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Molecular Characterization of a cDNA for a Cysteine-Rich Antifungal Protein from *Capsicum annuum*

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We have isolated a cDNA clone for the antifungal protein, *CaAFP*, from hot pepper, *Capsicum annuum* L. Its open reading frame encodes 85 amino acids, including 8 cysteine residues. CaAFP consists of three domains: a signal peptide, a chitin-binding domain, and a C-terminal peptide domain. The deduced amino acid sequence of the chitin-binding domain shows 92% and 85% similarity to the same domain from PnAMPs and hevein, respectively. Southern blot analysis indicated that *CaAFP* is present as a single copy, while the northern blots revealed that the clone is highly expressed in the leaves and flower buds, but not in the roots. However, wounding treatments and chemicals generally known to induce PR proteins did not stimulate its expression. *In situ* hybridization also showed that *CaAFP* is expressed in the parenchyma cells of the floral sepals. As seen in our functional analysis, this clone was expressed in *Escherichia coli*, and the fusion protein was purified using nickel-affinity column chromatography. This purified AFP fusion protein inhibited spore germination and appressoria formation in several plant pathogenic fungi, including *Fusarium oxisporum* and *Colletotrichum gloeosporioides*. Our results suggest CaAFP is an antifungal protein that defends developing seeds against pathogen invasion while also having a specific biological role during floral development.

Keyword: antifungal Protein, *Capsicum annuum*, chitin-binding domain, hevein

Plants and animals have developed highly systematic defense mechanisms against biotic stress, based on various antifungal proteins/antimicrobial peptides. Cysteine-rich proteins are key factors in providing that protection. In animals, these proteins are widely distributed among the arthropods and invertebrates (Dimarcq et al., 1998), and are characterized by low molecular weights (2 to 9 kD) and several disulfide bonds (Garcia-Olmedo et al., 1998). In plants, these numerous proteins include defensins (Broekaert et al., 1995), lipid transfer proteins (LTPs) (Garcia-Olmedo, 1998), knottin-type peptides (De Bolle et al., 1995), and hevein-type peptides (van Parijs et al., 1991).

Hevein, an abundant chitin-binding protein in the latex of the rubber tree (*Hevea brasiliensis*), consists of 43 amino acid residues, including 8 cysteines (Archer et al., 1969). It is known as a latex-allergen, which means it causes human allergies while also attacking pathogenic fungi (van Parijs et al., 1991). Several hevein-type peptides have been reported. PnAMP from *Pharbitis nil* has 8 cysteine residues in 40 amino acid residues, and possesses potent antifungal activity. This peptide penetrates very rapidly into the fungal hyphae and localizes at the septa and hyphal tips, causing the hyphae to burst (Koo et al., 1998). AcAMPs, from *Amaranthus caudatus*, and IWF4, from *Beta vulgaris*, have only 6 cysteine residues each; their amino acid sequences show less homology to hevein than do the PnAMPs (De Bolle et al., 1995; Nielsen et al., 1997).

Expression patterns vary in the antifungal proteins. For example, hevein is expressed in the leaf and stem but not in the root, and is induced by abscisic acid, ethylene, and wounding treatments (Broekaert et al., 1990). In contrast, AcAMP is expressed in the seed but not in leaves or roots, and it is not induced by either jasmonic acid or wounding (De Bolle et al., 1993). Because studies on the hevein-type peptides have begun only recently, and have concentrated only on those proteins purified from plants, genetic information is rather scarce. In the research presented here, our objectives were to isolate and characterize a cDNA clone for a hevein-type peptide from hot pepper, and then examine its expression patterns and antifungal activity.

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MATERIALS AND METHODS

Plant Materials and Pathogenic Fungi

F1 hybrid hot pepper plants (*Capsicum annuum* var. Dae Poong and var. Nok Kwang) were reared in a growth chamber under a 16-h photoperiod and a temperature cycle of 28°C/18°C (day/night). Their fruits were harvested at various developmental stages, and were designated as: ovary-type (OV), young-green (YG), growing-green (GG), mature-green (MG), and ripe-red (RR) (Kim et al., 1996). Several pathogenic fungi -- *Fusarium oxysporum f. sp. radicis-lycopersici, Phytophthora capsici, Bipolaris cohesis, Nectria radicicola, and Colletotrichum gloeosporioides* -- were kindly obtained from the Laboratory of Plant Mycology, Department of Agricultural Biology, Seoul National University.

