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A Novel Defensin Encoded by a Mungbean cDNA Exhibits Insecticidal Activity against Bruchid

KUAN-CHUNG CHEN,† CHING-YU LIN,† CHENG-CHUN KUAN,† HSIEN-YI SUNG,† AND CHING-SAN CHEN*,†,‡

Graduate Institute of Agricultural Chemistry, National Taiwan University, Taipei 106, Taiwan, and Institute of Botany, Academia Sinica, Nankang, Taipei 115, Taiwan

A cDNA encoding a small cysteine-rich protein designated *VrCRP* was isolated from a bruchid-resistant mungbean. *VrCRP* encodes a protein of 73 amino acids containing a 27 amino acid signal peptide and 8 cysteines. On the basis of the amino acid sequence similarity and conserved residues, it is suggested that VrCRP is a member of the plant defensin family. VrCRP protein was obtained by overexpression of *VrCRP* with a truncated signal peptide in an IMPACT system. Artificial seeds containing 0.2% (w/w) of the purified VrCRP-TSP were lethal to larvae of the bruchid *Callosobruchus chinensis*. VrCRP is apparently the first reported plant defensin exhibiting in vitro insecticidal activity against *C. chinensis*.

KEYWORDS: Defensin; bruchid resistance; mungbean; in vitro translation

INTRODUCTION

Plants have developed various defense mechanisms through evolution. The proteins that have been demonstrated to protect legume seeds from bruchid predation include arcelin (*1*), α-amylase inhibitor (*2*, *3*), vicilins (*4*), trypsin inhibitor (*5*), canatoxin (*6*), and soybean cystatins (*7*). In recent years, it has been reported that some small cysteine-rich proteins, including plant defensins, play an important role in protecting plants from pathogens (*8*−*10*). A group of such small cysteine-rich proteins, the purothionins, are lethal to spruce budworm cells (*11*). However, little is known about the role such peptides might play in protecting legume seeds from insect pests.

Mungbean (*Vigna radiata* [L.] Wilczek) is an important crop in many countries, particularly in Southeast Asia. The seeds of mungbean are used as an energy source and as food protein supplements. In addition, mungbean sprouts are a popular vegetable worldwide. The loss of mungbean seeds to insect pests during storage is a serious problem. One way to tackle the insect pests is to breed an insect-resistant mungbean variety. A wild accession of mungbean, *Vigna radiata* var. *sublobata* (Rovb.) Verdcourt, designated TC1966, is resistant to two major mungbean pests: the azuki bean weevil (*Callosobruchus chinensis*) and the cowpea weevil (*Callosobruchus maculatus*) (*12*, *13*). Investigators at the Asian Vegetable Research and Development Center (AVRDC) made crosses between TC1966 and the mungbean cultivar VC1973A, which is susceptible to *C. chinensis*, to produce a *C. chinensis*-resistant hybrid. The hybrid was backcrossed six times to VC1973A, and a bruchid-resistant, near-isogenic line (BC6F2), designated VC6089A, was developed.

To isolate genes responsible for bruchid resistance from VC6089A, suppression subtractive hybridization (SSH) was performed between VC6089A and matched VC1973A cDNAs. One of the full-length SSH positive clones, *VrCRP* (a small cysteine-rich protein from *V. radiata*) (GenBank Accession AF326687) encodes a predicted protein of 73 amino acids and shows sequence homology to the defensin family of small cysteine-rich proteins. We report here that VrCRP-TSP (i.e., VrCRP expressed in vitro with a truncated signal peptide) exhibited insecticidal activity in an artificial-seed bioassay and inhibits protein synthesis.

MATERIALS AND METHODS

Plant and Insect Materials. VC1973A, VC6089A, TC1966, and *C. chinensis* were obtained from AVRDC. *C. chinensis* was maintained on VC1973A at 25 °C in a growth chamber.

RNA Preparation. Total RNA was extracted from the developing seeds of VC1973A and VC6089A 15 days after flowering (DAF) by using the hot phenol method (*14*).

