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Purification and Characterization of an Antifungal Protein, C-FKBP, from Chinese Cabbage

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An antifungal protein was isolated from Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*) by buffer-soluble extraction and two chromatographic procedures. The results of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry revealed that the isolated Chinese cabbage protein was identical to human FK506-binding protein (FKBP). A cDNA encoding FKBP was isolated from a Chinese cabbage leaf cDNA library and named *C-FKBP*. The open reading frame of the gene encoded a 154-amino acid polypeptide. The amino acid sequence of C-FKBP exhibits striking degrees of identity with the corresponding mouse (61%), human (60%), and yeast (56%) proteins. Genomic Southern blot analyses using the full-length *C-FKBP* cDNA probe revealed a multigene family in the Chinese cabbage genome. The *C-FKBP* mRNA was highly expressed in vegetative tissues. We also analyzed the antifungal and peptidyl−prolyl *cis*−*trans* isomerase activity of recombinant C-FKBP protein expressed in *Escherichia coli*. This protein inhibited pathogenic fungal strains, including *Candida albicans*, *Botrytis cinerea*, *Rhizoctonia solani*, and *Trichoderma viride*, whereas it exhibited no activity against *E. coli* and *Staphylococcus aureus*. These results suggest that recombinant C-FKBP is an excellent candidate as a lead compound for the development of antifungal agents.

KEYWORDS: Antifungal protein; FKBP; Chinese cabbage; PPIase activity

INTRODUCTION

Plants produce compounds that act as natural defenses against pests and pathogens. During the past few years, several antimicrobial plant proteins and peptides that inhibit the growth of agronomically important pathogens have been isolated from various plant sources (*1*). These have been divided into the following subfamilies: chitinases, β-1,3-glucanases, thaumatin-like (TL) proteins, proteinase inhibitors, endoproteinases, peroxidases, ribonuclease-like proteins, γ-thionin as a plant defensin, oxalate oxidase, oxalateoxidase-like proteins, and other proteins of unknown biological properties (*2*−*8*).

Antifungal proteins protect plants from phytopathogenic fungal infections and have the potential to prevent massive economic losses. Many investigators continue to search for antifungal proteins and peptides, because transgenic plants expressing antifungal proteins are expected to be resistant to fungal infections (*2*).

On the basis of their affinities for the immunosuppressive drugs cyclosporin A and FK506, immunophilins are classified as cyclophilins and FKBPs (*9*, *10*). Cyclophilins bind to cyclosporin A (CsA), while FK506-binding proteins (FKBPs) bind to both FK506 and rapamycin. The cyclophilin−CsA and FKBP−FK506 complexes target a common protein, calcineurin (CN), a Ca^{2+}−calmodulin (CaM)-dependent protein phosphatase involved in numerous signaling processes (*11*−*14*). All immunophilins have peptidyl−prolyl *cis*−*trans* isomerase (PPIase) activity, regulate folding, assembly, and trafficking of substrate proteins, and act as molecular chaperones (*15*).

FKBPs comprise a growing family of highly conserved proteins that are widely distributed from *Escherichia coli* to mammals. FKBPs have also been found in plants such as horsebean, maize, wheat, and *Arabidopsis* (*16*−*19*). Although *FKBP* genes have been described in plants, *FKBP* cDNA clones and proteins have been isolated only recently. Though FKBP may play a general and important role, the exact biological importance of FKBPs in plants is poorly understood.

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Here, we describe the isolation and characterization of one protein with sequence homology to a FKBP and antifungal activity from Chinese cabbage. Using a bacterially expressed recombinant protein, named C-FKBP, we show that the protein displays PPIase activity and exhibits antifungal activity against various fungal strains.

MATERIALS AND METHODS

Plant Materials. Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*) was grown in a growth chamber with a 16/8 h light/dark cycle at 20 °C and 70% relative humidity. Various Chinese cabbage tissues were dissected and used to prepare genomic DNA and RNA.

