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Cloning and Functional Expression of a Mungbean Defensin VrD1 in *Pichia pastoris*

JI-JR CHEN,† GAN-HONG CHEN,‡ HUI-CHING HSU,§ SHIN-SHING LI,† AND CHING-SAN CHEN*,‡,§

Graduate Institute of Bioscience and Biotechnology, National Taiwan Ocean University, Keelung 202, Taiwan, Graduate Institute of Agricultural Chemistry, National Taiwan University, Taipei 106, Taiwan, and Institute of Botany, Academia Sinica, Nankang, Taipei 115, Taiwan

It was shown previously that a bacterially expressed mungbean defensin VrCRP exhibited both antifungal and insecticidal activities. To isolate this protein in a large quantity for its characterization, the defensin cDNA was expressed in *Pichia pastoris* and the recombinant defensin (rVrD1) was purified. The recombinant VrD1 was shown to inhibit the growth of fungi such as *Fusarium oxysporum*, *Pyricularia oryza*, *Rhizoctonia solani*, and *Trichophyton rubrum* and development of bruchid larva. The protein also inhibits in vitro protein synthesis. These biological activities are similar to that of the bacterially expressed defensin. Functional expression of VrD1 in *Pichia pastoris* provides a highly feasible system to study the structure−function relationship of VrD1 using the mutagenesis approach.

KEYWORDS: Defensin; insecticidal activity; mungbean; *Pichia pastoris*; protein synthesis

INTRODUCTION

Plant defensins formerly known as γ-thionins are a family of small and usually basic proteins containing 45−54 amino acids with four conserved disulfide bonds. These proteins are important components of innate immunity in plants and distributed widely throughout the plant kingdom. Most plant defensins exhibit antifungal activity against a broad range of fungi (*1*, *2*). Members of this family are less active against bacteria, but there are some exceptions. *Clitoria ternatea* plant defensin Ct-AMP1 is active against *Bacillus substilis* (*3*). A potato tuber plant defensin was shown to inhibit *Pseudomonas solanacearum* and *Clavibacter michiganensis* (*4*). Most plant defensins have not been shown to inhibit insect gut α-amylases (*3*). On the contrary, those from Poaceae seeds have been shown to inhibit either insect gut α-amylases (*3*, *5*, *6*) or protein synthesis in a cell-free system (*5*, *7*). Plant defensins have been characterized in various plant species and found to occur in leaves (*8*), tubers (*4*), flower organs (*4*, *9*, *10*), pods (*11*), and seeds (*3*, *5*, *7*, *12*). Many studies have been focused on seeds where plant defensins are prevalent. The mechanism of action of plant defensins is still unclear, and for most plant defensins, molecular components involved in signaling and putative intracellular targets remain unknown (*2*). Only for Dm-AMP1 has a putative binding target been identified in *Saccharomyces cerevisiae*. A gene encoding inositol phosphotransferase (*IPTI*) determines not only binding capacity but also Dm-AMP1-mediated permeabilization and growth inhibition, thus linking all three phenomena (*13*, *14*).

A bacterially expressed plant defensin named VrCRP (*Vigna radiata* cysteine-rich protein) encoded by a mungbean cDNA was shown to inhibit the development and growth of bruchid (*Callosobruchus chinensis*) larva (*15*, *16*). VrCRP was expressed as a fusion protein (VrCRP chitin-binding domain) in soluble form in *Escherichia coli*, but the expression level was low. Therefore, a high-level expression system is needed for isolation and further characterization of this protein. This paper describes functional expression of mungbean defensin VrD1 in *Pichia pastoris* and its characterization.

MATERIALS AND METHODS

Plant and Insect Materials. *V. radiata* VC1973A (hereafter VC1973A), *V. radiata* VC6089A (hereafter VC6089A), *V. radiata* var. *sublobata* (Rovb.) Verdcourt TC1966 (hereafter TC1966), and *C. chinensis* were obtained from AVRDC. *C. chinensis* was maintained on VC1973A at 25 °C in a growth chamber.