Suppression Subtractive Hybridization. SSH was performed between VC6089A cDNA and matched VC1973A cDNA, using the PCR Select cDNA subtraction kit (Clontech) according to the manufacturer's recommendations. A full-length cDNA, *VrCRP*, was isolated using this method.

Construction of the Bacterial Expression Vector. Because VrCRP was predicted to have a signal peptide of 22 amino acids according to SPScan of the Genetics Computer Group, oligonucleotide primers were designed to construct an expression vector harboring an N-terminal, 22 amino acid, truncated VrCRP insert. Two oligonucleotides were synthesized: *VrCR*F (5′-CAT GCC ATG GAG AGA GGA GAG GCT AGA AC-3′) and *VrCR*R (5′-TCC CCC GGG ACA GTT GAC GAG GCA ATA-3′). *VrCR*F corresponds to the degenerate codons of the

* Corresponding author (e-mail chingsan@gate.sinica.edu.tw; telephone 886-2-27899590, ext 222, 251; fax 886-2-27858936).
† National Taiwan University.
‡ Academia Sinica.

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  1  ACCTCAACAA TTCATCACTC ATGGAGAGAA AAACTTTCAG CTTCTTGTTC
                           M  E  R  K   T  F  S   F  L  F
 51  TCGCTCCTTC TCGTCTTAGC CTCTGATGTG GCCGTAGAGA GAGGAGAGGC
     S  L  L  L   V  L  A   S  D  V   A  V  E  R   G  E  A
101  TAGAACTTGT ATGATAAAGA AAGAAGGGTG GGGAAAATGC TTAATTGACA
     ↓R  T  C   M  I  K  K   E  G  W   G  K  C   L  I  D  T
151  CCACCTGTGC ACATTCGTGC AAGAACCGCG GTTACATAGG TGGAGATTGC
       T  C  A   H  S  C   K  N  R  G   Y  I  G   G  D  C
201  AAAGGCATGA CGCGCACCTG CTATTGCCTC GTCAACTGTT GAACCCTTTT
     K  G  M  T   R  T  C   Y  C  L   V  N  C  *
251  CGAATATCAT ATCATCTTAT CACAAATAAA TATAGCAGCA TCACTGCTAC

301  TAGTACCGCC CTCCGCACCA CGCCCT
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Figure 1. Nucleotide sequence of *VrCRP* and its deduced amino acid sequence. An arrow indicates the cleavage site for the signal peptide.

amino acids Glu^{23}−Arg^{28} (where the N-terminal amino acid methionine of the signal peptide is numbered as amino acid 1) of VrCRP and has a sense orientation and an *Nco*I site. *VrCR*R corresponds to the degenerate codons of the amino acids Tyr^{68}−Cys^{73} of VrCRP and has an antisense orientation and a *Sma*I site. PCR was performed following the method described by Saiki et al. (*15*), using 5 ng of *VrCRP* as target DNA, 10 pmol each of *VrCR*R and *VrCR*F, 250 nmol each of the dNTPs, and 2.5 units of *Taq* polymerase (Promega) in a total volume of 100 μL. The 171 bp amplification product was purified on a 1% agarose (FMC BioProducts) gel (*16*). The sequence of the amplification product was confirmed by DNA sequencing. The amplification product and a pTYB4 expression vector that has *Nco*I and *Sma*I sites on its multiple cloning sites (MCS) (BioLabs) were digested with *Nco*I and *Sma*I. The two restriction endonuclease-digested mixtures were combined and ligated with T4 DNA ligase (Promega). The nucleotide sequence of the insert in the construct was verified by DNA sequencing. The expression vector thus constructed is called pTYB4-*VrCRP*-TSP.

