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***Appl. Environ. Microbiol.* 2007, 73(3):956. DOI: 10.1128/AEM.02079-06.**
Published Ahead of Print 22 November 2006.

Applied and Environmental Microbiology, Feb. 2007, p. 956–961 Vol. 73, No. 3
0099-2240/07/$08.00+0 doi:10.1128/AEM.02079-06

Characterization of Chimeric *Bacillus thuringiensis* Vip3 Toxins[▽]

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Received 3 September 2006/Accepted 13 November 2006

***Bacillus thuringiensis* vegetative insecticidal proteins (Vip) are potential alternatives for *B. thuringiensis* endotoxins that are currently utilized in commercial transgenic insect-resistant crops. Screening a large number of *B. thuringiensis* isolates resulted in the cloning of *vip3Ac1*. Vip3Ac1 showed high insecticidal activity against the fall armyworm *Spodoptera frugiperda* and the cotton bollworm *Helicoverpa zea* but very low activity against the silkworm *Bombyx mori*. The host specificity of this Vip3 toxin was altered by sequence swapping with a previously identified toxin, Vip3Aa1. While both Vip3Aa1 and Vip3Ac1 showed no detectable toxicity against the European corn borer *Ostrinia nubilalis*, the chimeric protein Vip3AcAa, consisting of the N-terminal region of Vip3Ac1 and the C-terminal region of Vip3Aa1, became insecticidal to the European corn borer. In addition, the chimeric Vip3AcAa had increased toxicity to the fall armyworm. Furthermore, both Vip3Ac1 and Vip3AcAa are highly insecticidal to a strain of cabbage looper (*Trichoplusia ni*) that is highly resistant to the *B. thuringiensis* endotoxin Cry1Ac, thus experimentally showing for the first time the lack of cross-resistance between *B. thuringiensis* Cry1A proteins and Vip3A toxins. The results in this study demonstrated that *vip3Ac1* and its chimeric *vip3* genes can be excellent candidates for engineering a new generation of transgenic plants for insect pest control.**

Insecticidal *Bacillus thuringiensis* endotoxins have been extensively explored for biological control of insect pests. To date, over 350 *B. thuringiensis* endotoxins have been identified (3, 6, 7) (N. Crickmore, D. R. Zeigler, E. Schnepf, J. Van Rie, D. Lereclus, J. Baum, A. Bravo, and D. H. Dean, *Bacillus thuringiensis* toxin nomenclature, 2006, http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/), and some of their genes have been used to generate commercial transgenic insect-resistant crops (15, 16, 27). However, the prolonged application of *B. thuringiensis* formulations and the widespread planting of transgenic crops with *B. thuringiensis* endotoxins have raised concerns over the development of insect resistance (1, 12, 13, 17, 23, 24, 25, 26). Thus, alternative insecticidal proteins that do not share a mode of action identical with that of *B. thuringiensis* endotoxins are highly desirable for the development of the next generation of transgenic insect-resistant crops.

In addition to the endotoxins, *B. thuringiensis* also produces secreted insecticidal proteins during its vegetative growth stage, namely, vegetative insecticidal proteins (Vip). Since the discovery of the first Vip toxin, two major groups of Vip toxins have been identified in *B. thuringiensis*. One group of Vip toxins consists of binary toxins which are made of two components, Vip1 and Vip2 (27). The combination of Vip1 and Vip2 is highly insecticidal to an agriculturally important insect, the western corn rootworm (*Diabrotica virgifera*), but does not show any insecticidal activity for any lepidopteran insects (14). The other group consists of Vip3 toxins, which share no sequence similarity to Vip1 or Vip2. The first-identified Vip3 toxin, Vip3Aa1, is highly insecticidal to several major lepidopteran pests of maize and cotton, including the fall armyworm *Spodoptera frugiperda* and the cotton bollworm *Helicoverpa zea*, but shows no activity against the European corn borer *Ostrinia nubilalis*, a major pest of maize (11). The deletion of the *vip3Aa1* gene from a *B. thuringiensis* strain resulted in a significant reduction of the insecticidal activity of that *B. thuringiensis* strain, suggesting that Vip3 contributes to the overall toxicity of *B. thuringiensis* strains (9). It was also observed that Vip3Aa1 kills insects by lysing insect midgut cells (28) via cell membrane pore formation (18).

