JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Cloning and Characterization of a Plant Defensin VaD1 from Azuki Bean

GAN-HONG CHEN,[†] MING-PIN HSU,[‡] CHI-HSING TAN,[‡] HSIEN-YI SUNG,[†] C. GEORGE KUO,[#] MING-JEN FAN,[§] HUEI-MEI CHEN,[#] SHU CHEN,[§] AND CHING-SAN CHEN*[,†,‡,⊥]

Institute of Microbiology and Biochemistry and Department of Agricultural Chemistry, National Taiwan University, Taipei 106, Taiwan; Institute of Botany, Academia Sinica, Nankang, Taipei 115, Taiwan; Crop Genetic Resources Laboratory, Taiwan Agricultural Research Institute, Wu-Feng, Taichung 413, Taiwan; and Asian Vegetable Research and Development Center, Shanhua, Tainan 741, Taiwan

A recombinant mungbean defensin VrD1 was previously shown to exhibit antifungal and bruchid-resistant activity. To study the function and regulation of VrD1, genomic DNAs of plant defensins were isolated from *Vigna radiata* VC6089A and azuki bean *Vigna angularis* Kao Hsiung No. 6. The azuki bean defensin genomic DNA *VaD1* was sequenced and converted to *VaD1* cDNA. VaD1 defensin was purified from *Vigna angularis* Kao Hsiung No. 6 to apparent homogeneity. The complete amino acid sequence of the purified VaD1 was determined and was found to be exactly the same as the sequence deduced from *VaD1* cDNA. VaD1 is a basic protein containing 46 amino acids with four conserved disulfide bonds and shares high sequence homology (78.3%) with VrD1. VaD1 inhibited the growth of *Fusarium oxysporum*, *Fusarium oxysporum* f. sp. *pisi*, *Staphylococcus epidermidis*, and *Salmonella typhimurium*. VaD1 also inhibited in vitro protein synthesis and bruchid larval development, but was less active than the recombinant VrD1.

KEYWORDS: Antimicrobial activity; azuki bean (*Vigna angularis*); bruchid resistance; defensin; protein synthesis inhibition

INTRODUCTION

Plant defensins have been shown to be major constituents of the innate immune systems of plants and are apparently ubiquitous throughout the plant kingdom. This class of cysteine-rich peptides, which are usually basic and 45−54 amino acids in length, contains four conserved disulfide bridges (*1*). The study of the three-dimensional structures of some members of this family such as γ1-purothionin and γ-hordothionin from wheat and barley seeds, respectively (*2*), Rs-AFP1 from radish seeds (*3*, *4*), and a floral defensin NaD1 from *Nicotiana alata* (*5*) has revealed that the structure of plant defensins is dominated by a triple-stranded, antiparallel β-sheet and a single α-helix lying in parallel with the β-sheet. The α-helix is connected by two disulfide bonds to the third β-strand, forming a structure motif known as the cystine-stabilized αβ (CSαβ) motif (*6*). A smaller structural motif known as the cystine-stabilized α-helix (CSH) was found to occur in several ion-channel blocking neurotoxic peptides (*7*). It becomes apparent that all members of this family adopt a comparable global fold centered around the CSαβ motif (*5*), but relatively few amino acid residues are absolutely conserved between all members (*1*). The conserved residues are restricted to the eight cysteines, two glycines at positions 13 and 34, an aromatic residue or tryptophan at position 11, and a glutamic acid at position 29 (*1*, *5*, *8*, *9*) (numbering relative to Rs-AFP1). Whereas these residues are likely to play an important role in structure stabilization or in the protein-folding pathway, the CSαβ motif represents a robust and versatile scaffold that can incorporate a broad range of functional activities (*5*). The diverse biological functions of plant defensins include antifungal activities (*1*, *9*, *10*), inhibition of insect gut α-amylases (*11*) and bovine trypsin (*12*), inhibition of protein synthesis (*13*−*16*), antibacterial activities (*15*, *17*), blockage of the sodium channel (*18*), and a sweet taste (*19*).

A mungbean defensin VrD1 isolated from a bruchid-resistant, near-isogenic line of mungbean *Vigna radiata* VC6089A (hereafter VC6089A) has been shown to exhibit both antifungal and insecticidal activities (*16*, *20*, *21*). The flora defensin NaD1 from *N. alata*, like other plant defensins, has antifungal activity, but it was also shown to retard the growth and development of the Leptidopteran cotton pests *Helicoverpa punctigera* and

* Address correspondence to this author at the Institute of Botany, Academia Sinica, Nankang, Taipei 115, Taiwan (telephone +886-2-27899590, ext. 251; fax +886-2-27858936; e-mail chingsan@gate.sinica.edu.tw).
[†] Institute of Microbiology and Biochemistry, National Taiwan University.
[‡] Academia Sinica.
[§] Taiwan Agricultural Research Institute.
[#] Asian Vegetable Research and Development Center.
[⊥] Department of Agricultural Chemistry, National Taiwan University.

10.1021/jf0402227 CCC: $30.25
Published on Web 01/20/2005

Helicoverpa armigera when incorporated in an artificial diet or expressed in transgenic tobacco (*5*).

It is of interest to note that VrD1 and NaD1 share only 12 identical residues in their amino acid sequences. In addition to the eight cysteines and two glycines at positions 13 and 34, these two plant defensins also have two conserved arginine residues, one at the N-terminal position and another at position 39 (numbering relative to NaD1). In general, amino acid sequence homology between plant defensins is relatively low, mostly <45%. There are a few plant defensins isolated from seeds that have shown relatively high sequence identity with VrD1 such as γ1-purothionin (36.2% identity) and γ2-purothionin (38.3%) from wheat endosperm (*13*), a predominantly pistil-expressed γ-thionin-like protein PPT (40.4%) from petunia (*21*), γ-hordothionin (38.3%) from barley endosperm (*14*), and fabatin-2 (42.6%) from faba bean (*22*). To study the structure and regulation of the *VrD1* gene, we have isolated several genomic DNAs from *Vigna* species and found that a plant defensin cDNA *VaD1* from azuki bean *Vigna angularis* showed 78.3% sequence identity with VrD1. The high sequence homology between VrD1 and VaD1 prompted us to investigate the structure and function of VaD1 and compare them with that of VrD1 so as to furnish a clue to understand what amino acid residues in the global fold of VrD1 may be involved in bruchid resistance and/or antifungal activities. This paper describes cloning, characterization, and biological activities of VaD1 from azuki bean seeds.

