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Antimicrobial Activity of a Cys-Rich Peptide Derived from a *Centrosema virginianum* Vicilin

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Abstract

Many antimicrobial peptides (AMPs) have been identified in plants. These peptides are highly divergent at the primary sequence level and vary in their hierarchical structures. Some common biochemical features include the ability to form disulfide bonds, tandemly repeated amino acid sequences and a net charge at pH 7. Unusual Cysteine containing repeats has been identified in several plant seed storage proteins that may act as AMPs. We identified a Cys repeat within a vicilin (seed storage protein) of a wild legume, *Centrosema virginianum*. Cleavage of the vicilin protein during germination would generate a vicilin derived Cys peptide (VDCP). We investigated the antimicrobial properties of this VDCP and compared its efficacy as an antimicrobial agent to VDCPs from other species. We developed transgenic tobacco plants that expressed cloned sequences encoding the Cysteine repeat unit from *C. virginianum*, *Theobroma cacao* and *Gossypium hirsutum*. Extracts from fully expanded leaves were tested for antimicrobial activity against a fungal pathogen, *Botrytis cinerea*. The Cys motif from *C. virginianum* was also expressed in two *E. coli* cell lines (reducing or oxidizing cytoplasm) and peptide fusion protein fractions were tested for antimicrobial activity against a battery of fungal strains. The unique Cysteine repeat single unit from *C. virginianum* exhibited antimicrobial properties greater than or equal to the antimicrobial activity associated with expression of the multiple Cys-repeat VDCPs from *G. hirsutum* or *T. cacao* in transgenic tobacco. When expressed in bacteria, a *C. virginianum* VDCP fusion protein exhibited antifungal activity against 3 of the 4 fungi tested. Although the primary role of seed storage proteins is to provide a pool of amino acids and nitrogen for germinating seeds and developing plantlets, it is likely that seed storage protein proteolytic products also provide beneficial antimicrobial properties during germination and young plantlet development.

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Keywords

Seed Storage Proteins, Vicilins, Antimicrobial Peptides, *C. virginianum*

1. Introduction

Plants have evolved a variety of mechanisms to combat pathogen attacks. These include both active and passive responses. Examples of active responses are production of reactive oxygen species [1] [2], secondary metabolites (phytoalexins) [3] [4], hydrolytic enzymes [5], antimicrobial proteins [6]-[8] and peptides [9]. Passive strategies can include physical barriers to pathogen invasion such as thick cuticles and cell walls.

Antimicrobial peptides (AMPs) are widely distributed in higher plants and major groups including the thionins, defensins and Lipid Transfer Proteins (LTPs) [10]. The primary sequences of plant AMPs can be conserved within groups or sub-groups but are variable between classes. For example, thionins have a common structure [11] but are clearly different from other classes of AMPs such as defensins and LTPs in their primary sequences and hierarchical structures. Many AMPs contain Cysteine residues associated with two or more disulfide bonds, repeated sequences and are charged at neutral pH. Although many plant AMPs have been identified in seeds (such as Type I and Type IV thionins and most defensins), they can also be expressed in leaves, stems or nuts (Type II or Type III thionins, Lipid Transfer Proteins).

An unusual sequence with three repeats of a novel Cys-pattern, $CX_3CX_{10-15}CX_3C$ was first observed in the protein encoded by a *Gossypium hirsutum* vicilin DNA sequence [12]. Vicilins are a class of seed storage protein whose primary function is to serve as a source of nitrogen and amino acids for germinating seeds and developing seedlings. Upon imbibition and initiation of germination, the vicilin proteins are degraded. It was postulated that post-translational cleavage of the N-terminal proximal hydrophilic region of *G. hirsutum* protein could give rise to peptides that contain the unique Cys-pattern containing sequence [12]. It was proposed that this Cys-pattern motif might form disulfide bonds, which would stabilize the peptide [12]-[14]. Subsequently, it was shown that vicilin-derived peptides from *Macademia integrifolia* with four repeated segments of this $CX_3CX_{10-15}CX_3C$ pattern exhibited antimicrobial properties. The purified peptides from *Macademia integrifolia* inhibited the phytopathogens *Fusarium oxysporum*, *Verticillium dahliae* and *Clavibacter michiganenis* at peptide concentrations ranging from 5 $\mu g \cdot ml^{-1}$ to 50 $\mu g \cdot ml^{-1}$ [15]. This Cys-pattern was identified in other vicilin sequences and sequence alignments of the vicilin hydrophilic N-proximal regions of cotton, cocoa, maize basic protein, buckwheat trypsin inhibitor and pumpkin [16]-[20]. There was variation in the number of Cys-patterns in different species [15]. The potential VDCPs from these proteins exhibit a low level of identity in their primary sequences but there are some commonalities. The four hydrophobic Cysteines are conserved, there is a relatively higher proportion of positively charged arginine and lysine, the uncharged amino acid glutamine, and the negatively charged glutamate. Whether the numbers of Cys-patterns contained within a given vicilin or if different amino acid residues in the "X" position influence the spectrum or activity of the peptides has not been established.

We identified a VDCP in a wild Louisiana legume, *Centrosema virginianum.* Here we present evidence that expression of this peptide in plants confers resistance to microbes. Transgenic plant extracts showed significant antimicrobial activity against the filamentous fungus, *Botryris cinerea*. To further study the spectrum and efficacy of this peptide as an antimicrobial agent, we expressed it as a maltose binding protein fusion in K12 TB1 (reducing environment) and SHuffle (oxidizing environment) *E. coli* cells. Fusion proteins expressed in both cell lines exhibited antimicrobial activity. In some cases, the protein expressed in SHuffle cells (an oxidizing cytoplasm which would favor formation of disulfide bonds) exhibited greater antimicrobial activity than the fusion expressed in K12 TB1 cells. This supports the hypothesis that formation of disulfide bonds is important to the function of these VDCPs.

2. Materials and Methods

2.1. Extraction of Genomic DNA and PCR

Genomic DNA was extracted by established methods [21]. PCR reactions (25 μL) incorporating 40 ng of ge-

nomic DNA or 100 pg of plasmid DNA, 0.8 μM of each primer and 22.5 μl of Platinum PCR SuperMix High Fidelity (Invitrogen) were performed using the following profiles: 94˚C for 2 min; 35 cycles of 95˚C for 25 s; 25 s at optimized annealing temperature for primer pair; 1 min ramp to 72˚C, 72˚C for 1 min; then one incubation at 72˚C for 7 min. Primers, templates, predicted product size and annealing temperatures are listed in **Table 1**.

2.2. Identification of a Vicilin Sequence in *Centrosemsa virginianum*

Genomic DNA from *C. virginianum* was amplified as described above using primers designated vicilin promoter II and vicilin 4. The amplified fragment was cloned into pZERO (Invitrogen) and sequenced (ABI 310, Applied Biosystems) using Big Dye Terminator Reaction Ready Sequencing Mix 1.1 according to the manufacturer's recommendations (Applied Biosystems). This 1073 bp vicilin gene fragment (Genebank accession: FJ824190) was used as the template to amplify the *CvAFP*1 coding sequences CV1 and CV2 described below. A 42 residue segment of the corresponding protein sequence that contained the unique Cys motif was aligned with corresponding sequences from other plants using T-coffee [22].

