usually 19-27 kD) have been identified as permatins because of the proposed function of antifungal action to the target fungal cell [19]. Batalia *et al.* proposed that the antifungal activities of PR5 proteins indirectly mediate the water permeability of fungal cell membrane by the lysis of plasma membrane and electrostatic interaction with membrane ion channels or osmotic balance of a fungal cell [2]. The finding that TLPs have membrane-permeabilizing function with selectivity to fungal pathogens makes this class of proteins very attractive as components of antifungal defenses that can be deployed against pathogens that are not controlled by other PR proteins [15,22]. So far limited information on the mechanism of membrane permeability caused by the PR5 proteins and their interaction with fungal cell are available. Thus cloning and characterization of the PR5 genes from hot pepper will be requisite to investigate the interaction of the PR5 proteins with *Phytophthora capsici* causing a great damage to hot pepper.

In this study, three PR5 genes were cloned from hot pepper and their basic properties and phylogenetic clustering investigated. In order to determine the antifungal activities and specificities of the three PR5 proteins, the PR5 genes were artificially expressed in bacterial cells, and the proteins were extracted and treated to various fungi such as *Phytophthora capsici*, *Glomerella glycines*, and *Colletotrichum lagenarium*.

Materials and Methods

Plant material and total RNA isolation

The hot pepper (*Capsicum annuum*) seeds were kindly provided from the Young Yang Pepper Experiment Station (YYPES), Kyungsangpookdo, Korea. The cultivars of *Capsicum annuum*, Subicho and CM331, were sown in polyethylene pots and maintained on a growth chamber

for 6 weeks after seeding. After explanted leaves of Subicho and CM331 were irradiated using a Hitachi germicidal lamp (15 W) at the distance of 15 cm for 15 min, each sample was harvested at 12 h after UV-treatment. Total RNA of UV-irradiated Subicho and CM331 leaves was isolated using TRIzol reagents (GIBCO BRL, UK).

Isolation, cloning and sequencing of the gene encoding PR5

For RT-PCR, first strand cDNA synthesis was performed using 1 μg of total RNA with AMV reverse transcriptase and oligo(dT) primer (TaKaRa, Japan). To amplify the open reading frame (ORF) of CAPR5 genes, the specific primer was designed from already cloned *Capsicum annuum* PR5 gene (GenBank Accession No. AF082723). The PCR reaction was performed with a forward primer (5-GCC-ATA-TGG-GCT-ATT-TGA-GAT-CA-3) (*Nde*I restriction sites underlined) and a reverse primer (5-GCC-TCG-AGC-TAC-TTA-GCC-ACT-CC-3) harboring a *Xho*I site (underlined) as well as first strand cDNA as the template, and using the following program : 94℃, 2 min (for 1st cycle); 94℃, 30 sec; 55℃, 1 min; 72℃, 1 min 30 sec by 35 cycles. PCR products were electrophoresed on a 0.8% agarose gel and the fragments were eluted from gel using Gene-Clean Kit Ⅲ (Bio 101. Inc., USA).

Two 741-bp DNA fragments were obtained, named *Capsicum annuum* PR5-1 (CAPR5-1) from Subicho and *Capsicum annuum* PR5-3 (CAPR5-3) from CM331, and these fragments were cloned into the pGEM-T easy vector (Promega, USA) and used as a probe to screening the cDNA library. DNA sequencing was done by the method of chain termination. The sequences were analyzed for identity using the NCBI of the BLAST and aligned by DNASIS program (Hitachi, Japan).

Screening and Sequencing of cDNA library

Using cloned cDNA fragment as a probe, the *Capsicum annuum* cDNA library (constructed in λ ZAP vector) was screened for isolation of a homologous PR5 gene. The probe was labeled with α-^{32}P dCTP using the random priming method with a Megaprime Labeling kit (Amersham, UK). About 1×10^5 plaques were transferred onto the Hybond-N+ nylon membrane (Amersham, UK). The membranes were hybridized with ^{32}P-labeled CAPR5-1 as the probe using Quikhib-Solution (Stratagene, USA) at 65℃ for 2 h. After hybridization, the membrane was rinsed twice with 2×SSC, 0.1% SDS and 0.2×SSC, 0.1% SDS at 55℃ and exposed to X-ray film. A positive plaque was directly in vivo excised into pBluescript-SK phagemid vector according to the manufacturer's instructions (Stratagene, USA). The pBluescript SK vector DNA harboring the positive cDNA clone (CAPR5-2) was purified by Wizard Plus SV Minipreps Kit (Promega, USA) and used for sequencing.