Screening of cDNA Library and Nucleotide Sequencing

The method of Benton and Davis (1977) was followed for screening the cDNA library of hot pepper (Kim et al., 1996). *CaAFP* cDNA clones were sequenced as described by Sanger et al. (1977), using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemicals, USA) and [α-^{35}S] dATP (Amersham, UK). These sequences were then analyzed with the Blast search program, ExPASy system, and Clustal W Programs (Altschul et al., 1990; Higgins et al., 1992).

Southern and Northern Hybridizations

Genomic DNA was prepared from hot pepper leaves according to the method of Ausubel et al. (1987). We digested 20-μg samples of genomic DNA with restriction enzymes, separated them on 0.6% (w/v) agarose gel, and blotted them onto Hybond N membrane (Amersham). PCR product, generated with SP1 and SP2 primers, was labeled with [α-^{32}P] dCTP by random priming (Promega, USA) for use as a probe. Hybridization was performed in BEPS buffer (1 mM EDTA, 0.5 M sodium phosphate, 1% BSA, and 7% SDS) at 61°C for 24 h. Afterward, the filter was washed with 2×SSC/0.1% (w/v) SDS at 61°C for 30 min, 1X SSC/0.1% (w/v) SDS at 61°C for 30 min, and 0.5X SSC/0.1% (w/v) SDS at 61°C for 30 min. It was then visualized by autoradiography at –80°C.

Total RNA was extracted from the leaves, stems, roots, and fruits at five developmental stages using RNA Plus (QUANTUM, Canada) according to the manufacturer's instructions. RNA was dissolved in diethylpyrocarbonate (DEPC)-treated distilled water and separated on 1% formaldehyde-agarose gels in MOPS buffer. The gels were blotted onto Hybond-N membranes. Filters were pre-hybridized and hybridized using the same probe and method as with our Southern hybridization. Afterward, the filters were washed with 2X SSC at 61°C for 30 min and 1X SSC at 61°C for 30 min.

In Situ Hybridization

Tissues were prepared for hybridization as described by Cox and Goldberg (1988). Flower buds were fixed overnight in FAA solution under constant vacuum, dehydrated through a graded ethanol series, and embedded in Paraplast (Oxford, USA). Sections (10 μm thick) were applied to pre-cleaned glass slides treated with Vectabond (Vector Laboratory, USA). Pretreatment, hybridization, and slide-washing were performed as presented by McKhann and Hirsch (1993), except that RNase (50 μg mL^{-1} of NTE buffer) was added during the washing. Antisense and sense RNA probes were prepared from linearized plasmids with digoxigenin (DIG)-11-rUTP, according to the manufacturer's instructions (Boehringer, Germany), and hybridization was performed at 42°C for 16 h. After the post-hybridization treatment and incubation in antidigoxigenin conjugated with alkaline phosphatase, substrate was added and the color was allowed to develop in a dark cabinet for 1 to 2 d. This process was then stopped by immersing the slides in TE buffer (pH 8.0).

Induction of CaAFP Fusion Protein and Measurement of *E. coli* Growth Rate

To express the *CaAFP* cDNA in *E. coli*, the ORF of *CaAFP* cDNA was subcloned into a pET-30b (+) expression vector, under the control of the T7 promoter in the correct reading frame. After it was introduced into BL21 (DE3) *E. coli*, the cells carrying pET-*CaAFP* were cultured in 50 mL of an LB medium at 37°C(with shaking) until the OD_{600} reached 0.3. To induce expression of *CaAFP*, isopropyl-1-thio-β-D-galactoside (IPTG) was added to a final concentration of 0.5 mM, and the cells were further incubated for 3 h to promote recombinant protein synthesis. To analyze the effect of *CaAFP* expression on bacterial growth, the *E. coli* cells that carried pET-*CaAFP* were cultured in 50 mL of an LB medium at 37°C(with shaking) until the OD_{600} reached 0.3. Afterward, 0.5

mM IPTG was either added or not, and the cells were placed in culture. Absorbancies were measured at 600 nm for 9 h, at 1-h intervals.