2.3. Construction of the Binary Vectors Containing VDCPs, Transformation of *A. tumefaciens* and Generation of Transgenic Tobacco Plants

The pBI 221 and pBI 121 vectors were obtained from Clontech (Clontech). Cysteine containing peptide regions with or without the signal sequence from *C. virginianum*, *G. hirsutum* and *T. cacao* were amplified with primers that were engineered to contain Xba I and Sac I sites so that amplified products could be cloned into the pBI vectors (**Table 1**). To block the intrinsic *Xba-I* site of the CV1 coding sequence, the third codon of the sequence was mutated from TCA to TCT (no change in the amino acid sequence). Start codons were engineered into the forward primers for amplification of VDCP coding regions without the signal sequence. A stop codon was added to the 3' end of all six amplified VDCP coding sequences.

Agrobacterium tumefaciens, strains LBA4404 and C58C1 (PMP90) were transformed by the freeze –thaw method [23]. Axenic tobacco leaf discs (*Nicotiana tabaccum var xanthi*) were transformed with engineered *Agrobacterium* strains using vacuum infiltration, cocultivation and selection [24]. Plants were grown to maturity in the greenhouse and the presence of the introduced DNA was verified by PCR.

2.4. Extraction of Total RNA and Quantification of Expressed *C. virginianum* Cys Peptide Transcript

Total RNA was isolated as described [25]. Nucleic acid samples were quantified using a NanoDrop ND-1000

Table 1. Primers used for the PCR amplification of VDCP coding sequences and the construction of pBI121 binary vectors. The engineered start codons are underlined. The promoter-II primer is a degenerate PCR primer.

CV1-F	5' CGTCTAGAATGGGTTCAAGAGCGCGGTTTC 3'
CV2-F	5' GGTCTAGATGATTGCGTACTGGGAACAGGA 3'
CV-R	5' GGGAGCTCTTACTTAACGACCCTGCAACG 3'
TC1-F	5' CCTCTAGAATGGTGATCAGTAAGTCTCCTTTC 3'
TC2-F	5' CCTCTAGATGTATGGCAGAAAACAATATGAGCG 3'
TC-R	5' CCGAGCTCTTAATATTGCTCCCAGCATTTTC 3'
GH1-F	5' GGTCTAGAATGGTGAGGAATAAGTCAGCTTGC 3'
GH2-F	5' GGTCTAGATGAAAGACTTTCCCGGAAGAAGAG 3'
GH-R	5' GGGAGCTCTTAGTACCTTTCCCTGCATTC 3'
Promoter-II	5' GTAAGAGAAIICGGTGDAGWWA 3'
Vic-4	5' GAGGCCTCTAGAATATGCTTGCTGAA 3'

Spectrophotometer (NanoDrop Technologies, Inc.) and RNA sample integrity was visually verified by fractionation on formaldehyde agarose gels [26].

RNA samples (5 µg total RNA) were fractionated on denaturing formaldehyde agarose gels and transferred to either Magnagraph nylon membrane (MSI), or Hybond N+ membrane (Amersham Pharmacia Biotech) as described [26]. RNA was cross-linked to the membrane using the automatic setting on a Stratalinker (Stratagene). Northern hybridizations were performed in DIG Easy hybridization buffer (Roche) overnight at 37˚C in a Hybaid Mini Hybridization Oven (Hybaid Instruments). Digoxygienin dUTP labeled, randomly primed probes were generated as described (Genius Non-Radioactive Labelling and Detection System (Roche Biochemicals)). Filters were washed (stringency washes at 47˚C in 0.1 X SSC, 1% (w/v) SDS. Filters were blocked, incubated with primary antibody and washed as described by the manufacturer (Roche Biochemicals), hybridized probe detected with CDP-Star (Roche Biochemical) and chemiluminescence was detected using a Chemi-doc System (Bio-Rad).

2.5. Bacterial Expression, Purification and Protein Analysis

The pMAL-c2 vector (NEB) was engineered to only express the maltose-binding protein (MBP) as a control. The vector was digested with *Eco* RI, the ends filled in with T-4 DNA polymerase and then ligated with T4 DNA ligase [26]. A pMAL-c2 vector that expressed a MBP-VDCP fusion was also generated. The coding sequence of the single Cys-pattern bearing peptide ($Cys_{Repeat1}$) from *Centrosema virginianum* was amplified by PCR and cloned into the C-terminus of the *malE* gene of pMAL-c2 plasmid vector at the Xmn I site. All constructs were verified by DNA sequence analysis (ABI 310, Big Dye Terminator Chemistry). Both constructs were used to transform two *E. coli* strains: TB1 and SHuffle (NEB). MBP and the MBP-$Cys_{Repeat1}$ proteins were expressed and purified as described in the pMAL Protein Fusion and Purification System Manual (NEB). The purified protein fractions were dialyzed against 10 mM Tris-HCl, pH 7.5 and then the protein concentration was determined using a colorimetric dye binding assay (BioRad). Proteins fractions were analyzed on 12% denaturing acrylamide gels [27].

2.6. Antifungal Assays

A spore germination inhibition assay using crude protein extracts was adapted from Cary and Rajasekaran [28] [29]. Fresh leaf tissue was ground in a mortar and pestle in liquid nitrogen, the powder transferred to a microcentrifuge tube and then centrifuged at 1×10^4 g for 10 min at room temperature. Supernatants (225 µl) were incubated with 2.25×10^3 spores (25 µl) for 1 hour. The reaction mixtures were spread on PDA plates, incubated for 36 h at 28˚C and the fungal colonies were counted. The Dunnett Multiple Comparisons Test in the GraphPad Instat (V3.05) software was used as a post hoc test following one-way ANOVA to determine the significance of the effect of transgenic plant extracts on germinating conidia.

The antifungal activity of MBP or MBP-VDCP *C. virginianum* fusion was assayed using a microtiter tray plate assay. Pre-germinated spore solutions in synthetic media [30] were incubated with 10 mM Tris-HCl, pH 7.5 or 1.389 $mg \cdot mL^{-1}$, 0.833 $mg \cdot mL^{-1}$ and 0.278 $mg \cdot mL^{-1}$ of MBP or MBP-$Cys_{Repeat1}$ proteins expressed in both K12 TB1 and SHuffle *E. coli*. The microtiter plates were incubated in the dark and optical density determined at 590 nm (SPECTRA Fluor Plus, v4.23, Tecan) at 12 hr intervals. A heteroscedastic T-test with one-tailed distribution analysis was perfomed with a 95% confidence interval to compare the optical density observed with the MBP-$Cys_{repeat1}$ fusion expressed in MBP protein expressed in the same cell line.