However, the potential of Vip3 toxins for insect control has not been extensively explored. At present, the understanding of the diversity of the Vip3 toxins is very limited. Compared to over 300 *B. thuringiensis* crystal toxin genes cloned, only a dozen or so *vip3* genes have been cloned and characterized previously (20, 23; Crickmore et al., http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/), and many of these known Vip3 toxins have insecticidal activities similar to that of Vip3Aa1 (2, 4, 10). Enriching the diversity of available *vip3* genes will likely broaden the spectrum of activity of the Vip3 family and thus facilitate the application for control of various insect pests.

In this study, we used a PCR-based screening procedure to screen our collection of *B. thuringiensis* isolates for *vip3* genes. A new *vip3* gene was cloned, and chimeric genes were created by sequence swapping with a previously known *vip3* gene, *vip3Aa1*. We found that one chimeric Vip3 toxin gained novel properties of insecticidal activity. Further-

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▽ Published ahead of print on 22 November 2006.

more, we observed that the Vip3A toxins are highly active against a *B. thuringiensis*-resistant strain of *Trichoplusia ni*.

MATERIALS AND METHODS

PCR screening of *B. thuringiensis* isolates. *B. thuringiensis* isolates were cultured overnight at 37°C in Terrific broth medium in 24-well cell culture plates without shaking. One microliter of the overnight culture of each strain was used as the template for PCR analysis with two pairs of primers in a single reaction in 96-well PCR plates. The two primer pairs (V1F-V1R and V2F-V2R) were designed based on the *vip3Aa1* sequence (GenBank accession no. L48811) to amplify two DNA fragments of a *vip3* gene corresponding to nucleotide positions from 54 to 498 and from 2006 to 2370 of *vip3Aa1*. Analysis of the previously cloned novel *vip3* genes showed that the fragment from nucleotide 54 to 498 is well conserved whereas the fragment from nucleotide 2006 to 2370 is much less conserved (20, 23). The primers used were V1F (5′-TTATTTTAATGGCATT TATGGATTTGCC), V1R (5′-GCAGGTGTAATTTCAGTAAGTGTAGAG), V2F (5′-CTTCTGAAAAGTTATTAAGTCCAGAAT), and V2R (5′-TTACTT AATAGAGACATCGTAAAAA). The PCR products were examined by agarose gel electrophoresis. The successful amplification of two DNA fragments 444 and 364 bp in size, respectively, from a *B. thuringiensis* strain indicates the presence of a *vip3* gene that is likely highly similar to *vip3Aa1*, while strains showing only one DNA fragment in the PCR screening more likely carry novel *vip3* genes.

Cloning of *vip3Ac1*. Plasmids from *B. thuringiensis* isolate LG13, which was identified by PCR screening as carrying a putative novel *vip* gene, were isolated using the method described by Reddy et al. (21) and were partially digested with Sau3A. The digested DNAs were separated by 0.7% agarose gel electrophoresis, and fragments of approximately 1 to 3 kb were recovered from the gel with a DNA isolation kit from Sangon (Shanghai, China). The DNA fragments recovered were then treated with Klenow DNA polymerase to blunt the ends followed by a *Taq* polymerase reaction with 0.2 mM deoxynucleoside triphosphates at 72°C for 20 min to add an overhung A residue to their 3′ ends for subsequent ligation into the T vector (Sangon, Shanghai) to generate a minilibrary.

