

Vip3C, a Novel Class of Vegetative Insecticidal Proteins from *Bacillus thuringiensis*

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Three *vip3* genes were identified in two *Bacillus thuringiensis* Spanish collections. Sequence analysis revealed a novel Vip3 protein class (Vip3C). Preliminary bioassays of larvae from 10 different lepidopteran species indicated that Vip3Ca3 caused more than 70% mortality in four species after 10 days at 4 $\mu\text{g}/\text{cm}^2$.

Vegetative insecticidal proteins (Vip) are secretable proteins from *Bacillus thuringiensis* (4, 19) which do not share sequence homology with known Cry proteins and display insecticidal activity against a wide variety of lepidopterans (5, 21) and coleopterans (19) and some sap-sucking insect pests (17). The members of the Vip3 family characterized to date exhibit activity against lepidopterans, and several of them do not compete with Cry proteins for binding sites (12, 18). They are classified into two subfamilies (Vip3A and Vip3B), and some are especially toxic for species with little susceptibility to several Cry proteins (4, 14). All of these features have made Vips a research target for broadening the host-range of *B. thuringiensis*-based biopesticides and for the management of insect resistance to *B. thuringiensis* proteins (2).

This work aimed to identify novel *vip3*-like genes from two Spanish *B. thuringiensis* collections. Around 800 strains were previously screened by PCR with primers from conserved *vip3* gene sequences (*vip3-sc.fw* and *vip3-sc.rev*) (7). Most of the genes identified showed very high similarity to those previously described (7). Strains NaB8.1 and NaB8.3 were isolated from barley husk in Miranda de Arga (Navarra, Spain) and maize dust in Allo (Navarra, Spain), respectively, two localities located 30 km apart (11). Strain V-MU2.21 was isolated from a storehouse dust sample collected 810 km away from the other two, in Murcia (Spain) (7). Each of the three strains yielded a single amplicon using the *vip3*-specific primers and showed Sau3AI and AluI restriction endonuclease profiles distinct from those of other known *vip3* genes (7). Amplicons were cloned into pGEM-T Easy (Promega) and sequenced. Full-length open reading frames (ORFs) were obtained by a genome walking strategy, completely amplified from the original strains, and cloned into expression vectors pET-28b(+) (for strains NaB8.1 and NaB8.3) and pMaab10 (for strain V-MU2.21). Each full-length ORF was 2,412 bp long and encoded 803-residue proteins with 90.8-kDa predicted molecular masses and 99.8% identity among them. Sequence alignment with known Vip3 proteins revealed identities within the 45 to 78% range. They therefore constituted a new Vip3 subfamily designated Vip3C by the *B. thuringiensis* Nomenclature Committee (http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/), and the proteins (from strains NaB8.1, V-MU2.21, and NaB8.3, respectively) were named Vip3Ca1 (ADZ46178), Vip3Ca2 (AEE98106), and Vip3Ca3 (HQ876489). In the new Vip3 protein dendrogram, the novel Vip3Ca proteins were grouped in a separate branch (Fig. 1).

Differences between the Vip3Ca and Vip3Aa1 amino acid sequences were found over the entire length but were more frequent toward the C terminus, as was previously reported for differences between Vip3Ba1 and Vip3Aa1 (15). The signal peptide (SP), potentially involved in Vip3 secretion (4, 15), remained highly conserved and differed in only two amino acids. It is not clear whether these few changes modify the insecticidal properties of Vip3, but the N terminus seems to be required for the correct formation of the trypsin-resistant core fragment in some Vip3 proteins (13). The two proteolytic processing sites (PPSs), PPS1 and PPS2, were less conserved. Protease treatment of Vip3Aa1 has been reported to give rise to four major protein products with molecular masses of approximately 22, 33, 45, and 66 kDa (5). The 22-kDa fragment constitutes the N-terminal portion of Vip3Aa1, whereas the 66-kDa fragment comprises the rest of the protein. This fragment may vary in size from 62 to 66 kDa among different Vip3 proteins and is occasionally known as the “trypsin-resistant core” (13). Proteolytic processing of the 66-kDa fragment renders both the 33- and 45-kDa fragments (4, 5, 15). How the few changes in the Vip3Ca PPSs may modify either the protein activity or the host range is uncertain, but different Vip3 proteins have shown very distinct insecticidal properties (4, 15). Other remarkable differences were the presence of two insertions immediately downstream of PPS1 and PPS2 that, together with another one at the C terminus, may also increase the size of the trypsin-resistant core fragment described for Vip3Aa1 (4, 5, 15) from 66 kDa to approximately 69 kDa. Finally, Vip3C proteins maintained only one of the three residues of the C terminus stabilizing domain described for Vip3Aa1 (5). This higher divergence toward the C terminus might indicate lower functional constraints and, consequently, more permissibility to nonsynonymous substitutions (20).