All of the samples were tested in triplicate and the background absorbance from the media was subtracted prior to calculation of inhibition. The percent inhibition was calculated as 100 - (OD of the Cys-MBP Fusion/OD of the MBP*100). The background due to media alone was subtracted from all values prior to performing this calculation.

3. Results

3.1. Identification of *Centrosema virginianum* VDCP

A partial coding sequence for a vicilin from *C.virginianum* was amplified by PCR, cloned and sequenced. The amino acid sequence derived from this coding region is presented (**Figure 1**). The vicilin partial fragment encodes a leader peptide and the N-terminal portion of the vicilin protein. The predicted 26 amino acid signal peptide

```
ATG GGT TCA AGA GCG CGG TTT CCA CTG TTG TTG TTG CTG GGA GTT GTT TTC CTA GCA TCA
M   G   S   R   A   R   F   P   L   L   L   L   L   G   V   V   F   L   A   S

GTT TCT GTA TCA TTT GGC ATT GCG TAC TGG GAA CAG GAT AAC CCC AAT TAC GAC AAG TGC
V   S   V   S   F   G   I   A   Y   W   E   Q   D   N   P   N   Y   D   K   C

CTT CGG AGT TGC AAG AAC GAG AAT GAT TTG TTC AGG TTC AAA GCA TGC AAC ATT CGT TGC
L   R   S   C   K   N   E   N   D   L   F   R   F   K   A   C   N   I   R   C

AGG GTC GTT AAG GAC CCT GAC TTC GAG GTG AAA GAA CAA GAA AAA GAA CAT TAT TCA GAA
R   V   V   K   D   P   D   F   E   V   K   E   Q   E   K   E   H   Y   S   E

CAC GAA GAA AAG GAG GAA GAG GAA GAT CAA GGT TCA AAA TCA ATT ACA GAA ACA AAC CCC
H   E   E   K   E   E   E   E   D   Q   G   S   K   S   I   T   E   T   N   P

TAC CTT TTT AGG TCT TAC CGA ACT CTC TTC AGG AAC CAA CTC GGT CGG ATT CGT ATC CTT
Y   L   F   R   S   Y   R   T   L   F   R   N   Q   L   G   R   I   R   I   L

CAG GAG TTC AAC AGG AAC TCT AAG AAA CTT CAA AAT CTC GAA AAC TAC CGA ATG GTG GAG
Q   E   F   N   R   N   S   K   K   L   Q   N   L   E   N   Y   R   M   V   E

CTC GTG TCC GAC CCC AAC ACC TTG TAT CTC CCC CAC CAT GCT GGC GCT GAT TTC ATC TTC
L   V   S   D   P   N   T   L   Y   L   P   H   H   A   G   A   D   F   I   F

GTT GTA GTC GAG GGG AAA GCC TTA CTT ACT TTA GTG TAC CCC GAC CAA AGA CCA GTC CCT
V   V   V   E   G   K   A   L   L   T   L   V   Y   P   D   Q   R   P   V   P

TAC AAA CTT GAA CGC GGC CAA GGC ATT AGA ATT AAA CGA GGA ACC ACT TAC TAT TTG ATT
Y   K   L   E   R   G   Q   G   I   R   I   K   R   G   T   T   Y   Y   L   I

AAC AGA GAC CAA CGC GAC CGT CTC AGA GTA ATA AAA CTC GCC GTA CCT GTT AAC AAA CAA
N   R   D   Q   R   D   R   L   R   V   I   K   L   A   V   P   V   N   K   Q

GGA GAA TTC CGG CAT TTC TTC CCA TCT ACC ACT AAC GAG CAG AAA AGC TAC TTC CGC GGG
G   E   F   R   H   F   F   P   S   T   T   N   E   Q   K   S   Y   F   R   G

TTC AGC AAG CAT ATT CTA GAG GCC…
F   S   K   H   I   L   E   A
```

Figure 1. The nucleotide and predicted vicilin protein sequence from *C. virginianum* (GenBank Accession Number FJ824190.1). The third codon of the sequence was mutated from TCA to TCT by PCR directed mutagenesis to block the Xba I restriction site for cloning. The underlined sequence is the vicilin signal peptide predicted by SignalP 3.0. The CV1 peptide includes the signal sequence and the CV2 peptide. The shaded sequence denotes the CV2 peptide.

is located adjacent to an amino acid sequence that contains a single repeat of a predicted VDCP.

Alignment of the VDCP from *Centrosema virginianum* with VDCPs from *Glycine max* and *Arachis hypogea* (also legumes) shows absolute conservation of the Cys-motif residues and 9 additional amino acids out of the 38 - 43 residues used for the alignment (**Figure 2(a)**). An additional 8 amino acids are highly conservative substitutions (similar biochemical properties) and two more amino acids are moderately conservative substitutions. The high level of conservation observed when comparing these legume VDCPs (35%) is not observed when additional VDCPs from different plant families are added to the analysis. The only conserved residues are the Cysteines associated with the Cys-motif (**Figure 2(b)**).

```
A.
Glycine         1 YWEKENPKHNKCLQSCNSERDSYRNQACHARCNLLKVEKEECE   43
Arachis         1 YRKTENPCAQRCLQSCQQEPDDLKQKACESRCTKL-----EYD   38
Centrosema      1 YWEQDNPNYDKCLRSCKNENDLFRFKACNIRCRVVKDPD-FEV   42

cons            1 * : :**  ::**:**:.* *  : :**. **  :           43


B.
Arachis         PCAQRCLQSCQQEP-D-DLKQKACESRCTKLEY
Carya           WEFQQCQERCQHEE-RGQRQAQQCQRRCEEQLR
Carya_1         KQYEQCRETCEKQD---PRQQPQCERRCERQFQ
Carya_2         EQYRQCEEHCRRQG-QGQRQQQQCQSPCEERFE
Carya_3         EQYRQCQEHCRRQG-QGQRQQQQCQSRCEERLE
Carya_4         QRYEQCQQQCERQR-RG-QEQQLCRRRCEQQRQ
Carya_5         QQYHRCQRRCQTQE-QSPERQRQCQQRCERQYK
Centrosema      PNYDKCLRSCKNEN-D-LFRFKACNIRCRVVKD
Cucurbita       VDHDGCVNRCEELKGKNVDEFAACKKGCGVNQR
Cucurbita_1     AEYEVCRLRCQVAE-RGVEQQRKCEQVCEERLR
Glycine         PKHNKCLQSCNSER-D-SYRNQACHARCNLLKV
Gossypium       KRYEDCRRRCEWDT-RGQKEQQQCEESCKSQYG
Gossypium_1     RRYEECQQECRQQE-E--RQRPQCQQRCLKRFE
Gossypium_2     RQFQECQQHCHQQE-QRPERKQQCVRECRERYQ
Hordeum         HSLQQCVQRCRQER-P-RYSHARCVQECRDDQQ
Macadamia       QEYEECKRQCMQLE-T-SGQMRRCVSQCDKRFE
Macadamia_1     TDCQQCQRRCRQQE-SGPRQQQYCQRRCKEICE
Macadamia_2     QQYEQCQERCQRHE-TEPRHMQTCQQRCERRYE
Macadamia_3     REYEDCRRRCEQQE---PRQQYQCQRRCREQQR
Theobroma       QQYEQCQRRCESEA-TEEREQEQCEQRCEREYK
Theobroma_1     RQYQQCQGRCQEQQ-QGQREQQQCQRKCWEQYK
Zea             HKSGRCVRRCEDRP---WHQRPRCLEQCREEER

cons            *   *           *   *
```