Southern blot analysis

Genomic DNA from hot pepper leaves (Subicho) was isolated using CTAB method [18]. Genomic DNA (20 μg) was digested with various restriction enzymes for gel blot analysis, fractionated on 0.8% agarose gel, and transferred onto the Hybond-N+ nylon membrane (Amersham, USA), according to the manufacturer's instructions. The membranes were hybridized with CAPR5-1 and CAPR5-2 at 68℃ for 2 h. After hybridization, the membrane was washed twice with 2×SSC, 0.1% SDS and 0.2×SSC, 0.1% SDS at 55℃ and exposed to X-ray film.

Bacterial expression of CAPR5 genes

The nucleotides of CAPR5-1, CAPR5-2, and CAPR5-3 were amplified by PCR for harboring of the restriction enzyme sites. The PCR reaction for amplifying CAPR5-1 and CAPR5-3 was performed with a forward primer (5-GCC-ATA-TGG-GCT-ATT-TGA-GAT-CA-3) (*Nde*I restriction sites underlined) and a reverse primer (5-GCC-TCG-AGC-TAC-TTA-GCC-ACT-CC-3) harboring a *Xho*I site (underlined). For amplifying CAPR5-2, the specific primers were designed as a forward primer (5-GCC-ATA-TGA-

CGA-ACT-CGT-GCC-GA-3) (*Nde*I restriction sites underlined) and a reverse primer (5-GCC-TCG-AGT-TAC-TTA-GCA-ACA-TC-3) haboring a *Xho*I site (underlined) as well as the cloned CAPR5 cDNAs as the template respectively, and using the following program : 94℃, 2 min (for 1st cycle); 94℃, 30 sec; 55℃, 1 min; 72℃, 1 min 30 sec by 35 cycles. The amplified PCR products were digested with *Nde*I and *Xho*I, gel-purified, and ligated into the same restriction sites within the pET28c vector (Novagen, USA). The constructs were transformed into *E. coli* BL21 (DE3) strain.

SDS-polyacrylamide gel eletrophoresis

The CAPR5-1, CAPR5-2 and CAPR5-3 were expressed in *E. coli* by adding isopropylthio-β-D-galactoside (IPTG) to a final concentration of 1 mM to exponentially growing recombinant cell (OD_{600}=0.5) harboring the pET28c-CAPR5-1, pET28c-CAPR5-2 and pET28c-CAPR5-3 plasmids. One-mililiter aliquots of the cultures were subsequently removed and collected by centrifugation for 2min. The pellet was resuspended in 500 $\mu\ell$ of the buffer (50 mM Tris-HCl, pH 6.8, 10 mM DTT, 2% SDS, 0.01% bromophenol blue, and 10% glycerol). Twenty-microliter aliquots were electrophoresed on an 12.5% SDS-polyacrylamide gel and stained with Coomassie blue for protein detection.

In vitro antifungal assay

To perform the hyphal extension-inhibition assay, fungal mycelia of *Phytophthora capsici, Glomerella glycines* and *Colletotrichum lagenarium* were harvested from actively growing fungal plates and placed into the center of petri dishes containing potato dextrose agar (PDA) or V8 juice agar media, and it was subcultured to the same fresh medium, before using in assays. After fungal mycelium growth was allowed for 2 days at 30℃, the CAPR5 protein solution was applied to the filter discs laid on the agar surface in front of the advancing fungal mycelium. They were further incubated at 30℃ and investigated the inhibition of fungal growth. The amounts of CAPR5 proteins added onto the disc were 0, 5, 20, 50, 100 μg in disc #1, #2, #3, #4, #5, respectively. The extent of hyphal growth was measured each day. Antifungal activity was detected by the appearance of crescents of retarded growth around the discs.

Results and Discussion

Cloning and sequencing of the genes encoding CAPR5

The cloning of PR5 genes from *Capsicum annuum* was approached by RT-PCR and cDNA library screening methods in order to elucidate their alternative structure and function. By RT-PCR, 741 bp fragments were obtained using specific designed primers and one of them was designated as CAPR5-1 (Subicho) and another, CAPR5-3 (CM331). The sequences of the cloned PR5 genes were matched with that of PR5 gene in *Capsicum annuum* (GenBank Accession No. AF082723) and their amino acid homology was almost 80.6% (Fig. 1).