Purification of CaAFP Fusion Protein and Antifungal Activity Test

CaAFP fusion protein was purified by nickel-affinity column chromatography, using the His-Tag that originated from the pET vector (Novagen, USA). After the cultured cells were washed with cold 50 mM Tris-HCl (pH 8.0) and re-suspended in Bind-buffer [5 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl (pH 7.9)], they were sonicated and centrifuged. The supernatant was passed through a column charged with Ni^{2+}, then washed with Bind-buffer and Wash-buffer [60 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl (pH 7.9)]. To release the bound CaAFP fusion proteins, Elute-buffer [1 M imidazole, 0.5 M NaCl, and 20 mM Tris-HCl (pH 7.9)] was passed through the column. The purity of the eluted solution was analyzed on a 15% SDS-polyacrylamide gel, and protein content was determined by the method of Bradford (1976).

Antifungal activity was assayed using a spore germination test against several plant pathogens (Schirmbock et al., 1994). The purified CaAFP fusion protein solution was dialyzed with distilled water, and 1 ppm and 10 ppm of the dialyzed solutions were mixed with distilled water that contained 10^5 fungal spores per mL. These mixed solutions were incubated in the dark at 25°C. Spore germination and appressoria formation patterns were examined three times by optical microscopy.

RESULTS

Isolation of *CaAFP* by Library Screening

After screening a hot pepper leaf cDNA library (Kim et al., 1996), with aminoacyltransferase, glutamine synthetase, and the phenylalanine ammonia lyase cDNA clone as probes we isolated *CaAFP*, a cDNA clone whose nucleotide sequence is highly homologous with the chitin-binding domain of several hevein-type peptides. Its complete open reading frame (258 bp) was flanked by untranslated sequences -- 13 bp in the N terminus and 238 bp in the C terminus (Fig. 1). Two copies of a putative signal for poly $(A)^+$ addition (AATAA) were found at 195 bp and 160 bp upstream of the poly A residues.

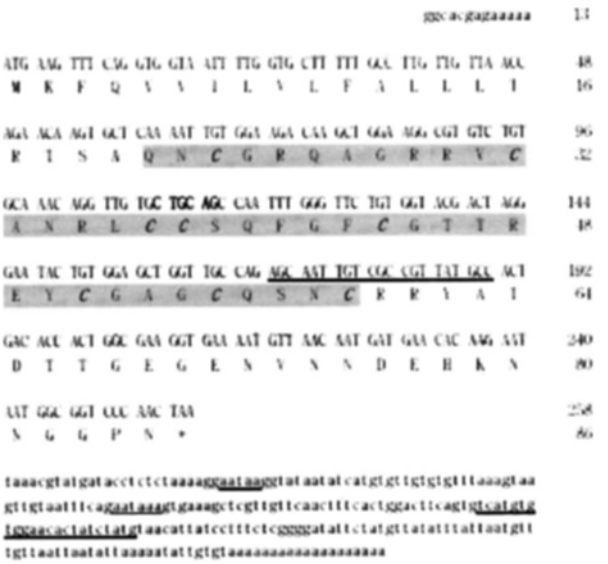

Figure 1. Nucleotide and deduced amino acid sequences of *CaAFP* cDNA clone from *C. annuum* (GenBank accession number AF333790). Amino acids are represented by standard one-letter abbreviations. Putative polyadenylation signals are underlined. Star indicates stop codon. Amino acids for putative chitin-binding domain are shaded. Cs, are cysteine residues found in other cysteine-rich peptides. Bold letters, 124-129 position, indicate *Pst*I restriction site. Primer sequences of SP1 and SP2 for gene-specific probe are underlined heavily.

