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Bacterial Vegetative Insecticidal Proteins (Vip) from Entomopathogenic Bacteria

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SUMMARY

Entomopathogenic bacteria produce insecticidal proteins that accumulate in inclusion bodies or parasporal crystals (such as the Cry and Cyt proteins) as well as insecticidal proteins that are secreted into the culture medium. Among the latter are the Vip proteins, which are divided into four families according to their amino acid identity. The Vip1 and Vip2 proteins act as binary toxins and are toxic to some members of the Coleoptera and Hemiptera. The Vip1 component is thought to bind to receptors in the membrane of the insect midgut, and the Vip2 component enters the cell, where it displays its ADP-ribosyltransferase activity against actin, preventing microfilament formation. Vip3 has no sequence similarity to Vip1 or Vip2 and is toxic to a wide variety of members of the Lepidoptera. Its mode of action has been shown to resemble that of the Cry proteins in terms of proteolytic activation, binding to the midgut epithelial membrane, and pore formation, although Vip3A proteins do not share binding sites with Cry proteins. The latter property makes them good candidates to be combined with Cry proteins in transgenic plants (*Bacillus thuringiensis*-treated crops [Bt crops]) to prevent or delay insect resistance and to broaden the insecticidal spectrum. There are commercially grown varieties of Bt cotton and Bt maize that express the Vip3Aa protein in combination with Cry proteins. For the most recently reported Vip4 family, no target insects have been found yet.

INTRODUCTION

Entomopathogenic bacteria have enormous potential for insect control, and they can provide us with an arsenal of insecticidal compounds (1). By far, the most widely used and best-known insecticidal proteins are the Cry proteins from *Bacillus thuringiensis*. These proteins accumulate in the parasporal crystal at the time of sporulation and are released into the culture medium only after the cell wall disintegrates. Formulations based on *B. thuringiensis* crystals and spores have been successfully used to control a wide range of lepidopteran pests as well as some coleopteran, blackfly, and mosquito species (2, 3). The insecticidal potency of some Cry proteins is such that their respective *cry* genes have been transferred to plants, conferring total or very-high-level protection against the most damaging pests (4–6).

Despite the wide success of Cry proteins in insect control, some important pests were found to be highly tolerant to Cry proteins, such as *Agrotis ipsilon* (Lepidoptera: Noctuidae) and *Diabrotica* spp. (Coleoptera: Chrysomelidae), which cause significant dam-

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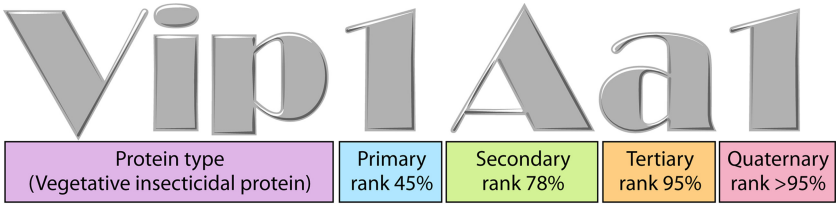

FIG 1 Nomenclature system for Vip proteins. The system consists of four ranks based on amino acid sequence identity (9). The primary, secondary, and tertiary ranks distinguish proteins with less than ~45, 78, and 95% sequence identities, respectively. The quaternary rank distinguishes proteins sharing >95% sequence identity, which can be considered products of "allelic" forms of the same gene but can also have the same sequence that originated from different isolates.

age to corn. Screening programs that aimed to evaluate active insecticidal components in culture supernatants from *Bacillus* isolates identified a culture supernatant from *Bacillus cereus* AB78 that induced 100% mortality of *Diabrotica virgifera virgifera* and *Diabrotica longicornis barberi* larvae (7). The active component of this supernatant was found to be proteinaceous. Anion exchange chromatography followed by SDS-PAGE showed that the insecticidal activity was due to two different proteins of 80 and 45 kDa, which were named Vip1Aa and Vip2Aa, respectively (for vegetative insecticidal protein). Sequences with homology to the respective *vip1Aa* and *vip2Aa* genes were found in ~12% of the 463 *B. thuringiensis* strains tested (7). In that same study, the vegetative culture supernatant from *B. thuringiensis* strain AB88 contained an 88.5-kDa protein that was highly toxic to *A. ipsilon* and other lepidopteran larvae, which was named Vip3Aa. More recently, Vip4Aa was reported (NCBI GenBank accession number AEB52299). *In silico* analysis predicted a molecular mass of ~108 kDa for Vip4Aa (8).

Alternative names for Vip proteins were also given before standardization by the Bt Toxin Nomenclature Committee (9) (Fig. 1), such as insecticidal secreted proteins (Isp), with the classes Isp1, Isp2, and Isp3 (NCBI GenBank accession numbers AJ871923, AJ871924, and AJ872070, respectively), which are homologous to Vip1, Vip2, and Vip3, respectively. It should be mentioned that another secreted insecticidal protein from *B. thuringiensis*, named Sip, has been reported. This protein shares no homology with the Vip proteins and should not be mistaken for one of them (10).

To date, 15 Vip1 proteins, 20 Vip2 proteins, 101 Vip3 proteins, and 1 Vip4 protein have been reported (9). Figure 2 shows a dendrogram with the hierarchy of the Vip proteins based on their degree of amino acid identity. Vip1 and Vip2 act as binary toxins for some members of the Coleoptera and Hemiptera (7, 11–14), and Vip3 is active against a wide range of species of Lepidoptera (15, 16). No target insects have as yet been found for Vip4. Vip1, Vip2, Vip3, and Vip4 share almost no sequence homology with each other, with Vip1 and Vip4 being the most similar (34% amino acid identity).

THE BINARY Vip1/Vip2 TOXIN

In addition to *B. cereus* and *B. thuringiensis*, *vip1* and *vip2* genes have also been found in other bacterial species, such as *Lysinibacillus sphaericus* (formerly *Bacillus sphaericus*) and *Brevibacillus laterosporus* (17, 18). Studies on the distribution of *vip1* and *vip2* genes have shown that they are found in ~10% of *B. thuringiensis* strains (7, 19–22). These two genes have been found in the same operon and with two different open reading frames separated by an intergenic spacer of 4 to 16 bp within a 4- to 5-kb genomic sequence (7, 23, 24) and in a megaplasmid (~328 kb in length) in *B. thuringiensis* strain IS5056 (25). At the time of writing of this review, the *Bacillus thuringiensis* Toxin Nomenclature database lists the following *vip1* and *vip2* genes: 3 *vip1Aa*, 1 *vip1Ab*, 1 *vip1Ac*, 1 *vip1Ad*, 2 *vip1Ba*, 3 *vip1Bb*, 1 *vip1Bc*, 2 *vip1Ca*, and 1 *vip1Da*, and 3 *vip2Aa*, 1 *vip2Ab*, 2 *vip2Ac*, 1 *vip2Ad*, 3 *vip2Ae*, 2 *vip2Af*, 2 *vip2Ag*, 2 *vip2Ba*, and 4 *vip2Bb* genes (9).

Vip1 and Vip2 proteins are expressed concomitantly, and translation from the same transcript appears to be essential to ensure high levels of both proteins. They are produced during the vegetative growth phase of *B. thuringiensis*, and their levels remain high until after the sporulation stage. Gene transcripts are detected at the start of the logarithmic phase, reaching their maximum expression levels in the stationary phase and remaining at high levels in the sporulation stage (15, 23, 24, 26).

Protein Structure and Function

Classical binary bacterial toxins of the "A-B" type, such as cholera toxin, interact with cells as a complex composed of one or several polypeptides associated in solution (in the case of cholera toxin, the A component is surrounded by 5 B polypeptides). Alternatively, Gram-positive bacilli from the genera *Clostridium* and *Bacillus* produce proteins with a synergistic binary mode of action in which the two proteins do not form an aggregate before binding to the cell surface (binary toxins of the "A+B" type) (27). The Vip1/Vip2 toxin is an example of an A+B toxin related to mammalian toxins from *Clostridium* spp. (*C. botulinum*, *C. difficile*, *C. perfringens*, and *C. spiroforme*) and *Bacillus anthracis*. Sequence homology with the mammalian toxins, the lack of toxicity of the individual proteins, data from translational frameshift mutation experiments with the *vip1* gene, along with data from toxicity bioassays against susceptible insects confirmed the binary mode of action of these proteins (7).

Sequence analysis of the Vip1Aa and Vip2Aa proteins revealed the presence of N-terminal signal peptides of ~30 and 50 amino acids, respectively (23, 26, 28). The signal peptide was shown to be cleaved during secretion, rendering mature proteins of ~82 kDa (for Vip1Aa) and 45 kDa (for Vip2Aa) (7, 24). Sequence alignment revealed that the N terminus of Vip1 is highly conserved (75 to 91% identity) (Fig. 3). In contrast, the C terminus of Vip1 is much less conserved (23 to 35% identity) (7, 23, 26).

Vip1 has moderate sequence identity with the binding component C2-II of the C2 *C. botulinum* toxin (29%) and the Ib component of iota-toxin from *C. perfringens* (31%). It also shares 33 to 38% identity with the *C. spiroforme* toxin, the *B. anthracis* protective antigen, and toxin B of *C. difficile* at amino acids 142 to 569 (23, 26, 29). Vip2 shares >30% sequence identity with the clos-

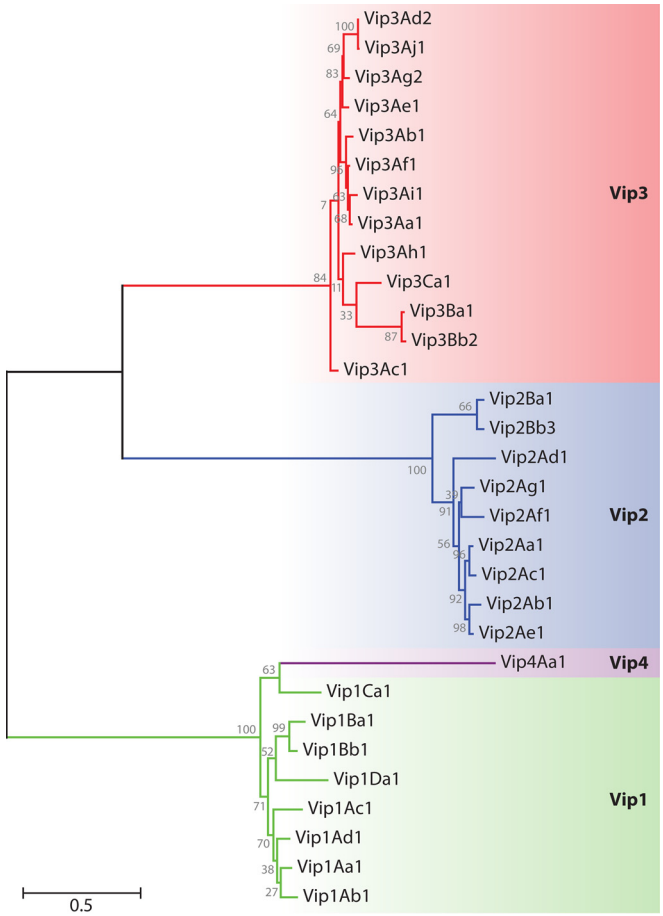

FIG 2 Dendrogram showing the relationships among Vip proteins based on their degree of amino acid identity. Amino acid sequences were aligned by using the Clustal X interface (120). The evolutionary distance was calculated by maximum likelihood analysis, and the tree was constructed by using the MEGA5 program (121). The proteins used in this analysis are as follows: Vip1Aa1 (sequence identification number [Seq. ID no.] 5 in reference 28), Vip1Ab1 (Seq. ID no. 21 in reference 28), Vip1Ac1 (GenBank accession number HM439098), Vip1Ad1 (accession number JQ855505), Vip1Ba1 (accession number AAR40886), Vip1Bb1 (accession number AAR40282), Vip1Ca1 (accession number AAO86514), Vip1Da1 (accession number CAI40767), Vip2Aa1 (RCSB Protein Data Bank accession number 1QS1_A), Vip2Ab1 (Seq. ID no. 20 in reference 28), Vip2Ac1 (accession number AAO86513), Vip2Ad1 (accession number CAI40768), Vip2Ae1 (accession number EF442245), Vip2Af1 (accession number ACH42759), Vip2Ag1 (accession number JQ855506), Vip2Ba1 (accession number AAR40887), Vip2Bb3 (accession number AIA96500), Vip3Aa1 (accession number AAC37036), Vip3Ab1 (accession number AAR40284), Vip3Ac1 (named PS49C; Seq. ID no. 7 in K. Narva and D. Merlo, U.S. patent application 20,040,128,716), Vip3Ad2 (accession number CAI43276), Vip3Ae1 (accession number CAI43277), Vip3Af1 (accession number CAI43275), Vip3Ag2 (accession number ACL97352), Vip3Ah1 (accession number ABH10614), Vip3Ai1 (accession number KC156693), Vip3Aj1 (accession number KF826717), Vip3Ba1 (accession number AAV70653), Vip3Bb2 (accession number ABO30520), Vip3Ca1 (accession number ADZ46178), and Vip4Aa1 (accession number HM044666).