Expression and Purification of VrCRP-TSP. The construct (pTYB4-*VrCRP*-TSP) was transferred to *Escherichia coli* BL21(DE3), and the expressed VrCRP-TSP was purified by an intein-mediated purification system with an affinity chitin-binding tag (IMPACT) according to the method described previously by Chong et al. (*17*). The *E. coli* transformants were cultured in Luria Bertani (LB) liquid medium containing 100 μg mL^{-1} ampicillin at 37 °C. β-D-Thiogalactopyranoside was added to the culture at a final concentration of 0.3 mM, and the culture was incubated at 24 °C for 6 h. The cell suspension was homogenized with Microfluidizer, and cell debris was removed by centrifugation at 12000*g* for 10 min. The supernatant was filtered through a 0.45 mm membrane filter. The filtrate containing the VrCRP-TSP-chitin binding domain (CBD) fusion protein was passed through a chitin affinity column (16 × 100 mm, bed volume = 30 mL). The column was washed, and the VrCRP-TSP−CBD fusion protein bound to the affinity column was cleaved with 1,4-dithiothreitol (DTT) by introducing a 2.5-fold bed volume of a cleavage buffer (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1 mM EDTA, and 30 mM DTT) into the column and kept at 4 °C for 16 h. VrCRP-TSP was eluted from the column with an elution buffer (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 0.1 mM EDTA). VrCRP-TSP was further purified with an FPLC system using a Superdex peptide HR 10/30 column (10 × 300−310 mm, bed volume = 24 mL) (Pharmacia) to remove small amounts of contaminating proteins. The purified VrCRP-TSP was homogeneous as examined by the gradient PAGE. Protein concentration was determined with the dye binding method (*18*).

Bioassay of VrCRP-TSP. To determine whether VrCRP-TSP has a role in bruchid resistance, we produced artificial seeds containing different amounts of the purified VrCRP-TSP (0.01−0.25%, w/w) and tested these seeds in bioassays with *C. chinensis.* Intact seeds of VC1973A, VC6089A, the bruchid-susceptible mungbean cultivar Tainan Select No. 5 (TN5), and artificial seeds made from the flour of these three mungbean varieties were tested for comparison. The artificial seeds were prepared according to the method of Shade et al. (*19*) with modification. The artificial seeds have an average volume and weight of 95 μL and 98.6 mg, respectively. Each seed is sufficient to hatch six eggs of *C. chinensis*. Thirty-six artificial seeds were divided into six groups for bioassay of VrCRP-TSP for a six-replicate experiment. Ten pairs of *C. chinensis* with equal numbers of male and female bruchids were introduced into the growth chamber and allowed to lay eggs on the seeds for 24 h. The number of eggs on the surface of each seed was strictly confined to six by destroying the extra eggs with a small needle. The seeds were incubated at 25 °C and 60% relative humidity and examined for within-seed development time (WSDT) and percentage emergence at proper time intervals.

In Vitro Translation Inhibition Assay. The reaction mixture contained 0.75 μL of potassium acetate (1 M), 0.5 μL of brome mosaic virus (BMV) RNA (0.5 mg μL^{-1}), 2 μL of an unlabeled amino acid mixture except methionine (1 mM), 1.9 μL of L-[^{35}S]methionine (15 μM, [15 μCi mL^{-1}], Amersham), 12.5 μL of wheat germ extract (Promega), 0.5 μL of RNasin (40 units μL^{-1}), and 10−40 μM VrCRP-TSP. The final volume was adjusted to 25 μL with nuclease-free water. The reaction mixture was incubated at 25 °C for 90 min. Aliquots of the reaction mixture were taken, spotted onto a glass-fiber filter, and dried at 45 °C for 3 min. The filters were dipped in 10% trichloroacetic acid (TCA) and washed thoroughly with 5% TCA before finally being washed with 95% ethanol and dried. Radioactivity was measured in a liquid scintillation counter.