Figure 2. Alignment of representative VDCPs. Panel A shows a comparison of VDCPs from three legume species. Panel B shows the multiple sequence alignment of representative VDCP sequences from different genera. Multiple sequence alignments were generated using T-COFFEE [22]. Sequences used for the alignments and their GenBank accession numbers are: Glycine (*Glycine max*, BAE44299.1), Arachis (*Arachis hypogea*, P43238.1), Centrosema (*Centrosema virginianum*, ACZ51236.1), Carya (*Carya illinoiensis*, ABV49590.1), Cucurbita (*Curcurbita maxima*, BAA34056.1), Gossypium (*Gossypium hirsutum*, P09801.1), Hordeum (*Hordeum vulgare*, AAA32936.1), Macadamia (*Macadamia integrifolia*, Q9SPL4.1), Theobroma (*Theobroma cacao*, Q43358.1), Zea (*Zea mays*, ACZ74248.1). When multiple VDCPs could be identified within a single protein sequence, the sequence closest to the N-terminal is shown without a numeric identifier and additional VDCPs are listed with numbers in ascending order. Amino acids that are identical in all alignments are indicated by a "*", those with similar biochemical properties are indicated by a ":" and semi-conservative substitutions are indicated by a "." below the alignment.

3.2. Generation of Transgenic Tobacco Plants That Express VDCPs from *C. virginianum*, *G. hirsutum* and *T. cacao*

Transgenic tobacco plants that contained VDCP coding sequences from *C. virginianum* (*CvAFP*1); *T. cacao* (*TcAFP*1); and *G. hirsutum* (*GhAFP*1) with or without signal peptides were generated. A total of 108 well-rooted, kanamycin-resistant tobacco plants including 20 pBI121 control transgenic lines were generated from individual calli, thus each line represents an independent, random transformation event. PCR assays were used to identify VDCP transformed plants. The transgenic tobacco plants grew normally: no differences were observed when compared to pBI121 or non-transformed controls.

VDCP mRNA was detected in 89% of the PCR positive transgenic lines at variable levels of expression

(**Figure 3**). In the *C. virginianum* constructs, *CvAFP*1 mRNA was detected in two pBI121-35S-CV1 lines and five pBI121-35S-CV2 lines. The highest level of expression was seen in the pBI121-CV2-4. Five pBI121-35S-GH1 lines and four pBI121-35S-GH2 lines expressed the *GhAFP*1 mRNA. The highest levels of expression were seen in transgenic lines pBI121-35S-GH1-4 and 13 and pBI121-35SGH2-7. All *Theobroma cacao* transgenic lines that we tested expressed the corresponding *TcAFP*1 RNAs and TC2-12 showed the highest level of expression. Cys sequences were not detected in any of the non-transformed or vector only control plants. The presence or absence of the signal peptide coding sequence did not appear to impact the level of VDCP mRNA in a predictable manner. We attribute the observed variation in expression between independently transformed lines to integration position effects. There was no obvious correlation between copy number and level of expression (data not shown).

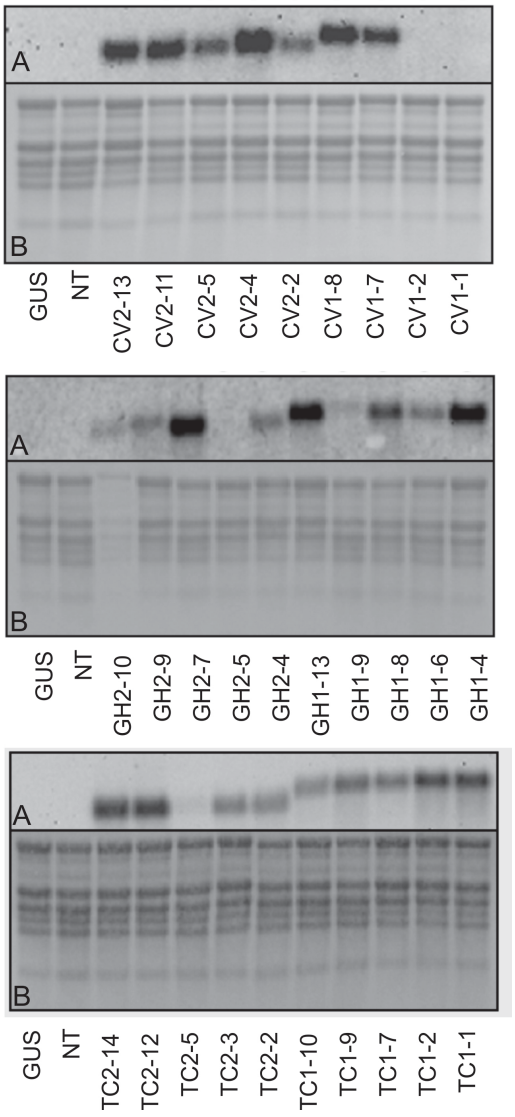

Figure 3. Northern blot analysis of transgenic tobacco lines to detect expression of VDCPs. Top Panel A: Total RNA from pBI121-35S-CV1, pBI-121-35S-CV2, GUS (pBI121control) transgenic lines and NT (non-transformed plants) probed with CV2. B: Methylene blue stained filter after transfer of the RNA to the filter. Middle Panel A: Total RNA from pBI121-35S-GH1, pBI-121-GH2, GUS (pBI121 control) transgenic lines and NT (non-transformed plants) probed with GH2. B: Methylene blue stained filter after transfer of the RNA to the filter. Bottom Panel A: Total RNA from pBI121- 35S-TC1, pBI-121-TC2, GUS (pBI121 control) transgenic lines and NT (non-transformed plants) probed with TC2. B: Methylene blue stained filter after transfer of the RNA to the filter.