Cloning of *VrD1* cDNA. A cDNA encoding mungbean defensin VrCRP was previously isolated from a bruchid resistant isogenic line of mungbean *V. radiata* VC6089A by suppressive subtractive hybridization (*15*). To isolate VrCRP genomic DNA, the total cellular DNA was prepared by the method previously described (*17*) and used as a template, and oligonucleotides with sequences 5′-ACCTCAACAATTCATCACTCATG and 5′-TAGCAGTGATGCTGCTATATTTATTTGTGA, based on 5′- and 3′-nucleotide sequences of *VrCRP*, were used as primers for polymerase chain reaction (PCR) amplification. A 402 bp DNA fragment was obtained and cloned into pGEM-T easy vector and sequenced. Comparison of this nucleotide sequence 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. This genomic DNA was named *VrD1* genomic DNA. The strategy of cloning *VrD*1 cDNA was to eliminate the 103 bp intron by inverse PCR using *VrD1* genomic DNA as a template, and an antisense primer complimentary to the last 30 bp of the 3′-end of exon 1 in VrD1

* To whom correspondence should be addressed. Tel: +886-2-27899590 ext. 251. Fax: +886-2-27858936. E-mail: chingsan@gate.sinica.edu.tw.
† National Taiwan Ocean University.
‡ National Taiwan University.
§ Academia Sinica.

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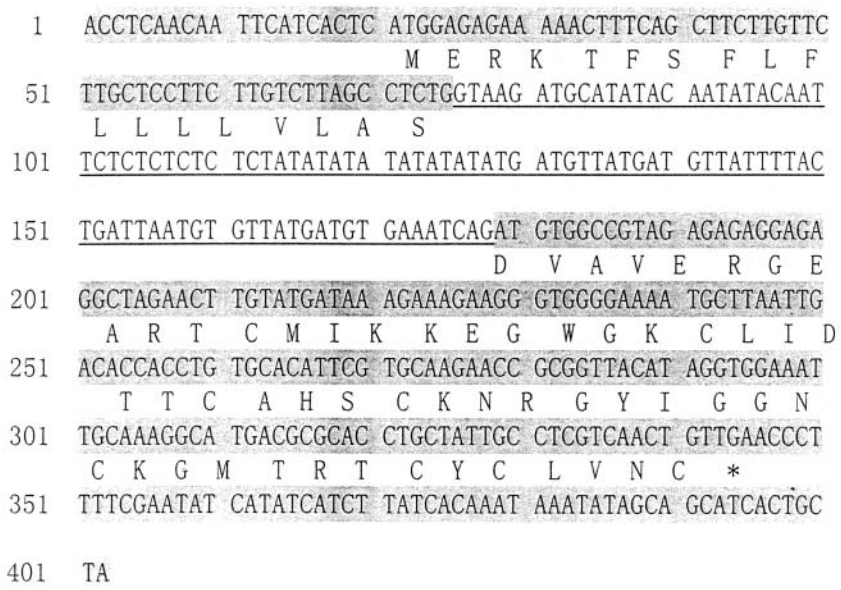


Figure 1. Nucleotide sequence of *VrD1* genomic DNA from *V. radiata* VC6089A and VC1973A and its deduced amino acid sequence. The underline indicates the intron region (103 bp). The amino acids of the gene product are shown below the corresponding codons of the coding region, and the termination codon is indicated by an asterisk. The two shaded sequences represent *VrD1* cDNA derived from its genomic DNA.

genomic DNA: 5′-AGAGGCTAAGACAAGAAGGAGCAAGAACAA and a sense primer corresponding to first 30 bp of the 5′-end of exon 2: 5′-GATGTGGCCGTAGAGAGAGGAGAGGCTAGA were used for inverse PCR. The PCR product was circularized by T4 ligase, and the *VrD1* cDNA insert in the pGEM-T easy vector (*VrD*1/pGEM-T easy vector) was verified by colony PCR and nucleotide sequencing.

Construction of *VrD*1/pPIC9K Expression Vector. The 154 bp fragment encoding mature VrD1 was amplified by PCR using *VrD*1/pGEM-T easy vector as a template and a sense primer 5′-**TACGTA**A-GAACTTGTATGATAAAG with *Sna*B I cutting site and an antisense primer 5′-**GAATTC**CTCAACAGTTGACGAGGCAATAGC with *Eco*R I cutting site. The PCR amplified fragment was digested with *Sna*B I and *Eco*R I enzymes and ligated into the *Sna*B I/*Eco*R I digested pPIC9K in-frame to the α-factor secretion signal peptide, downstream of the alcohol oxidase promoter. The resultant plasmid (*VrD*1/pPIC9K) was transformed into *E. coli* TOP10′F, and recombinant *E. coli* was screened on an ampicillin containing LB plate. The plasmid (*VrD*1/pPIC9K) was purified from the recombinant *E. coli* and subjected to DNA sequence analysis to ensure the *VrD*1 cDNA fragment in the correct reading frame. This plasmid was then linearized with *Pme* I, to favor integration at the *his* 4 locus of *P. pastoris* SMD1168 genome, and transformed into yeast by electroporation.