MATERIALS AND METHODS

Plant and Insect Materials. *V. radiata* VC1973A (hereafter VC1973A), *V. radiata* VC6089A, and *Callosobruchus chinensis* were obtained from the Asian Vegetable Research and Development Center (AVRDC). Azuki bean (*V. angularis* Kao Hsiung No. 6) seeds were provided by the Taichung Agricultural Research Institute (TARI) and AVRDC. *C. chinensis* was maintained on VC1973A at 25 °C in a growth chamber. Seven plant pathogenic fungi, *Fusarium oxysporum* f. sp. *pisi*, *F. oxysporum*, *F. oxysporum* CCRC 35270, *Pyricularia oryzae*, *Trichophyton rubrum*, *Stemphylium solani*, and *Rhizoctonia solani*, were obtained from the Bioresources Collection and Research Center, Food Industry Research and Development Institute (FIRDI), Hsinchu, Taiwan. Fifteen food pathogenic bacteria, *Bacillus cereus* CCRC 10446, *Staphylococcus epidermidis* CCRC 10785 ATCC 14990, *Enterobacter aerogenes* ATCC 13048, *Escherichia coli* ATCC 11229 CCRC 11549, *Klebsiella oxytoca* ATCC 13182 CCRC 13985, *Proteus vulgaris* ATCC 13315 CCRC 11039, *Pseudomonas aeruginosa* ATCC 10145 CCRC 10944, *Salmonella enteritidis* ATCC 13076 CCRC 10744, *Salmonella typhimurium* ATCC 14028 CCRC 10747, *Listeria monocytogenes* CCRC 14845, *Staphylococcus aureus* subsp. *aureu* CCRC 12652, *Vibrio cholera*, *Vibrio damsela* CCRC 15428, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* CCRC 15430, were kindly provided by Professor Shann-Tzong Jiang, Department of Food Science, National Taiwan Ocean University, Keelung, Taiwan. Three plant pathogenic bacteria, *Pseudomonas syringae* pv. *syringae*, *Erwinia carotovora* subsp. *carotovora*, and *Xanthomonas campestris* pv. *vesicatoria*, were kindly provided by Professor Teng-Yung Feng, Institute of Botany, Academia Sinica, Taipei, Taiwan.

Cloning of *VrD1* and *VaD1* Genomic DNAs. A cDNA encoding mungbean defensin VrD1 was previously isolated from a bruchid-resistant isogenic line of mungbean *V. radiata* VC6089A by conversion of *VrD1* genomic DNA to *VrD1* cDNA (*21*). VrD1 has one amino acid residue different from VrCRP. Whereas VrD1 has asparagines at position 32, VrCRP has an aspartic acid at this position (*21*). To isolate plant defensin genomic DNAs from mungbean and azuki bean, the total cellular DNAs were prepared from these two *Vigna* species according to the method previously described (*24*), and each cellular DNA was used as a template; oligonucleotides with the sequences 5′-ACCTCAACAATTCATCACTCATG and 5′-TAGCAGTGATGCTGCTATATTTATTTGTGA, based on 5′- and 3′-nucleotide sequences of *VrCRP*, were used as primers for PCR amplification. Two DNA fragments of ~0.4 kb were obtained from mungbean and azuki bean genomes and separately cloned into pGEM-T easy vectors (Promega) and sequenced. Comparison of these two nucleotide sequences with that of *VrD1* cDNA revealed a single 103 bp intron in the *VrD1* genomic DNA and a 97 bp intron in the azuki bean genomic DNA, both introns located between serine 18 and aspartic acid 19 in the 27 amino acid signal peptide. These two genomic DNAs were named *VrD1* genomic DNA and *VaD1* genomic DNA, respectively.

Cloning of *VaD1* cDNA. *VaD1* cDNA was converted from *VaD1* genomic DNA by elimination of the single 97 bp intron from the genomic DNA using inverse PCR as previously described (*21*).

Purification of VaD1 from Azuki Bean Seeds. Eight hundred grams of dry seeds of azuki bean were ground in a mill, and the seed powder was extracted with 4 L of an extraction buffer (10 mM NaH_2PO_4, 15 mM Na_2HPO_4, 500 mM NaCl, 100 mM KCl, 2 mM EDTA, 1.5% polyvinylpolypyrrolidone, and 1 mM phenylmethanesulfonyl fluoride, pH 6.4) at 4 °C for 16 h under continuous stirring. Solid materials were subsequently removed by centrifugation at 11000*g* for 30 min, and solid ammonium sulfate was added to the supernatant to obtain a 30−80% saturated ammonium sulfate fraction (30−80% AS fraction), which contained most of the azuki bean defensin. The 30−80% AS fraction was dissolved in distilled water and dialyzed against 20 mM Tris-HCl, pH 7.0 (buffer A). The dialyzed solution was passed through a CM-Sepharose CL-6B (Pharmacia) column (2.6 cm × 30 cm) previously equilibrated with buffer A. The column was washed with 10 volumes of buffer A and eluted with a linear gradient (3 L total volume) of 0−0.5 M NaCl in buffer A. Fractions of 12 mL each were collected at a flow rate of 4 mL/min. Proteins in fractions were monitored at 280 nm. Aliquots of protein fractions were analyzed on NuPAGE 4−12% Bis-Tris gel (Invitrogen) (hereafter gradient PAGE), and fractions showing a protein band with M_r about 5.2 kDa were pooled and concentrated with a MasterFlex ultrafiltration apparatus (Millipore). The concentrated protein solution was applied on a Superdex Peptide HR 10/30 column (Pharmacia) and eluted with buffer A containing 150 mM NaCl at a flow rate of 0.5 mL/min. Protein peaks eluted from the gel filtration column were also analyzed with the gradient PAGE. Protein concentration was determined according to the Lowry method as modified by Alam (*25*).