3.3. Antifungal Activity of Extracts from Plants That Express VDCPs

A total of 27 *CvAFP*1 (CV1/2), *TcAFP*1 (TC1/2) and *GhAFP*1 (GH1/2) expressing transgenic tobacco lines were tested for antifungal activity (**Figure 4**). The number of fungal colonies arising from germinating conidia of *B. cinerea* after incubation with leaf extracts from 8 transgenic lines was significantly reduced ($p < 0.05$,

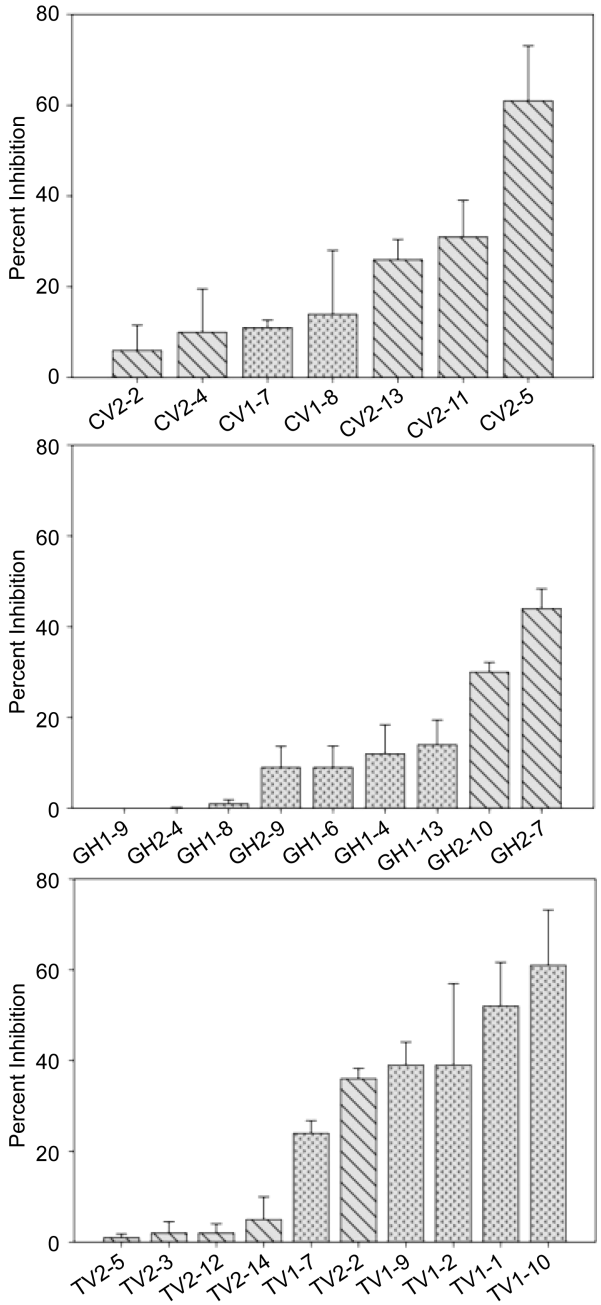

Figure 4. Inhibition of *Botrytis cinerea* spore germination by leaf extracts of plants that express VDCPs. Transgenic tobacco plants that express the GUS protein (pBI121 transgenics) were used for comparison. The percent inhibition was calculated as the percent inhibited compared to the control. The top panel shows the results using leaf extracts from plants transformed with *C. virginianum* CV-1 or CV-2, the middle panel shows the inhibition observed using leaf extracts from plants transformed with *G. hirsutum* GH-1 or GH-2 and the bottom panel shows the inhibition observed with leaf extracts from plants transformed with *T. cacao* TC-1 or TC-2. The error bar is the standard error, n = 3.

Dunnett test) after one-hour co-incubation at 28˚C. Two pBI121-35S-CV1 expressing lines (no leader sequence) showed ≤20% inhibition compared to the controls. However, two *CvAFP*1 lines, pBI121-35S-CV2-5 and pBI121-35S-CV2-11 showed significant inhibition of 61% ($p < 0.01$, Dunnett test) and 31% respectively.

Of the five pBI121-35S-GH1 lines tested (with leader peptide), pBI121-35SGH1-13 exhibited the highest level of activity: 20% inhibition of *B. cinerea* spore growth. Two GhAFP2 lines, pBI121-35SGH2-7 and 10 showed 44% ($p < 0.05$, Dunnett test) and 30% inhibition, respectively. Five TC1 lines and one TC2 line exhibited between 24% to 61% inhibition. This includes transgenic lines expressing the leaderless *T. cacao* VDCP sequence (pBI121-35S-TC1-1, pBI121-35S-TC1-2, pBI121-35S-TC1-7, pBI121-35S-TC1-9 and pBI121-35S-TC1-10) and one signal peptide plus line (pBI121-35S-TC2-2). The highest level of inhibition was observed for the *TcAFP*1 transgenic lines pBI121-35S-TC1-1 and pBI121-35S-TC1-10, in which 52% and 61% inhibition was observed ($p < 0.01$, Dunnett test). The pBI121-35S-TC2-2 transgenic line showed 36% inhibition, which is significant ($p < 0.05$) compared to the control.

3.4. Expression of VDCP Fusion Protein in *E. coli*

Control (MBP) and test proteins (MBP-Cys fusion proteins) were expressed and purified from and SHuffle *E. coli* cells. Protein yields were highest in extracts from the *E. coli* K12 TB1—MBP construct—over 174 mg/L (**Table 2**). Crude protein yields of MBP expressed in *E. coli Shuffle* and of the MBP-$Cys_{Repeat\ 1}$ expressed in *E. coli* K12 TB1 or *E. coli* Shuffle cells were similar. Optimal protein binding to the amylose resin was achieved by overnight batch incubation in binding buffer. After washing the resin with binding buffer, specifically bound proteins were eluted with binding buffer containing 10 mM maltose. Fractions were analyzed on 12% SDS denaturing polyacrylamide gels [27] to determine the molecular weights of the constituents and the purity of the fractions (**Figure 5**). Proteins that correspond to the predicted molecular weights for MBP or the MBP-$Cys_{Repeat\ 1}$ proteins were observed in the induced and eluted fractions.

We observed two major differences in the amount of purified protein recovered that depended on the construct and the cell line used for expression. First, we consistently recovered less MBP than MBP-$Cys_{Repeat1}$ fusion protein from either *E. coli* K12 TB1 cells or SHuffle cells. The yield was between 3 and 4.5 times more for the fusion protein than MBP. Secondly, we recovered more purified expressed protein per liter from induced K12 TB1 cell extracts: the yield of recovered fusion protein was 1.8 fold higher and the yield of MBP was 1.2 fold higher than the amount of protein recovered from SHuffle cells.

3.5. Antifungal Activity of Bacterially Expressed VDCP Fusion Protein

Antifungal activity was determined by comparing the growth of test fungi in the presence of MBP-fusion protein to the growth of fungus in the presence of MBP alone (**Figure 6**). After 24 hours co-incubation, the single Cys-pattern bearing peptide from *Centrosema virginianum* exhibited antifungal activity against three of the four fungi tested. The raw optical density values used to calculate the percent inhibition of the test fungi were statistically analyzed (Heteroscedastic T test with one-tailed distribution). The difference in optical density observed between the Cys-fusion MBP and MBP proteins was statistically significant ($p < 0.05$) at the two higher test concentrations of protein expressed in either TB-1 cells or SHuffle cells.