Purification and Identification of Antifungal Protein. Chinese cabbages were homogenized in buffer A (25 mM Tris−HCl, 1 mM dithiothreitol, 1 mM EDTA, pH 7.5). The homogenate was centrifuged and ultrafiltered with a 30 000 molecular weight membrane. The ultrafiltered material was then applied to a DEAE-Sepharose column (1.5 × 8 cm) previously equilibrated with buffer A. To obtain more positively charged proteins, the unadsorbed proteins were collected and dialyzed with buffer B (25 mM sodium phosphate buffer, pH 6.5). The dialyzed fraction was loaded on a CM-Sepharose column (1.5 × 8 cm). The adsorbed proteins were eluted with a linear gradient of 0−500 mM NaCl. Antifungal assay against *Candida albicans* was performed with the collected fractions. After PAGE, protein bands were identified by MALDI-TOF-MS according to standard protocols (*20*).

Isolation and Sequencing of cDNAs Encoding Chinese Cabbage C-FKBP. Partial *FKBP* genes were obtained from *B. campestris* ESTs (expressed sequence tags). Using the partial *FKBP* cDNAs as probes, full-length *FKBP* genes were isolated from a Chinese cabbage cDNA library and ligated into the *Eco*RI and *Xho*I sites of pBluescript II SK+/− vector. After the 3′ and 5′ ends of the clone were sequenced and confirmed, serial deletion constructs were generated for full sequencing using the PCR method and an automated sequencing apparatus (ABI, Foster City, CA). Homology searches were performed with BLAST, and the amino acid sequence alignment was computed with the CLUSTAL program in the PCGENE software package.

Genomic Southern and Northern Blot Analysis. Chinese cabbage genomic DNA was prepared from leaves as described by Dellaporta et al. (*21*). A 10 μg sample of genomic DNA was digested overnight at 37 °C with *Bgl*II, *Hin*dIII, or *Xba*I, phenol/chloroform-extracted, ethanol-precipitated, and separated by 0.8% agarose gel electrophoresis. For Northern blot analysis, total RNAs were isolated from various tissues using the phenol/LiCl method as described (*22*). A 20 μg sample of total RNA was separated by 1.2% formaldehyde agarose gel electrophoresis. Both Southern and Northern gels were transferred to a nitrocellulose membrane in 10× SSC and probed with full-length *C-FKBP* cDNA randomly labeled with [α-^{32}P]dATP.

Expression of C-FKBP in *E. coli*. The GST-fusion system (Pharmacia) was used to express C-FKBP. To generate an active form of C-FKBP, the putative ER signal sequence was deleted from *C-FKBP* by PCR. The cDNA encoding the truncated, N-terminal-deleted C-FKBP (amino acids 27−164) was amplified by PCR from the pBluesript−*C-FKBP* plasmid with two mutagenic primers: sense, 5′-TTGGATCCAAGAAGTCAGG-3′, and antisense, 5′-AAGTCGACG-CAATCAAAGC-3′. After confirmation of the sequence of the correctly sized PCR product, the amplified DNA was cloned into the pGEX-2T-linker expression vector. The resulting plasmid (pGEX−*C-FKBP*) was transformed into *E. coli* strain BL21 (pLysS) and grown in LB medium containing 100 μg/mL ampicillin. The GST-fusion protein GST−C-FKBP was purified on a glutathione−agarose column, and C-FKBP was cleaved from the GST moiety by on-column thrombin digestion. The C-FKBP protein was separated from thrombin by Mono-Q column chromatography.

Peptidyl−Prolyl *cis*−*trans* Isomerase Assays. PPIase activity was measured in a coupled assay with α-chymotrypsin (Sigma) utilizing the substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma) in 88% *trans*, 12% *cis* configuration (*23*). The PPIase activity was measured as the *cis*−*trans* isomerization of the proline−alanine peptide bond in the peptide. The *trans* form of the substrate is cleaved by α-chymotrypsin to release *p*-nitroanilide, while the *cis* form is not cleaved; released *p*-nitroanilide is quantified spectrophotometrically at 395 nm. The assays were conducted at ambient temperature. The substrate was prepared daily as a 1.7 mM stock solution in methanol. The assay buffer was 25 mM Hepes (pH 7.8) with 100 mM NaCl. Chymotrypsin was prepared as a 100 μg/mL stock solution and was maintained on ice. In each assay, 910 μL of the assay buffer and 30 μL of chymotrypsin were equilibrated in an E-tube. The enzyme was then added to the E-tube followed by addition of 60 μL of substrate. Absorbance changes were measured by recording 0.1 s spectral integrations at 0.5 s intervals for 5 min at 395 nm.