tridial Rho-ADP-ribosylating exotoxin C3 (30). These similarities suggested that the Vip1 protein is the "B" component and that the Vip2 protein is the "A" component of the binary toxin (27). Thus, Vip1 acts as the binding and translocation component (channel-forming protein) (31–33), and Vip2 enters the cell and exerts its toxic effect.

Vip2 is a NAD-dependent actin-ADP-ribosylating toxin (34) that has two distinctive domains: the N-terminal domain, composed of amino acids 60 to 265, and the C-terminal domain, composed of amino acids 266 to 461, which is the NAD-binding domain (Fig. 4) (30). Despite their limited sequence homology to each other, crystallography structure analysis of the Vip2 N- and C-terminal domains showed homology in their structures (Fig. 5). Each domain core is formed mainly by the perpendicular packing of a five-stranded mixed β-sheet with a three-stranded antiparallel β-sheet. The three-stranded sheet is flanked by four consecutive α-helices, and the five-stranded sheet is flanked by an additional α-helix (30). The overall fold of each domain resembles the catalytic domains of classical A-B toxins. In fact, crystal structure superposition of Vip2 and the clostridial toxin C3, along with sequence alignment, suggests that the class of Vip2 toxins has arisen by a single gene duplication of an ancestral ADP-ribosyltransferase. This duplication event would have been followed by further divergence by which the N-terminal domain would have lost catalytic function and evolved into a binding component, to finally give rise to a new protein family with the ability to bind to other carrier proteins (e.g., Vip1) and thereby act as binary toxins (30, 35, 36).

Insecticidal Activity

The toxicity of Vip1, Vip2, and their combination has been tested against a number of insect species belonging to the orders Coleoptera, Lepidoptera, Diptera, and Hemiptera as well as nematodes (Table 1). So far, toxicity against 10 coleopteran species (7, 11, 12, 24, 37, 38) and the hemipteran species *Aphis gossypii* (13, 14) has been found.

Testing of individual Vip1 or Vip2 proteins against a number of insect species from different orders confirmed the fact that these proteins must act together to be toxic, since neither protein alone displayed any toxic activity against the species tested (Table 1). Another interesting feature of these toxins comes from experiments combining different pairs of proteins. The Vip1Aa/Vip2Aa binary toxin (carried on and expressed from the same operon) is active against *D. virgifera virgifera*, but Vip1Ab/Vip2Ab (carried on and expressed from the same operon) has no activity against this insect. Interestingly, the Vip1Aa/Vip2Ab combination is active, whereas its counterpart Vip1Ab/Vip2Aa is not, suggesting that the lack of toxicity of the Vip1Ab/Vip2Ab pair to *D. virgifera virgifera* is due to the Vip1Ab component (7).

Mode of Action

The molecular mechanism of the insecticidal activity of the Vip1/Vip2 toxin is not totally understood (Fig. 6). The multistep process begins with the ingestion of the toxin by the larva, followed by proteolytic activation in the midgut by trypsin-like proteases. The activated monomer of Vip1Ac has been shown to form oligomers containing seven Vip1 molecules (29). These oligomers recognize specific receptors in the midgut brush border membrane, where the toxin is then inserted into the membrane. Evidence that the Vip1 component is involved in receptor recognition was in part provided by the finding that Vip1Aa cannot be replaced by Vip1Ab without losing toxicity to *D. virgifera virgifera* (7). The first Vip1-binding protein described was identified in *A. gossypii* by ligand blot analysis and was ~50 kDa; concomitantly, no binding of Vip1 to brush border mem-

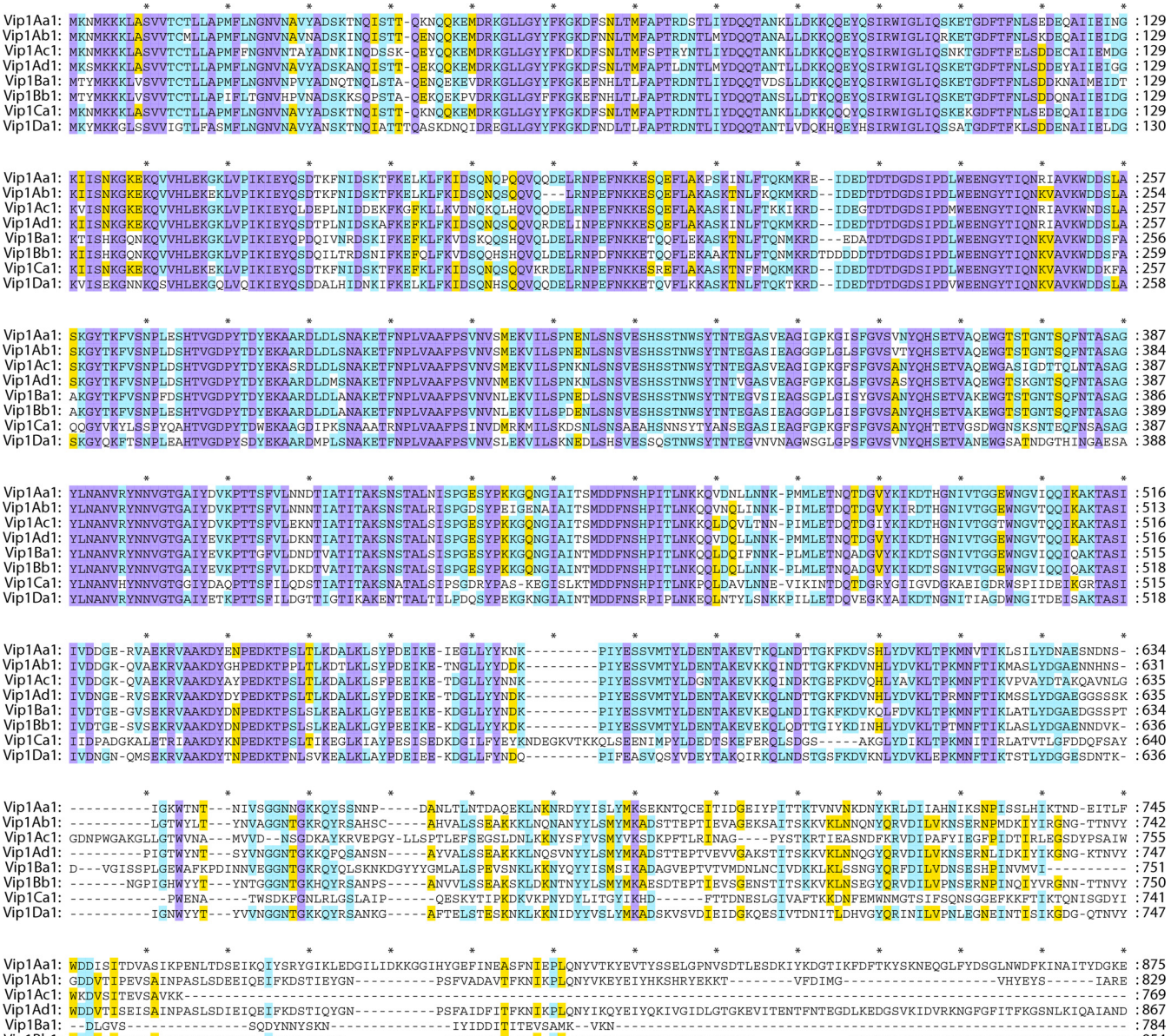

FIG 3 Multiple-sequence alignment of the Vip1 proteins. Sequence identity is indicated by shading, where violet is 100% sequence identity, pale blue is 80 to 100%, yellow is 60 to 80%, and white is <60%. Intervals of 10 amino acids are marked with "*." SP, signal peptide. Proteins used in this analysis are as follows: Vip1Aa1 (Seq. ID no. 5 in reference 28), Vip1Ab1 (Seq. ID no. 21 in reference 28), Vip1Ac1 (GenBank accession number HM439098), Vip1Ad1 (accession number JQ855505), Vip1Ba1 (accession number AAR40886), Vip1Bb1 (accession number AAR40282), Vip1Ca1 (accession number AAO86514), and Vip1Da1 (accession number CAI40767).

brane vesicles (BBMVs) from nonsusceptible insect species was observed (14).

In vitro experiments showed that Vip1 formed membrane pores in artificial lipid bilayers (29). The pores had two different conductance states, suggesting the simultaneous formation of two different channels. Vip1Ac channels are asymmetric and moderately anion selective. The putative channel-forming domain of Vip1 contains two negatively charged (E340 and E345) and two