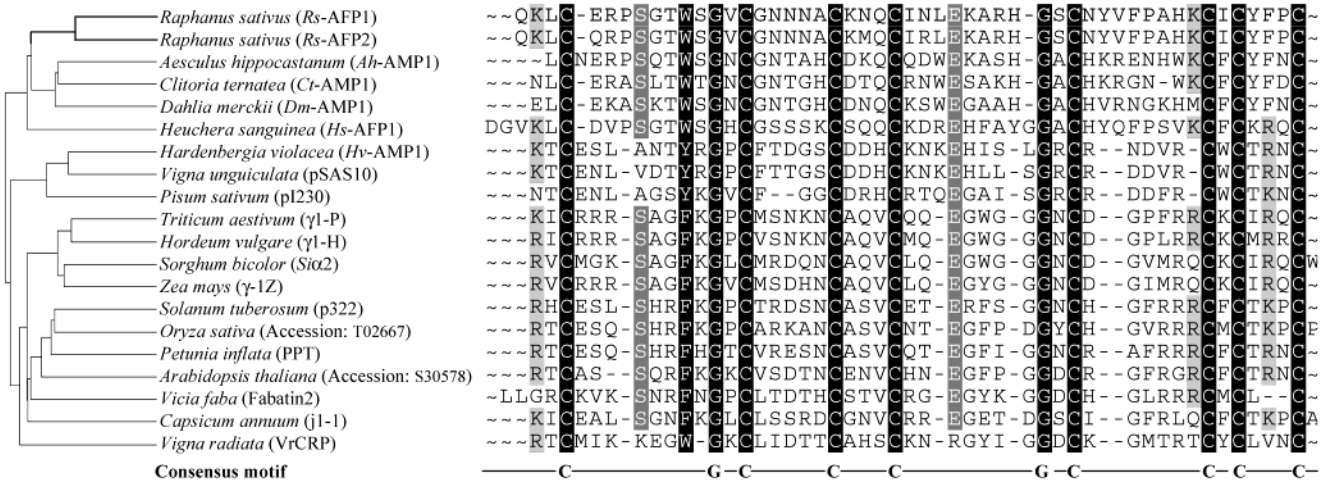


Figure 2. Amino acid sequences of 20 plant defensins. Dashes indicate gaps introduced to maximize homology by multiple alignment program. Dark columns indicate all residues are identical or similar. Shaded columns indicate most, but not all, residues are identical or similar. The following sequences were derived from protein sequencing: *Rs*-AFP1, *Rs*-AFP2 (*24*, *25*); *Ah*-AMP1, *Ct*-AMP1, *Dm*-AMP1, *Hs*-AFP1 (*26*); *Hv*-AMP1 (*22*); γ1-P (*27*); γ1-H (*28*); Siα2 (*29*, *30*); γ-1Z (*31*); Fabatin 2 (*32*); and j1-1 (*33*). The following sequences were deduced from cDNA clones with omission of the putative signal peptides: pSAS (*34*); pI230 (*35*); p322 (*36*); and PPT (*37*). The VrCRP sequence was aligned with sequences of related peptides, obtained from the Swiss-Prot database. Alignment of peptide sequences and determination of clustering relationships was carried out using PileUp.

RESULTS AND DISCUSSION

Isolation of VrCRP cDNA from a Bruchid-Resistant, Near-Isogenic Line of Mungbean VC6089A. Bioassays of *C. chinensis* on the seeds of VC1973A and VC6089A revealed that the developing seeds of VC6089A were resistant to bruchid approaching a maximum around 15 DAF (data not shown). To isolate mRNAs preferentially displayed in 15 DAF developing seeds, cDNAs prepared from total RNA of 15 DAF seeds of VC1973A and VC6089A were subjected to SSH (Clontech, PCR-Select). Of the isolated preferentially expressed cDNA clones, 18 gave positive results in VC6089A but not in VC1973A in Northern blot analysis. The clones showed sequence homology (34−96.6%) with albumin (96.6%), phaseolin (86.4%), late embryogenesis abundant (LEA) protein (85.4%), β-conglycinin α-chain precursor (78%), 1L-*myo*-inositol 1-phosphate synthase (69.9%), glycinin (63.1%), pectin methylesterase (60.8%), dehydration inducible protein (55.6%), basic 7S globulin (55.4%), conglutin γ gene (39.7%), and γ1- and γ2-purothionins (34 and 36.2%, respectively). Some clones that showed no sequence homology with previously described proteins were also obtained (data not shown). VrCRP was chosen from these 18 candidate clones for this study because it shares sequence homology with wheat γ1- and γ2-purothionins (*20*). This cDNA encoded a predicted protein of 73 amino acids containing 8 cysteine residues (**Figure 1**). The VrCRP has a predicted cystine-stabilized α-helix motif, as do a number of cysteine-rich proteins (*21*), and shows amino acid sequence homology to plant defensins (*8*−*10*). We therefore suggest that VrCRP is a member of the plant defensin family. Plant defensins have the conserved residues 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 (*8*, *22*, *23*) (numbering relative to *Rs*-AFP1) (**Figure 2**). It is of interest to note that VrCRP has all of these conserved residues except the residues 8 and 29. Whereas all other plant defensins have a conserved glutamic acid at position 29, VrCRP has an arginine at this position. In addition, unlike other plant defensins having a nonionizable side-chain amino acid (mostly serine) at residue 8, VrCRP has a lysine at this position. Whether the substitutions at residue 8 or 29 are associated with the insecticidal activity of VrCRP remains to be investigated.