CaAFP Is Highly Homologous with Hevein

The ORF of *CaAFP* encodes a protein with a molecular weight of 9.3 kD and a pI of 8.73. Its deduced amino acid sequence comprises three distinct domains totaling 85 amino acid residues -- a putative signal sequence of 20 amino acids, a chitin-binding domain (CBD) of 39 amino acids, and a carboxyl-terminal domain of 26 amino acids (Fig. 1). This structure is common to other chitin-binding plant proteins, e.g., hevein, AcAMP, and IWF4. The nucleotide sequence of *CaAFP* shows 58% identity with *IWF4*, as well as 55% and 52% with *AcAMP* and *hevein/CBP20*, respectively. The CBD contains the chitin-binding motif, C-x(4,5)-C-C-S-x(2)-G-x(4)-[FYW]-C, and eight cysteine residues are also conserved. This domain is very similar to that of other chitin-binding proteins, sharing 72% and 63% identity and 92% and 85% similarity with those of PnAMPs from *Pharbitis nil* and hevein from the rubber tree, respectively. The CBD is preceded by a putative signal sequence that is highly hydrophobic in its profile analysis (data not shown). This domain is followed by a C-terminal peptide that shows no sequence homology with other proteins, as

determined by our data-bank search.

CaAFP Is a Single-Copy Gene

Genomic DNA from leaves of the 'Dae Poong' cultivar was digested with three restriction enzymes -- *Eco*RI, *Hin*dIII, and *Bam*HI -- and hybridized with a specific probe (Fig. 2). Because this probe hybridized strongly to one band in each lane, we believe that *CaAFP* exists as a single copy in the hot pepper genome. However, the presence of several weakly hybridizing bands suggested that related genes existed in that genome. Nevertheless, when the filter was washed under high-stringency conditions, all the weak bands disappeared (data not shown).

CaAFP Is Highly Expressed in Leaves and Young Fruits

We used RNA blot analysis to analyze the expression patterns of *CaAFP* mRNA in different organs and at several fruiting stages. A high level was found in the leaves, whereas the amount was very low in the stems and undetectable in the roots (Fig. 3A). Transcript levels were high in dry seeds (data not shown). Differential accumulation of *CaAFP* mRNA was also observed during fruit development, with transcripts being highly abundant in the ovary-type (OV) fruit, then decreasing gradually with maturation so that no expression was detected in the ripe-red (RR) fruit (Fig. 3B). Prior treatments with methyl jasmonate, which is known to induce PR proteins, or even 2,4-D or wounding, did not increase *CaAFP* expression (Fig. 4).

Figure 2. Genomic Southern hybridization of *CaAFP*. Twenty μg of hot pepper DNA was digested with three restriction enzymes and separated on 0.6% agarose gel. DNA fragments transferred to Hybond N filter were hybridized with a 234-bp gene-specific probe labeled with [α-^{32}P] dCTP. E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI.

CaAFP Transcripts Are Localized in Sepals of Flower Buds

In situ hybridization of the flower buds demon-

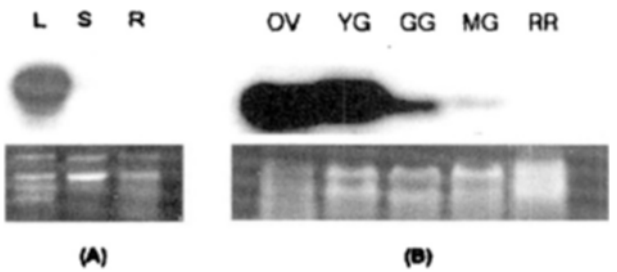

Figure 3. RNA gel blot analyses of *CaAFP* transcripts at different organ (**A**) and stages of fruit development (**B**). Twenty μg of hot pepper RNA separated on 1.0% formaldehyde-agarose gel and blotted on Hybond N filter was hybridized with a gene-specific probe. L, leaf; S, stem; R, root; OV, ovary-type; YG, young-green; GG, growing-green; MG, maturing-green; RR, ripe-red fruit.