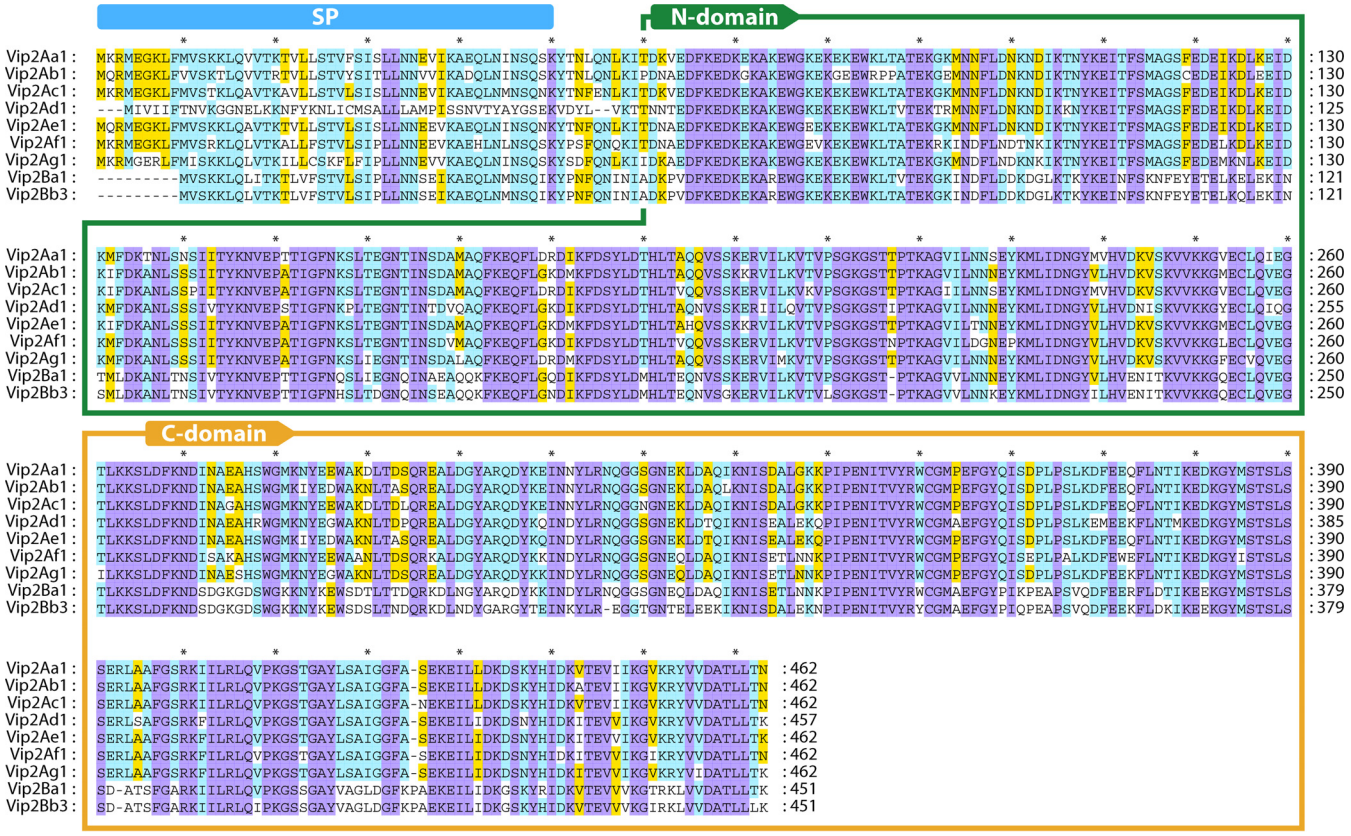

FIG 4 Multiple-sequence alignment of the Vip2 proteins. Sequence identity is indicated by shading, where violet is 100% sequence identity, pale blue is 80 to 100%, yellow is 60 to 80%, and white is <60%. Intervals of 10 amino acids are marked with "*." SP, signal peptide. The N-terminal domain (N-domain) and C-terminal domain (C-domain) are framed within boxes. The protein sequences used in this analysis are as follows: Vip2Aa1 (RCSB Protein Data Bank accession number 1QS1_A), Vip2Ab1 (Seq. ID no. 20 in reference 28), Vip2Ac1 (GenBank accession number AAO86513), Vip2Ad1 (accession number CAI40768), Vip2Ae1 (accession number EF442245), Vip2Af1 (accession number ACH42759), Vip2Ag1 (accession number JQ855506), Vip2Ba1 (accession number AAR40887), and Vip2Bb3 (accession number AIA96500).

positively charged (K351 and H363) amino acids, which are hypothesized to contribute to the selectivity of the channel (29).

The mechanism by which Vip2 enters the cell is still unknown, but based on its homology with the C2-I component of the C2 clostridial binary toxin, it seems likely that Vip2 enters the cell via receptor-mediated endocytosis (27). Leuber et al. (29) proposed a second possibility, in which the strong outward proton gradient across the midgut brush border membrane of insect cells (maintained by the highly alkaline midgut fluids of the larvae) could favor Vip2Ac being "directly" delivered into the cytoplasm of the midgut cells via the channel formed by Vip1Ac. Experimental evidence favoring either one of these mechanisms is lacking. Once inside the cytosol, the catalytic Vip2 domain would catalyze the transfer of the ADP-ribose group from NAD to actin, preventing its polymerization and thus inhibiting microfilament network formation (30, 34).

Expression in Plants

Despite the economic importance of Vip1 and Vip2 as effective toxins against the major corn pest *D. virgifera virgifera*, expression of the binary toxin *in planta* has not been possible due to the cytotoxic activity of the Vip2 protein. In fact, Vip2 expression in yeast resulted in serious developmental pathology and phenotypic alterations (34). To overcome this problem, Jucovic et al. (34) designed a new zymogene strategy that consisted of the expression of a zymogenic form of Vip2 called "ProVip2." The Vip2 proenzyme was obtained by extension of the C-terminal portion of the protein in such a way that it masked the enzymatic activity. The additional C-terminal peptide was effectively eliminated by the proteolytic action of *D. virgifera virgifera* midgut enzymes, and insects on a diet containing ProVip2 transgenic corn and Vip1 were all killed. Transformed plants had a phenotype unrecognizable from that of controls.

THE Vip3 LEPIDOPTERAN-ACTIVE PROTEIN

Similarly to the Vip1 and Vip2 proteins, Vip3 proteins are produced during the vegetative growth phase of *B. thuringiensis* and can be detected in culture supernatants from 15 h postinoculation to beyond sporulation, which reflects their high stability (15, 39). A study of the *vip3Aa16* gene reported that the transcription start point was located 101 bp upstream of the start codon and that the −35 and −10 promoter regions were very similar to the *B. subtilis* promoters that are under the control of the σ^E holoenzyme. These results strongly suggested that the *vip3Aa16* gene is transcribed by a σ^{35} holoenzyme, the *B. thuringiensis* homolog of σ^E (39).

Genes coding for Vip3 proteins are commonly found among *B. thuringiensis* strains, and hence, some studies have even found them in 50% and up to 87% of the strains tested and in >90% of

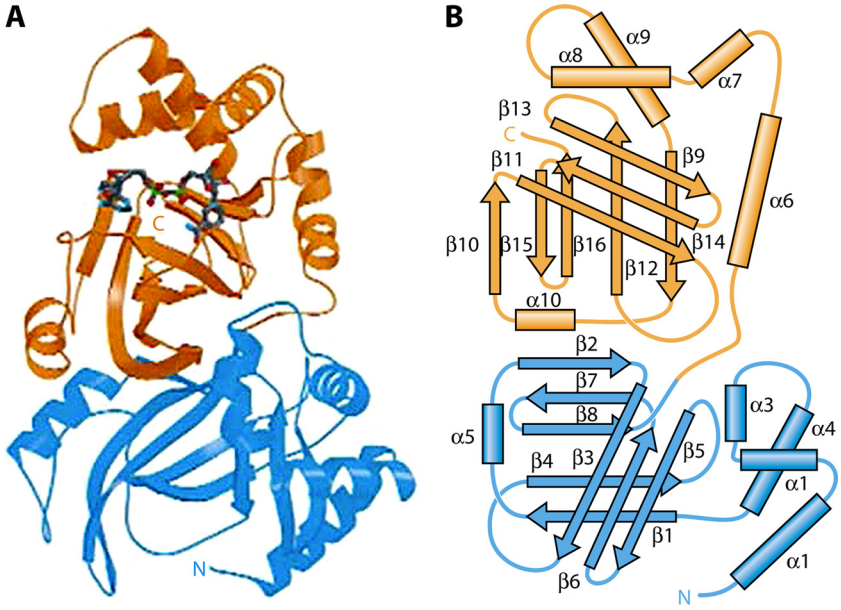

FIG 5 Tridimensional structure of Vip2 showing the two domains in different colors (N-terminal domain in blue and C-terminal domain in orange). (A) Schematic ribbon representation showing the NAD molecule (in blue) bound to the C-terminal domain. (B) Schematic drawing with secondary structure nomenclature. (Reprinted from reference 30 by permission from Macmillan Publishers Ltd.)

strains carrying *cry1* and *cry2* genes (20, 21, 40–44). *vip3* genes are ~2.4 kb in length, and they are normally carried on large plasmids (43, 45), although in some cases, they have been proposed to be located in the bacterial chromosome (46). Many strategies for screening of *B. thuringiensis* isolates have been performed with the aim of isolating new *vip3* genes (19, 20, 25, 42, 46–52). At the time of writing of this review, there have been 54 *vip3Aa*, 2 *vip3Ab*, 1 *vip3Ac*, 4 *vip3Ad*, 1 *vip3Ae*, 3 *vip3Af*, 15 *vip3Ag*, 1 *vip3Ah*, 1 *vip3Ai*, 2 *vip3Ba*, 3 *vip3Bb*, and 4 *vip3Ca* genes reported (9). It is not surprising that most studies on the Vip3 proteins have been carried out with the most abundant Vip3Aa proteins, and hence, very little information is available on the Vip3B and Vip3C proteins and other less common proteins of the Vip3A family (Vip3Ab and Vip3Ac, etc.). Unfortunately, early papers omitted the tertiary rank for the Vip3 proteins, referring just to Vip3A. Although these studies were most likely carried out on Vip3Aa, in this review, we follow the nomenclature provided by the authors whenever we found that it was not possible to identify the protein by accession number or by any other means.

Protein Structure and Function

The number of amino acids in any particular Vip3 protein is ~787, and the protein has an average molecular mass of ~89 kDa. The N terminus of Vip3 is highly conserved, while the C-terminal region is highly variable (16, 50, 53) (Fig. 7); thus, the C-terminal region was proposed to be involved in target specificity (53).

Vip3A proteins contain three cysteine residues. Point mutations in each of these three residues resulted in a loss of activity. However, this loss of activity was related to trypsin sensitivity rather than to the disruption of potential disulfide bonds (54).

The N terminus of Vip3 proteins contains a signal peptide that is responsible for the translocation of the protein across the cell membrane. It consists of a few positively charged amino acids, followed by a hydrophobic region, which are not removed after secretion from the bacterial cell (15, 55, 56). Without a clear putative cleavage site, the size of the signal peptide varies depending on the protein sequence itself and on the program used for prediction and ranges from 11 to 28 amino acids (15, 55, 56). Since the secretion of proteins commonly implies the excision of the signal peptide, the secretion mechanism for the Vip3 proteins is still unclear.

The highly conserved amino acid sequence of the N-terminal region of Vip3A proteins suggests that this region likely plays an important role in protein structure and insecticidal activity. However, contradictory results have been obtained in experiments testing the insecticidal activity of mutant Vip3A proteins with deletions at the N-terminal end. Deletion of the first 198 amino acids (which corresponds to the 22-kDa proteolytic fragment described by Estruch and Yu [57]) abolished toxicity to *Helicoverpa armigera* (Lepidoptera: Noctuidae) and *Spodoptera exigua* (Lepidoptera: Noctuidae) (58). Deletion of the first 27 N-terminal amino acids from Vip3Aa rendered the protein inactive due to a total loss of solubility (56). The deletion of the first 39 N-terminal amino acids from Vip3Aa differentially affected the toxicity of this protein toward the two susceptible insect species *Spodoptera litura* (Lepidoptera: Noctuidae) and *Chilo partellus* (Lepidoptera: Crambidae) (59). Contrary to the above-described results, Gayen et al. (60) found that a deletion of the first 200 N-terminal amino acids enhanced the insecticidal potency of the core active toxin ~2 to 3-fold against *H. armigera*, *A. ipsilon*, *Spodoptera littoralis* (Lepidoptera: Noctuidae), and *Scirpophaga incertulas* (Lepidoptera: Pyralidae). Similarly, in another study (49), a deletion of 33 amino acids from the Vip3Aa N terminus caused no loss of toxicity against *S. litura*, *Plutella xylostella* (Lepidoptera: Plutellidae), and *Earias vitella* (Lepidoptera: Noctuidae).