Bioassay of the Purified VaD1. Antifungal and antibacterial activities were assessed on several phytopathogenic fungal and bacterial strains and on food pathogenic bacterial strains using previously described methods (*26*, *27*). Bacterial cell suspension, fungal spores, or mycelial fragments were used to inoculate the cultures. Absorbance was measured at 595 nm with a microplate reader after an appropriate incubation period. To determine bruchid-resistant activity, artificial seeds containing the purified VaD1 were tested in bioassays with azuki bean weevil *C. chinensis* as described previously (*16*). The artificial seeds were prepared according to the method of Shade et al. (*28*) with modification.

In Vitro Translation Inhibition Assay. Inhibition of protein synthesis was determined with wheat germ extract as previously described (*16*).

RESULTS AND DISCUSSION

Cloning of *VaD1* Genomic DNA and cDNA. A cDNA encoding an antifungal and insecticidal plant defensin *VrCRP* was previously isolated from VC6089A by suppression subtractive hybridization (*16*). To study the expression and regulation of the *VrCRP* gene, a *VrCRP* genomic DNA fragment (402 bp) was isolated using total cellular DNA of VC6089A as a template and a pair of oligonucleotides designed on the basis of 5′- and 3′-nucleotide sequences of *VrCRP* as primers for PCR amplification (*21*). Comparison of the nucleotide sequence of the 402 bp DNA fragment with that of *VrCRP* cDNA revealed a single 103 bp intron located between serine 18 and aspartic acid 19 of the 27 amino acid signal peptide of VrCRP. In addition, in the coding region of this 402 bp DNA fragment there is a single

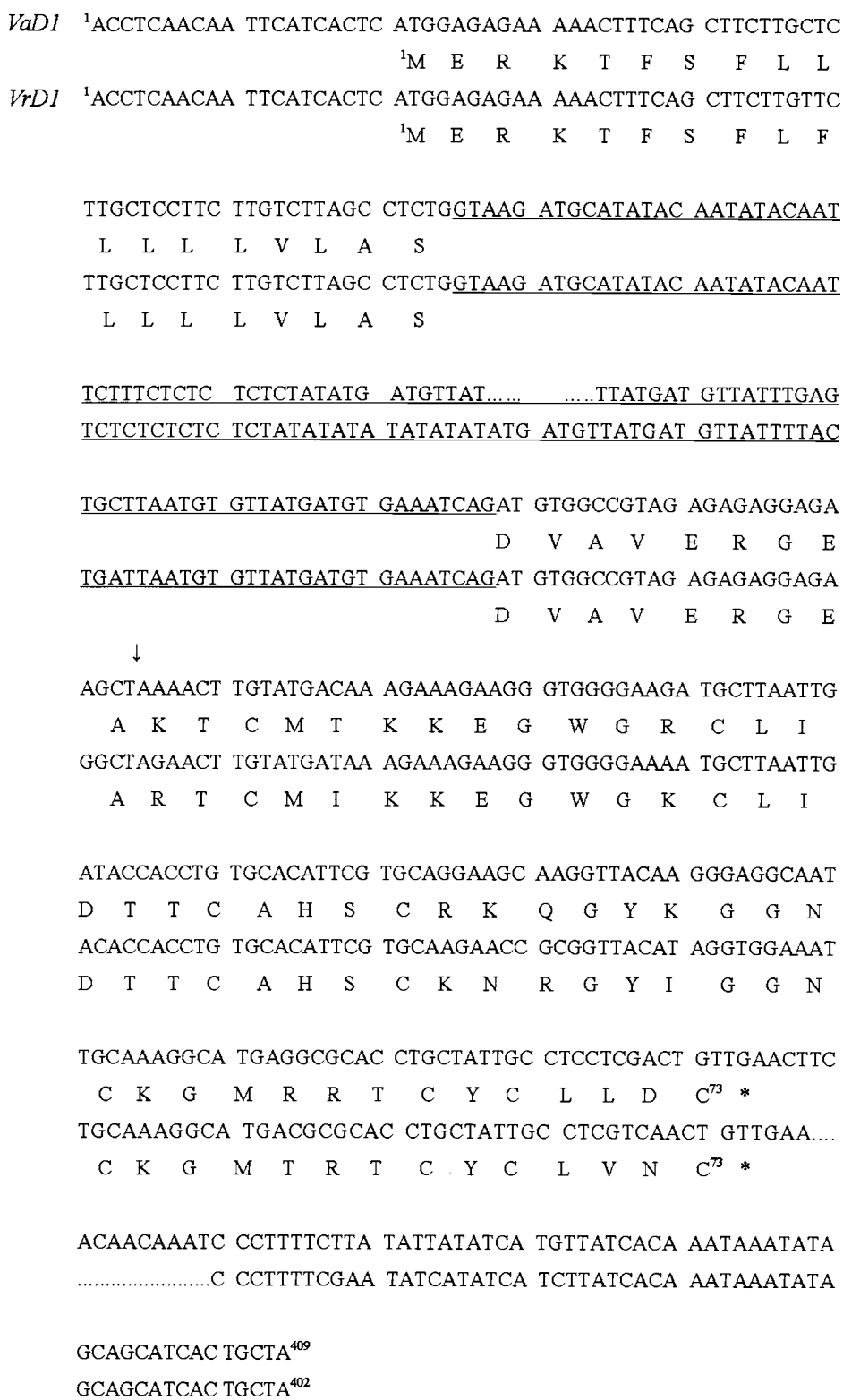


Figure 1. Comparison of the nucleotide sequences of azuki bean defensin (*VaD1*) and mung bean defensin (*VrD1*) genomic DNAs and their deduced amino acid sequences. The intron regions 97 bp and 103 bp of *VaD1* and *VrD1*, respectively, are underlined. The amino acids of the gene products are shown below the corresponding codons of the coding region, and the termination codon is indicated by a ∗. The predicted signal peptide cutting site is indicated by an arrow.