Antifungal Assay. To compare and visualize the antifungal activities of purified and recombinant C-FKBP for pathogenic fungi, we performed a radial growth inhibition assay. Fungal fragments were placed in the center of potato dextrose broth (PDB; Difco Lab.) agar plates, and the plates were incubated for 72 h at 28 °C. After incubation, sterilized blank paper disks were placed at an appropriate distance around the fungal fragment, and an aliquot of protein in 25 mM Hepes buffer (pH 7.2) was then placed on each disk. The plates were incubated for 72 h at 28 °C until mycelial growth from the central disk had enveloped peripherally. We also used a 96-well microtiter plate assay to evaluate the antifungal effects of the recombinant protein. The following fungal strains were used: *Trichoderma viride* (Korean Collection for Type Cultures, KCTC 6047), *Trichoderma harzianum* (KCTC 6043), *Rhizoctonia solani* (Korean Agricultural Culture Collection, KACC 40138), *Fusarium solani* (KCTC 6326), *Fusarium oxysporum* (KCTC 16909), *Botrytis cinerea* (KACC 40573), *Aspergillus flavus* (KCTC 6905), and *C. albicans* (KCTC 7270). Conidial suspensions were filtered through two layers of sterile muslin. The density of each conidial suspension was determined with a hemocytometer and adjusted to 10^4 conidia mL^{-1} in PDB. Two-fold serial dilutions of protein (20 μL) in 25 mM Hepes buffer containing 1% PDB were added to the conidial suspension (80 μL). The plate was incubated at 25 °C for an appropriate period (12−48 h) specific to each fungus. Fungal growth was monitored microscopically and evaluated by measuring the optical density of the culture at 595 nm using a microplate reader. Each measurement was performed in triplicate.

Antibacterial Assay. *E. coli* (American Type Culture Collection, ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were cultured with agitation in trypticase soy broth (TSB) at 37 °C. The antimicrobial activity of the protein was determined using microdilution assays. Briefly, microorganisms were collected in mid-log phase and suspended in 25 mM Hepes, pH 7.2. Two-fold serial dilutions of protein were distributed in sterile 96-well plates. An aliquot of cell suspension (1×10^5 CFUs/mL) was then added to each well, and the plates were incubated at 37 °C for 2 h. At the end of this incubation, 50 μL portions of the 20-fold diluted samples were plated on TSB agar plates and incubated overnight, after which the colonies were counted.

RESULTS AND DISCUSSION

Understanding plant defensive responses and devising new methods to protect plants from pathogens and pests is one of the largest fields of research in plant science. Proteins with antimicrobial activity are classified into various subgroups on the basis of their structural/functional properties in the plant. Most antimicrobial proteins are capable of defense toward plant pathogens. In the present work, we isolated an antifungal protein, C-FKBP, from Chinese cabbage and characterized its molecular function on the basis of its antifungal and enzymatic activity.

Cloning and Sequence Analyses of C-FKBP in Chinese Cabbage. After several purification steps, MALDI-TOF-MS analysis confirmed that the purified protein shares high sequence identity with human FKBP (data not shown).

A partial *C-FKBP* cDNA clone was obtained from *Brassica* ESTs, and full-length cDNAs of *C-FKBP* genes were isolated from a cDNA library of Chinese cabbage leaves. The full-length *C-FKBP* nucleotide sequence contains 508 bp's with a 465-bp open reading frame predicted to encode a 154-amino acid protein (**Figure 1**). When the amino-terminal peptide sequence of the