The function of some C-terminal modifications has also been studied, without leading to a general conclusion. Usually, the ef-

TABLE 1 Spectrum of activity of individual Vip1 and Vip2 protoxins and their combinations as binary toxins

Protein	Insect order	Insect species	Activity[a] (LC_{50})	Reference(s)
Vip1Aa	Coleoptera	*D. virgifera virgifera*	NA	7
Vip1Ac	Coleoptera	*C. suppressalis*, *Holotrichia oblita*	NA	13
		Tenebrio molitor	NA	26
	Lepidoptera	*H. armigera*, *S. litura*	NA	13
		S. exigua	NA	26
	Diptera	*C. quinquefasciatus*	NA	26
	Hemiptera	*A. gossypii*	NA	13, 26
Vip1Ad	Coleoptera	*Anomala corpulenta*, *H. oblita*, *Holotrichia parallela*	NA	24
Vip1Ae	Hemiptera	*A. gossypii*	NA	14
Vip1Da	Coleoptera	*D. virgifera virgifera*	NA	37
Vip2Aa	Coleoptera	*D. virgifera virgifera*	NA	7
Vip2Ac	Coleoptera	*T. molitor*	NA	26
	Lepidoptera	*H. armigera*, *S. exigua*, *S. litura*	NA	26
Vip2Ad	Coleoptera	*D. virgifera virgifera*	NA	37
Vip2Ae	Coleoptera	*H. oblita*, *T. molitor*	NA	13
	Lepidoptera	*C. suppressalis*, *H. armigera*, *S. exigua*	NA	13
	Diptera	*C. quinquefasciatus*	NA	13
	Hemiptera	*A. gossypii*	NA	13, 14
Vip2Ag	Coleoptera	*A. corpulenta*, *H. oblita*, *H. parallela*	NA	24
Vip1Aa + Vip2Aa	Coleoptera	*Diabrotica longicornis barberi*	+++ (NI)	7
		Diabrotica undecimpunctata howardi	+ (NI)	7
		D. virgifera virgifera	+++ (40/20[b] ng/g diet)	7
		Leptinotarsa decemlineata, *T. molitor*	NA	7
	Lepidoptera	*A. ipsilon*, *H. virescens*, *H. zea*, *M. sexta*, *O. nubilalis*, *S. exigua*, *S. frugiperda*	NA	7
	Diptera	*Culex pipiens*	NA	7
Vip1Aa + Vip2Ab	Coleoptera	*D. virgifera virgifera*	+++ (NI)	7
Vip1Ab + Vip2Aa	Coleoptera	*D. virgifera virgifera*	NA	7
Vip1Ab + Vip2Ab	Coleoptera	*D. virgifera virgifera*	NA	7
Vip1Ac + Vip2Ac	Coleoptera	*T. molitor*	NA	26
	Lepidoptera	*H. armigera*, *S. exigua*, *S. litura*	NA	26
Vip1Ac + Vip2Ae	Coleoptera	*H. oblita*, *T. molitor*	NA	13
	Lepidoptera	*C. suppressalis*, *H. armigera*, *S. exigua*	NA	13
	Diptera	*C. quinquefasciatus*	NA	13
	Hemiptera	*A. gossypii*	+++ (87.5 ng/ml)	13
Vip1Ad + Vip2Ag	Coleoptera	*A. corpulenta*	+++ (220 ng/g soil)	24
		H. oblita	+++ (120 ng/g soil)	24
		H. parallela	+++ (80 ng/g soil)	24
Vip1Ae + Vip2Ae	Hemiptera	*A. gossypii*	++ (96/481[b] ng/ml)	14
Vip1Ca + Vip2Aa	Coleoptera	*T. molitor*	NA	23
	Lepidoptera	*H. armigera*, *S. exigua*, *S. litura*	NA	23
	Diptera	*C. quinquefasciatus*	NA	23
Vip1Da + Vip2Ad	Coleoptera	*Anthonomus grandis*	+ (207 μg/ml)	37
		D. longicornis barberi	+++ (213 ng/ml)	37

(Continued on following page)

TABLE 1 (Continued)

Protein	Insect order	Insect species	Activity[a] (LC_{50})	Reference(s)
		D. undecimpunctata howardi	++ (4.91 μg/ml)	37
		D. virgifera virgifera	+++ (437 ng/ml)	37
		L. decemlineata	+++ (37 ng/ml)	37
	Lepidoptera	*H. virescens*, *H. zea*, *M. sexta*, *O. nubilalis*, *Sesamia nonagrioides*, *S. littoralis*, *S. frugiperda*	NA	37
Vip1Ac-like/Vip2Ac-like	Coleoptera	*Sitophilus zeamais*	++ (NI)	38
Vip1/Vip2	Nematoda	*Caenorhabditis elegans*, *Pristionchus pacificus*	NA	122
Vip1Ba1-Vip2Ba1	Coleoptera	*D. virgifera virgifera*	+++ (NI)	11
Vip1Aa2-Vip2Aa2	Coleoptera	*D. virgifera virgifera*	+++ (NI)	12
	Lepidoptera	*H. virescens*, *H. zea*	NA	12
Vip1Bb1-Vip2Bb1	Coleoptera	*D. virgifera virgifera*	+++ (NI)	12
	Lepidoptera	*H. virescens*, *H. zea*	NA	12

[a] The number of "+" symbols reflects the activity level. NA, not active; NI, no information on the LC_{50}.
[b] Proportion of Vip1/Vip2 that gives 50% mortality.

fect of the same change varies among different insect species, preventing a consensus about the contribution of certain regions or amino acid positions to the toxicity of Vip3A proteins (49, 56, 58–60). There is generally agreement in that the last amino acids of the C terminus are critical for the activity and stability of Vip3 proteins, since their deletion, their replacement by nonconservative residues, or the addition of amino acids to the end of the protein completely abolishes protein activity (59, 60) and increases susceptibility to proteases (57, 58). A triple mutation at the C terminus of Vip3Aa1 resulted in an unstable protein that was completely hydrolyzed by the midgut juice of *A. ipsilon* larvae but retained toxicity against Sf9 cells (57).

An analysis of Vip3 protein sequences using the NCBI CDD database (61) which we conducted revealed the presence of a carbohydrate-binding motif (CBM) (CBM_4_9 superfamily; pfam02018) in all Vip3 proteins with the exception of Vip3Ba (Fig. 8). The CBM spans from position 536 to a position near amino acid 652, with a consistent E value of between 10 e^{-4} and 10 e^{-17}, depending on the Vip3 protein being considered. Analysis of Vip3 sequences also revealed positive hits with dif-

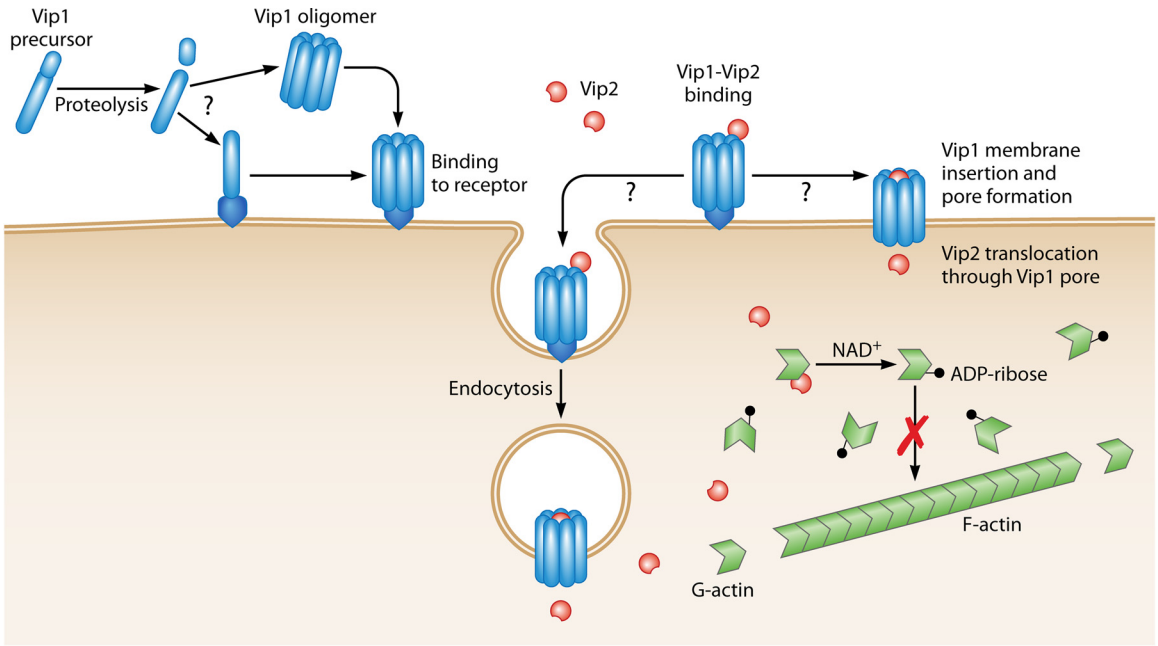

FIG 6 Proposed mode of action of the binary Vip1/Vip2 toxin. The Vip1 protoxin is proteolytically processed by midgut proteases. The activated toxin binds to specific receptors either as a monomeric form or after oligomerization. Vip2 then binds to the oligomeric Vip1 protein and enters the cell either by endocytosis of the whole complex or directly through the pore formed by Vip1. Once inside the cytosol, Vip2 catalyzes the transfer of the ADP-ribose group from NAD to the actin monomers, preventing their polymerization.

FIG 7 Multiple-sequence alignment of the Vip3A proteins. Sequence identity is indicated by shading, where violet is 100% sequence identity, pale blue is 80 to 100%, yellow is 60 to 80%, and white is <60%. SP, signal peptide (50); "T," 65-kDa fragment after proteolysis; "PPS1" and "PPS2," first and second processing sites, respectively (50). Intervals of 10 amino acids are marked with "*." The protein sequences used in this analysis are as follows: Vip3Aa1 (GenBank accession number AAC37036), Vip3Ab1 (accession number AAR40284), Vip3Ac1 (named PS49C; Seq. ID no. 7 in Narva and Merlo, U.S. patent application 20,040,128,716), Vip3Ad2 (accession number CAI43276), Vip3Ae1 (accession number CAI43277), Vip3Af1 (accession number CAI43275), Vip3Ag2 (accession number ACL97352), Vip3Ah1 (accession number ABH10614), Vip3Ai1 (accession number KC156693), Vip3Aj1 (accession number KF826717), Vip3Ba1 (accession number AAV70653), Vip3Bb2 (accession number ABO30520), and Vip3Ca1 (accession number ADZ46178).