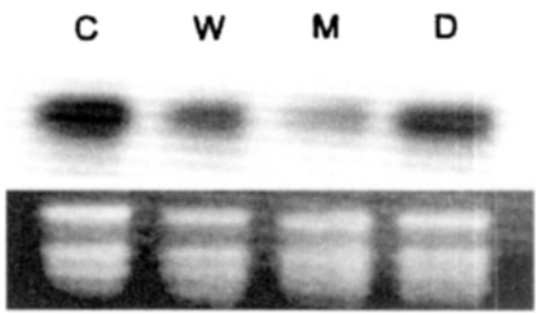

Figure 4. Expression patterns of *CaAFP* mRNA during several treatments. Leaf fragments were floated on MS liquid medium (W), MS liquid medium containing 10 μM methyljasmonate (M), or MS liquid medium containing 1 μM 2,4-D (D). Northern blots with 10 μg total RNA per lane were hybridized with a gene-specific probe.

strated that transcripts of the *CaAFP* gene were localized in the sepals, but not in the petals, anthers, or ovules (Fig. 5A). In those sepals, transcripts were detected mostly in the cells just below the epidermis, especially in its outer region, but not in the epidermis per se (Fig. 5B). However, De Bolle et al. (1996) have used an immunocytochemical technique to show that AcAMP2, the antimicrobial peptide from *Amaranthus caudatus*, is localized in the intercellular space of the epidermis in transgenic tobacco. Therefore, further examination is needed to determine whether CaAFP transcripts truly are localized in the hot pepper epidermis as well.

Purified CaAFP Fusion Protein Shows Antifungal Activity

BL21 *E. coli* cells carrying either pET or pET-*CaAFP* were grown in liquid LB media with or without IPTG. Their growth rates were monitored at 1-h intervals by measuring their absorbance at 600 nm. Uninduced *E. coli* cells showed standard sigmoid growth curves, while the induced *E. coli* (with pET-*CaAFP*) decreased in growth immediately after IPTG was added, to a rate that was one-third that of the uninduced cells (data not shown; see also Koo et al., 1995). This result suggests that *CaAFP* expression inhibited *E. coli* cell growth.

Expression in *E. coli* BL21 (DE3) was detected by SDS-polyacrylamide-gel electrophoresis. Although the anticipated size of the CaAFP fusion protein was about 14.3 kD (9.3 kD of CaAFP plus 5 kD of the vector's peptide), we instead detected a 23-kD protein on the 15% SDS-PAGE gel (Fig. 6A). This size difference, which has been reported in the antifungal protein isolated from radish seed (Terras et al., 1992), is caused by oligomer formation (Broekaert et al., 1990). Our fusion protein was purified by affinity column chromatography, using the His-tag originated from the pET vector. It was then confirmed both by SDS-PAGE gel electrophoresis (data not shown) and by immuno-detection using the S-tag originated from the pET (Fig. 6B).

We also examined the activity of the purified CaAFP fusion protein against five fungal species. Compared with the control, the rate of spore germination in *F. oxysporum* f. sp. *radicis-lycopersici* was reduced by about 50% when treated with 1 ppm fusion protein (Fig. 7A). Similar degrees of inhibition were observed with *N. radicicola* and *B. cohesis* when 1 ppm and 10 ppm, respectively, of the CaAFP solution was applied (data not shown). However, 1 ppm *CaAFP* did not affect the germination of *P. capsici* and *C. gloeosporioides* spores. Instead, unidentified structures formed at the tips of the growing hyphae in 35% of the *P. capsici* (Fig. 7B). Moreover, the potential for appressoria formation dropped by half in *C. gloeosporioides* (Fig. 7C). These results clearly demonstrate that the CaAFP fusion protein inhibited spore germination and appressoria formation in sev-

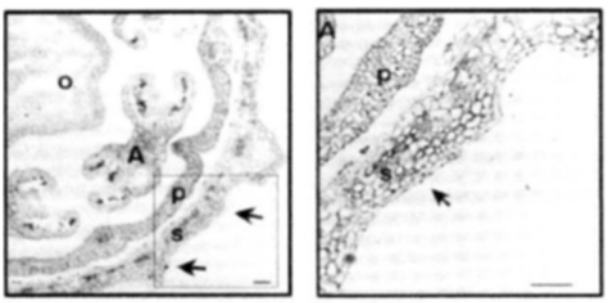

Figure 5. *In situ* localization of *CaAFP* transcripts in transverse sections of hot pepper flower bud. RNA probes for *in situ* hybridization were prepared from linearized plasmids with DIG-11-rUTP labelling. Arrows indicate hybridization signals having purple color, which is darkened here. Boxed region in left panel (**A**) is detailed in right panel (**B**). A, anther; O, ovary; P, petal; S, sepal. Bar represents 100 µm.