Screening of Recombinant *P. pastoris* Clones. The transformed *P. pastoris* was screened for His^+ transformants on MD medium (1.34% YNB, 4×10^{-5}% biotin, 2% dextrose, and 1.5% agar). The His^+ transformants were further verified by colony PCR. Of the 17 recombinant *P. pastoris* clones harboring *VrD*1/pPIC9K, the number 4 clone (*VrD*1/pPIC9K-4) was found to possess the highest copy number (~6) according to G418 titration. This clone was therefore used for expression and purification of recombinant VrD1 (rVrD1).

Expression and Purification of rVrD1. The highest copy number colony (*VrD*1/pPIC9K-4) was grown in 25 mL of BMG medium (100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, and 1% glycerol), for 2 days at 30 °C at 200 rpm. The culture was centrifuged, and the cells were resuspended in 1 L of BMMY medium (100 mM potassium phosphate, pH 6.0, 1% yeast extract, 2% peptone, 1.34% YNB, 4×10^{-5}% biotin, and 0.5% methanol) and shaken at 80 rpm at 30 °C for 5 days with daily supplementation of 0.5% methanol. The culture was centrifuged to remove the cells. The culture medium was dialyzed against 20 mM Tris-HCl, pH 7.6 (buffer A), and applied to a CM-Sepharose column (2.6 cm × 10 cm) previously equilibrated with buffer A. The column was washed with buffer A and eluted with a linear gradient (0.8 L total volume) of 0−0.5 M NaCl in buffer A, and the absorbance of the fractions was measured at 280 nm. Fractions of 8 mL each were collected at a flow rate of 0.5 mL/min. Protein peaks eluted from the column were analyzed with the gradient PAGE. Protein concentration was determined with the dye binding method (*18*).

Bioassay of rVrD1. Antifungal activity of the purified rVrD1 was assessed on several phytopathogenic fungal strains using a previously described method (*12*, *19*). 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 rVrD1 were tested in bioassays with azuki bean weevil *C. chinensis* as described previously (*15*). The artificial seeds were prepared according to the method of Shade et al. (*20*) with modification.

In Vitro Translation Inhibition Assay. Inhibition of protein synthesis was determined with wheat germ extract as previously described (*15*).

RESULTS AND DISCUSSION

cDNA Cloning of Mungbean Defensin *VrD1*. A cDNA encoding a cysteine-rich protein VrCRP was previously isolated from VC6089A, a bruchid resistant isogenic line of mungbean, by suppressive subtractive hybridization. VrCRP was shown to exhibit both antifungal (*16*) and bruchid resistant activities and was identified as a member of plant defensin family (*15*). It was also shown that both seeds of VC6089A and the bruchid susceptible isogenic line *V. radiata* VC1973A might contain an identical defensin, and the defensin content in VC6089A seed is approximately 3.5 times that of VC1973A (data not shown). It was therefore suggested that the quantitative difference of mungbean defensins in these seeds may partly account for why VC6089A seeds are resistant to *C. chinensis*, whereas VC1973A seeds are not (*15*). To clarify whether these two mungbean isogenic lines contain a common defensin gene, defensin genomic DNAs of VC6089A and VC1973A were amplified by genomic PCR using total cellular DNA of VC6089A or VC1973A as a template and oligonucleotides designed on the basis of the 5′- and 3′-nucleotide sequences as primers as described in the Materials and Methods. The two defensin genomic DNAs thus obtained were shown to have exactly the same nucleotide sequence shown in **Figure 1**. This common genomic DNA was named *VrD1*. It is likely that *VrD1* gene in VC6089A was originated from VC1973A because VC6089A