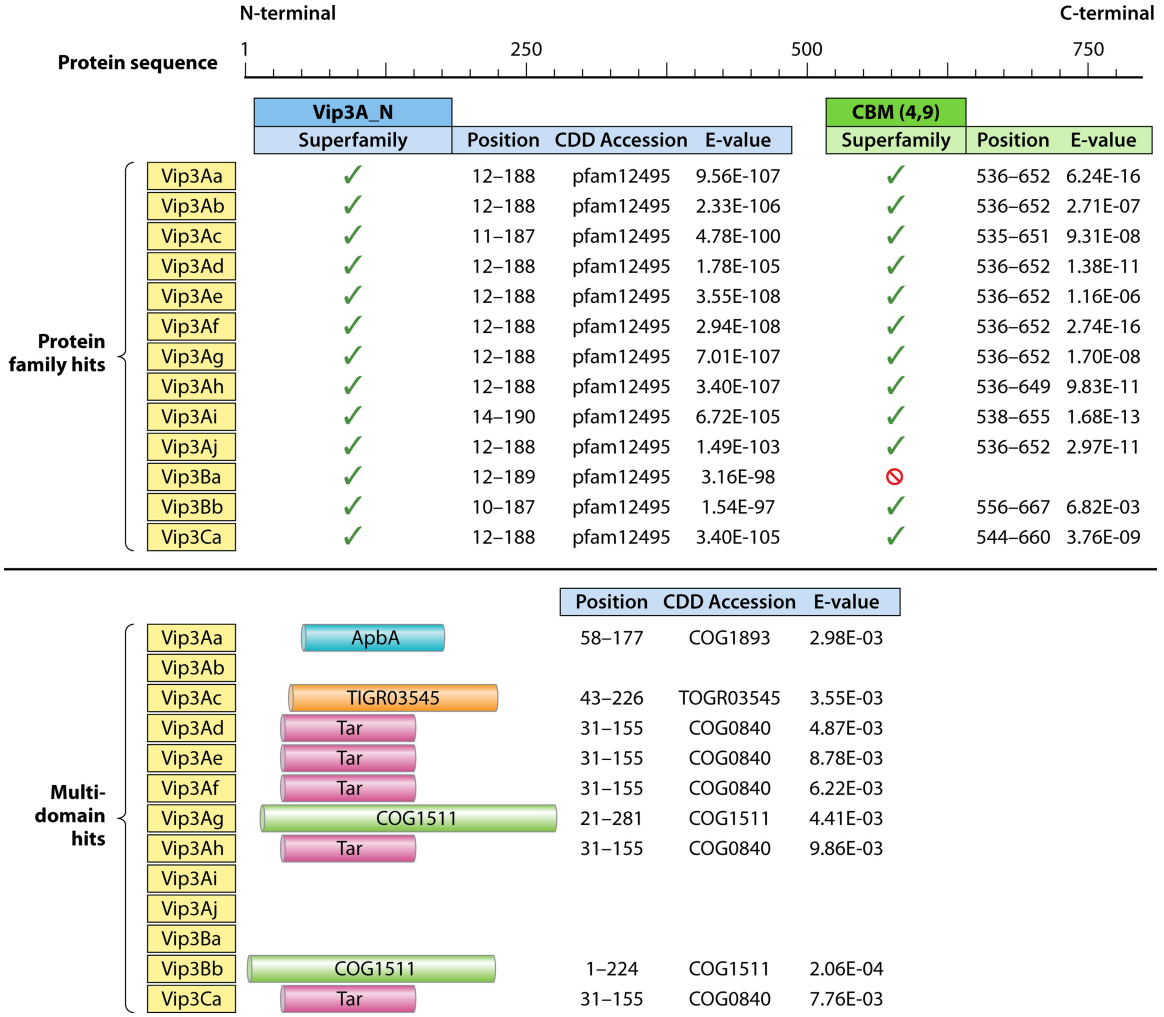

FIG 8 Conserved Domain Database (CDD) analysis of representative Vip3 proteins. The same sequences as those shown in Fig. 4 were used. CBM, carbohydrate-binding motif; ApbA, ketopantoate reductase motif; Tar, methyl-accepting chemotaxis protein motif; COG1511, motif of a predicted protein membrane of unknown function. TIGR03545 represents a relatively rare but broadly distributed uncharacterized family of proteins, distributed in 1 to 2% of bacterial genomes.

ferent multidomains in the N-terminal region, with lower E values of ~10 e^{-4} and with differences depending on the Vip3 protein being considered. We did not detect any hydrophobic region susceptible to forming a transmembrane domain other than the short succession of hydrophobic amino acids in the signal peptide (15, 55).

Comparison of the Vip3Aa1 sequence with those of the Vip3B- and Vip3C-type proteins reveals differences distributed throughout the length of the protein (Fig. 7). Nevertheless, maximum divergence was found at the C terminus, as occurs among Vip3A family proteins. The N termini of the putative signal sequences of Vip3B and Vip3C are almost identical to those of all Vip3A proteins. The proteolytic processing sites are less conserved among the three Vip3 proteins, but major differences in the middle of the protein sequence are found: an insertion of 5 amino acids downstream of the first processing site for Vip3Ca1 and an insertion of 17 amino acids downstream of the second processing site for Vip3Ba1 are responsible for the change in the expected size of the toxin "active form" from 66 kDa to 69 kDa. The inserted Vip3B sequence consists of three repetitions of the pattern DCCEE, which is characterized by its high content of negatively charged amino acids (D and E) and cysteine residues. Of a total of 11 cysteine residues found in Vip3B proteins, 8 (78%) are located in this inserted sequence (50, 62). Whether the insertion of this repetitive pattern contributes to the limited insecticidal activity of the Vip3B proteins is not known.

The conformational three-dimensional (3D) structure of Vip3 proteins has not yet been elucidated. Secondary structure prediction suggests that the N terminus is composed mainly of α-helix structures, whereas the essential components of the C terminus are β-helix structures and coils, which would be consistent with its proposed role in insect specificity (50, 53). The fact that Vip3 proteins do not show homology to any protein outside their group prevents *in silico* modeling based on structure homology. Only a partial tertiary structure of the Vip3 protein corresponding to the last 200 amino acids has been modeled by homology to domain II of the Cry proteins (53).

Insecticidal Activity

Most of the information on the insecticidal activity of Vip3 proteins has been obtained with the most abundant variants of the Vip3Aa subfamily, and there are very few data on the toxicity of Vip3B, Vip3C, and other Vip3A proteins outside the Vip3Aa subfamily.

Insecticidal spectrum of Vip3 proteins. Vip3A proteins are toxic to a large number of lepidopteran species. It is worth mentioning that Vip3A proteins are very active against insect species of the genus *Agrotis*, which are known to be tolerant to Cry proteins, and also against species of the genus *Spodoptera*, which display low susceptibility to Cry proteins (63). In this regard, it has been shown that deletion of the *vip3A* gene from the *B. thuringiensis* HD1 strain significantly decreased this strain's toxicity to *A. ipsilon* and *S. exigua* (64). On the other hand, other species susceptible to Cry proteins, such as *Ostrinia nubilalis* (Lepidoptera: Crambidae), *Culex quinquefasciatus* (Diptera: Culicidae), and *Chironomus tepperi* (Diptera: Chironomidae), are marginally or not susceptible to any Vip3A protein tested (15, 55, 65, 66). With Vip3 proteins, depending on the Vip3 protein and the insect species being considered, it is not uncommon to find that while mortality is reached with a high concentration of Vip3 protein, strong growth inhibition (or even complete growth arrest) is observed with lower concentrations (16, 62, 67–69). Therefore, "functional mortality" (dead insects plus those remaining at larval instar L1) better represents the effectiveness of the Vip3 protein in these cases (16, 70, 71).

Table 2 summarizes the results that have been reported on the insecticidal activity of proteins of the Vip3Aa subfamily. Only the values for the protoxin form are given, since there are no reports indicating relevant differences in the insecticidal activities of the protoxin and the activated forms (16), with the exceptions of the activities of Vip3Aa16 against *S. exigua* and Vip3Af1 against *Spodoptera frugiperda* (Lepidoptera: Noctuidae) (71, 72). Despite the very small differences among Vip3Aa sequences, some proteins may exhibit significant differences in toxicity to the same insect species (16, 59, 73). For example, among all Vip3Aa proteins tested, only Vip3Aa1 and Vip3Aa14 have been described to have low or no activity against *H. armigera* (Table 2). Nonetheless, considering that most of the data in Table 2 were obtained in different laboratories, insecticidal differences are likely to come from factors other than slight differences in protein sequence, such as the protocol used for protein preparation, purity of the sample, method of quantification, bioassay conditions, or variability among insect populations. Independent laboratories have observed a decrease in the toxicity of some Vip3A proteins after purification with metal chelate chromatography (47, 72). The effect of the method of purification on toxicity depends on both the type of protein and the insect species tested. The duration of the bioassay can also drastically affect the final outcome for some proteins, as has been shown for Vip3Aa16 with *S. exigua* and *S. frugiperda*, for which the 50% lethal concentrations (LC_{50}s) decreased by a factor of 10 when mortality was scored at 10 days instead of 7 days (71). Ali and Luttrell (70) found that the insecticidal responses of *Helicoverpa zea* and *Heliothis virescens* (Lepidoptera: Noctuidae) to Vip3Aa varied greatly among different batches of the same protein as well as with the buffer used.

Table 3 summarizes the bioassay data on Vip3A proteins other than those of the Vip3Aa subfamily, and Table 4 summarizes the bioassay data on Vip3 proteins other than those of the Vip3A family.

Interactions with other insecticidal proteins. Synergism has been observed between the Vip3Aa and Cyt2Aa proteins against *Chilo suppressalis* (Lepidoptera: Crambidae) and *S. exigua* after their coexpression in *Escherichia coli*; contrarily, this protein combination was slightly antagonistic against *C. quinquefasciatus* (66). Bergamasco et al. (74) reported synergism between Vip3A and Cry1Ia in three *Spodoptera* species (*S. frugiperda*, *S. albula*, and *S. cosmioides* [Lepidoptera: Noctuidae]) but slight antagonism in *Spodoptera eridania* (Lepidoptera: Noctuidae). Antagonism between the Vip3A and Cry1A or Cry1Ca proteins in *H. virescens* was described (75): antagonism was found for the combination of Cry1Ca and Vip3Aa, Vip3Ae, or Vip3Af and for the combination of Vip3Af and either Cry1Aa or Cry1Ac. In that same study, Vip3Aa and Cry1Ca showed antagonism in *S. frugiperda*, whereas the same combination was synergistic in *Diatraea saccharalis* (Lepidoptera: Crambidae).

The mechanisms underlying synergism and antagonism are unknown. For the antagonism between the Vip3A and Cry1C proteins, Lemes et al. (75) hypothesized that a physical interaction of the two proteins impairs the access of the binding epitopes to the membrane receptor. Hetero-oligomer formation with an increased ability for membrane insertion or pore formation was proposed to explain the synergism between Cry1Ac and Cry1Aa (76). However, this possibility for the Vip3 and Cry1 proteins seems very unlikely because of their lack of homology.

Genetically engineered *vip3A* genes. Genetic engineering allows the construction of chimeric genes that code for parts of different proteins to obtain new proteins with novel or improved properties. Knowledge of the domains of a protein is of great advantage in the design of chimeric proteins. Despite the lack of information on the tertiary structure of Vip3A proteins, two chimeras have been created by sequence swapping between the *vip3Aa* and *vip3Ac* genes with the aim of increasing host specificity (77). These chimeras were created by combining ~600 amino acids from the N terminus of one protein and ~180 amino acids of the C terminus of the other (Table 5). The two chimeric proteins exhibited new toxicity properties: Vip3AcAa (with the N terminus of Vip3Ac) was more toxic to all the insects tested than the two original proteins, and it even caused growth inhibition of Vip3A-tolerant *O. nubilalis*. In contrast, Vip3AaAc was less toxic than its counterpart and the original proteins, and it even completely lost activity against *Bombyx mori* (Lepidoptera: Bombycidae) (77) (Table 5). Li et al. (58) achieved an 18-fold increase in toxicity against *S. exigua* by changing the last two amino acids of the chimeric Vip3AcAa protein (from IK to LR).