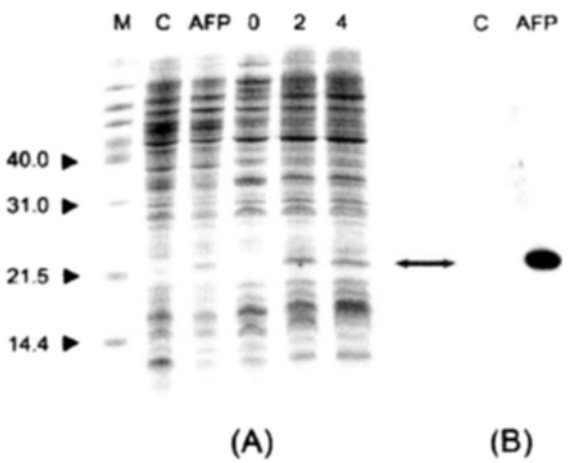

Figure 6. Expression of CaAFP in *E. coli* BL21 (DE3). Total proteins were loaded in SDS PAGE gel and stained with Coomassie blue (**A**). Cells containing pET (C; Control) or pET-*CaAFP* (AFP) were cultured for 4 h in LB medium without IPTG, while cells containing pET-*CaAFP* were cultured for 0, 2, or 4 h with IPTG. CaAFP fusion protein purified by nickel affinity column was subjected to immuno-detection using S-tag originated from pET vector (**B**). Arrows indicate CaAFP fusion proteins.

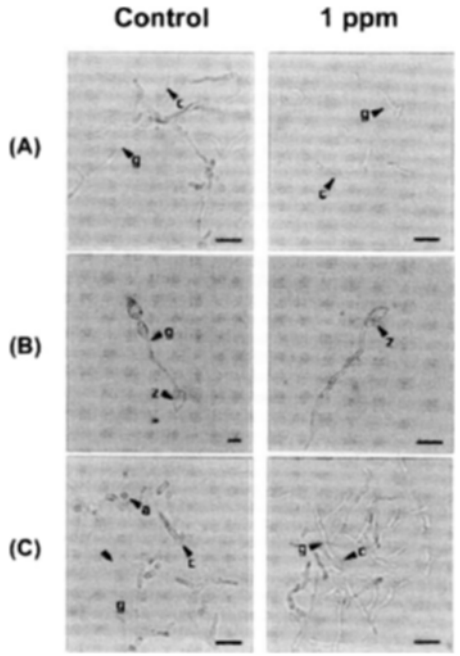

Figure 7. Optical micrographs of *Fusarium oxysporum* f. sp. *radicis-lycopersici* (**A**), *Phytophthora capsici* (**B**), and *Colletotrichum gloeosporioides* (**C**) treated with CaAFP. Distilled water (Control) or distilled water containing 1 ppm CaAFP fusion protein was mixed with 10^5 spores. a, appressorium; c, conidium; g, germ tube; z, zoosporangium. Scale bars indicate 20 μm.

eral plant pathogenic fungi.

DISCUSSION

We have isolated a cDNA for the antifungal protein, *CaAFP*, from hot pepper. Among its three distinct domains (Fig. 1), the chitin-binding domain is well conserved, having also been found in a variety of chitin-binding proteins, such as hevein, cereal lectins, and several chitinases (Chrispeels and Raikhel, 1991). The unique processing of transcripts means that the hevein-type peptides comprise only that chitin-binding domain. In hevein, the *HEV1* cDNA clone encodes a putative signal sequence of 17 amino acids followed by a polypeptide of 187 amino acids. It is co- and post-translationally processed to produce an amino-terminal portion corresponding to mature hevein as well as a carboxyl-terminal polypeptide with unknown function (Lee et al., 1991). This type of processing also occurs during the formation of AcAMPs and IWF4 (De Bolle et al., 1995; Nielsen et al., 1997). These examples suggest that *CaAFP* can also undergo co- and post-translation processings to form mature (i.e., chitin-binding domain) CaAFP.