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                                                    GGTGAGCAAGTAAGATAGATCGAAGACG      28
ATGAGGAGCTCTGCATCATCCGCCATGAAAGCTGCTGGAGTTTTGGTTCTCCTTGCCGTC                      88
 M  R  S  S  A  S  S  A  M  K  A  A  G  V  L  V  L  L  A  V                     (20)
TTAACATCAGTTTATGCGAAGAAGTCAGGTGATGTGACAGAGTTGCAGATTGGTGTTAAG                     148
 L  T  S  V  Y  A  K  K  S  G  D  V  T  E  L  Q  I  G  V  K                     (40)
TTCAAGCCGAAGACATGTGATGTTCAGGCTCACAAAGGGGACAAGATCAAGGTCCACTAT                     208
 F  K  P  K  T  C  D  V  Q  A  H  K  G  D  K  I  K  V  H  Y                     (60)
CGAGGAAAGTTAACAGATGGAACTGTGTTTGATTCAAGTTTTGAGAGAGGTGATCCTATT                     268
 R  G  K  L  T  D  G  T  V  F  D  S  S  F  E  R  G  D  P  I                     (80)
GAGTTTGAGCTTGGGAGTGGTCAAGTCATTCCAGGATGGGACCAGGGTCTACTTGGAGCT                     328
 E  F  E  L  G  S  G  Q  V  I  P  G  W  D  Q  G  L  L  G  A                    (100)
TGTGTAGGTGAGAAGAGGAAGCTCAAAATCCCGTCCAAGCTTGGTTATGGTGACAACGGC                     388
 C  V  G  E  K  R  K  L  K  I  P  S  K  L  G  Y  G  D  N  G                    (120)
TCACCACCGAAAATCCCCGGTGGGGCGACATTGATATTTGACACCGAGCTAGTTGCTGTG                     448
 S  P  P  K  I  P  G  G  A  T  L  I  F  D  T  E  L  V  A  V                    (140)
AACGGGAAACCATCCAGCGAAGGAAAGACAAAGAATGAGCTTTGATTGCATCCATTTCCT                     508
 N  G  K  P  S  S  E  G  K  T  K  N  E  L  *                                    (154)
ACTTTTTTTCATTTCAAATGAAACCTGTTAAGTCGTGTTCATTTGCATTTGAATCTCGAA                     568
CATGGTAGAGACTAGAGTCGTTTACTTTTTGGCTGGTTAAGGACAATAATTGCGTGTTTG                     628
GTCATAACATTAGTTATGTTGGCACTTGGCAGTTGGAAAATAACGTGGAAGCGCAGAAAG                     688
TCGGTCTATAAATCCATTTTACTGTGTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                      748
AA                                                                               750
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Figure 1. Nucleotide sequence and deduced amino acid sequence of Chinese cabbage *C-FKBP* cDNA. The predicted amino acid sequence is given below the nucleotide sequence. An asterisk marks the stop codon. The amino-terminal sequence obtained by peptide sequencing is underlined, and the 26 amino acids upstream of this sequence are presumed to be a signal sequence for protein targeting.

Figure 2. Alignment of the deduced amino acid sequence of Chinese cabbage C-FKBP with FKBPs from other organisms. Abbreviations for the organisms are as follows (database accession number in parentheses): Hs, *Homo sapiens* (P26885); Mm, *Mus musculus* (P45878); Sc, *Saccharomyces cerevisiae* (P32472). Black shading indicates identical amino acid residues, and dashes indicate gaps. The respective lengths of the polypeptides are indicated on the right.

purified protein was compared with that deduced from the cDNA, it was seen that the preprocessed protein contains a 26-amino acid signal peptide, presumably for protein targeting. This 26-amino acid sequence contains highly hydrophobic amino acids and is typical of signal peptides found on proteins translocated across the ER membrane.

Figure 2 displays the comparison of the amino acid sequence of the C-FKBP protein with those of other eukaryotic FKBP-type immunophilins. The amino acid sequence of C-FKBP shares high sequence identity with those of mouse (61%), human (60%), and yeast (56%).

Southern and Northern Blot Analysis of *C-FKBP*. Southern blot analysis was performed with a full-length *C-FKBP* cDNA hybridization probe to determine the copy number of *FKBP* genes in the Chinese cabbage genome. The hybridization of the [α-^{32}P]-labeled *C-FKBP* cDNA to Chinese cabbage genomic DNA is shown in **Figure 3A**. Although the membrane hybridization and washes were performed under high stringency conditions, several hybridizing bands were detected in the Southern blot analysis. The presence of several bands indicates the complexity in genes encoding plant FKBP, although the exact number of *FKBP* genes in the Chinese cabbage genome remains to be determined.

To investigate the transcriptional expression levels of *C-FKBP*, Northern blot analysis was carried out using the *FKBP* cDNA insert as a probe. As seen in **Figure 3B**, *C-FKBP* mRNA was highly expressed in vegetative tissues, and relatively low expression was detected in the cotyledon, mature leaf, and floral leaf; however, little transcript was found in the seed. This suggests that *C-FKBP* may play an important role in vegetative plant growth.