Table 2. Purification of Fusion Proteins by Affinity Chromatography. Percentage yield was calculated as the amount of protein recovered divided by the amount of input protein multiplied by 100.

	Protein (mg) recovered per liter bacterial cells			
	Input	Non-bound	Eluted	% Yield
Expressed in TB1 *E. coli*				
MBP	81.8 ± 4.8	66.52 ± 4.72	0.98 ± 0.02	1.2 ± 0.05
MBP CysRepeat1	174.45 ± 0.45	123.54 ± 1.74	9.48 ± 0.4	5.43 ± 0.21
Expressed in SHuffle *E. coli*				
MBP	84.4 ± 3.1	67.54 ± 1.42	0.85 ± 0.04	1.01 ± 0.01
MBP CysRepeat1	81.25 ± 22	64.12 ± 16.2	2.51 ± 1.01	2.97 ± 0.44

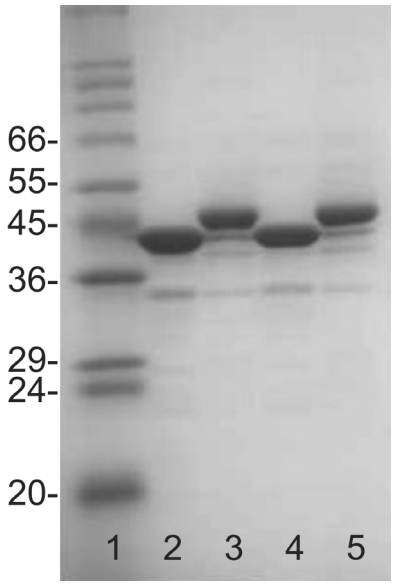

Figure 5. Purification of MBP and MBP-VDCP fusion proteins expressed in *E. coli*. Lane 1: molecular weight markers, Lane 2: MBP purified from *E. coli* TB-1 cells, Lane 3: MBP-VDCP fusion purified from *E. coli* TB-1 cells, Lane 4: MBP purified from *E. coli* SHuffle cells, Lane 5: MBP-VDCP fusion purified from *E. coli* SHuffle cells.

At 4.2 μM, statistical analysis of the optical density values observed in the *B. cinerea* assays were significant for the fusion protein expressed in SHuffle cells ($p < 0.05$), but not for fusion protein expressed in TB1 cells. In contrast, analysis of the *F. verticuloides* optical densites observed in the 4.2 mM assays showed that the differences observed were statistically significant for the fusion expressed in TB1 cells but not SHuffle cells. At 4.2 μM, comparison of the optical density values for *R. solani* were not statistically significant at the $p < 0.05$ confidence level.

We calculated the percent inhibition of fungal growth (**Figure 6**). Growth of *B. cinerea* was inhibited by at least 40% in all test concentrations of the purified peptide-MBP fusion expressed in Shuffle cells (4.23, 12.68 and 21.14 μM). The purified MBP-$Cys_{Repeat1}$ protein expressed in K12 TB1 cells was less potent and exhibited less than 18% inhibition at 4.2 μM and more than 65% inhibition at 12.68 μM and almost completely inhibited the growth of *B. cinerea* at 21.14 μM.

The fusion protein expressed in SHuffle cells more effectively inhibited the growth of *B. cinerea* than the fusion protein expressed in TB1 cells. Fungal growth was completely inhibited by addition of 12.68 and 21.14 μM MBP-$Cys_{Repeat1}$ protein expressed in SHuffle. The effect was dramatic in that the optical density of the well was less than that observed for the media alone. For our plate-based assay analysis, the background absorbance of the media was subtracted from the absorbance readings prior to calculation of the percent inhibition to account for lot to lot variation in the growth medium. In the *B. cinerea* replicate assays, when normalized values were used to calculate percent inhibition, the percent inhibition exceeded 100% due to the total clearing of the media at the higher test concentrations. This phenomenon was observed in all of our *B. cinerea* assays using 12.68 and 21.14 μM MBP-CysRepeat1 protein expressed in SHuffle.

Fusarium verticulloides was inhibited by purified MBP-$Cys_{Repeat1}$ expressed in K12 TB1 at 4.23 μM, 12.68 μM and 21.14 μM. At 4.23 μM the amount of inhibition of growth is approximately the same for the fusion protein expressed in either K12 TB1 or SHuffle cells. However, fusion protein expressed in SHuffle *E. coli* showed enhanced inhibition compared to the same amount of fusion protein expressed in TB1 cells at the two higher concentrations. The highest mean maximal inhibition values for the fusion protein's effect on *F. verticulloides* were ~60% for the TB1 expressed and ~90% for the SHuffle expressed fusion.

Although a small amount of growth inhibition of *R. solani* was observed in the reactions with 4.23 μM fusion protein (from either TB1 or SHuffle), variation in the data was high so it is difficult to quantify the effect. However, growth of *R. solani* was clearly inhibited by 12.68 μM (50% TB1, 85% SHuffle) and 21.14 μM MBP-$Cys_{Repeat1}$ fusion protein (75% TB1 and 90% SHuffle).