The diverse homologous PR5 genes were screened from the cDNA library generated from hot pepper irradiated by UV irradiation using the cloned CAPR5-1 as a probe. The screening of 100,000 recombinant plaques using random primed ^{32}P-labeled CAPR5-1 as a probe resulted in identification of two positive clones from *Capsicum annuum* cDNA library. One of them showing the larger insert of 864 bp designated as CAPR5-2, was selected and sequenced. The CAPR5-2 had 741 bp of open reading frame and 123 bp of 3′ UTR.

The nucleotide sequence analysis of CAPR5-1, CAPR5-2, and CAPR5-3 indicated that there was a little difference from previously reported thaumatin-like protein in *Capsicum annuum* (CAPR5). Sequence analysis revealed that CAPR5, CAPR5-1, CAPR5-2, and CAPR5-3 contained inserts of 246 amino acid residues with calculated molecular mass of 23 kD. High identity (> 80%) was found in the coding regions between CAPR5 and the three CAPR genes, CAPR5-1, CAPR5-2, and CAPR5-3 (Fig. 1).

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CAPR5      1 ATGGGCTATT TGAGATCATC TTTTGTTCTC TTTCTTCTAG CTTTTGTGAC GTTACACTTA
CAPR5-1    1 ATGGGCTATT TGAGATCATC TTTTGTTCTC TTTCTTCTAG CTTTTGTGAC GTTACACTTA
CAPR5-2    1 -------ACT CGTGCCGAAT TCGGCAGCAC TTCCTCCTTG CCTTTGTGAC GTTATACTTA
CAPR5-3    1 ATGGGCTATT TGAGATCATC TTTTGTTCTC TTTCTTCTAG CTTTTGTGAC GTTACACTTA

CAPR5     61 TGCTGCCACT TTCGAGGGCC GAAACAACTG TCCATACACC TTTGGGCGGC ATCGACCCCC
CAPR5-1   61 TGCTGCCACT TTCGAGGTCC GAAACAACTG TCCATACACC TTTGGGCGGC ATCGACCCCC
CAPR5-2   61 TGCTGCCACT ATCGAGGTCC GAAACAATTG CCCGTACACC TTTGGGCGGC ATCAACTCCG
CAPR5-3   61 TGCTGCCACT TTCGAGGTCC GAAACAACTG TCCATACACC TTTGGGCGGC ATCAACTCCG

CAPR5    121 GTAGGTGGCG GTCGACGTCT TGATCGAGGC CAGACCTGGA CCATCAATGC CCCACCAGGG
CAPR5-1  121 GTAGGTGGCG GTCGACGTCT TGATCGAGGC CAGACCTGGA CCATCAATGC CCCACCAGGG
CAPR5-2  121 GTAGGCGGAG GCAGACGACT CAATCGGGGC CAAACGTGGG TCATCAATGC ACCGAGGGGC
CAPR5-3  121 GTAGGCGGAG GCAGACGACT CAATCGGGGC CAAACGTGGG TCATCAATGC ACCGAGGGGC

CAPR5    181 ACAGCGATGG CACGTATATG GGGTCGTACT AATTGCAACT TCGATGGTTC TGGCAGAGGT
CAPR5-1  181 ACAGCGATGG CACGTATATG GGGTCGTACT AATTGCAACT TCGATGGTTC TGGCAGAGGT
CAPR5-2  181 ACTAAGATGG CACGTATATG GGGCCGGACA GGTTGCAACT TCAATGCTGC AGGCAGAGGC
CAPR5-3  181 ACTAAGATGG CACGTATATG GGGTCGTACT AATTGCAACT TCGACGGTTC TGGCAGAGGT

CAPR5    241 TCGTGCCAGA CTGGTGATTG CGGTGGAGTC TTGCAGTGCA CCGGGTGGGG CAAACCACCA
CAPR5-1  241 TCGTGCCAGA CTGGTGATTG CGGTGGAGTC TTGCAGTGCA CCGGGTGGGG CAAACCACCA
CAPR5-2  241 TCGTGTCAGA CCGGCGATTG TGGTGGAGTC TTGCAGTGCA CTGGGTGGGG CAAACCACCA
CAPR5-3  241 TCGTGCCAGA CTGGTGATTG CGGTGGAGTC TTGCAGTGCA CCGGGTGGGG CAAACCACCA