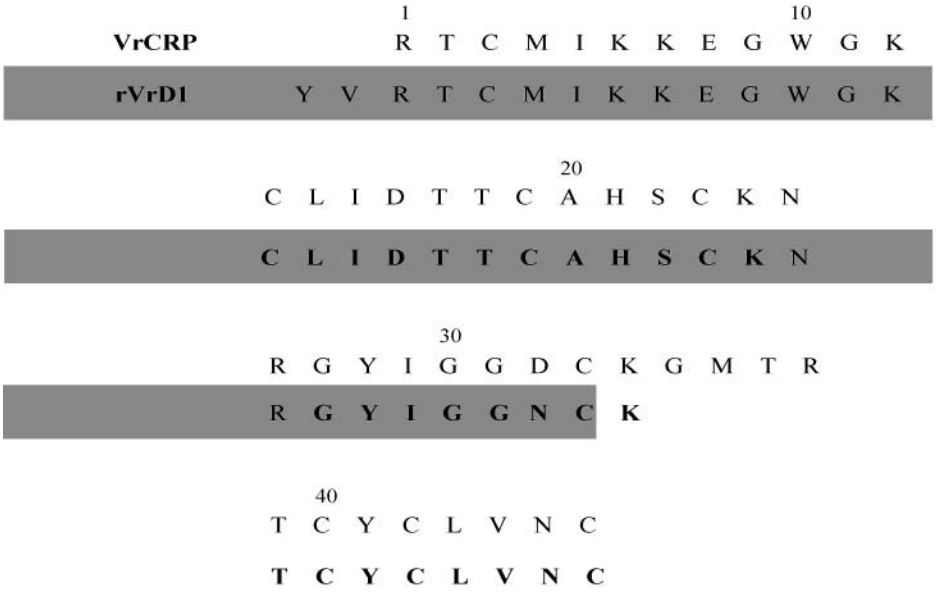


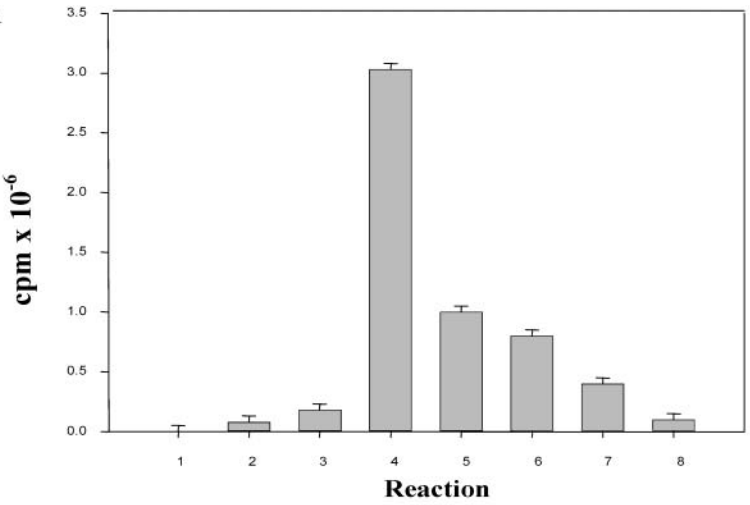
Figure 2. Amino acid sequence of rVrD1. The amino acid sequence of rVrD1 was determined by N-terminal amino acid sequencing (shaded region) and tandem mass spectrometry (boldface).

was produced by crossing between the mungbean cultivar VC1973A and a bruchid resistant wild accession of mungbean TC1966, and the resultant bruchid resistant hybrid was backcrossed six times to VC1973A to develop a bruchid resistant, near-isogenic line (BC6F2), namely, VC6089A. The bruchid resistance in TC1966 is controlled by a single dominant gene (*Br*) and has been successfully incorporated into a bruchid susceptible mungbean cultivar Osaka-ryokutou. As a result, a *C. chinensis* resistant isogenic line BC20F4 was derived (*21*, *22*). *Br* gene has not been isolated. As mentioned above, the VrD1 content in VC6089A seed is approximately 3.5 times that of VC1973A. Whether *Br* gene has been integrated into VC6089A genome and plays a role in regulating the expression of *VrD1* gene awaits further investigation. *VrD1* cDNA was obtained by elimination of the 103 bp intron by inverse PCR. The *VrD1* cDNA was subcloned into pGEM-T easy vector and sequenced (**Figure 1**, shaded region). Comparison of the nucleotide sequence of *VrD*1 cDNA with that of *VrCRP* cDNA revealed a single amino acid substitution at residue 32 (N-terminal arginine of the mature VrCRP protein as residue 1). Whereas VrCRP has aspartic acid at position 32, VrD1 has an asparagine at this position. Because both VrD1 of VC6089A and VC1973A have asparagine at position 32 and a defensin gene cloned from another legume azuki bean also has an asparagine at this position (data not shown), it is reasonable to suggest that the naturally occurring mungbean defensin has the asparagine residue at position 32.