amino acid substitution at residue 32 (N-terminal arginine of the mature VrCRP protein as residue 1). Whereas VrCRP has an aspartic acid at position 32, the coding region of the 402 bp DNA fragment has an asparagine at this position. Several lines of evidence indicated that naturally occurring mungbean defensin has the asparagine residue at position 32 (*21*). The 402 bp genomic DNA therefore was named *VrD1* instead of *VrCRP* (*21*). Similarly, a genomic DNA encoding azuki bean defensin VaD1 (*V. angularis* defensin *1*) was also isolated using the same approach mentioned above. It was found that *VaD1* genomic DNA has a single 97 bp intron located exactly at the same site as *VrD1* genomic DNA (**Figure 1**). Thus, elimination of the

Figure 2. Amino acid sequences of eight plant defensins. Dashes indicate gaps introduced to maximize homology by multiple alignment programs. Amino acids of each plant defensin identical to that of VrD1 are shaded. Connecting lines between cysteine residues represent disulfide bonds (*1*, *9*, *10*, *29*). The spiral and arrows indicate the location of the α-helix and β-strands, respectively (*10*). The following sequences were derived from protein sequencing: γ-purothionins (γ1-P, γ2-P) of wheat (*Triticum turgidum* L.) endosperm (*13*); γ-hordothionin (γ-H) of barley (*Hordem vulgure*) endosperm (*14*); fabatin-2 of *Vicia faba* (*23*); and VrD1 (*21*). The following sequences were deduced from cDNA clones with omission of the putative signal peptides: PPT of *Petunia inflate* pistil (*22*); P322 of potato (*Solanum tuberosum*) tuber (*30*).

two introns from *VaD1* and *VrD1* created nucleotide sequences of *VrD1* (*21*) and *VaD1* cDNAs. Both of these cDNAs are 73 amino acids in length. The deduced amino acid sequences of these two cDNAs are very similar, showing 87% amino acid sequence similarity and 78.3% sequence identity in the mature defensin molecules. Of the previously described plant defensins, several have been shown to have highest amino acid sequence identity to VrD1. They are γ1- and γ2-purothionins (γ1-P and γ2-P) of wheat (*Triticum turgidum* L.) endosperm (36.2 and 38.3%) (*13*), PPT of *Petunia inflate* pistil (40.4%) (*21*), fabatin-2 of faba bean (*Vicia faba*) (42.6%) (*22*), and P322 of potato (*Solanum tuberosum*) (36.2%) (*30*) (**Figure 2**). The sequence identity of VaD1 (78.3%) compared to that of VrD1 is much higher than these published sequences of plant defensins. These observations thus prompt us to study the structure and function of mungbean and azuki bean defensins to provide insight into the structure/function relationship of these two legume defensins and to provide a molecular basis for improving insect-resistant activity of legume defensins. There are 10 amino acid substitutions between VrD1 and VaD1: R1K (VrD1 to VaD1), I5T, K12R, K24R, N25K, R26Q, I29K, T37R, V44L, N45D (N-terminal R or K as residue 1).

Studies of the effect of these substitutions on the structure and/or biological activities of these two *Vigna* defensins would shed light on the key amino acid residues involved in the molecular function of *Vigna* defensins.

Purification and Characterization of VaD1 from Azuki Bean Seeds. Cation exchange chromatography and gel filtration were employed for the purification of VaD1 on the basis of the findings that plant defensins are usually basic and small proteins. The 30−80% AS fraction was chromatographed on the CM-Sepharose CL-6B column, and three protein peaks were eluted from the column (**Figure 3A**). Analysis of proteins in each peak by the gradient PAGE indicated that peak 3 contained a 5.2 kDa protein. Fractions of peak 3 therefore were pooled, concentrated, and fractionated on the Superdex Peptide HR 10/30 column. A single protein peak was eluted from the column (**Figure 3B**). Five micrograms of protein in the peak was separated on the gradient PAGE, and the gel was subjected to

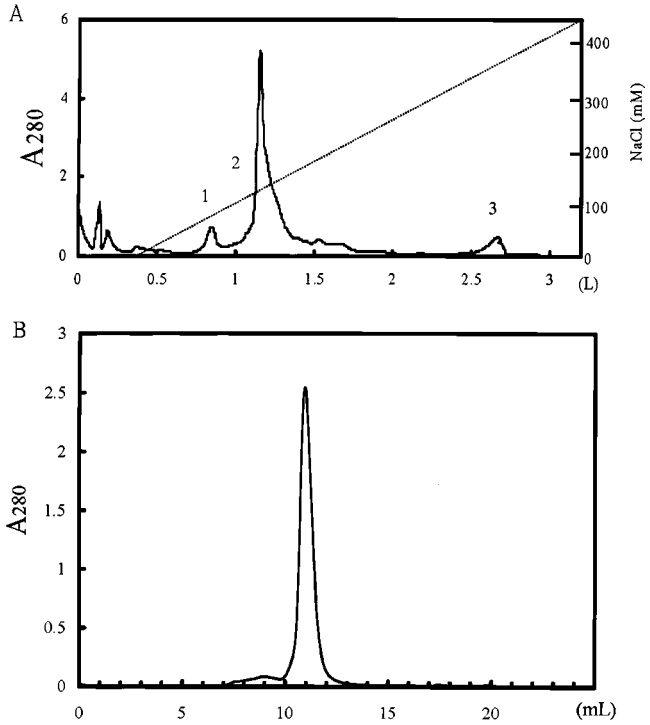


Figure 3. Purification of VaD1 from azuki bean. The experimental details are given under Materials and Methods. The 30–80% ammonium sulfate fraction was chromatographed on CM-Sepharose (**A**). Fractions of peak 3 from the CM-Sepharose column were pooled and separated by gel filtration (**B**).