CAPR5    301 AACACCCTAG CCGAGTACGC CTTGAACCAA TTCAACAACC TAGATTTCTG GGACATTTCT
CAPR5-1  301 AACACCCTAG CCGAGTACGC CTTGAACCAA TTCAACAACC TAGATTTCTG GGACACTTCT
CAPR5-2  301 AACACCCTGG CCGAATACGC CTTGGACCAA TTCAGTAACC TAGATTTCTG GGACATTTCT
CAPR5-3  301 AACACCCTAG CCGAGTACGC CTTGAACCAA TTCAACAACC TAGATTTCTG GGACATTTCT

CAPR5    361 TTAGTCGATG GATTCAACAT ACCGATGACT TTCGCACCGA CCAATCCTAG TGGTGGGAAA
CAPR5-1  361 TTAGTCGATG GATTCAACAT ACCGATGACT TTCGCACCGA CCAATCCTAG TGGTGGGAAA
CAPR5-2  361 TTGGTCGATG GATTCAACAT TCCAATGACT TTTGCCCCAA CCAAACCTAG TGGTGGAAAA
CAPR5-3  361 TTTGTCGATG GATTCAACAT ACCGATGACT TTCGCACCGA CCAATCCTAG TGGTGGGAAA

CAPR5    421 TGCCACGCAA TTCAATGCAC GGCCAATATA AATGGTGAAT GCCCTGGTTC ACTCAGGGTA
CAPR5-1  421 TGCCACGCAA TTCAATGCAC GGCCAATATA AATGGTGAAT GCCCTGGTTC ACTCAGGGTA
CAPR5-2  421 TGCCACGCGA TCCATTGCAC GGCCAATATA AATGGTGAAT GCCCTCGCGC CCTTAAGGTG
CAPR5-3  421 TGCCACGCAA TTCAATGCAC GGCCAATATA AATGGTGAAT GCCCTGGTTC ACTCAGGGTA

CAPR5    481 CCAGGAGGAT GTAACAACCC TTGTACAACG TTTGGAGGAC AACAATATTG TTGCACCCAA
CAPR5-1  481 CCAGGAGGAT GTAACAACCC TTGTACAACG TTTGGAGGAC AACAATATTG TTGCACCCAA
CAPR5-2  481 CCCGGAGGAT GCAACAACCC TTGTACCACG TTCGGAGGAC AACAATATTG TTGCACCCAA
CAPR5-3  481 CCAGGAGGAT GTAACAACCC TTGTACAACG TTTGGAGGAC AACAATATTG TTGCACCCAA

CAPR5    541 GGTCCATGTG GTCCTACTGA GTTGTCAAAA TTTTTCAAGA AAAGATGCCC TGATGCCTAT
CAPR5-1  541 GGTCCATGTG GTCCTACTGA GTTGTCAAAA TTTTTCAAGA AAAGATGCCC TGATGCCTAT
CAPR5-2  541 GGTCCATGTG GTCCTACAGA GTTGTCAAAA TTTTTCAAGA AAAGATGTCC TAATGCCTAT
CAPR5-3  541 GGTCCATGTG GTCCTACTGA GTTGTCAAAA TTTTTCAAGA AAAGATGCCT TGATGCCTAT

CAPR5    601 AGCTACCCAC AAGATGATGC TACTAGCACA TTTACTTGCC CAAGTGGTAG TACAAATTAT
CAPR5-1  601 AGCTACCCAC AAGATGATGC TACTAGCACA TTTACTTGCC CAAGTGGTAG TACAAATTAT
CAPR5-2  601 AGCTACCCAC AAGATGATCC TACTAGCACA TTTACTTGTC CAAGTGGTAG TACAAACTAT
CAPR5-3  601 AGCTACCCAC AAGATGATGC TACTAGCACA TTTACTTGCC CAAGTGGTAG TACAAATTAT

CAPR5    661 AGGGTAGTGT TCTGTCCTAA TGGTGTTACT GGCCCAAATT TTCCATTGGA GATGCCTG--
CAPR5-1  661 AGGGTAGTGT TCTGTCCTAA TGGTGTTACT GGCCCAAATT TTCCATTGGA GATGCCTG--
CAPR5-2  661 AGGGTTGTCT TTTGTCCTAA TGGTGTTGCT GATCCAAATT TCCCCTTGGA GATGCCTACA
CAPR5-3  661 AGGGTAGTGT TCTGTCATAA TGGTGTTACT GGCCCAAATT TTCCATTGGA GATGCCTGGT

CAPR5    719 -GTAGTGATG GAGTGGCTAA GTAG  741
CAPR5-1  719 -GTAGTGATG GAGTGGCTAA GTAG  741
CAPR5-2  721 AGTACTGATG AAGTTGCTAA GTAA  744
CAPR5-3  721 AGTGATGGAG TGGCTAAGTA GTAA  744
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Fig. 1. The cDNA ORF sequence of diverse PR5s from *Capsicum annuum*. Translation initiation and termination codons were represented by bold characters. The aligned CAPR5-1 and CAPR5-3 showed over 99% and 93% of sequence affinity with CAPR respectively, meanwhile the sequence identity between CAPR5 and CAPR5-2 was 73.3% consensus sequence onto the ORF region.