Expression and Purification of C-FKBP. To date, all FKBPs characterized from other organisms possess PPIase enzyme activity. To confirm the size and mobility of C-FKBP on SDS−PAGE, a GST−C-FKBP fusion protein was expressed in the pGEX vector. As shown in **Figure 4**, the major band in the crude extract prepared from a transformant cultured with IPTG corresponded to a 15 000 increase in molecular weight compared with that of the purified protein. Using the purified C-FKBP protein, we performed PPIase assays, which showed that C-FKBP is an active PPIase that can catalyze the *cis*−*trans* interconversion of the Ala−Pro bond in the substrate

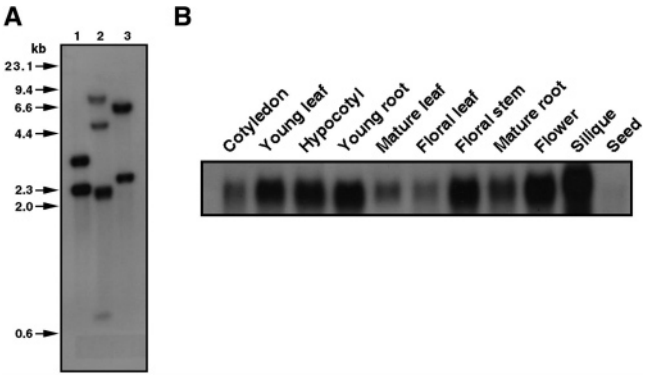

Figure 3. Genomic Southern and Northern blot analyses of *C-FKBP*. (**A**) Analyses of the *C-FKBP* gene copy number. Genomic DNA extracted from Chinese cabbage plants was digested with *Bgl*II (lane 1), *Hin*dIII (lane 2), and *Xba*I (lane 3) restriction endonucleases. After agarose gel electrophoresis, DNA was blotted onto nylon membranes and hybridized with the α-^{32}P-labeled *C-FKBP* probe. After hybridization and washing, the membranes were exposed to an X-ray film. (**B**) *C-FKBP* gene expression in various young and adult plant tissues. Total RNAs (20 μg/lane), extracted from the indicated tissues of young and adult Chinese cabbage, were separated on formaldehyde–agarose gels, transferred onto membranes, and hybridized with the α-^{32}P-labeled *C-FKBP* insert.

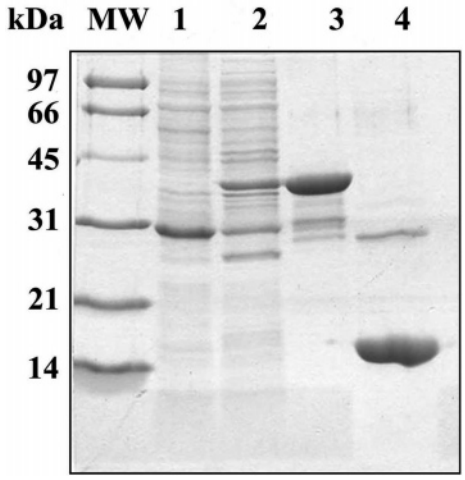

Figure 4. SDS–PAGE of proteins isolated by affinity chromatography. A 12% polyacrylamide gel was loaded with proteins obtained from the lysate of cells transformed with the pGEX vector (lane 1) and from the lysate of cells overexpressing the GST–C-FKBP fusion protein induced with IPTG (lane 2). Lane 3 shows the fusion protein purified by affinity chromatography with a glutathione–agarose affinity column. A portion of GST was removed by thrombin treatment (lane 4). MW indicates molecular standards. The proteins were stained with Coomassie blue.

compared to the level of spontaneous isomerization in the absence of C-FKBP (**Figure 5**). Also, the C-FKBP PPIase activity was comparable to that of calf CyP.

C-FKBP Antifungal Activity. To compare the antifungal activities of the purified and recombinant proteins, we performed antifungal assays against *C. albicans* (**Figure 6A**), *R. solani* (**Figure 6B**), and *B. cinerea* (**Figure 6C**). The results showed that purified and recombinant C-FKBPs had similar antifungal activities. The in vitro antifungal activity of recombinant C-FKBP was also evaluated against various microbial cells (**Table 1**). Recombinant C-FKBP exerted inhibitory effects on all fungal cells except *A. flavus*, but it had no activity against bacterial cells.