About 300 colonies of the minilibrary were screened by PCR using two pairs of primers. One primer pair (V1F-V1R) was the same one as described above, and the other primer pair was V3F-V3R (V3F, 5′-GATTCTTCTACAGGAGA AATTGACTTAA; V3R, 5′-CACTCCGCCTGTATGATCTACATAC), which, like V3F-V3R, was designed based on the *vip3Aa1* sequence, although in this case the primer pair was designed to amplify a DNA fragment from nucleotide 1330 to 1690. Both pairs of primers are located in conserved regions of *vip3* genes. Two clones from the minilibrary were selected as representing positive results by PCR amplification of both pairs of primers. The DNA sequences of these two clones indicate that one clone contained the 5′ end of a *vip3* gene and the other contained the 3′ end. According to the sequences of these two clones, we designed a pair of primers to amplify the complete open reading frame of the *vip3* gene. One primer was designated Vip3Ac1-BglII (5′-GGCTAGATCTATG AATAATACTAAATTAAAC), and the other was designated Vip3Ac1-XhoI (5′-GGCTCTCGAGTTACTTAATTGAAAAATCTCGGAAA) (the restriction sites are underlined). *Pfu* DNA polymerase was used for the PCR for high-fidelity amplification. The PCR product was digested with BglII and XhoI and cloned into the vector pET28a digested with BamHI and XhoI (BamHI and BglII are compatible). The full-length sequence was then obtained by sequencing both strands of the DNA.

Cloning of *vip3Aa1*. The *vip3Aa1* gene was amplified from *B. thuringiensis* isolate LG01 by use of two primers designed from the *vip3Aa1* sequence (GenBank accession no. L48811) and anchored with a BglII and an XhoI restriction digestion site, respectively (Vip3Aa1-BglII, 5′-GGCTAGATCTATGAACAAGAATAATACTA AATTAAGC; Vip3Aa1-XhoI, 5′-TCCGCTCGAGTTATACTAATAGAGACAT CGTAAAA) (the restriction sites are underlined). The PCR product was digested with both BglII and XhoI and then cloned into pET28a predigested with BamHI and XhoI. The identity of the cloned *vip3Aa1* was confirmed by DNA sequencing.

Construction of chimeric genes of *vip3Aa1* and *vip3Ac1*. An overlap PCR method was used to generate the chimeric genes of *vip3Aa1* and *vip3Ac1*. To generate the chimeric gene *vip3AaAc*, two overlapping DNA fragments were amplified by PCR. One was the 1.8-kb fragment of the 5′-end portion of *vip3Aa1*, amplified using primers Vip3Aa1-BglII (5′-GGCTAGATCTATGAACAAGAA TAATACTAAATTAAGC) and V3Aa1834R (5′-TTCTCATTTTTTTATCTTT AAATGAATAGAAGG), and the other was the 0.5-kb fragment of the 3′-end portion of the *vip3Ac1*, amplified with primers Vip3Ac1-XhoI (5′-GGCTCTCG AGTTACTTAATTGAAAAATCTCGGAAA) and Vip3Ac1810F (5′-CTTCTA TTCATTTAAAAGATAAAAAAAATGAGAA). The above-named two overlapping PCR products were gel purified and mixed as the template for the second PCR to generate the full-length hybrid gene with primers Vip3Aa1-BglII and Vip3Ac1-XhoI.

The same strategy was used to generate the chimeric gene *vip3AcAa*. The primers used for PCR amplification of the 5′-end portion of *vip3Ac1* were Vip3Ac1-BglII (5′-GGCTAGATCTATGAATAATACTAAATTAAAC) and V3Ac1834R (5′-CC AGTATTTTCATCTTTTAAATAAATAGAAG), and the primers for amplification of the 3′-end fragment of *vip3Aa1* were Vip3Aa1-XhoI (5′-TCCGCTCGAG TTATACTAATAGAGACATCGTAAAA) and Vip3Aa1810F (5′-GGAAAA GCTTCTATTTATTTAAAAGATGAAAATAC). The full-length chimeric gene *vip3AcAa* was generated by PCR using the mixture of the above-named two PCR products as the template and V3Ac1-BglII and Vip3Aa1-XhoI as the primers.