The deduced amino acid sequences of CAPR5-1, CAPR5-2, and CAPR5-3 were compared with that of the thaumain-like protein gene, CAPR5. There was a high level of identity (80 to 90%) among these four CAPRs (Fig. 2). The 16 cysteine residues involved in the formation of disulfide bonds conserved in osmotin-like protein were all presented in these proteins. Putative N-terminal signal sequence cleavage site was found between YA and AT at 21st deduced amino acid sequence, in addition, putative C-terminal peptide cleavage site was also found between NG and VT at the 288th site of deduced amino acid region [24].

Second structural analysis and phylogenetic clustering of CAPR5s

Some parts of secondary structure in CAPR5 are putatively crucial on forming the cleavage site to burst fungal cells. Although the mechanistic mode of CAPR5 was not clarified and argued by many researcher, the different formation on α-helix and β-sheet of CAPR5-1, CAPR5-2 and CAPR5-3 might adjust to perceive a specific fungus and mediate degree of antifungal activity [2].

The phylogenetic relationship of the three PR5s in hot pepper represented that the genes belong to various clustering group. Data base search with the predicted amino acid sequences revealed a significant sequence similarity with PR5 and thaumatin-like protein in *Capsicum annuum* and other plants. Well defined thirteen osmotin and thaumatin amino acid sequences from the GeneBank and the three CAPR5s were clustered by DNASIS program. CAPR5-1 represented over 91.5% of homology with osmotin and thaumatin of pepper and CAPR5-3, 90.4% (Fig. 3).

CAPR5-1 and CAPR5-3 showed the highest sequence similarity to taumatin-like protein of *Capsicum annuum*, while CAPR5-2 showed the highest sequence similarity to osmotin-like protein of *Lycopersicom esculentum* and *Solanum dulcamara* (Fig. 3). The deduced amino acids

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CAPR5      1  MGYLRSSFVL FLLAFVTYTY AATFEGRNNC PYTVWAASTP VGGGRRLDRG
CAPR5-1    1  MGYLRSSFVL FLLAFVTYTY AATFEVRNNC PYTVWAASTP VGGGRRLDRG
CAPR5-2    1  ---SCRIRHD FLLAFVTYTY AATIEVRNNC PYTVWAASTP IGGGRRLNRG
CAPR5-3    1  MGYLRSSFVL FLLAFVTYTY AATFEVRNNC PYTVWAASTP VGGGRRLDRG

CAPR5     51  QTWTINAPPG TAMARIWGRT NCNFDGSGRG SCQTGDCGGV LQCTGWGKPP
CAPR5-1   51  QTWTINAPPG TAMARIWGRT NCNFDGSGRG SCQTGDCGGV LQCTGWGKPP
CAPR5-2   51  QTWVINAPRG TKMARIWGRT GCNFNAAGRG SCQTGDCGGV LQCTGWGKPP
CAPR5-3   51  QTWTINAPPG TAMARIWGRT NCNFDGSGRG SCQTGDCGGV LQCTGWGKPP

CAPR     101  NTLAEYALNQ FNNLDFWDIS LVDGFNIPMT FAPTNPSGGK CHAIQCTANI
CAPR5-1  101  NTLAEYALNQ FNNLDFWDTS LVDGFNIPMT FAPTNPSGGK CHAIQCTANI
CAPR5-2  101  NTLAEYALDQ FSNLDFWDIS LVDGFNIPMT FAPTKPSGGK CHAIHCTANI
CAPR5-3  101  NTLAEYALNQ FNNLDFWDIS FVDGFNIPMT FAPTNPSGGK CHAIQCTANI

CAPR5    151  NGECPGSLRV PGGCNNPCTT FGGQQYCCTQ GPCGPTELSK FFKKRCPDAY
CAPR5-1  151  NGECPGSLRV PGGCNNPCTT FGGQQYCCTQ GPCGPTELSK FFKKRCPDAY
CAPR5-2  151  NGECPRALKV PGGCNNPCTT FGGQQYCCTQ GPCGPTELSK FFKKRCPNAY
CAPR5-3  151  NGECPGSLRV PGGCNNPCTT FGGQQYCCTQ GPCGPTELSK FFKKRCLDAY