Expression of VrCRP in *E. coli*. The expressed VrCRP-TSP fusion protein had a molecular mass of ∼60.9 kDa and was purified by chitin affinity chromatography. The fusion protein was cleaved with DTT to release VrCRP-TSP with a molecular mass of ∼5.9 kDa from the chitin-binding domain (**Figure 3A**). The eluted VrCRP-TSP was purified further on a Superdex Peptide HR 10/30 column. Four A280 peaks were eluted from the column (**Figure 3B**), but only peak 2 had protein that could be visualized by silver staining after separation on NuPAGE 4−12% Bis-Tris gel (Invitrogen) (hereafter "gradient PAGE") (**Figure 3C**). Because 4 mg of peak 2 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, it is reasonable to conclude that the protein in peak 2 is essentially homogeneous. The purified protein was confirmed to be VrCRP-TSP by N-terminal sequencing and mobility on the gradient PAGE. The purified VrCRP-TSP had a relative molecular mass of ∼5.9 kDa on the gradient PAGE (**Figure 3C**) corresponding to the calculated molecular weight 5944.

Bruchid-Resistant Activity of Bacterially Expressed VrCRP-TSP. The results of the seed bioassays are shown in **Table 1**. There was no adult emergence from intact VC6089A seeds or artificial VC6089A seeds after up to 90 days of incubation, indicating that some component(s) in VC6089A was (were) responsible for inhibiting the growth of bruchid larvae. There was a ∼10-day delay in adult emergence (WSDT) in artificial seeds of bruchid-susceptible VC1973A and TN5 compared to the intact seeds of these two cultivars. The percentage emergence in these two artificial seeds was about half of that observed in the corresponding intact seeds. Similar results have been reported previously for intact versus artificial seeds of the common bean (*Phaseolus vulgaris* L.) (*1*). Regardless of whether intact or artificial seeds of bruchid-