The PCR products of the chimeric genes *vip3AaAc* and *vip3AcAa* were purified after agarose gel electrophoresis and then digested with BglII and XhoI. The digested PCR products were subsequently ligated into pET28a digested with BamHI and XhoI. The constructs were confirmed by sequencing. Vip3AaAc is a chimeric protein consisting of the N-terminal 610 amino acid residues of Vip3Aa1 and the C-terminal 179 amino acid residues of Vip3Ac1, while Vip3AcAa is a chimeric protein consisting of the N-terminal 600 amino acid residues of Vip3Ac1 and the C-terminal 189 amino acid residues of Vip3Aa1.

Expression of Vip3 proteins. The plasmid constructs based on expression vector pET28a were transformed into *Escherichia coli* strain BL21 Star (Stratagene) for expression of the Vip3 proteins. A single colony was picked for starting the culture by use of LB medium at 250 rpm in a shaking incubator at 37°C. A final concentration of 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to induce expression when the LB culture just began to become turbid, with an optical density of about 0.6 at 600 nm. After 3 h of induction at 37°C, the *E. coli* cells were collected by centrifugation for 10 min at 3,000 × *g* and resuspended in 20 mM Tris-HCl buffer (pH 7.5) and then sonicated for bioassay for insecticidal activities. The total sonicated cell lysate of each sample was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, and the Vip3 proteins in the samples were densitometrically quantified in gel using a VersaDoc imaging system and Quantity One image analysis software from Bio-Rad Laboratories (Hercules). A series of concentrations of bovine serum albumin (0.1, 0.2, 0.4, 0.8, 1.0, and 2.0 μg per lane) was used as the standards.

Bioassays for insecticidal activities. For the insecticidal activity assays against European corn borer *O. nubilalis*, fall armyworm *S. frugiperda*, cotton bollworm *H. zea*, and silkworm *B. mori*, an aliquot of a 50-μl sample of Vip3 protein was spread on the surface of an artificial diet prepared in 24-well plates. A preparation from the culture of *E. coli* strain BL21 Star without an insecticidal *vip* gene was used as the negative control. After the samples on the diet surfaces were air dried on the rockers for 2 to 3 h at room temperature, neonate larvae were transferred into the wells and then covered with a permeable Breathe-Easy film (E&K Scientific Products). Gradient concentrations of each expressed protein were used for determining the 50% lethal concentration (LC_{50}) of a Vip3 toxin for a given insect species. Two wells with five insects each were set up for each concentration. The assays were repeated three times. Larval mortality was recorded on day 7 after each assay started.

A *T. ni* strain resistant to *B. thuringiensis* endotoxin Cry1Ac and its near-isogenic susceptible strain were used to test their susceptibilities to the Vip3 proteins following the methods described by Kain et al. (17). Briefly, five to six concentrations of each toxin and five cups (replications) for each concentration were included in each bioassay. An aliquot of 0.2 ml of toxin solutions of different concentrations was applied evenly over the diet surface (surface area, ~7 cm^2) of 30-ml plastic cups with 5 ml of a high-wheat-germ diet. Larval growth inhibition (defined as inhibition when larvae did not develop into a second instar within 4 days of culture) was scored after 4 days of assay. The 50% inhibitory concentration (IC_{50}) is the concentration of toxin that caused growth inhibition of 50% of the assay neonates and was calculated based on probit analysis using the statistical software POLO as described by Kain et al. (17). Most of the neonates that did not reach the second instar after 4 days eventually died before pupation.

Nucleotide sequence accession number. The full-length *vip3Ac1* sequence was deposited in GenBank under accession number DQ054848.