CAPR5    201  SYPQDDATST FTCPSGSTNY RVVFCPNGVT GPNFPLEMPG S-DGVAK*
CAPR5-1  201  SYPQDDATST FTCPSGSTNY RVVFCPNGVT GPNFPLEMPG S-DGVAK*
CAPR5-2  201  SYPQDDPTST FTCPSGSTNY RVVFCPNGVA DPNFPLEMPT STDEVAK*
CAPR5-3  201  SYPQDDATST FTCPSGSTNY RVVFCHNGVT GPNFPLEMPG S-DGVAK*
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Fig. 2. Comparison of the deduced amino acid sequence among the different PR5 genes in *Capsicum annuum*. Numbers indicate the amino acid residues in the sequence. Gaps in the alignmnet are designated by dashes. The 16 cysteine residues which might involve in the formation of osmotin-like protein were all presented as bold characters.

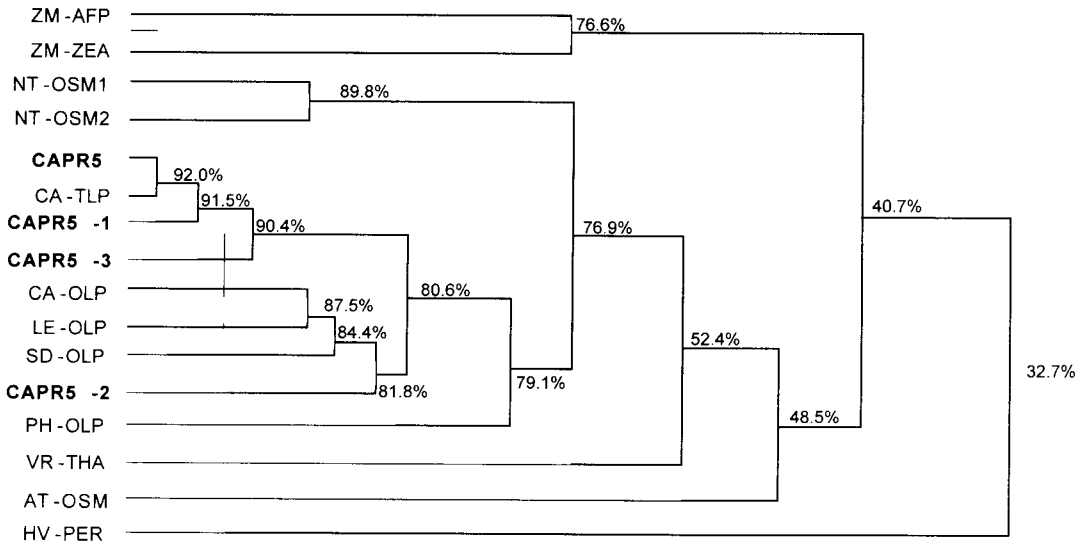

Fig. 3. Phylogenetic relationship of the three PR5s of *Capsicum annuum* and thirteen osmotin and thaumatin amino acid sequences. ZM; *Zea mays* (T02075, P33679), NT; *Nicotiana tabacum* (X65701, X657000), CA; *Capsicum annuum* (AF294847, AF297646, AJ287410), LE; *Lycopersicom esculentum* (X66416), SD; *Solanum dulcamara* (AY007309), PH; *Petunia hybrida* (AF376058), VR; *Vitis riparia* (AF178653), AT; *Arabidopsis thaliana* (AL049500), HV; *Hordeum vulgare* (T05973).

showed a high homology with the OLP and TLP reported in other plants [6,24]. The results may suggest that PR5 genes existing as multi-gene family will be expressed by alternative stimuli and through different signals. The antifungal activity and specificity of the CAPRs may also have closer relationship among them, however, the possibility must be clarified by correlational experiment on their antifungal activity and specificity.