Expression and Characterization of VrD1. Expression of biologically active VrCRP with truncated signal peptide in *E. coli* has been described (*15*). The *E.coli*/IMPACT system requires the use of a reducing agent to release VrCRP-TSP from the fusion protein (VrCRP-TSP/chitin-binding domain) at the final step of purification. In addition, VrCRP-TSP was expressed as an intracellular protein; consequently, disruption of cells is needed for protein purification. A cost effective expression system therefore is needed for a larger scale protein purification. The *P. pastoris* expression system was chosen because (i) the expressed proteins are secreted into the culture medium, (ii) expression of recombinant protein is inducible, and (iii) multiple copies of target gene can be integrated into expression vector thereby enhancing expression level (*23*, *24*). The *VrD*1/pCIP9K expression vector was transformed into *P. pastoris*, the recombinant *P. pastoris* was grown, and rVrD1 was purified from the culture medium by chromatography on a CM-Sepharose column. Four protein peaks were eluted from the column, and peak 4 was shown to contain rVrD1 by analysis on NuPAGE 4−12% Bis-Tris gel (Invitrogen) (hereafter gradient PAGE). The protein in peak 4 is essentially homogeneous because when 5 μg of peak 4 protein was analyzed on the gradient PAGE and stained with silver nitrate, only one single protein band corresponding to molecular weight 5385 determined by tandem mass spectrometry was shown on the gel (data not shown). The purified protein was confirmed to be rVrD1 by amino acid sequence determined by N-terminal amino acid sequencing and tandem mass spectrometry (**Figure 2**) and Western blot analysis using antiserum raised against VrCRP (data not shown). Thus, rVrD1 can be purified rapidly with a single CM-Sepharose column. An average of 3 mg of purified rVrD1 was recovered from 1 L of culture medium. The yield of rVrD1 potentially can be improved by optimizing the fermentation condition, particularly the composition of medium. Almeida et al. (*24*) previously described an optimal condition for expression of *Pisum sativum* defensin 1 by *P. pastoris*, yeilding 13.8 mg/L. The amino acid sequence of rVrD1 exactly matched the cDNA-derived sequence, except the four undetermined consecutive amino acids GMTP (residues 35−38, relative to N-terminal R as residue 1). In *P. pastoris*, rVrD1 was expressed with two additional N-terminal amino acids tyrosine and valine. This is a result of construction and expression of the *VrD*1/pCIP9K *Pichia* expression vector. These two additional amino acids seemed to have no effect on the conformation of the rVrD1 molecule as judged from the findings that biological activities of rVrD1 shown in the next section are essentially unchanged as compared to that of VrCRP previously described (*15*).

Biological Activity of the Recombinant VrD1. Most plant defensins isolated to date exhibit a broad specificity of antifungal activity, including a variety of plant pathogens. We assayed antifungal activity of rVrD1 against some plant pathogens. The antifungal activity was expressed as IC_{50} that represents concentration of rVrD1 required for 50% inhibition of fungal growth. IC_{50} was in the range of 5.4−90.7 μg/mL among the six fungus strains tested (**Table 1**). On the other hand, rVrD1 exhibited very little or no antibacterial activity against *E. coli* TOP10'F, *Vibrio harveyi*, and *Vibrio alginolyticus* (data not

A



B

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

Figure 3. Inhibition of protein synthesis by rVrD1. The experimental details of in vitro translation are given in the 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, and 8 also contained 10, 20, 30, and 40 μM rVrD1, 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.

Table 1. Antifungal Activity of rVrD1[a]

fungus	IC_{50} (μg/mL)
F. oxysporum	5.4
F. oxysporum CCRC 35270	17.5
F. oxysporum f.sp.pisi	12.1
P. oryzae	20.7
R. solani	90.7
Trichophyton rubrum	63.8

[a] Concentration of rVrD1 required for 50% inhibition was defined as IC_{50}, which was determined from dose–response curves (percent growth inhibition vs protein concentration).