RESULTS

Cloning of *vip3Ac1*. Over 300 *B. thuringiensis* isolates were screened by PCR using the two pairs of primers that enable amplification of two DNA fragments (444 bp and 364 bp) of the first-discovered and also the most frequently identified *vip*

TABLE 1. Insecticidal activities of Vip3 proteins to the neonates of fall armyworm, cotton bollworm and silk worm[a]

Vip3 tested and insect species[b]	Gradient doses (ng/cm^2)	Probit analysis				LC_{50} (range) with 95% fiducial limits (ng/cm^2)
		Slope (SE)	χ^2	df	P	
Vip3Ac1						
FAW	2.5–80	2.93 (0.42)	2.26	4	0.52	11.6 (9.4–14.1)
CBW	20–320	3.94 (0.57)	5.90	3	0.12	133.7 (110.1–177.4)
SW	0.5×10^4–10×10^4	2.65 (0.39)	8.35	5	0.14	44,776.0 (33,841–63,923)
Vip3Aa1						
FAW	2.5–40	4.02 (0.57)	0.09	3	0.99	6.9 (5.4–8.2)
CBW	10–80	5.76 (0.94)	0.49	2	0.78	27.7 (23.8–32.0)
SW	500–5,000	4.00 (0.87)	2.00	4	0.73	1,986.1 (1,524.1–2,592.5)
Vip3AcAa						
FAW	1.25–10	3.28 (0.49)	4.94	2	0.08	3.5 (2.8–4.2)
CBW	10–160	5.37 (0.84)	1.31	3	0.73	31.3 (25.7–36.3)
SW	100–4,000	3.86 (0.49)	5.19	4	0.27	1,208.8 (1,029.5–1,450.2)
Vip3AaAc						
FAW	80–640	2.83 (0.47)	3.11	2	0.21	156.5 (115.1–195.3)
CBW	800–6,400	3.08 (0.48)	0.86	2	0.65	1,778.4 (1,382.6–2,176.9)
SW	0.5×10^4–10×10^4	NA[c]	NA	NA	NA	NA

[a] The toxins were applied on the surface of the artificial diets. The death rates were recorded on day 7 after the start of the assays.
[b] FAW, fall armyworm; CBW, cotton bollworm; SW, silkworm.
[c] NA, no detectable activity.

gene, *vip3Aa1*, in *B. thuringiensis* strains (11). While both fragments were amplified from most of the *B. thuringiensis* isolates screened, only one of the two DNA fragments was amplified from a few isolates. The PCR products from these few isolates were sequenced. One of the isolates, named LG13, was particularly interesting, since the DNA sequence of its *vip* fragment amplified is significantly different from that of *vip3Aa1*. Subsequently, the *vip3* gene harbored in LG13 was cloned. It turned out that the sequence of the *vip3* is identical to that of a toxin gene that was described in a U.S. patent application as "*Sup*" (K. E. Narva and D. J. Merlo, U.S. patent application 20040128716) and was named *vip3Ac1* according to the nomenclature system of Vip toxins (Crickmore et al., http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/). Vip3Ac1 shares 85% overall amino acid sequence identity with Vip3Aa1, and the similarity between Vip3Aa1 and Vip3Ac1 was not evenly distributed over the entire length of the protein. The N-terminal region of about 600 amino acid residues of Vip3Ac1 is highly conserved, sharing 94% identity with that of Vip3Aa1, while the C-terminal region of about 190 amino acid residues is much less conserved, exhibiting only 51% sequence identity with Vip3Aa1.