Similar attempts have been conducted by combining the *vip* and *cry* genes. Fusion of the *vip3Aa* gene with *cry1A* rendered a fusion protein that retained the toxicity of Cry1Ac but partially lost that of Vip3Aa, possibly due to incorrect Vip3A folding (78). In another study, the *vip3Aa* gene was fused with the 5′ region of *cry9Ca*, and the resultant chimeric protein was more toxic than the individual proteins and the mixture of them, probably because Vip3Aa increased the solubility of the Cry9Ca protein (79). In an attempt to improve the Vip3Aa yield, a mutant *vip3Aa* gene (with the signal peptide deleted) was fused with the promoter and the 3′-terminal half of *cry1C*, with the result of a 9-fold increase in the

TABLE 2 Spectrum of activity and toxicity of the Vip3Aa subfamily proteins

Protein	Insect species	Larval instar[c]	Assay type	LC_{50} (ng/cm^2)[a]	Scoring time (days)	Reference(s)
Vip3Aa	*A. ipsilon*	2nd–3rd	Diet incorporation	<200.0	2	65
	O. nubilalis	2nd–3rd	Diet incorporation	NA	2	65
	S. frugiperda	2nd–3rd	Diet incorporation	<200.0	2	65
	H. armigera	Neonate	Surface contamination	155	7	123
	Helicoverpa punctigera	Neonate	Surface contamination	22	7	123
	H. virescens	Neonate	Diet incorporation	NI	7	123
	H. zea	Neonate	Diet incorporation	NI	7	123
	A. ipsilon	1st	Surface contamination	17.1	5	87
	Danaus plexippus	1st	Surface contamination	NA	5	87
	H. zea	1st	Surface contamination	112.5	5	87
	M. sexta	1st	Surface contamination	176.3	5	87
	O. nubilalis	1st	Surface contamination	NA	5	87
	S. frugiperda	1st	Surface contamination	55.9	5	87
	H. armigera	Neonate	Diet incorporation	89	5	60, 118
	A. ipsilon	Neonate	Diet incorporation	63	5	60, 118
	S. littoralis	Neonate	Diet incorporation	36	5	60, 118
	S. incertulas	Neonate	Diet incorporation	60	5	60
Vip3Aa1	*A. ipsilon*	Neonate	Diet incorporation	<28	6	15
	H. virescens	Neonate	Diet incorporation	<420	6	15
	H. zea	Neonate	Diet incorporation	≥420	6	15
	O. nubilalis	Neonate	Diet incorporation	>420	6	15
	S. exigua	Neonate	Diet incorporation	<28	6	15
	S. frugiperda	Neonate	Diet incorporation	<70	6	15
	B. mori	Neonate	Surface contamination	1,986	7	77
	H. zea	Neonate	Surface contamination	27.7	7	77
	S. frugiperda	Neonate	Surface contamination	6.9	7	77
	S. frugiperda	Neonate	Surface contamination	49.3	7	95
	A. ipsilon	Neonate	Surface contamination	14	7	72
	S. frugiperda	Neonate	Surface contamination	620	7	72
	H. armigera	Neonate	Surface contamination	1,660	7	16
	Lobesia botrana	Neonate	Diet incorporation	1.3 μg/ml	7	16
	Mamestra brassicae	Neonate	Surface contamination	14.4	7	16
	S. littoralis	Neonate	Surface contamination	4.0	7	16
Vip3Aa7	*H. armigera*	Neonate	Leaf dip	35.6 ng/ml	3	80
	P. xylostella	3rd	Diet incorporation	28.9 ng/ml	3	80
	S. exigua	Neonate	Diet incorporation	46.1 ng/ml	7	80
	P xylostella	3rd	Leaf dip	4.9	3	54, 79
Vip3Aa9	*A. ipsilon*	1st	Leaf dip	2,165	1	59
	C. partellus	1st	Leaf dip	8	1	59
	Phthorimaea operculella	1st	Leaf dip	370	1	59
	P xylostella	1st	Leaf dip	36	1	59
	S. litura	1st	Leaf dip	5	1	59
Vip3Aa10	*A. ipsilon*	Neonate/1st	Surface contamination	80.7	6	55
	B. mori	Neonate/1st	Surface contamination	NA	6	55
	C. quinquefasciatus	Neonate/1st	In water	NA	6	55
	H. armigera	Neonate/1st	Surface contamination	325.2	6	55
	P. xylostella	Neonate/1st	Leaf dip	220.7	6	55
	S. litura	Neonate/1st	Surface contamination	45.4	6	55
Vip3Aa11	*H. armigera*	1st	Diet incorporation	25.7 ng/mg	7	42
	Ostrinia furnacalis	1st	Diet incorporation	720 μg/ml	7	42
	P. xylostella	1st	Leaf dip	4.2 mg/ml	4	42
	S. exigua	1st	Diet incorporation	1.3 ng/mg	7	42
Vip3Aa13	*H. armigera*	Neonate	Diet incorporation	160 ng/ml	2	56
	S. exigua	Neonate	Diet incorporation	740 ng/ml	2	56
	S. litura	Neonate	Diet incorporation	270 ng/ml	2	56

(Continued on following page)

TABLE 2 (Continued)

Protein	Insect species	Larval instar[c]	Assay type	LC_{50} (ng/cm^2)[a]	Scoring time (days)	Reference(s)
Vip3Aa14	*Earias vitella*	Neonate	Leaf dip	794	3	49
	H. armigera	Neonate	Leaf dip	NA	3	49
	Pieris brassicae	Neonate	Leaf dip	NA	3	49
	P. xylostella	Neonate	Leaf dip	120	3	49
	S. litura	Neonate	Leaf dip	12	3	49
	H. armigera	Neonate	Diet incorporation	NA	3	78
	P. xylostella	Neonate	Leaf dip	NA	3	78
	S. litura	Neonate	Leaf dip	0.1	3	78
Vip3Aa16	*P. oleae*	3rd	Leaf dip	NI	5	97
	S. littoralis	1st	Surface contamination	305	6	68
	E. kuehniella	1st	Diet incorporation	36	6	88
	S. exigua	Neonate	Surface contamination	2,600	7	71
				290	10	71
	S. frugiperda	Neonate	Surface contamination	340	7	71
				24	10	71
	A. segetum	1st	Surface contamination	86	6	69
	Tuta absoluta	3rd	Leaf dip	335	3	90
	Ectomyelois ceratoniae	Neonate	Diet incorporation	40[b]	5	91
Vip3Aa19	*H. armigera*	1st	Diet incorporation	24.1 ng/mg	7	42
	O. furnacalis	1st	Diet incorporation	>100 μg/ml	7	42
	P. xylostella	1st	Leaf dip	59.8 μg/ml	4	42
	S. exigua	1st	Diet incorporation	1.4 ng/mg	7	42
	H. virescens	1st	Diet incorporation	1.35 μg/ml	7	124
	P. xylostella	1st	Leaf dip	2236 μg/ml	5	124
	H. zea	Neonate	Surface contamination	500	7	109
Vip3Aa29	*C. quinquefasciatus*	—	In water	NA	2	66
	C. suppressalis	—	Diet incorporation	24.0 μg/ml	5	66
	C. tepperi	—	In water	NA	2	66
	H. armigera	—	Diet incorporation	22.6 μg/ml[d]	5	66
	S. exigua	—	Diet incorporation	36.6 μg/ml	5	66
Vip3Aa43	*S. albula*	Neonate	Surface contamination	3.9	7	74
	S. cosmioides	Neonate	Surface contamination	2.8	7	74
	S. eridania	Neonate	Surface contamination	3.4	7	74
	S. frugiperda	Neonate	Surface contamination	24.7	7	74
Vip3Aa45	*Chrysodeixis chalcites*	Neonate	Surface contamination	1,044.6	7	73
	L. botrana	Neonate	Diet incorporation	1.96 μg/ml	7	73
	M. brassicae	Neonate	Surface contamination	39.7	7	73
	S. exigua	Neonate	Surface contamination	119.7	7	73
	S. littoralis	Neonate	Surface contamination	18.7	7	73
Vip3Aa50	*Anticarsia gemmatalis*	Neonate	Surface contamination	20.3	7	125
	S. frugiperda	Neonate	Surface contamination	79.6	7	125
Vip3Aa58	*S. exigua*	Neonate	Surface contamination	160	10	47
	Cydia pomonella	Neonate	Surface contamination	2,380	10	47
	Dendrolimus pini	2nd	Leaf dip	23,550	10	47
Vip3Aa59	*S. exigua*	Neonate	Surface contamination	190	10	47
	C. pomonella	Neonate	Surface contamination	2,750	10	47
	D. pini	2nd	Leaf dip	16,260	10	47

[a] Unless otherwise stated, LC_{50}s are given in nanograms per square centimeter and refer to the protoxin form of the proteins. NA, not active; NI, no information on the LC_{50} is available, although the protein was active.
[b] Although the LC_{50} is given in nanograms per square centimeter, the bioassay was performed by using diet incorporation.
[c] —, not specified.
[d] The 50% inhibitory concentration is shown instead of the LC_{50}.

TABLE 3 Spectrum of activity and toxicity of Vip3A proteins other than those of the Vip3Aa subfamily

Protein	Insect species	Larval instar	Assay type	LC_{50} (ng/cm^2)[a]	Reference
Vip3Ab1	*A. ipsilon*	Neonate	Surface contamination	62	16
	S. exigua	Neonate	Surface contamination	597	16
	S. frugiperda	Neonate	Surface contamination	2,020	16
	S. littoralis	Neonate	Surface contamination	163	16
Vip3Ac1	*Anopheles gambiae*	—[b]	—	NA	77
	B. mori	Neonate	Surface contamination	44.8	77
	D. virgifera virgifera	—	—	NA	77
	H. zea	Neonate	Surface contamination	133.7	77
	O. nubilalis	Neonate	Surface contamination	NA	77
	S. frugiperda	Neonate	Surface contamination	11.6	77
Vip3Ad2	*A. ipsilon*	Neonate	Surface contamination	>4,000	72
	S. frugiperda	Neonate	Surface contamination	>4,000	72
Vip3Ae1	*A. ipsilon*	Neonate	Surface contamination	4	72
	S. frugiperda	Neonate	Surface contamination	28	72
	S. exigua	Neonate	Surface contamination	11.1	94
	S. frugiperda	Neonate	Surface contamination	20	94
	H. armigera	Neonate	Surface contamination	4,460	16
	L. botrana	Neonate	Diet incorporation	0.2 μg/ml	16
	M. brassicae	Neonate	Surface contamination	258	16
	S. littoralis	Neonate	Surface contamination	8	16
Vip3Af1	*S. frugiperda*	Neonate	Surface contamination	49.3	95
	A. ipsilon	Neonate	Surface contamination	18	72
	S. frugiperda	Neonate	Surface contamination	60	72
	H. armigera	Neonate	Surface contamination	840	16
	L. botrana	Neonate	Diet incorporation	0.8 μg/ml	16
	M. brassicae	Neonate	Surface contamination	6	16
	S. littoralis	Neonate	Surface contamination	43.2	16
Vip3Ag4	*C. chalcites*	Neonate	Surface contamination	45.5	73
	L. botrana	Neonate	Diet incorporation	1.1	73
	M. brassicae	Neonate	Surface contamination	>2,500	73
	S. exigua	Neonate	Surface contamination	265.2	73
	S. littoralis	Neonate	Surface contamination	34.9	73

[a] LC_{50}s refer to mortality at 7 days for the protoxin form of the proteins and are given in nanograms per square centimeter unless otherwise stated; NA, not active.
[b] —, information not available.

expression of the recombinant protein, which was concentrated in inclusion bodies. Unfortunately, this protein showed lower insecticidal activity against the insects tested than the original Vip3Aa protein, probably due to low solubilization or improper folding of the protein (80) (Table 5).

Another type of approach has been the introduction and expression of *vip3A* genes in *B. thuringiensis* strains expressing different *cry* genes, to create new strains to be used in insecticidal formulations with a broader spectrum of action. Commercial formulations of *B. thuringiensis* strains contain small amounts of Vip proteins, since these proteins are secreted into the growth medium, which is mostly discarded during the processing of the formulation (81). This problem can be alleviated by directing the expression of the *vip3A* gene to the sporulation stage by using sporulation-dependent promoters and specific transcription sequences from different *cry* genes (82–85). The engineered strains in all these cases showed improved production of Vip3A proteins and higher toxicity to the insects tested. Cloning and expression of the *vip3Aa* gene in *Pseudomonas fluorescens* have also been accomplished with the aim of producing spray insecticides based on the Vip3A protein, either combined with Cry proteins or not (86). The heterologously expressed Vip3Aa protein, which was not secreted into the medium and remained "encapsulated" within the bacterial cell, retained full toxicity.