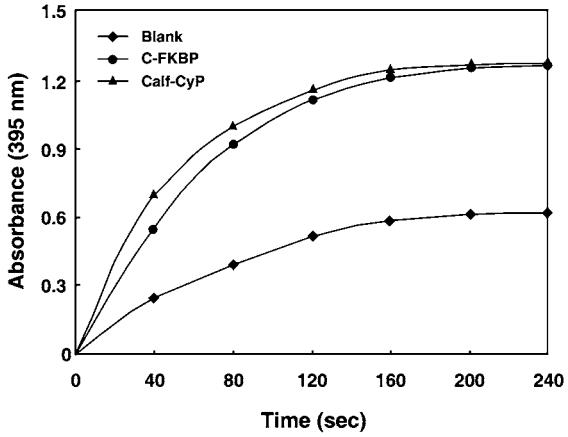

Figure 5. *cis–trans* peptidyl isomerization activity of C-FKBP. Spectrophotometric proline isomerization assays were performed with either C-FKBP or calf CyP or in the absence of any protein. The graph is representative of several independent experiments.

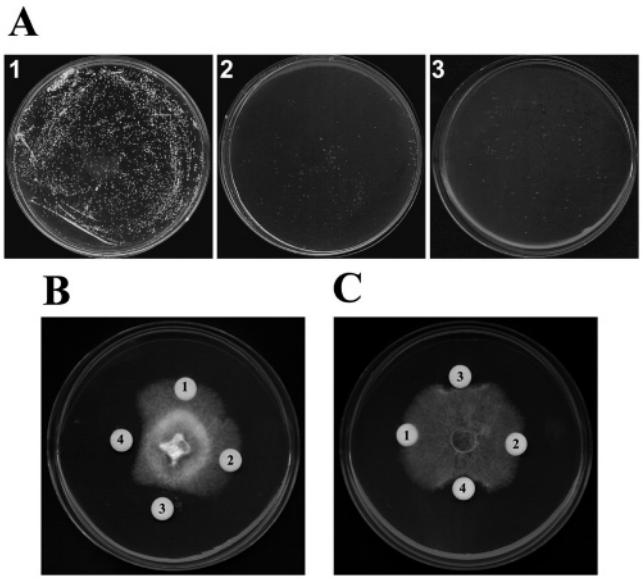

Figure 6. Inhibitory effects of purified and recombinant C-FKBP toward fungal cells. (**A**) Purified (2; 100 μg) and recombinant (3; 100 μg) C-FKBPs were mixed with *C. albicans*. Buffer alone (25 mM HEPES buffer, pH 7.2) served as a negative control (1). (**B**, **C**) Inhibition tests against *B. cinerea* (**B**) and *R. solani* (**C**) were performed using a radial growth inhibition method. Buffer (25 mM Hepes buffer, pH 7.2) (1) and BSA (2) served as controls. Purified (3; 200 μg) and recombinant (4; 200 μg) C-FKBPs were injected onto paper disks.

Table 1. Inhibitory Effects of Recombinant C-FKBP against Fungal and Bacterial Cells

pathogen	protein EC_{50}[a] (μM)	pathogen	protein EC_{50}[a] (μM)
	Fungi		
B. cinerea	4	*T. hazianum*	8
R. solani	8	*T. viride*	8
F. oxysporum	32	*A. flavus*	NA
F. solani	32	*C. albicans*	16
	Bacteria		
E. coli	NA	*S. aureus*	NA

[a] EC_{50} is the effective concentration for 50% inhibition. NA indicates not active at a concentration of <128 μM.

The presence of FKBPs in almost every compartment of the cell suggests a possible role for FKBPs in protein-folding pathways. In the endoplasmic reticulum (ER), secreted and

membrane proteins are synthesized and modified. To that end, the ER contains multiple molecular chaperones to promote the correct folding and assembly of proteins (*24*−*28*). Immunophilins emerge as an important multifunctional gene family that is probably involved in this basic regulatory system. Further studies on their biological functions in plant systems will contribute to understanding their specific roles.

Furthermore, C-FKBP has antifungal activity against various fungal pathogens at micromolar concentrations, but has no effect on bacterial pathogens. We suggest that FKBPs belong to a novel group of bifunctional proteins. Further investigations regarding FKBP's mechanism of action will greatly enhance our research of its role in plant disease resistance or signal transduction. Genetically manipulating its expression in plants could also confer effective resistance against pests and pathogens.

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