Insecticidal activity of Vip3Ac1. Due to the significant amino acid sequence differences between Vip3Ac1 and Vip3Aa1, it is interesting to compare their biological activities to illustrate the relationship between sequence and activity. Both Vip3Ac1 and the previously identified Vip3Aa1 were expressed in *E. coli* for biological activity assays (Table 1). Vip3Ac1 was highly active against fall armyworm and cotton bollworm, with LC_{50}s under 200 ng/cm^2, showing that it was almost as active as Vip3Aa1 against the two pests. However, Vip3Ac1 showed 22.5-fold lower activity against the silkworm than Vip3Aa1. No activity against the European corn borer was detected even at a dose as high as 200 μg/cm^2 at the diet surface. Activity assays of the mosquito *Anopheles gambiae* and the corn rootworm *D. virgifera* revealed no activity at high doses either. Thus, Vip3Ac1 also appears to be active against certain species of lepidopteran insects.

Insecticidal activities of chimeric proteins generated from sequence swapping between Vip3Aa1 and Vip3Ac1. To evaluate the roles of the conserved and the variable regions of the Vip3A proteins in their toxicity and host specificity and to explore the possibility for toxin improvement by artificial gene recombination, chimeric genes of *vip3Aa1* and *vip3Ac1* were created. The two chimeric proteins Vip3AcAa and Vip3AaAc, together with Vip3Aa1 and Vip3Ac1, were expressed by *E. coli* (Fig. 1).

Both chimeric toxins, Vip3AcAa and Vip3AaAc, were biologically active. Vip3AcAa was active against fall armyworm, cotton bollworm, and silkworm, while Vip3AaAc was active

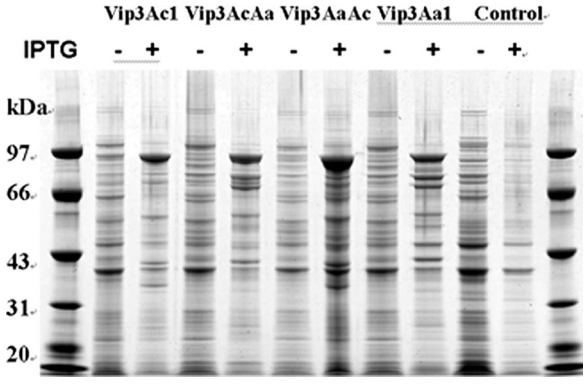

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of recombinant insecticidal proteins from *E. coli*. *vip3Ac1*, *vip3AcAa*, *vip3AaAc*, and *vip3Aa1* were all cloned into pET28a for expression in *E. coli* strain BL21 Star. The samples from *E. coli* culture with or without induction by 0.5 mM IPTG were analyzed. The *E. coli* with the empty vector pET28a served as the negative control.

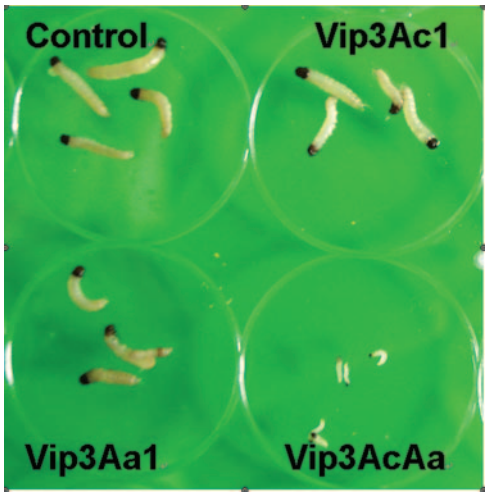

FIG. 2. Activity assays of Vip3Aa1, Vip3Ac1, and Vip3AcAa against European corn borer (*Ostrinia nubilalis*). All three Vip3 proteins were applied to the surface of artificial diet at a dose of 20 μg/cm^2. The neonates were then transferred to the surface of the feeding wells that had been air dried. After incubation for 7 days at room temperature, living larvae were picked out for photography. *E. coli* strain BL21 Star with empty plasmid vector served as the negative control.

against fall armyworm and cotton bollworm but not silkworm (Table 1). Interestingly, Vip3AcAa showed higher activities than either Vip3Aa1 or Vip3Ac1 against fall armyworm and silkworm, while Vip3AaAc was much less active against all tested insects. However, the most striking activity of Vip3AcAa was its novel activity against European corn borer. The corn borer larvae were inhibited from growth and development by Vip3AcAa at 20 μg/cm^2 of diet surface (Fig. 2). In contrast, the corn borer larvae grew normally when fed a diet containing Vip3Aa1 or Vip3Ac1 at the same dosage. The novel activity of Vip3AaAc against European corn borer may be of value for corn insect control.