The only differences among the three novel Vip3Ca proteins consisted of two point mutations rendering nonsynonymous substitutions. The first one, at position 3, modified the predicted sec-

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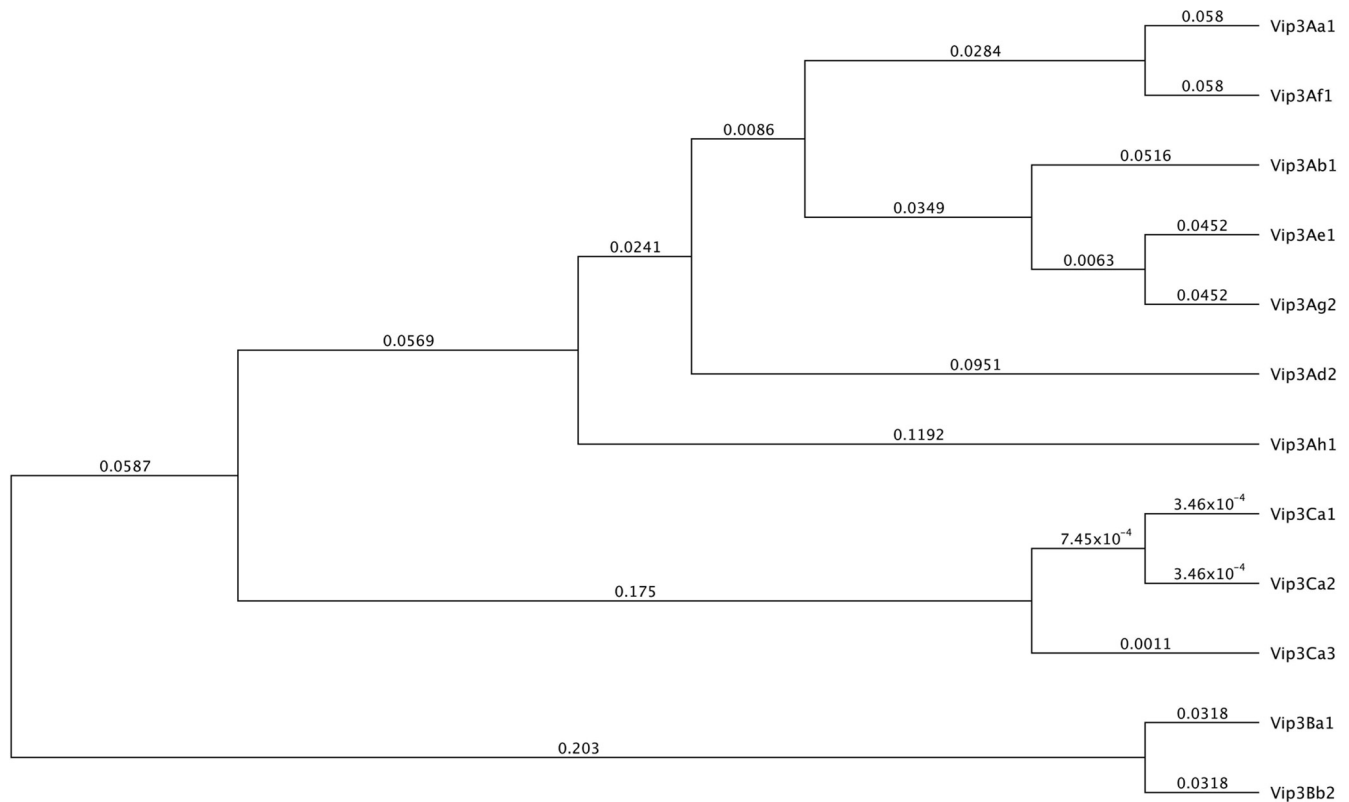