silver staining and Western blotting. The same amount of VrD1 purified from VC6089A was also separated on the same gel. Polyclonal antibody raised against the purified VrCRP-TSP (*16*) was used for the Western blot analysis. The results indicated that the protein in the single peak eluted from the Superdex Peptide HR 10/30 column has been purified to apparent homogeneity because 5 μg of protein was used for this analysis and the sensitivity of silver staining is generally accepted as being in the range of 0.05−0.1 μg of protein (data not shown). The purified protein was identified as VaD1 by mobility on the gel and Western blot analysis (data not shown). The purified VaD1 had a relative molecular mass of ∼5.2 kDa on the gradient PAGE corresponding to the molecular mass of 5200 Da determined by MALDI-TOF MS. The complete amino acid sequence determined by N-terminal amino acid sequencing and tandem mass spectrometry further confirmed the identity of VaD1 (**Figure 4**). The N-terminal 20 amino acids were identical to the deduced amino acid sequence of KTCMTKKEGWGR-CLIDTTGA shown in **Figure 4**, indicating that the signal peptide of VaD1 has 27 amino acids the same as that of VrD1 (*16*). Western blot analyses of VrD1 and VaD1 are available as Supporting Information. The finding that VaD1 also cross-reacted with anti-VrD1 antiserum indicated that VaD1 and VrD1 are immunologically similar to each other (data not shown). This is reminiscent of the high sequence homology (78.3%) between VrD1 and VaD1.

Biological Activity of VaD1. The purified VaD1 was assayed for antifungal and antibacterial activity. The antimicrobial activity was expressed as IC_{50}, which represents the concentration of VaD1 required for 50% inhibition of fungal or bacterial growth. VaD1 was shown to inhibit the growth of both fungi and bacteria (**Table 1**). IC_{50} values of antifungal activity were in the range of 30−53.2 μg/mL. Interestingly, IC_{50} values of antibacterial activity, in the 36.6−143.4 μg/mL range, resembled

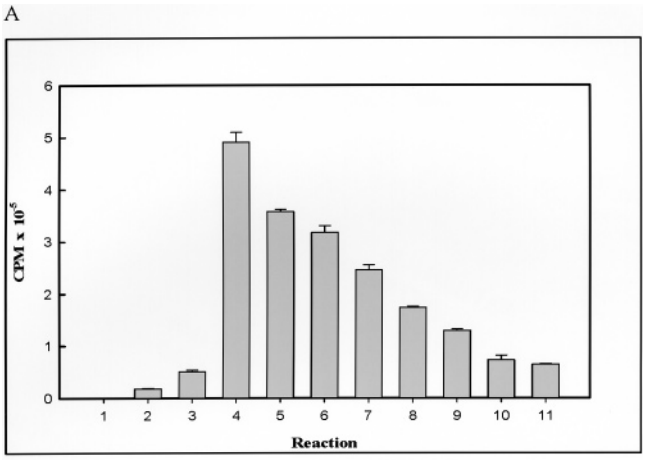
1										10
K	T	C	M	T	K	K	E	G	W	
K	T	C	M	T	K	K	E	G	W	
11										20
G	R	C	L	I	D	T	T	C	A	
G	R	C	L	I	D	T	T	C	A	
21										30
H	S	C	R	K	Q	G	Y	K	G	
H	S	C	R	**K**	**Q**	**G**	**Y**	**K**	**G**	
31										40
G	N	C	K	G	M	R	R	T	C	
G	**N**	**C**	**K**	**G**	**M**	**R**	**R**	T	C	
41						46				
Y	C	L	L	D	C					
Y	C	L	L	D	C					

Figure 4. Complete amino acid sequence of VaD1. The amino acid sequence of VaD1 determined by N-terminal amino acid sequencing (shaded region) and tandem mass spectrometry is shown below the amino acid sequence deduced from *VaD1* cDNA. Boldfaced and underlined regions indicate peptide fragment(s) obtained by endoproteinase Arg-C and trypsin digestion, respectively.

Table 1. Antimicrobial Activity of VaD1[a]

organism	IC_{50} (μg/mL)
fungus	
Fusarium oxysporum	30
Fusarium oxysporum f. sp. *pisi*	53.2
Trichophyton rubrum	>500
bacterium	
Staphylococcus epidermidis	36.6
Xanthomonas campestris pv. *vesicatoria*	40.8
Salmonella typhimurium	143.4
Bacillus cereus	>500
Escherichia coli	>500
Erwinia carotovora pv. *carotovora*	1000
Proteus vulgaris	>1000
Salmonella enteritidis	>1000
Pseudomonas syringae pv. *syringae*	>1000

[a] Concentration of VaD1 required for 50% inhibition was defined as IC_{50}, which was determined from dose–response curve (percent growth inhibition versus protein concentration).