Insecticidal activity of Vip3A toxins against Cry1Ac-resistant cabbage looper. To determine whether an endotoxin-resistant insect would also be cross-resistant to Vip3A proteins, the four toxins studied in this report, Vip3Aa1, Vip3Ac1, Vip3AaAc, and Vip3AcAa, were assayed for their activity against a *B. thuringiensis*-resistant cabbage looper strain which is highly resistant to Cry1Ac as well as to *B. thuringiensis* formation Dipel (17). The resistant strain showed a Cry1Ac resistance ratio over 2,000-fold greater than that seen with the susceptible strain (Table 2). However, the *B. thuringiensis*-resistant strain had a resistance ratio for the Vip3 proteins only 1.0- to 3.2-fold greater than that seen with the susceptible strain (Table 2). The results demonstrated that the *B. thuringiensis*-resistant cabbage looper showed practically no cross-resistance to any of the Vip3A proteins tested in this study.

DISCUSSION

Vip represents a major discovery among insect toxins and shares no amino acid sequence similarity with the widely used *B. thuringiensis* endotoxins. Vip toxins and *B. thuringiensis* endotoxins may be equally valuable for crop transgenic insect control. However, Vip toxins are still at the early stage of discovery and utilization, while *B. thuringiensis* endotoxins have been extensively explored and utilized in the last several decades. The huge diversity of *B. thuringiensis* strains may imply the virtual certainty of discovering more novel Vip toxins with desirable insecticidal activities. The identification of Vip3Ac1

TABLE 2. Susceptibility of Cry1Ac-sensitive and -resistant strains of *T. ni* to Vip3 proteins[a]

Protein and insect strain Cry1Ac sensitivity[b]	*n*	Slope (SE)	IC_{50} (95% CI[c]) (mg/liter)	χ^2 (df)	rr[d]	tr[e]
Cry1Ac						
SS	200	1.24 (0.16)	0.129 (0.089–0.191)	0.35 (3)	1	26
RR	200	2.34 (0.29)	265 (167–470)	5.21 (3)	2,054	
Vip3Ac1						
SS	160	3.60 (0.62)	3.31 (2.63–3.97)	0.26 (2)	1	1
RR	160	3.52 (0.60)	3.37 (2.69–4.05)	0.72 (2)	1.0	
Vip3AcAa						
SS	160	2.96 (0.40)	0.120 (0.099–0.144)	0.36 (2)	1	27
RR	200	2.24 (0.27)	0.386 (0.311–0.478)	0.69 (3)	3.2	
Vip3AaAc						
SS	200	1.70 (0.27)	432 (316–582)	0.15 (3)	1	0.01
RR	200	1.86 (0.27)	791 (591–1,035)	0.10 (3)	1.8	
Vip3Aa1						
SS	200	2.94 (0.36)	0.351 (0.299–0.418)	1.42 (2)	1	9.4
RR	200	3.57 (0.68)	0.746 (0.591–0.884)	1.22 (2)	2.1	

[a] For each concentration, an aliquot of 0.2 ml of toxin solution was applied over the artificial diet surface (surface area, ~7 cm^2). Larval growth inhibition (defined as inhibition when larvae did not develop into a second instar within 4 days of culture) was scored. IC_{50} is the concentration of the toxin solution that caused 50% inhibition of assay neonates.
[b] SS, Cry1Ac-sensitive strain; RR, Cry1Ac-resistant strain.
[c] CI, confidence interval.
[d] rr, resistance ratio (IC_{50}/IC_{50} of SS for each protein).
[e] tr, toxicity ratio (for SS) = IC_{50} of Vip3Ac1/IC_{50} of strain examined.