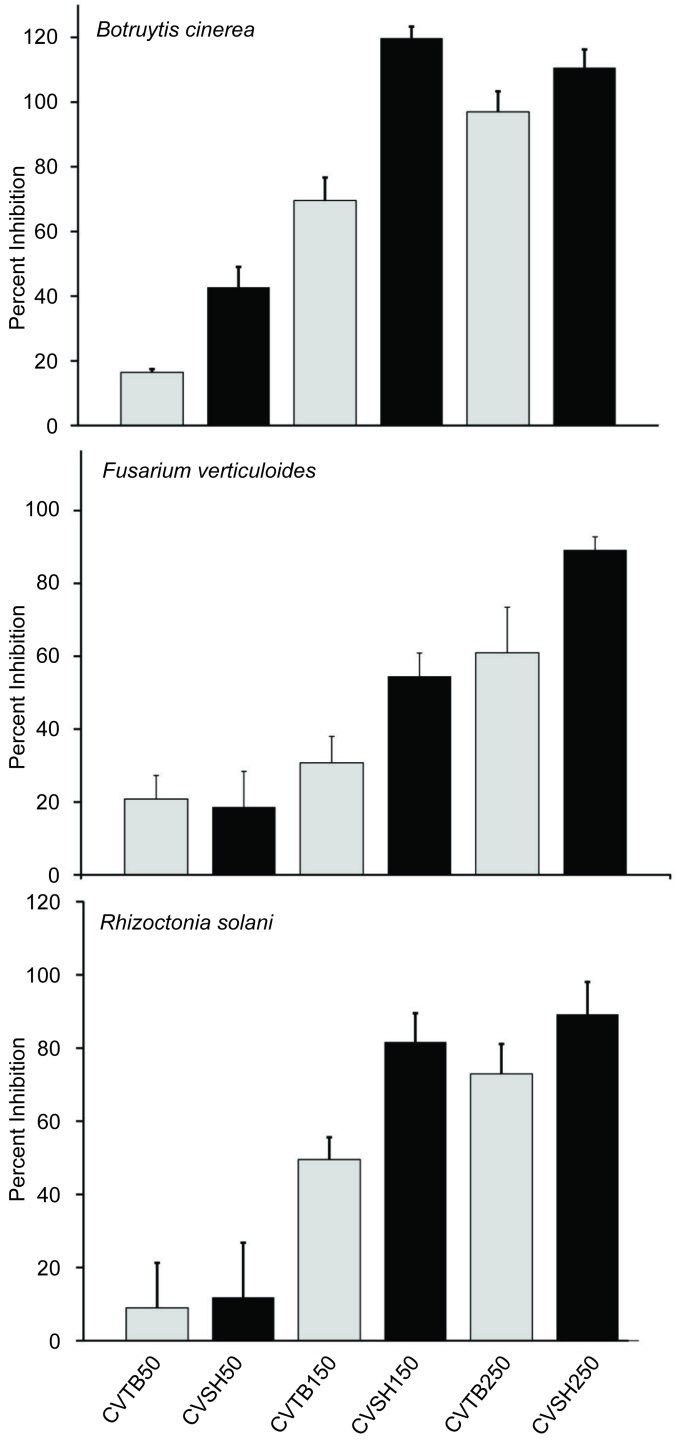

Figure 6. Inhibition of the growth of *B. cinerea* (top panel), *F. verticuloides* (middle panel) and *R. solani* (bottom panel) after 24 hours incubation with different amounts of MBP-VDCP fusion protein expressed in TB-1 cells (white bars) or SHuffle cells (black bars). CVTB50 and CVSH50 values correspond to 4.23 μM VDCP, CVTB150 and CVSH150 values correspond to 12.68 μM VDCP and CVTB250 and CVSH250 correspond to 21.14 μM VDCP. The Y-axis is percent inhibition. The optical density observed with MBP was compared to that observed with the MBP-VDCP fusion protein. Error bars show the standard error, n = 3.

4. Discussion

Seed storage proteins are synthesized during embryogenesis and deposited in protein bodies within the developing cotyledons of dicotyledonous plants. It is estimated that seed storage proteins can account for up to 70% of the total protein in seeds. During germination, the seed storage proteins are degraded and processed to supply essential nutrients (nitrogen and amino acids) for germinating seeds and developing seedlings.

Analysis of vicilin genes from *G. hirsutum* showed the presence of a unique repeat that contained Cysteine residues [12]. Similar Cys-repeat motifs were identified in some other plant vicilins [14] and all of the characterized plant proteins that contain the Cys-pattern motifs were either vicilins or vicilin-like proteins. We have found other proteins that are either uncharacterized or classified as vicilin-like proteins that share similar Cys-pattern motifs. The number of VDCP repeats varies greatly between species, but is consistent within species. Previously, the antimicrobial role of the Cys-pattern has been demonstrated in *M. integrifolia* and *Z. mays* [15] [16]. Because seeds germinate in warm, moist environments, antimicrobial peptides associated with seed storage protein degradation products provide protection from infection with fungal and bacterial pathogens. To date, peptides with the same Cys-repeat pattern have been identified in diverse plant species. The peptides from other plants with the same Cys-pattern are predicted to have antimicrobial activity based on the sequence similarity with the characterized peptides demonstrated to be an AMP.

We identified and cloned a new one Cys-pattern VDCP sequence, *CvAFP*1, from a wild Louisiana legume *C. virginianum.* We compared the antimicrobial activity of leaf extracts from plants that expressed this motif with antimicrobial activity of leaf extracts from plants that express either the double Cys-pattern *TcAFP*1 from *T. cacao* [17] or the triple Cys-pattern *GhAFP*1 coding sequences from *G. hirsutum* vicilin [16]. Constitutive expression of the three different VDCP coding sequences in transgenic tobacco plants inhibited germinating conidia of *B. cinerea.* The degree of inhibition was not dependent upon the number of Cys-repeats: the greatest inhibition was observed in single and double VDCP transgenic tobacco lines. The VDCP sequences for all of the repeats differed in the amino acid composition between the conserved residues. This may be responsible for the observed differences in activity. It is also possible that the differences in antifungal activity observed in our studies are associated with different integration locations as *Agrobacterium*-mediated transformation is not site specific. Indirect evidence suggests that post-transcriptional regulation may also impact expression of the VDCP sequences as some transgenic lines with the highest RNA expression levels (ie CV2-4) do not exhibit high levels of antifungal activity.

VDCPs are located at the N-terminus of vicilin precursors between the vicilin signal peptide and the first cupin-2 domain. To understand whether a signal peptide is essential to produce a biologically active form of the VDCP, we constructed transgenic tobacco lines that express VDCPs either with or without a corresponding vicilin signal peptide. Presumably, VDCPs with the vicilin signal peptide sequence would be delivered to the endoplasmic reticulum and subsequently transported to other intracellular organelles for further modification and storage. Activity assays of constructs with or without the signal sequence provide indirect evidence that targeting of the VDCP was not required for antifungal activity.

To obtain enough VDCP from *Centrosema virginianum* to test activity against a panel of fungi, we expressed the peptide in bacteria. We opted to express the predicted VDCP as a fusion protein because previous efforts in our lab to express and purify active, non-degraded, *Centrosema virginianum* VDCP in *E. coli* TB-1 cells were unsuccessful. Since the Cys residues in the VDCP have the potential to form disulfide bonds which could stabilize the structure of the peptide, we expressed the fusion protein in *E. coli* TB-1 cells (standard reducing cytoplasmic environment) and in *E. coli* SHuffle cells (a mutant line with an oxidizing environment). In both cases, we were able to express and purify a stable fusion protein that was tested for antimicrobial activity. The IC_{50} values for the *C. virginianum* VDCP fusion expressed in both cell lines were comparable to values for other plant-derived antimicrobial peptides (**Table 3**).

Botrytis cinerea is most susceptible to the *C. virginianum* VDCP fusion protein. This supports our findings with transgenic plant leaf extracts that showed high levels of inhibition in several of the transgenic lines. Calculated IC_{50} values for the *C. virginianum* fusion VDCP expressed in Shuffle *E. coli* was 0.6 times of that for the fusion protein expressed in *E. coli* K12 TB1 cells. Since the IC_{50} values associated with peptides isolated from *E. coli* cells with an oxidizing cytoplasmic environment (favors disulfide bond formation) are lower than those for peptides isolated from *E. coli* cells with a with reducing cytoplasmic environment, the formation of disulfide bonds enhances the antimicrobial activity. This could be due to formation of a secondary structure that either enhances antimicrobial activity or increased peptide stability.