B

Reaction	1	2	3	4	5	6	7	8	9	10	11
Wheat germ extract, 12.5 µl	-	-	+	+	+	+	+	+	+	+	+
BMV RNA, 0.25 µg	-	+	-	+	+	+	+	+	+	+	+
Amino acid mixture (-Met), 2 nmol	-	+	+	+	+	+	+	+	+	+	+
Potassium acetate, 0.75 µg	-	+	+	+	+	+	+	+	+	+	+
L-[^{35}S]Met, 0.029 nmol (31.7 µCi)	-	+	+	+	+	+	+	+	+	+	+
RNasin, 20 U	-	+	+	+	+	+	+	+	+	+	+
VaD1 (µM)	-	-	-	-	10	20	30	40	60	80	-
rVrD1 (µM)	-	-	-	-	-	-	-	-	-	-	40

Figure 5. Inhibition of protein synthesis by VaD1. The experimental details of in vitro translation are given under Materials and Methods. Counts per minute (CPM) of TCA insoluble fraction were measured. Values are from triplicate results (**A**). The complete reaction mixture (reaction 4) in a final volume of 25 μL contained all six components shown in the table (B). Reactions 5, 6, 7, 8, 9, and 10 also contained 10, 20, 30, 40, 60, and 80 μM VaD1, respectively, in addition to the complete reaction mixture. As the control, wheat germ extract (reaction 2) or BMV RNA (reaction 3) was eliminated from the reaction mixture. For comparison of the inhibitory activity on protein synthesis, 40 μM recombinant VrD1 (*21*) was added to the complete reaction mixture (reaction 11).

that of antifungal activity (**Table 1**). Members of the plant defensin family are either inactive or less active against bacteria, but there are some exceptions. *Clitoria ternatea* plant defensin Ct-AMP1 is active against fungi in the 2−20 μg/mL range and is also active against the Gram-positive bacterium *Bacillus subtilis* at 15 μg/mL (*15*). Thus, VaD1, like Ct-AMP1, has both antifungal and antibacterial activities, although VaD1 was less active than Ct-AMP1. The antifungal activity of VaD1 was also weaker than that of the recombinant VrD1 (rVrD1) expressed by *Pichia pastoris*. The rVrD1 inhibited the growth of *F. oxysporum* and *F. oxysporum* f. sp. *pisi* with IC_{50} values at 5.4 and 12.1 μg/mL, respectively (*21*). It was shown previously that the recombinant VrD1 inhibited in vitro protein synthesis (*21*). VaD1 therefore was also tested for protein synthesis inhibition using the same cell-free system derived from wheat germ. VaD1 at 80 μM almost completely inhibited in vitro protein synthesis, and a significant dosage response was observed between 10 and 80 μM (**Figure 5**). The rVrD1 at 40 μM has completely inhibited in vitro protein synthesis (**Figure 5**), whereas VaD1 required a 2-fold higher concentration (80 μM) to exert the same degree of inhibition. Inhibition of protein synthesis by thionins isolated from wheat endosperm (*31*), both γ- and ω-hordothionin from barley endosperm (*14*), and HvAMP1 from Australian native *Hardenbergia violacea* (*8*) has been reported. Whether translational inhibition provides intracellular targets for the action of plant defensins remains to be elucidated. The bacterially expressed VrCRP-TSP (*16*) and the rVrD1 (*21*) exhibit insecticidal activity against azuki bean weevil *C. chinensis* in bioassay with artificial mungbean seeds. VaD1 was assayed for bruchid-resistant activity using the same method. The intact seed of azuki bean *V. angularis* Kao Hsiung No. 6 was also assayed. The results are shown in **Table 2**. The percent emergence and within seed development time (WSDT) of intact azuki bean seeds were 100% and 26.8 days, respectively. These data closely resembled those of two bruchid-susceptible mungbean varieties, *V. radiata* L. Tainan Select No. 5 (TN5) and *V. radiata* VC1973A (VC1973A) (*16*, *21*). Thus, azuki bean is susceptible to *C. chinensis* as expected.

Artificial seeds containing 0.1% VaD1 exhibited no or very low bruchid-resistant activity as compared to the control VC1973A artificial seed, which had 36.6 days of WSDT and 53.3% adult emergence. The percent emergence of the artificial seeds decreased from 51 to 32.5% as the content of VaD1 in the artificial seed increased from 0.1 to 0.8%. A significant dosage effect was observed between the artificial seeds contain-

Table 2. Bruchid-Resistant Activities of Intact and Artificial Seeds with and without the Addition of Purified VaD1[a]

material examined	WSDT (days)	% emergence
intact seed		
azuki bean	26.8 (± 0.4)	100 (± 0)
artificial seed		
VC1973A	36.6 (± 1.3)	53.3 (± 5.5)
experimental artificial seed		
VC1973A + 0.1% VaD1	38.7 (± 0.7)	51.0 (± 5.3)
VC1973A + 0.2% VaD1	37.9 (± 0.9)	49.8 (± 7.2)
VC1973A + 0.4% VaD1	39.5 (± 0.8)	37.5 (± 4.5)
VC1973A + 0.8% VaD1	39.6 (± 1.0)	32.5 (± 2.1)
VC1973A + 0.8% VrD1		0
VC1973A + 0.8% BSA	39.9 (± 0.9)	49.4 (± 5.4)

[a] Preparation of artificial seeds and bioassay of bruchid resistance are given under Materials and Methods. Values represent the mean (± SEM) of six replicates containing six seeds each.

ing 0.1 and 0.8% VaD1. Artificial seeds containing 0.8% of VrD1 purified from VC6089A were shown to completely arrest bruchid larval development (**Table 2**). Thus, the bruchid-resistant activity of VaD1 is weaker than that of VC6089A VrD1. It is of interest to note that VaD1 also exhibited weaker protein synthesis inhibitory activity than rVrD1 (*21*). Whether there is a correlation between bruchid resistance and protein synthesis inhibition remains to be investigated. Approximately 161.2 mg of VaD1 was purified from 800 g of azuki bean (data not shown). The content of VaD1 in dry azuki bean seeds estimated from this datum is ~0.02% (w/w), which is higher than VrD1 in dry seeds of VC6089A (0.012%) and VC1973A (0.0033%) (*16*).