CaAFP is most similar to hevein in its gene size, amino acid sequences, and antifungal potential, although the signal sequence and C-terminal peptide domains of CaAFP share little homology with comparable hevein domains. The tissue-specific expression pattern of *CaAFP* (Fig. 3) is also very similar to that of hevein in that both are expressed in the leaf and stem but not in the root (Broekaert et al., 1990). In separate experiments (data not shown), we obtained essentially the same results for three varieties of hot pepper. However, wounding treatments, which normally induce the hevein gene, did not elicit *CaAFP* expression (Fig. 4). Therefore, its overall expression pattern is more similar to that of *IWF4*, in terms of tissue-specific expression and non-inducibility by wounding and chemicals known to affect PR protein activities (Nielsen et al., 1997).

The genes involved in plant defenses generally are up-regulated during fruit development. For example, in grape, antimicrobial peptides such as thaumatin-like protein (grape osmotin), lipid transfer protein, and basic/acidic chitinases, accumulate as ripening proceeds (Horsfall and Dimond, 1957; Salzman et al., 1998). Likewise, in hot or bell peppers, the thionin-like protein and defensin genes are induced according to the stage of maturation (Meyer et al., 1996; Oh et al., 1999). Interestingly, *CaAFP* in this study was highly expressed in early-stage fruits, but the level of transcript then decreased gradually and was not detected in fully-matured, red fruits (Fig. 3).

In situ hybridization results showed that *CaAFP* is expressed in the sepals of young flower buds (Fig. 5). In contrast, *CaLTP*, the lipid transfer protein gene for another antifungal protein, is expressed exclusively in hot pepper anthers and ovules (Dr. S.R. Kim, Sogang Univ., Korea, personal communication). Numerous data have demonstrated that antifungal protein genes are expressed at different locations in the flower buds of that species. Similar results have been reported for PR proteins during floral development in tobacco (Lotan et al., 1989; Cote et al., 1991). The data from our northern and *in situ* hybridization experiments suggest that CaAFP may play a defensive role in protecting the exposed portions of a plant, e.g., its leaves and flower buds, against pathogen attack. However, the non-inducibility we observed when tissues were subjected to wounding or methyl jasmonate treatments, as well as strong expression by *CaAMP* in the placenta rather than in the pericarps of green fruits (data not shown), implies it has other, unknown biological functions in hot pepper.

Hevein shows inhibitory properties against phytopathogens (van Parijs et al., 1991). We also found here that recombinant CaAFP exhibits strong antifungal activities across a broad range of plant pathogenic fungi, including *P. capsici*, which has no chitin in its cell walls. At the 1 μg mL^{-1} concentration (1 ppm), the CaAFP fusion protein inhibited conidial germination by 50% in *F. oxysporum* (Fig. 7A) and *N. radicola*. But this same treatment had no effect on spore germination rates in *P. capsici* and *C. gloeosporioides*. Instead, an unidentified structure was formed at the hyphal tip of the former (Fig. 7B), while appressoria formation was diminished by 50% in the latter (Fig. 7C). This antifungal activity of CaAFP is comparable to that of PnAMP, which shows IC_{50} values in these ranges, i.e., 1 to 10 μg mL^{-1} (1 to 10 ppm), in tests with eight different fungi (Koo et al., 1995). The antifungal potential of hevein, however, is significantly weaker, with IC_{50} values of 90 to 1,250 μg mL^{-1}, depending on the fungal species (eight fungi; van Parijs et al., 1991). Finally, the recombinant MiAMP, an antimicrobial peptide from the nut kernels of *Macadamia integrifolia*, also exhibits such antifungal properties (Harrison et al., 1999).

In this study, we have characterized the cDNA for *CaAFP*, a hevein-type antifungal protein from hot pepper, and have examined its expression patterns and antifungal activity. To further understand its functioning *in planta*, we are currently focusing our research on its purification, amino acid sequencing, and localization, and are also constructing transgenic plants.

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