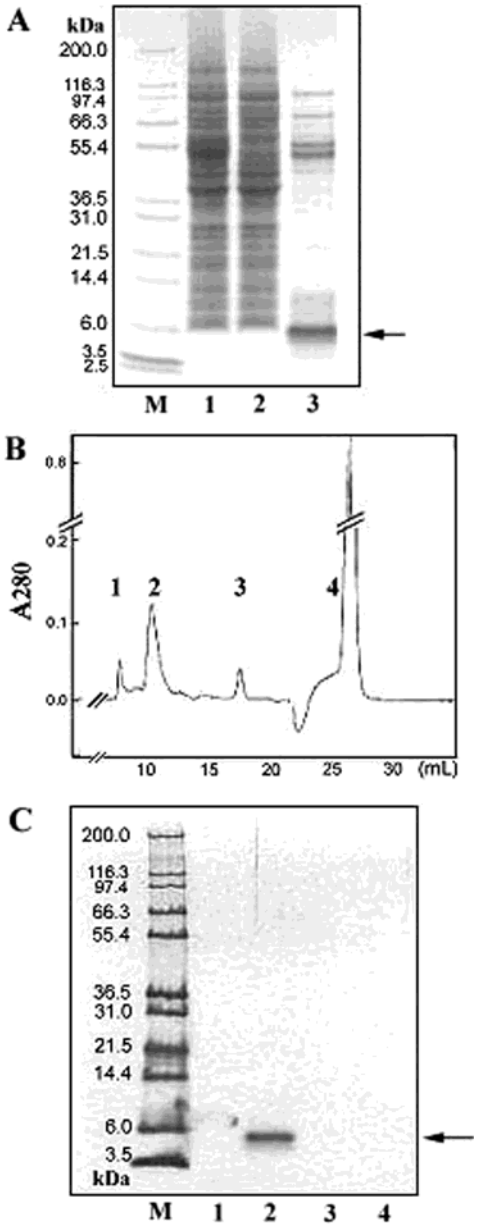


Figure 3. Expression and purification of VrCRP-TSP. Experimental details are given under Materials and Methods. *E. coli* cells were homogenized after induction. The crude extract was applied to a chitin column, and the column was washed. VrCRP-TSP was eluted from the column. The crude extract, the column flow-through, and eluate, lanes 1, 2, and 3, respectively, were resolved on NuPAGE 4–12% Bis-Tris gel (Invitrogen) and stained with silver nitrate (A). VrCRP-TSP obtained from the chitin column (A, lane 3) was further purified by FPLC on a Superdex HR 10/30 column. Four peaks, 1–4, were eluted from the column (B). Four micrograms of protein in peak 2 was analyzed by the gradient PAGE and stained with silver nitrate (C). M, molecular weight marker. Arrow indicates position of VrCRP-TSP.

Table 1. Bruchid-Resistant Activities of Intact and Artificial Seeds with and without the Addition of Purified VrCRP-TSP[a]

material examined	within seed development time (days)	% emergence
intact seed		
TN5	26.3 (±0.6)	100 (±0)
VC1973A	25.9 (±0.9)	100 (±0)
VC6089A		0
artificial seed		
TN5	35.4 (±1.6)	51.3 (±9.8)
VC1973A	36.4 (±1.6)	53.1 (±5.0)
VC6089A		0
experimental artificial seed		
VC1973A + 0.01% VrCRP-TSP	40.7 (±2.1)	12.5 (±5.9)
VC1973A + 0.09% VrCRP-TSP	48	2.1 (±5.9)
VC1973A + 0.20% VrCRP-TSP		0
VC1973A + 0.25% VrCRP-TSP		0
VC1973A + 0.25% BSA	41.0 (±2.6)	52.6 (±7.9)

[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. TN5, *V. radiata* L. Tainan Select No. 5.

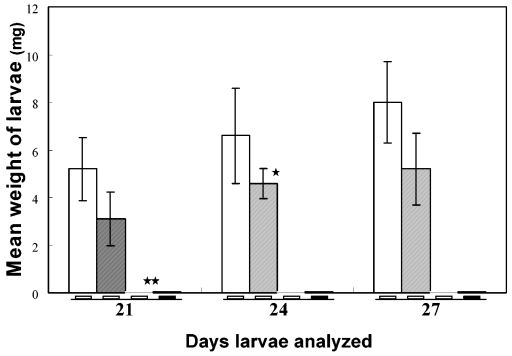
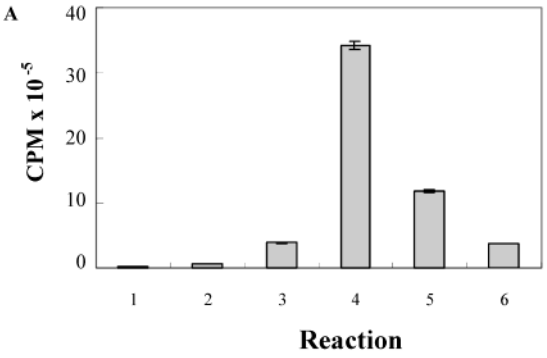


Figure 4. Performance of *C. chinensis* larvae reared on artificial seeds containing VrCRP-TSP. Six artificial seeds containing six eggs each were prepared for each dose of VrCRP-TSP. Two capsules were opened for determination of body weight and number of larvae after incubation at 25 °C and 60% relative humidity for 21, 24, and 27 days. Results shown are mean ± SEM of six replicates of each condition and subjected to statistical analysis using an unpaired Student's *t* test. ★, $P < 0.035$, indicating significant difference between control and artificial seeds containing 0.01% VrCRP-TSP at 24 days. ★★, $P < 0.001$, indicating significant difference between artificial seeds containing 0.01% VrCRP-TSP and 0.2 or 0.25% VrCRP-TSP at 21 days. Percentage of VrCRP-TSP in the artificial seeds: white bars, 0; slashed bars, 0.01; lightly shaded bars, 0.2; black bars, 0.25.