Table 3. Comparison of IC50 Values of Representative Plant Antimicrobial Peptides The peptides, Rs-AFPs [31] [32], *Ac*-AMPs [33], *Mj*-AMPs [30], Ah-AMP1, Ct-AMP1, Dm-AMPs, Hs-AFP1, SIα1 [34], Ib-AMPs [35], MiAMP1 [36], MiAMP2c [15] and $Cys_{Repeat1}$ were isolated from *Raphanus sativus*, *maranthus caudatus*, *Mirabilis jalapa*, *Aesculus hippocastanum*, *Clitora ternatea*, *Dahlia merckii*, *Heuchera sangiunea*, *Sorghum bicolor*, *Impatiens balsamina*, *Macademia integrifolia*, *Theobroma cacao*, *Centrosema virginianum* respectively. The microbial inhibition by MBP-$Cys_{Repeat1}$ fusion protein was estimated after incubating the fungi and the protein for 24 hrs. The concentrations (μM) of only the $Cys_{Repeat1}$ peptide was used in this calculation to get a direct comparison of its IC_{50} values with that of the other peptide. N.A inhibition is not available.

Fungus	Antifungal peptide	IC50 (μM)
Botrytis cinerea	*Rs*-AFPs	0.4 to 1.8
	Ac-AMPs	2.58 to 3.22
	Mj-AMPs	0.5 to 15
	Ah-AMP1	5
	Ct-AMP1	4
	Dm-AMPs	2 to 2.4
	Hs-AFP1	1.2
	SIa1	20
	Ib-AMPs	2.39 to 9.95
	MiAMP1	0.6 to 1.23
	CysRepeat1 (reducing environment)	0.78
	CysRepeat1 (oxidizing environment)	0.49
Fusarium sp.	*Rs*-AFPs	0.4 to 6
	Ac-AMPs	0.64
	Ah-AMP1	2.4
	Ct-AMP1	2
	Dm-AMPs	0.6 to 1
	Hs-AFP1	0.2
	Cotton basic protein	0.6
	Ib-AMPs	0.4 to 2.39
	MiAMP1	0.25 to 0.6
	MiAMP2c	1.66
	CysRepeat1 (reducing environment)	1.65
	CysRepeat1 (oxidizing environment)	1.02
Rhizoctonia solani	Rs-AFPs	>20
	Mj-AMPs	3.75 to 15
	CysRepeat1 (reducing environment)	1.36
	CysRepeat1 (oxidizing environment)	0.8
Verticillium sp.	Rs-A F P s	0.3 to 1
	Ac-AMPs	1.93 to 2.58
	Mj-AMPs	0.13 to 3
	Ah-AMP1	1.2
	Ct-AMP1	0.4
	Dm-AMPs	0.4 to 0.8
	Hs-AFP1	2.4
	Ib-AMPs	1.19 to 4.78
	MiAMP1	0.25
	MiAMP2c	0.83 to 1.66
	CysRepeat1 (reducing environment)	N.A
	CysRepeat1 (oxidizing environment)	N.A

The Cys-pattern bearing peptide from *C. virginianum* exhibited antimicrobial activity against two major phyla of fungi: the Basidiomycota (*R. solani*) and the Ascomycota (*B. cinerea* and *F. verticilloides*). The fungi in the Basidiomycota phylum have no asexual spores and generally invade the host by the formation of multinucleate mycelia. Members of the phylum Ascomycota propagate by asexual spores (conidia) on specialized branches called conidiophores. The fact that the peptide inhibited fungal growth from both of these phyla implies that the peptide has activity on both the mycelia and spore formation of the fungi. The vegetative propagation of mycelial fungi is very fast. That may explain why the growth of *R. solani* was faster than that of *B. cinerea* or *F. verticilloides* and exceeded the linear range of the spectrophotometric optical density by 36 hr (data not shown).

The fungus *F. verticilloides* belongs to the class Sordariomycetes, which produces spores in a perithecal fruiting body made up of hard tissue. *B. cinerea* belongs to the class Lectiomycetes which produces spores in apothecial or cleistothecal fruiting bodies with fleshy, soft walls of tissue. The ease of penetration of the putative AMP molecule through the soft apothecial wall in contrast to hard perithecal layer may be the reason why *B. cinerea* is more susceptible to the Cys-pattern bearing peptide from *C. virginianum* compared to *R. solani* or *F. verticilloides*.

We did not observe inhibition of *Verticillium dahliae* fungal growth when incubated with the Cys-pattern containing peptide expressed in either oxidizing or reducing environments. Other plant AMPs, including some that contain the unique Cys-pattern [15] [36], have shown inhibition of *V. dahliae* growth. The Cys-pattern bearing peptide from *C. virginianum* is different from other characterized peptides demonstrated to have antimicrobial activity on *V. dahlia*. The amino acids surrounding the four Cysteine residues of the unique pattern differ. All four Cys-pattern motifs of MiAMP2 family peptides from *M. integrifolia* have glutamine residues between the four Cysteine residues but the *C. virginianum* Cys-pattern motif does not. Instead, there is a single asparagine residue between the Cysteine residues in the second part of the repeat and no glutamine or asparagine in the first repeat. In the eleven amino acid spacer between the two half-Cys repeats, there are two to five asparagine or glutamine residues in the Cys-pattern in *Macadamia integrifolia*. In *Centrosema virginianum* there are two asparagine residues. The *Centrosema virginianum* Cys-pattern repeat has fewer charged amino acids following the second half repeat (five out of ten compared to seven out of ten, nine out of ten and six out of eight) compared to the *M. integrifolia* Cys-pattern repeat. These differences may account for the observed variability in activity against different fungi. After 24 hr, observed inhibition declined probably due to degradation of the VDCP thus enabling enhanced fungal growth. Under the conditions used for this study, the VDCP fusions are stable in buffer based on 12% SDS denaturing polyacrylamide gels, however, fungal proteases could have degraded the proteins during the time course of the assay.

Our studies of the vicilin-derived peptide from *C. virginianum* further support the role of vicilins as the source of nutrition for the developing plant and seedling, and as a source of antimicrobial peptides. The vicilin-derived antimicrobial peptides help protect the germinating plant from microbes abundant in the moist, nutrition-rich soil. It is interesting to note that these unique VDCP are not characteristic of all vicilins or vicilin-like proteins nor are they always found in closely related species. In the Phaseoleae tribe of legumes, we identified the predicted Cys peptide sequence in *G. max* and *C. virginianum* but not in *P. vulgaris*, *V. luteola*, *V. radiata*, *V. angluaris*, *C. ensiformis* or *G. soja*. In the Fabaeae tribe, a Cys peptide sequence occurs in vicilin-like sucrose binding protein sequences of *P. sativum* and *V. faba* but cannot be identified in *V. narbonensis* or *L. culinaris* vicilins. In contrast, the Cys peptide occurs more consistently in vicilins of members of the Malvaceae. It is present in four species of *Gossypium* (*hirsutum*, *arboretum*, *raimondii* and *herbaceum*) and is present in *T. cacao* vicilin. As more full-length vicilin sequences become available, it will be possible to track the occurrence of this unique sequence which may provide general insights into the evolution of multifunctional seed storage proteins and facilitate elucidation of the origin and evolution of the unique Cys peptide sequence.

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