Mode of Action

Study of the mode of action of the Vip3 proteins started soon after their discovery in 1996 by Estruch et al. (15), who proposed that Vip3 proteins would exert their toxicity via a process different from that of the Cry proteins, based on the lack of structural homology of these two types of proteins. Despite being so different, both types of toxins exert their toxic action through apparently the same sequence of events: activation by midgut proteases, crossing the peritrophic membrane, binding to specific proteins in the apical membrane of the epithelial midgut cells, and pore formation (87) (Fig. 9). So far, all reported studies on the mode of action of Vip3 proteins have been carried out with those of the Vip3A family, mostly those of the Vip3Aa subfamily. Ongoing studies on the Vip3Ca protein indicate that this protein has a mode of action similar to that of the Vip3A proteins (J. Gomis-Cebolla, I. Ruiz de

TABLE 4 Spectrum of activity and toxicity of Vip3B and Vip3C protein families

Protein	Insect species	Larval instar[b]	Assay type	LC_{50} (ng/cm^2)[a]	Scoring time (days)	Reference
Vip3Ba1	*O. nubilalis*	Neonate	Surface contamination	NA	7	50
	P. xylostella	2nd	Leaf dip	NA	7	50
Vip3Bb2	*A. gossypii*	Nymph	Diet incorporation	NA	7	40
	C. tepperi	4th	Liquid solution	NA	4	40
	H. armigera	Neonate	Surface contamination	NI	7	40
	H. punctigera	Neonate	Surface contamination	NI	7	40
	Tribolium castaneum	—	Diet incorporation	NA	10	40
Vip3Ca3	*A. ipsilon*	Neonate	Surface contamination	>4,000	10	62
	C. chalcites	Neonate	Surface contamination	<400	10	62
	H. armigera	Neonate	Surface contamination	<4,000	10	62
	L. botrana	Neonate	Diet incorporation	>100 μg/ml	10	62
	M. brassicae	Neonate	Surface contamination	<4,000	10	62
	O. nubilalis	Neonate	Surface contamination	>4,000	10	62
	S. exigua	Neonate	Surface contamination	>4,000	10	62
	S. frugiperda	Neonate	Surface contamination	>4,000	10	62
	S. littoralis	Neonate	Surface contamination	<4,000	10	62
	T. ni	Neonate	Surface contamination	<4,000	10	62

[a] Unless otherwise stated, LC_{50}s are given in nanograms per square centimeter and refer to the protoxin form of the proteins. NA, not active; NI, no information on the LC_{50} is available, although the protein was active.
[b] —, not specified.

Escudero, M. Chakroun, N. M. Vera-Velasco, P. Hernández-Martínez, C. S. Hernández-Rodríguez, Y. Bel, B. Escriche, P. Caballero, and J. Ferré, presented at the 48th Annual Meeting of the Society for Invertebrate Pathology, Vancouver, BC, Canada, 8 to 13 August 2015).

Behavioral and histopathological effects. The behavioral symptoms observed in susceptible insects after ingestion of the Vip3Aa protein resemble the ones observed after Cry intoxication: feeding cessation, loss of gut peristalsis, and overall paralysis of the insect (65). Analysis of gut cross sections of susceptible insects after ingestion of the Vip3Aa protein shows extensive damage in the midgut, with disrupted, swollen, and/or lysed epithelial cells and leakage of cellular material into the lumen (65, 68, 69, 88–91). No damage was observed in either the foregut or the hindgut, nor was damage observed in the midgut of nonsusceptible insects (65).

Proteolytic processing. *In vitro* proteolysis of full-length Vip3Aa proteins using insect midgut juice showed that they are processed to several major proteolytic products, generally of ~62 to 66, 45, 33, and 22 kDa (65, 68, 69, 88, 89). The 22-kDa fragment corresponds to the N terminus of the protein (amino acids 1 to 198 in Vip3Aa1), the 66-kDa fragment corresponds to the remainder of the protein (from amino acid 199 to the end of Vip3Aa1), and the 45- and 33-kDa fragments are thought to be derived from the 66-kDa portion (57).

The minimal toxic fragment of the Vip3Aa protein has also

TABLE 5 Genetically engineered Vip3A proteins and effects on insect toxicity

Protein	Modification type(s)	Description[b]	Effect(s) of modification[a]	Reference
Vip3AcAa	Domain swapping	Chimera of Vip3Ac N terminus (600 aa) and Vip3Aa C terminus (189 aa)	Gain of toxicity against *O. nubilalis*; IA against *S. frugiperda*, *H. zea*, and *B. mori*	77
Vip3AaAc	Domain swapping	Chimera of Vip3Aa1 N terminus (610 aa) and Vip3Ac C terminus (179 aa)	DA against *S. frugiperda* and *H. zea*; LA against *B. mori*	77
Vip3Aa14	Protein fusion	Chimera of Vip3Aa14 and Cry1Ac	As effective as Cry1Ac against *H. armigera* and *P. xylostella* but DA compared to Vip3Aa against *S. litura*	78
Vip3Aa7	Gene promoter change and protein fusion	Chimera of Cry1C promoter with truncated Vip3Aa7 (39 aa deleted at N terminus) and Cry1C C-terminal region	Higher yield of Vip3Aa7, Vip relocation in Bt inclusion bodies but DA against *P. xylostella*, *H. armigera*, and *S. exigua*	80
Vip3Aa7	Protein fusion	Chimera of Vip3Aa7 and Cry9Ca N terminus	IA against *P. xylostella*	79

[a] DA, decrease of activity; IA, increase of activity; LA, loss of activity.
[b] aa, amino acids.

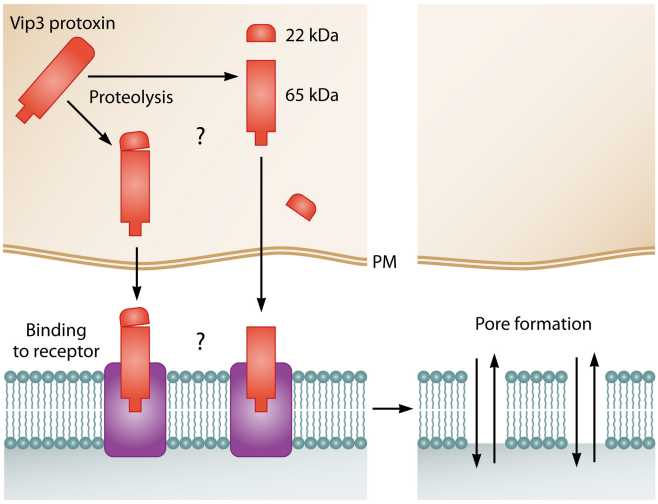

FIG 9 Proposed mode of action of the Vip3 proteins. The full-length protoxin is proteolytically processed by midgut proteases. The 65-kDa fragment binds to specific receptors (with the 22-kDa fragment still bound or not). Pores are then formed, which leads to the death of the cell.

been studied. Although an early study claimed that the minimal fragment that retained insecticidal activity after proteolysis was the 33-kDa fragment (57), all subsequent studies are in favor of the 62- to 66-kDa fragment being the Vip3Aa toxic core (58, 60, 68, 69, 71, 72, 87–89, 92–94). Interestingly, the 20-kDa fragment produced upon proteolytic processing of the Vip3Aa16 protoxin copurifies with the 62-kDa fragment, suggesting that after activation of the full-length protein, the two fragments remain together (89).

Compared to Cry proteins, the 62- to 66-kDa toxic core of the Vip3A proteins is more susceptible to the action of proteases. Incubation of either Vip3Aa or Vip3Ae with commercial serine proteases or insect midgut juice showed the unstable nature of the 62-kDa fragment, which started to break down even before all the protoxin was processed (65, 69, 88, 89, 94). Partial purification of peptidases from *S. frugiperda* midgut juice showed that cationic trypsin-like and anionic chymotrypsin-like peptidases are involved in the formation of the Vip3A 62-kDa fragment, whereas cationic chymotrypsin-like peptidases participated in its further processing (94).

In general, proteolytic activation does not seem to be a critical step in determining Vip3A insect toxicity and specificity. It has been shown that the midgut juice of a nonsusceptible insect (*O. nubilalis*) could process Vip3A *in vitro* into a 65-kDa fragment that was fully toxic when fed to susceptible insects (65). However, in some cases, the rate of processing of the full-length protein was proposed to account for differences in the toxicity of a given Vip3A protein to different insect species (71, 88, 94). Indeed, some studies have shown that differences in mortality disappeared when the trypsin-activated protein was used instead of the full-length protein (71, 72).

Binding to the larval midgut epithelium. *In vivo* immunolocalization studies have shown that Vip3A binds to the apical microvilli of midgut epithelial cells (65, 89) (Fig. 10). Specific binding to brush border membrane vesicles (BBMVs) prepared from susceptible insects was first shown by using biotin-labeled Vip3Aa and Vip3Af (68, 69, 74, 87, 92, 93, 95). Interestingly, Vip3Aa also binds specifically to BBMVs of the nonsusceptible insect *O. nubilalis* (87), which indicates that specific binding is not sufficient to produce toxicity.

Quantitative binding parameters were obtained by using ^{125}I-labeled Vip3Aa and *S. frugiperda* BBMVs. This binding was found to be saturable, mostly irreversible, and differentially affected by the presence of divalent cations (89). Vip3A proteins were also found to have lower affinities but higher numbers of binding sites than the Cry1A and Cry2A proteins. Interestingly, homologous competition showed that both the 62-kDa and the 20-kDa fragments of trypsin-activated ^{125}I-labeled Vip3Aa bound to BBMVs, and both fragments were displaced by the addition of nonlabeled Vip3Aa. In contrast, using biotin-labeled Vip3Aa, Liu et al. (93) found that only the 62-kDa fragment was able to bind to *H. armigera* BBMVs and also that the 20-kDa fragment was found exclusively in the supernatant of the binding reaction mixture.

Competition binding assays showed the absence of shared binding sites between Vip3A and Cry proteins. This has been shown for Vip3Aa with Cry1Ac, Cry1Ab, Cry1Fa, Cry2Ae, and

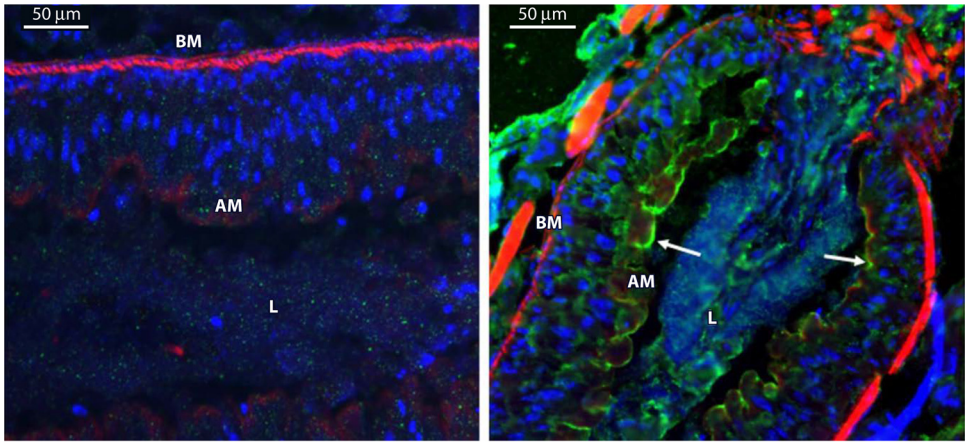

FIG 10 Immunolocalization of Vip3Aa in midgut tissue sections after ingestion by *S. frugiperda* larvae. (Left) Control larvae. (Right) Larvae that ingested Vip3Aa. Nuclei were stained blue, and the apical and basal membranes were stained red. Binding of Vip3Aa to the apical membrane is shown in green. BM, basal membrane; AM, apical membrane; L, gut lumen. (Reprinted from reference 89.)