Southern blot analysis

Southern blot of *Capsicum annuum* genomic DNA probed with full-length CAPR5-1 and CAPR5-2 genes was shown in Fig. 4. In genomic DNA gel blot analysis, two hybridized bands with *Eco*RI+*Kpn*I of CAPR5-1 and *Eco*RI+*Pst*I of CAPR5-2 were expected because CAPR5-1 cDNA had *Kpn*I sites and CAPR5-2 cDNA has *Pst*I sites. However, in the *Eco*RI digested lane, four hybridized bands were detected, suggesting that high nucleotide similarity existes between CAPR5-1 and CAPR5-2, and the two genes are a member of PR5 gene families (Fig. 4). The

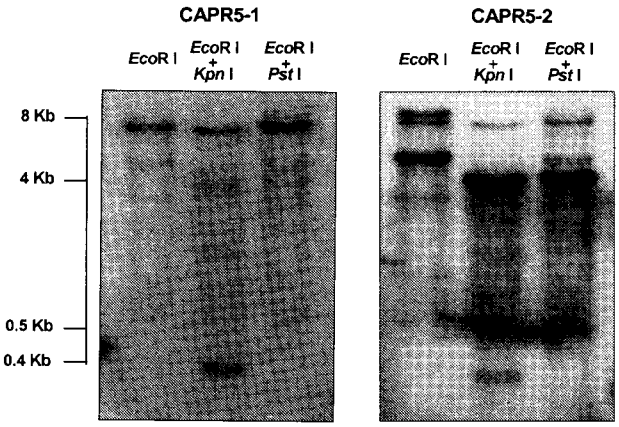

Fig. 4. DNA gel blot analysis of PR5-like genes in hot pepper genomes. Genomic DNA (20 μg) was digested with the restriction enzymes, *Eco*RI, *Eco*RI+*Kpn*I, and *Eco*RI+*Pst*I, respectively, separated on 0.8% agarose gel, transferred to nylon membrane, and hybridized with ^{32}P-labeled CAPR5-1 and CAPR5-2 cDNA fragments, as probes. The size marker are indicated in kb.

divergence in nucleotide sequence between different thaumatins from the same systems is common. Altogether,

this result revealed that at least four homologous genes of CAPR5 putatively exists in the genome of *Capsicum annuum*.

Expression of CAPR5 genes in *E. coli*

The entire coding regions of the CAPR5 genes were cloned into bacterial expression vector pET28c. The constructs of vector were confirmed by *Nde*I + *Xho*I double digestion. The vectors of pET28c-CAPR5-1, pET28c-CAPR5-2, and pET28c-CAPR5-3 were transformed into bacterial host BL21 for expressing recombinant CAPR5 proteins. The CAPR5 proteins were induced by adding 1 mM IPTG.

Total proteins isolated from the cultured cell before and after IPTG induction were separated on 12.5% SDS-PAGE gel. The expressed CAPR5 proteins of 23 kD were matched with the predicted size of CAPR5-1, CAPR5-2, and CAPR5-3. There was no corresponding protein in pET28c without CAPR5 gene (Fig. 5).

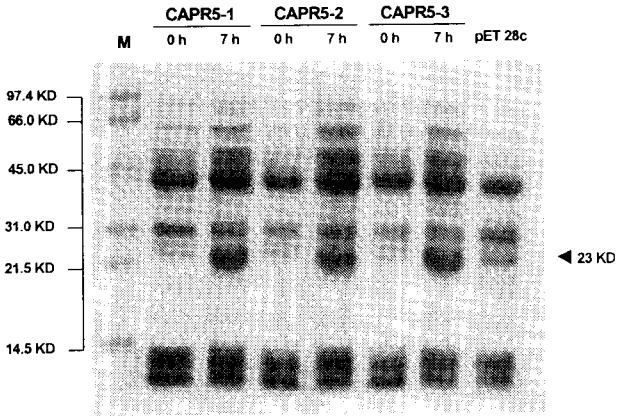

Fig. 5. Total protein of artificially expressed CAPR5 proteins in *E.coli* strain BL21 (DE3) was detected by SDS-PAGE stained with Coomassie blue. The twenty three kD proteins in cells harboring three recombinant plasmids for CAPR5-1, CAPR5-2 and CAPR5-3 genes were accumulated at 7 h after addition of 1 mM IPTG, respectively. M, Molecular mass markers; pET28-c, total protein form BL21 with harboring the intact bacterial expression vector (pET28-c).

Antifungal assay with the CAPR5 proteins

To investigate the antifungal activity of CAPR5-1, CAPR5-2, and CAPR5-3 proteins, hyphal growth inhibition of the *Phytophthora capsici, Glomerella glycines* and *Colletotrichum lagenarium* were performed by treatment with the extracted CAPR5 proteins.