shown). The antifungal activity of rVrD1 is comparable to Rs-AFP1 (*Raphanus sativus* L.) assayed against *Fusarium oxysporum* f. sp. pisi (IC_{50} = 15 μg/mL) and both Rs-AFP1 (IC_{50} = 100 μg/mL) and Rs-AFP2 (IC_{50} > 100 μg/mL) against *Rhizoctonia solani* but is weaker than Rs-AFP2 against *F. oxysporum* f. sp. pisi (IC_{50} = 2 μg/mL). These two radish seed antifungal proteins have higher antifungal activity toward *Pyricularia oryzae* (IC_{50} = 0.3−0.4 μg/mL) (*12*) as compared to rVrD1. Inhibition of in vitro protein synthesis was assayed with a cell-free system derived from wheat germ. The recombinant VrD1 at 40 μM has completely inhibited in vitro protein synthesis. A significant dosage response was observed between 10 and 40 μM (**Figure 3**). Inhibition of protein synthesis by thionins isolated from wheat endosperm (*25*) and both γ- and ω-hordothionin from barley endosperm (*7*) has been reported. At low concentrations (1−10 μM), the plant defensins (γ- and ω-hordothionin) seem to affect mainly the polypeptide chain initiation process. At higher concentrations (20−80 μM), this inhibitor induces activation of an eukaryotic polypeptide chain initiation factor 2 α-subunit (eIF-2α) kinase in hemin-supplemented reticulocyte lysates. Whether translational inhibition provides intracellular targets for the action of plant defensins remains to be elucidated. The bacterially expressed VrCRP-TSP exhibits insecticidal activity against azuki bean weevil *C. chinensis* in bioassay with artificial mungbean seeds (*15*). The recombinant VrD1 was assayed for bruchid resistant activity using the same method, and the results are shown in **Table 2**. Artificial seeds containing 0.2 or 0.4% rVrD1 completely arrested bruchid larval development. The results indicated that the *Pichia* expressed rVrD1 exhibited bruchid resistant activity similar to the bacterially expressed VrCRP-TSP (*15*). These results, together with above-mentioned antifungal activities and

Table 2. Bruchid Resistant Activities of Intact and Artificial Seeds with and without the Addition of Purified rVrD1[a]

material examined	WSDT (days)	% emergence
intact seed		
VC 6089A	27.9 (±0.56)	100 (±0)
VC 1973A		0
artificial seed		
VC 1973A	34.1 (±1.63)	46.8 (±16.3)
VC 6089A		0
experimental artificial seed		
VC 1973A + 0.2% r*P*VrCRP		0
VC 1973A + 0.4% r*P*VrCRP		0
VC1973A + 0.2% BSA	35.7 (±2.6)	41.1 (±12.4)

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

inhibitory activity on in vitro translation, suggest that the *Pichia* expressed rVrD1 has a properly folded structure. It was also noted that the substitution of N^{32} in VrD1 by D^{32} apparently had no effect on all of the biological activities assayed in this study as compared with that of VrCRP (*15*). It became evident that plant defensins are important components of innate immunity, protecting plants from pathogen attack (*1*, *2*). However, little is known about whether defensins are also involved in insect resistance in plants. It was described recently that transgenic expression of a floral defensin from *Nicotiana alata* NaD1 in tobacco resulted in an increased mortality rate and developmental effects on the insect pests, *H. armigera* and *H. punctigera* (*10*). In vivo systems such as the transgenic technology would be a useful approach to verify that VrD1 plays a role in protecting mungbean against pathogen and herbivory. In addition, development of transgenic technology would pave the way to augment the defenses of commercially important crops by incorporation of VrD1 gene or its mutated gene into these crops.

In summary, the *VrD1* cDNA cloned from its genomic DNA was subcloned into pPIC9K expression vector and transformed into *P. pastoris*. The expressed rVrD1 was in its active form and exihibited biological activities similar to that of the bacterially expressed VrCRP-TSP. The *Pichia* expression system potentially can also be used for functional expression of VrD1 mutants, therefore providing a highly feasible system to study the structure−function relationship of the mungbean defensin using the mutagenesis approach.

ABBREVIATIONS USED

AVRDC, Asian Vegetable Research and Development Center; IMPACT, intein-mediated purification with an affinity chitin-binding tag; VrCRP, a cysteine-rich protein from *V. radiata*; VrD1, *V. radiata* defensin 1 (*VrD*1 represents cDNA, and VrD1 represents protein); WSDT, within seed development time; YNB, yeast nitrogen base with ammonium sulfate without amino acids.

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