in this study serves as a good example for the discovery of such novel Vip toxins. The diversity of spectra of activities is desirable for selectively controlling different insect pests for different crops or for the same crops in different geographic areas. Although the activities of both Vip3Aa1 and Vip3Ac1 are limited to lepidopteran insects, their activities are nevertheless not identical, suggesting that the sequence variation is responsible for the difference in insecticidal properties. It is possible that novel Vip3 toxins with activities against non-*Lepidoptera* insect species will be discovered in the future, assuming that *vip3* evolution is somehow parallel to that of the endotoxins which were first discovered as lepidopteran toxins. New homologues of endotoxins discovered later showed activities against coleopterans and dipterans (6, 7, 22).

In addition to the discovery of novel insecticidal toxins from *B. thuringiensis* strains, it has been shown that the chimeric toxins generated artificially by sequence swapping can also enrich the diversity of the toxins in the laboratory. For instance, enhanced toxin activities were obtained by domain swapping in *B. thuringiensis* endotoxins (8, 19). This is also true for Vip toxins, as was demonstrated in this study. The chimeric Vip3AcAa not only exhibited a higher activity against the fall armyworm but also gained a novel activity against the European corn borer. Thus, the domain substitution and sequence-swapping approach may be broadly used to create chimeric Vip3 toxins to improve or to create activities. Gene-shuffling methods, a technology that could swap sequences among homologous genes efficiently (5), might be useful to fully explore this opportunity.

Compared to Vip3Aa1, Vip3Ac1 has much lower activity against silkworm, a major industrial insect species widely being raised in southern China, where mulberry trees are planted along with rice fields. It has been a concern that pollens of transgenic rice plants could contaminate mulberry leaves to endanger silkworm. Therefore, *vip3Ac1*, with very low activity against silkworm, is apparently a better choice than *vip3Aa1* for development of transgenic rice for insect control.

The development of insect resistance to endotoxins has become a major concern since the wide release of insect-resistant transgenic crops. Implementing insect resistance management measures is important for keeping the long-term effectiveness of transgenic crops. In addition to implementation of refuges in transgenic crop planting areas, gene stacking and crop rotating with two different insecticidal proteins without cross-resistance may dramatically slow the development of resistance (29). This study unambiguously demonstrated that there is virtually no cross-resistance between Cry1Ac and Vip3As in *B. thuringiensis*-resistant *T. ni*. The *cry1* genes, including *cry1Ab*, *cry1Ac*, and *cry1F*, are currently the leading genes used in transgenic crops planted worldwide. Thus, *vip3* genes may be excellent candidates for stacking with *B. thuringiensis* Cry1 genes in field transgenic crop applications for resistant management.

The members of the major class of the *B. thuringiensis* endotoxins discovered are homologous and are thus likely also to share a similar three-dimensional structure forming the base for their similar modes of action. In contrast, Vip toxins share no sequence similarity with any known *B. thuringiensis* endotoxins and thus likely have modes of action different from those of *B. thuringiensis* endotoxins. Thus, it is plausible that cross-resistance may be less likely to develop between a *B. thuringiensis* endotoxin and a Vip toxin than among two homologous *B. thuringiensis* endotoxins. This notion was supported by the observation that Vip3Aa1 does not bind to the insect midgut aminopeptidase N-like and the cadherin-like molecules, both of which are considered to be the receptors of *B. thuringiensis* endotoxins (18).

ACKNOWLEDGMENTS

This research was supported by grants from the National Science Foundation of China (grant no. 30425044) and the 973 Program of the Ministry of Science and Technology of China (grant no. 2003CB114203) and by the IRT program of the Ministry of Education of China (IRT0535).

We also express our thanks to Athenix Corporation (Durham, NC) for providing part of the supplies for the insect bioassay.

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