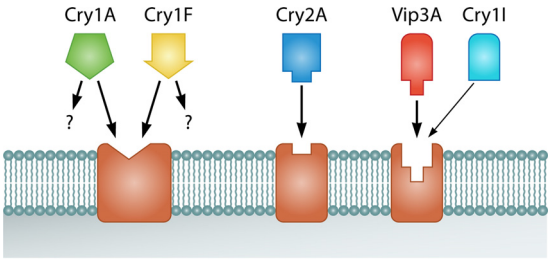

FIG 11 General binding site model for the Cry and Vip proteins in the midgut epithelial membrane of lepidopteran larvae. Cry1Fa and Cry1A proteins, in addition to the shared binding site, may have other sites depending on the insect species considered. Recognition of Vip3Aa sites by Cry1Ia has been found only in *S. eridania* (of four *Spodoptera* species tested).

Cry2Ab in all insect species tested and for Vip3Af with Cry1Ab and Cry1F in *S. frugiperda* (69, 89, 92, 93, 95, 96). However, Bergamasco et al. (74) reported partial competition of Cry1Ia for the Vip3Aa-binding sites in *S. eridania* BBMVs but not in *S. frugiperda*, *S. albula*, and *S. cosmioides* BBMVs. Competition among proteins of the Vip3A family has been tested only with *S. frugiperda* (89). Vip3Ae, Vip3Af, and even the nonactive Vip3Ad protein competed for the Vip3Aa-binding sites, with no significant differences in their binding parameters. A general model of the binding sites of Vip3A proteins in relation to Cry proteins is shown in Fig. 11.

The interaction of Vip3Aa with BBMVs of susceptible insects involves specific binding molecules different from the ones recognized by Cry1A proteins. Ligand blot analyses revealed that Vip3Aa recognized 80- and 110-kDa proteins in *Manduca sexta* (Lepidoptera: Sphingidae), while Cry1Ab bound to proteins of 120 and 210 kDa (87). That same study showed that Vip3Aa was unable to bind to purified aminopeptidase N (APN) and the cadherin ectodomain toxin-binding region (TBR) from *M. sexta*, both membrane proteins known to bind Cry proteins (87). In *Prays oleae* (Lepidoptera: Yponomeutidae) and *Agrotis segetum* (Lepidoptera: Noctuidae), Vip3Aa bound to a 65-kDa protein, while Cry1Ac bound to a 210-kDa band in *P. oleae* and to a 120-kDa band in *A. segetum* (69, 97). In *S. littoralis*, Vip3Aa bound proteins of 55 and 100 kDa (68), and in *Ephestia kuehniella* (Lepidoptera: Pyralidae), *S. frugiperda*, *S. albula*, *S. cosmioides*, and *S. eridania*, Vip3Aa bound to a protein of 65 kDa (74, 88), to which Cry1Ia also bound in the four *Spodoptera* species (74).

Very few studies have addressed the identity of the Vip3A-binding molecules in the insect midgut. Two Vip3Aa-binding molecules have been identified so far by using the yeast two-hybrid system. The first one was a 48-kDa protein from *A. ipsilon* with homology to a family of extracellular glycoproteins called tenascins, which could be associated with apoptotic processes (57). The second binding molecule was the S2 ribosomal protein from *S. litura*, identified as a Vip3A receptor in Sf21 cells (98). Silencing of the *S2* gene reduced the toxicity of Vip3A to both Sf21 cells and fifth-instar *S. litura* larvae. Both S2 and Vip3Aa colocalized on the surface and in the cytoplasm of Sf21 cells, suggesting that the interaction takes place on the cell surface and, once pores are produced, that the Vip3-S2 complex internalizes (98). How this S2-Vip3A protein interaction could trigger the lysis of cells was not explained and remains unknown. In *H. armigera*, the molecules that bind to Vip3Aa were found to be slightly associated with lipid rafts (93).

In an attempt to understand how midgut cells respond to intoxication by Vip3 proteins, gene expression profiles of *S. exigua* larvae treated with a sublethal dose of Vip3Aa were obtained by using a genome-wide microarray that included >29,000 unigenes (unique assembled sequences obtained from a transcriptome) (99). No alteration in the expression levels of the two Vip3A-binding proteins described above (S2 and the tenascin of the X-tox type) was found, nor were there alterations in the transcription levels of genes related to the mode of action of the Cry proteins. It was concluded that the lack of significant changes in the transcription levels of the above-mentioned genes was most likely either due to the fact that they were not involved in the Vip3 mode of action or because the mechanisms of defense against Vip3A toxins do not rely on the regulation of the members involved in the mode of action.

Pore formation. Despite the absence of any predicted pore-forming structure in the Vip3 proteins, the pore formation activity of the Vip3Aa protein activated with trypsin or midgut juice has been demonstrated by voltage clamping assays with dissected midguts of *M. sexta* (Lepidoptera: Sphingidae) and also in planar lipid bilayers (87). In contrast, the full-length Vip3Aa protein was unable to form pores. The ion channels were able to destroy the transmembrane potential, and they were voltage independent and cation selective (87). The pore-forming ability of activated Vip3Aa was also demonstrated by fluorescence quenching using *H. armigera* BBMVs (93). The formation of Vip3Aa ion channels was restricted to susceptible insects, and they have been found to have biophysical properties that differ from those of Cry1Ab in *M. sexta* (87).

Resistance and Cross-Resistance

Very few cases of resistance to Vip3 proteins have been reported. Laboratory selection of a *H. virescens* colony led to 2,040-fold resistance to Vip3Aa compared to the unselected population (100). Resistance was found to be polygenic, with possible paternal influence, and ranged from almost completely recessive to incompletely dominant; fitness costs were temperature dependent, with reduced mating success, fecundity, and fertility (101). After 12 generations of selection with Vip3A, a freshly established laboratory colony of *S. litura* reached a resistance level of 285-fold compared to a susceptible colony (102). The resistant insects were found to lack two casein-degrading bands in nondenaturing electrophoretic gels and to have reduced proteolytic activity (~2-fold) toward several protease substrates.

The presence of Vip3Aa resistance alleles in field populations of *H. armigera* and *Helicoverpa punctigera* (Lepidoptera: Noctuidae) was studied in Australia by using the F_2 screening method (103). The results showed that resistance alleles in both insect species existed as natural polymorphisms at a relatively high frequency (0.027 and 0.008, respectively), above mutation rates normally encountered (103). Interestingly, within each species, the resistance of two different F_2 families was due to alleles at the same locus, and resistance was found to be essentially recessive, most probably conferred by a single gene, and did not result in cross-resistance to Cry1Ac or Cry2Ab (103). The frequency of resistant alleles in *H. armigera* did not increase over the following four seasons (until 2014/2015), and resistant insects were found to activate the Vip3Aa protoxin more slowly than susceptible insects, although no significant differences in binding to membrane receptors were found (M. Chakroun, N. Banyuls, T. Walsh, S.

Downes, B. James, and J. Ferré, submitted for publication). Further studies on the resistant *H. punctigera* strain confirmed that there was no linkage between the Vip3A and the Cry2Ab resistance loci (104). A study on the presence of Vip3Aa resistance alleles in field populations of *S. frugiperda* from different states of Brazil, using the F_2 method, estimated an overall frequency of 0.0009, which is relatively low (105).

The increased use of Vip3 toxins in pyramided *B. thuringiensis*-treated crops (Bt crops) to improve both pest control and resistance management sparked interest in the evaluation of cross-resistance between Cry and Vip3A proteins (106). So far, no significant cross-resistance between these two classes of proteins has been described. Vip3Aa was found to be equally toxic to one susceptible and three Cry-resistant *H. virescens* strains (YHD2, resistant to Cry1Ac and Cry1F and slightly cross-resistant to Cry2A, and CXC and KCBhyb, both resistant to Cry1Ac, Cry1Aa, Cry1Ab, Cry1F, and Cry2Aa2) (107). Two studies on Cry1Ac-resistant strains of *H. zea* showed no significant cross-resistance to Vip3A or Cry2Ab (108, 109). A study on two *H. armigera* populations from Cry1Ac-cotton planting regions in China showed a lack of significant correlation between the responses to Vip3Aa and those to Cry1Ac, suggesting little or no cross-resistance between these two toxins (110). Cross-resistance to Vip3A has also been studied in two *S. frugiperda* Cry1F-resistant populations, one collected from Bt maize fields in Puerto Rico and the other collected from the southeast United States. Both populations were very susceptible to Vip3Aa, indicating the absence of cross-resistance between the Vip3Aa and Cry1F proteins (111, 112). A study using a different Vip3A protein, Vip3Ac, showed that it was equally toxic to susceptible and Cry1Ac-resistant *Trichoplusia ni* (Lepidoptera: Noctuidae) strains (77). However, in this case, the resistant strain was slightly less susceptible to Vip3Aa (resistance ratio of 2.1) and to two Vip3A chimeric proteins (resistance ratios of 1.8 and 3.2) (77).

Expression in Plants

The *vip3Aa* gene has been successfully introduced into cotton and corn and was later combined with other *cry* genes to confer higher-level protection and delay insect resistance (http://www.epa.gov/ingredients-used-pesticide-products/current-previously-registered-section-3-plant-incorporated). VipCot and Agrisure Viptera were registered in the United States in 2008 and 2009, respectively (Syngenta Seeds, Inc.). The former is the result of the transformation event COT102 in cotton, which produces the Vip3Aa19 protein [see http://www.isaaa.org/gmapprovaldatabase/gene/default.asp?GeneID=24&Gene=vip3A(a) and http://en.biosafetyscanner.org/schedaevento.php?evento=208], whereas the latter is the result of event MIR162 in corn, which produces the Vip3Aa20 protein (http://iaspub.epa.gov/apex/pesticides/f?p=CHEMICALSEARCH:30#p). Both events were pyramided with *cry1Ab* (VipCot Vip3Aa plus mCry1Ab and Agrisure Viptera Vip3Aa plus Cry1Ab) and later with *cry1Fa* (VipCot Vip3Aa plus Cry1Ac plus Cry1Fa and Agrisure Viptera Vip3Aa plus Cry1Ab plus Cry1Fa) to confer wider and more robust protection against Lepidoptera (113–115). Furthermore, corn event MIR162 has been stacked with other *cry* genes expressing proteins that are active against Coleoptera (Cry3A and eCry3.1Ab) to confer protection against these two insect orders (116). A 3-year study on the field performance of VipCot expressing just the Vip3Aa protein indicated that the plants were highly efficacious against *H. armigera* early in the season but that efficacy declined as the season progressed although not so drastically as Cry1Ac in Bollgard or Ingard cotton (117). In 2015, the first modified Vip3A protein, with improved toxicity, was introduced into tobacco, conferring almost total protection against *H. armigera*, *A. ipsilon*, and *S. littoralis* (118).

Cotton has also been transformed with a synthetic *vip3A* gene fused to a chloroplast transit peptide coding sequence (119). The Vip3A protein accumulated in chloroplasts, and its concentration in plants was higher than that in plants transformed with just the synthetic gene. Transformed plants provoked 100% mortality in larvae of *S. frugiperda*, *S. exigua*, and *H. zea*.

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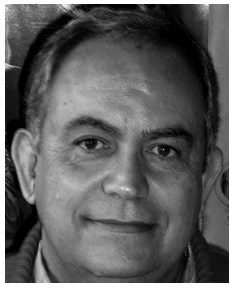

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