susceptible TN5 or VC1973A were used as a control, the bruchid resistance of artificial seeds containing VrCRP-TSP was easily distinguishable. Artificial seeds containing 0.2% VrCRP-TSP completely arrested bruchid development. A significant dosage response was observed between 0.01 and 0.2% of VrCRP-TSP. Complete growth arrest was observed with seeds containing either 0.2 or 0.25% VrCRP-TSP (**Figure 4**).

To compare the structure of bacterially expressed VrCRP-TSP with that of naturally occurring VrCRPin VC6089A seeds, we purified defensin(s) from seeds of VC6089A and VC1973A using a procedure previously used for purification of several plant defensins (*22*, *24*). We found that both VC6089A and VC1973A seeds contained a defensin similar in molecular mass but quantitatively different. VC6089A contained 114.7 μg of defensin g^{-1} of dry seed, whereas VC1973A had 32.9 μg of defensin g^{-1} of dry seed, equivalent to 0.012 and 0.0033% (w/w) of dry seed, respectively. Thus, defensin content in



B

Reaction	1	2	3	4	5	6
Wheat germ extract, 12.5 µL	-	-	+	+	+	+
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, 20 µU	-	+	+	+	+	+
VrCRP-TSP (µM)	-	-	-	-	10	40

Figure 5. Inhibition of protein synthesis by the purified VrCRP-TSP. Experimental details of in vitro translation are given under Materials and Methods. Counts per minute (cpm) of TCA insoluble fraction was 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 and 6 also contained 10 and 40 μM VrCRP-TSP, 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.

VC6089A seed is approximately 3.5 times that of VC1973A (data not shown). 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. Both defensins also cross-reacted with anti-VrCRP-TSP antiserum. In addition, their N-terminal 15 amino acids were identical to the deduced amino acid sequence of RTC-MIKKEGWGKCLI shown in **Figure 1** and corresponding to $Arg^{7}-Ile^{21}$ in the bacterially produced VrCRP-TSP (data not shown). These results indicate that the signal peptide of VrCRP has 27 amino acids instead of 22 amino acids as initially predicted by comparison to other plant defensins (*33*).

Whether both seeds of the two near-isogenic mungbean lines contain an identical defensin remains to be analyzed. The molecular basis of the insecticidal activity of VrCRP-TSP is not known, although it is an inhibitor of protein synthesis (**Figure 5**). Inhibition of protein synthesis by thionins isolated from wheat (*38*) and γ-hordothionin from barley (*28*) has been reported. 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 (*12*, *13*). Two novel cyclopeptide alkaloids denoted vignatic acids A and B were isolated from BC20F4, and vignatic acid A was shown to resist bruchid infestation (*39*). It is also suggested that the vignatic acids are not the principle factors responsible for bruchid resistance in *V. radiata* (*40*). In light of the findings that both VC6089A and VC1973A seeds contain defensins and that these genes occur as a single copy in the VC6089A and VC1973A genomes as indicated in Southern analysis (data not shown), it is suggested that VrCRP is not a product of the *Br* gene. The antimicrobial activities of plant defensins have been the subject of intensive study. However, little is known about whether defensins are also involved in

insect resistance in plants. VrCRP may provide a good system for studies that will eventually add insect resistance to a lengthy list of defensin activities.

ABBREVIATIONS USED

AVRDC, Asian Vegetable Research and Development Center; CBD, chitin binding domain; DAF, days after flowering; DTT, 1,4-dithiothreitol; IMPACT, intein-mediated purification with an affinity chitin-binding tag; SSH, suppression subtractive hybridization; TCA, trichloroacetic acid; TSP, truncated-signal-peptide; VrCRP, a cysteine-rich protein from *Vigna radiata* (*VrCRP* represents cDNA, VrCRP represents protein); WSDT, within seed development time.

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