Because azuki bean seed is susceptible to *C. chinensis* (**Table 2**), it is suggested that the major role of VaD1 in host defense may be directed torward phytopathogenic microorganisms. Considering the high amino acid sequence homology (78.3%) shared by VrD1 and VaD1, it is likely that development of VaD1 variants by mutagenesis may furnish a method to enhance the protein synthesis inhibition and/or bruchid resistance of VaD1. The production of mutant VaD1 is highly feasible as it has previously been reported that overexpression of plant defensins in *Pichia pastoris* has resulted in a reasonably high yield of recombinant plant defensins and that the secreted defensins are soluble and have a properly folded structure (*21*, *32*−*34*).

ABBREVIATIONS USED

AVRDC, Asian Vegetable Research and Development Center; BSA, bovine serum albumin; FIRDI, Food Industry Research and Development Institute; rVrD1, a recombinant VrD1 expressed by *Pichia pastoris*; TARI, Taichung Agricultural Research Institute; VaD1, *Vigna angularis* defensin 1; VrCRP, a cysteine-rich protein from *Vigna radiata*; VrCRP-TSP, VrCRP with truncated signal peptide expressed by *Escherichia coli*; VrD1, *Vigna radiata* defensin 1; WSDT, within seed development time.

ACKNOWLEDGMENT

We thank AVRDC for providing us with VC6089A, VC1973A, *Vigna angularis* Kao Hsiung No. 6 and *C. chinensis*. We also thank TARI for providing *V. angularis* Kao Hsiung No. 6. We are grateful to Professor Shann-Tzong Jiang, Department of Food Science, National Taiwan Ocean University, and Professor Teng-Yung Feng, Institute of Botany, Academia Sinica, for providing the fungal and bacterial species.

Supporting Information Available: Western blot analysis of VrD1 and VaD1. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Broekaert, W. F.; Terras, F. R. G.; Cammue, B. P. A.; Osborn, R. W. Plant defensins: novel antimicrobial peptides as components of the host defense system. *Plant Physiol.* **1995**, *108*, 1353−1358.

(2) Bruix, M.; Jiménez, M. A.; Santoro, J.; González, C.; Colilla, F. J.; Méndez, E.; Rico, M. Solution structure of γ1-H and γ1-P thionins from barley and wheat endosperm determined by ^{1}H NMR: a structure motif common to toxic arthropod proteins. *Biochemistry* **1993**, *132*, 715−724.

(3) Fant, F.; Vranken, W. V.; Martins, J. C.; Borremans, F. A. M. Solution structure of *Raphanus sativus* antifungal protein 1 (Rs-AFP1) by ^{1}H nuclear magnetic resonance. Resonance assignments, secondary structure and global fold. *Bull. Soc. Chim. Belg.* **1997**, *106*, 51−57.

(4) Fant, F.; Vranken, W. V.; Broekaert, W. F.; Borremans, F. A. M. Determination of the three-dimensional solution structure of *Raphanus sativus* antifungal protein 1 by ^{1}H-NMR. *J. Mol. Biol.* **1998**, *279*, 257−270.

(5) Lay, F. T.; Schirra, H. J.; Scanlon, M. J.; Anderson, M. A.; Craik, D. J. The three-dimensional solution structure of NaD1, a new floral defensin from *Nicotiana alata* and its application to a homology model of the crop defense protein alfAFP. *J. Mol. Biol.* **2003**, *325*, 175−188.

(6) Cornet, B.; Bonmatin, J. M.; Hetru, C.; Hoffmann, J. A.; Ptak, M.; Vovelle, F. Refined three-dimensional solution structure of insect defensin A. *Structure* **1995**, *3*, 435−448.

(7) Kobayashi, Y. H.; Takashima, H.; Tamaoki, H.; Kyogoku, Y.; Lambert, P.; Kuroda, H.; Chino, N.; Watanabe, T. X.; Kimura, T.; Sakakibara, S.; Moroder, L. The cystine-stabilized α-helix: a common structural motif of ion-channel blocking neurotoxic peptides. *Biopolymers* **1991**, *31*, 1213−1220.

(8) Harrison, S. J.; Marcus, J. P.; Goulter, K. C.; Green, J. L.; Maclean, D. J.; Manners, J. M. An antimicrobial peptide from the Australian native *Hardenbergia violacea* provides the first functionally characterized member of a subfamily of plant defensins. *Aust. J. Plant Physiol.* **1997**, *24*, 571−578.

(9) Garcia-Olmedo, F.; Molina, A.; Alamillo, J. M.; Rodriguez-Palenzuela, P. Plant defense peptides. *Biopolymers* **1998**, *47*, 479−491.

(10) Thomma, B. P. H. J.; Cammue, B. P. A.; Thevissen, K. Plant defensins. *Planta* **2002**, *216*, 193−202.

(11) Bloch, C., Jr.; Richardson, M. A new family of small (5 kDa) protein inhibitors of insect α-amylases from seeds of sorghum (*Sorghum bicolor* (L) Moench) have sequence homologies with wheat γ-purothionins. *FEBS Lett.* **1991**, *279*, 101−104.

(12) Wijaya, R.; Neumann, G. M.; Condron, R.; Hughes, A. B.; Polya, G. M. Defense proteins from seed of *Cassia fistula* include a lipid transfer protein homologue and a protease inhibitory plant defensin. *Plant Sci.* **2000**, *159*, 243−255.

(13) Colilla, F. J.; Rocher, A.; Mendez, E. γ-Purothionins: amino acid sequence of two polypeptides of new family of thionins from wheat endosperm. *FEBS Lett.* **1990**, *270*, 191−194.

(14) Mendez, E.; Moreno, A.; Colilla, F. J.; Pelaez, F.; Limas, G. G.; Mendez, R. Primary structure and inhibition of protein synthesis in eukaryotic cell-free system of a novel thionin, γ-hordothionin, from barley endosperm. *Eur. J. Biochem.* **1990**, *194*, 533−539.