As shown in Fig. 6, the hypha growth of two fungus stains, *G. glycines* and *C. lagenarium* causing anthracnose on soybean and water melon, respectively were not affected by CAPR5-1, CAPR5-2, and CAPR5-3 proteins. However, the hypha growth of *P. capsici* causing pepper root rot was inhibited. Clear inhibition zone was seen around the discs treated with 5, 20, 50, 100 μg of CAPR5-1, CAPR5-2, and CAPR5-3 proteins as determined at 24-48 h after incubation at 37℃, while no inhibition zone was seen around the control disc (Fig. 6).

Even though the action mechanism of the PR-5 protein family has not been completely elucidated, it seems that PR-5 protein may form a membrane pore causing water influx, and subsequently fungal membrane rupture. Cheong *et al.* reported that PR5 protein of pumpkin

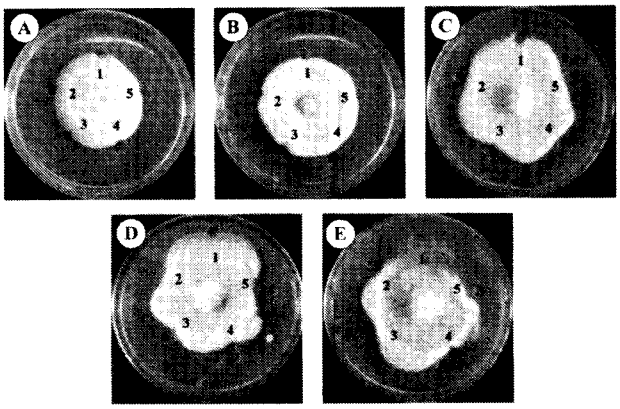

Fig. 6. Antifungal activities of CAPR5s protein was assayed on reducing the hyphae growth of fungal pathogens, *G. glycines* (A), *C. lagenarium* (B) and *P. capsici* (C, D, E). *P. capsici* appeared to be sensitive on CAPR5-1 (c), CAPR5-2 (D) and CAPR5-3 (E), while *G. glycines* and *C. lagenarium* seemed to be insensitive. Discs: 1, water; 2, 5 μg; 3, 20 μg; 4, 50 μg; 5, 100 μg of CAPR5 proteins.

rapidly ruptured the fungal hyphae and released clouds of cytoplasm [6]. They also represented that NaCl concentration is very important to hyphae bursting. It suggests that pumpkin PR5 protein controls the osmotic pressure of fungal cytoplasm through forming of membrane.

Based on the results, CAPR5-1, CAPR5-2, and CAPR5-3 had a specific antifungal activity against *P. capsici*. Considering the high affinity of amino acid sequence between pumpkin PR5 genes and CAPR5-1, CAPR5-2, CAPR5-3, this may suggest that a similar mechanism of the antifungal activity against *P. capsici* may be existed in CAPR5s and pumpkin PR5 proteins.

Acknowledgements

This work was supported by Korea Research Foundation Grant (KRF-2000-005-G00002). We appreciate Young-Yang Pepper Experiment Station for providing hot pepper seeds.

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(Received March 28, 2002; Accepted May 13, 2002)

초록 : 고추(*Capsicum annuum*)의 항균성 단백질(PR-5) 유전자의 클로닝과 발현 분석

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식물은 병원균이나 여러 가지 환경스트레스에 대하여 자기 방어기작을 가지며, 특히 PR 단백질은 병원균의 침입시에 동물의 면역반응과 유사한 생체방어반응을 나타내는 중요한 역할을 하는 것으로 알려져 있다. 본 연구에서는 고추에서 항균 특성을 나타내는 PR5 유전자를 클로닝하고 이들의 특성을 구명하였다. 고추에서 서로 다른 3종의 PR5 유전자, CAPR5-1, CAPR5-2, CAPR5-3를 클로닝하였다. 이들 유전자의 특성을 조사하고 아미노산 수준에서 유사성을 비교하여 본 결과, 서로간에는 90% 이상의 상동성을 나타내었고 이들의 2차구조를 비교한 결과 중요한 domain은 높은 상동성을 나타내어 PR5 유전자들이 항균 특성을 나타내는데 매우 중요한 motif로 작용할 것으로 사료된다. CAPR5-1, CAPR-2, CAPR5-3 유전자들의 항균성 정도를 조사하기 위하여 이들 유전자를 대장균에서 발현시켜 단백질을 분리하여 고추 역병균인 *Phytophthora capsici*에 처리한 결과, 균사의 성장이 억제되어 CAPR5-1, CAPR5-2, CAPR5-3 단백질들이 항균성을 지니고 있는 것으로 나타났다.