(15) Osborn, R. W.; De Samblanx, G. W.; Thevissen, K.; Goderis, I.; Torrekens, S.; Van Leuven, F. V. Isolation and characterization of plant defensins from seeds of Asteraceae, Hippocastanaceae and Saxifragaceae. *FEBS Lett.* **1995**, *368*, 257−262.

(16) Chen, K. C.; Lin, C. Y.; Kuan, C. C.; Sung, H. Y.; Chen, C. S. A novel defensin encoded by a mungbean cDNA exhibits insecticidal activity against bruchid. *J. Agric. Food Chem.* **2002**, *50*, 7258−7263.

(17) Moreno, M.; Segura, A.; Garcia-Olmedo, F. Pseudothionin-St1, a potato peptide active against potato pathogens. *Eur. J. Biochem.* **1994**, *223*, 135−139.

(18) Kushmerick, C.; Castro, M. D.; Cruz, J. S.; Bloch, C.; Beirao, P. S. L. Functional and structural features of γ-zeathionins, a new class of sodium channel blockers. *FEBS Lett.* **1998**, *440*, 302−306.

(19) Caldwell, J. E.; Abildgaard, F.; Dzakula, E.; Ming, D.; Hellekant, G.; Markley, J. L. Solution structure of the thermostable sweet-tasting protein brazzin. *Nat. Struct. Biol.* **1998**, *5*, 427−431.

(20) Chen, K. C.; Lin, C. Y.; Chung, M. C.; Kuan, C. C.; Sung, H. Y.; Tsou, S. C. S.; Kuo, C. G.; Chen, C. S. Cloning and characterization of a cDNA encoding an antimicrobial protein from mungbean seeds. *Bot. Bull. Acad. Sin.* **2002**, *43*, 251−259.

(21) Chen, J. J.; Chen, G. H.; Hsu, H. C.; Li, S. S.; Chen, C. S. Cloning and functional expression of a mungbean defensin VrD1 in *Pichia pastoris*. *J. Agric. Food Chem.* **2004**, *52*, 2256−2261.

(22) Karunanandaa, B.; Singh, A.; Kao, T. H. Characterization of a predominantly pistil-expressed gene encoding a γ-thionin-like protein of *Petunia inflate*. *Plant Mol. Biol.* **1994**, *26*, 459−464.

(23) Zhang, Y.; Lewis, K. Fabatins: new antimicrobial plant peptides. *FEMS Microbiol. Lett.* **1997**, *149*, 59−64.

(24) Rogers, S. O.; Bendich, A. J. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Mol. Biol.* **1985**, *5*, 69−76.

(25) Alam, A. A model for formulation of protein assay. *Anal. Biochem.* **1992**, *203*, 121−126.

(26) Terras, F. R. G.; Schoofs, H. M. E.; De Bolle, M. F. C.; Leuven, F. V.; Reeds, S. B.; Vanderleyden, J.; Cammue, B. P. A.; Broekaert, W. F. Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativus* L.) seeds. *J. Biol. Chem.* **1992**, *267*, 15301−15309.

(27) Broekaert, W. F.; Terras, F. R. G.; Cammue, B. P. A.; Vanderlyden, J. An automated quantitative assay for fungal growth inhibition. *FEMS Microbiol. Lett.* **1990**, *9*, 55−60.

(28) Shade, R. E.; Murdock, L. L.; Foard, D. E.; Pomeroy, M. A. Artificial seed system for bioassay of cowpea weevil (Coleoptera: Bruchidae) growth and development. *Environ. Entomol.* **1986**, *15*, 1286−1291.

(29) Broekaert, W. F.; Cammue, B. P. A.; De Bolle, M. F. C.; Thevissen, K.; De Sarablanx, G. W.; Osborn, R. W. Antimicrobial peptide from plants. *Crit. Rev. Plant Sci.* **1997**, *16*, 297−323.

(30) Stiekema, W. J.; Heidekamp, F.; Dirkse, W. G.; van Beckum, J.; de Haan, P.; ten Bosch, C.; Louwerse, J. D. Molecular cloning and analysis of four potato tuber mRNAs. *Plant Mol. Biol.* **1988**, *11*, 255−269.

(31) Garcia-Olmedo, F.; Carbonero, P.; Hernandez-Lucas, C.; Paz-Ares, J.; Ponz, F.; Vicente, O.; Sierra, J. M. Inhibition of eukaryotic cell-free protein synthesis by thionins from wheat endosperm. *Biochim. Biophys. Acta* **1983**, *740*, 52−56.

(32) Kristensin, A. K.; Brunstedt, J.; Neilsen, J. E.; Mikkelsen, J. D.; Roepstorff, P.; Nielsen, K. K. Expressing, disulfide pattern and biological activity of a sugar beet defensin, AX2, expressed in *Pichia pastoris*. *Protein Expression Purif.* **1999**, *16*, 377−387.

(33) Almeida, M. S.; Cabral, K. M. S.; de Medeiros, L. N.; Valente, A. P.; Almeida, F. C. L.; Kurtenbach, E. cDNA cloning and heterologous expression of functional cysteine-rich antifungal protein Psd1 in the yeast *Pichia pastoris*. *Arch. Biochem. Biophys.* **2001**, *395*, 199−207.

(34) Cabral, K. M. S.; Almeida, M. S.; Valente, A. P.; Almeida, F. C. L.; Kurtenbach, E. Production of the active antifungal *Pisum sative* defensin 1 (Psd1) in *Pichia pastoris*: overcoming the inefficiency of the STE13 protease. *Protein Expression Purif.* **2003**, *31*, 115−122.

Received for review May 3, 2004. Accepted October 16, 2004. This work was supported by the National Science and Technology Program for Agricultural Biotechnology (Grant NSC 92-2317-B-001-019).

JF0402227