FIG 1 Dendrogram of Vip3 proteins from different subfamilies, including novel Vip3Ca1, Vip3Ca2, and Vip3Ca3 proteins. Branch lengths represent the number of substitutions per site of the multiple-sequence alignment as a measure of divergence.

ondary structure of the putative SP from a turn (Vip3Ca1 and Vip3Ca3) to an alpha-helix (Vip3Ca2). The second one, at position 215, caused a slight enlargement of the predicted secondary structure (alpha-helix) of the trypsin-resistant core in Vip3Ca3. The three Vip3Ca proteins had identical PPSs. These few differences are not attributable to laboratory contamination or PCR failure since they were cloned in two different laboratories and obtained in independent replications.

For a preliminary screening of the insecticidal properties of Vip3Ca (host range and toxicity), Vip3Ca3 was picked because of its high yields, expressed in *Escherichia coli*, column purified, quantified by densitometry, and used to challenge larvae of 10 different lepidopteran species (Table 1). Bioassays were conducted with neonate larvae that were placed over a surface-contaminated artificial diet. Two protein concentrations (0.4 and 4 $\mu\text{g}/\text{cm}^2$) were tested for all species except *Lobesia botrana*, which was challenged with 10 and 100 $\mu\text{g}/\text{ml}$ of diet (16). Water was used as a negative control. Sixteen larvae were used for each protein concentration, and each bioassay was repeated twice. Bioassays were conducted at 25°C, 60% \pm 5% relative humidity, and a 16:8-h (light/dark) photoperiod. Absolute and functional mortality rates (dead larvae plus larvae remaining in the first instar) were scored after 10 days. *Chrysodeixis chalcites*, *Helicoverpa armigera*, *Mamestra brassicae*, *Trichoplusia ni*, and *Spodoptera littoralis* seemed to be moderately to highly susceptible to the toxin, particularly *C. chalcites*. Compared with previously characterized Vip3 or Cry proteins, several differences in their insecticidal activity could be detected (4, 15; <http://www.glfrc.forestry.ca/bacillus>

/BtSearch.cfm). For example, in *H. armigera*, higher mortality rates have been obtained with other Vip3 or Cry toxins (3, 8), whereas *M. brassicae* mortality rates obtained with Vip3Ca3 were in a range similar to that of the mean lethal concentration (LC_{50}) of several Cry1 proteins (9). Nevertheless, determination of the LC_{50} of the three Vip3Ca proteins will allow more reliable comparisons among the Vip3C proteins and between Vip3C proteins and other *B. thuringiensis* toxins.

Strong growth inhibition was observed in *S. exigua* and *S. fru-*

TABLE 1 Effect of toxin Vip3Ca3 on larvae of 10 lepidopteran species

Insect species	Mean mortality rate ^b (%) \pm SD			
	Absolute		Functional	
	0.4 $\mu\text{g}/\text{cm}^2$	4 $\mu\text{g}/\text{cm}^2$	0.4 $\mu\text{g}/\text{cm}^2$	4 $\mu\text{g}/\text{cm}^2$
<i>A. ipsilon</i>	16 \pm 1.5	22 \pm 3.5	16 \pm 1.5	22 \pm 3.5
<i>C. chalcites</i>	73 \pm 2.9	79 \pm 5.9	73 \pm 2.9	79 \pm 5.9
<i>H. armigera</i>	15 \pm 2.9	65 \pm 2.9	38 \pm 5.9	83 \pm 5.9
<i>L. botrana</i> ^a	5 \pm 0.00	12 \pm 4.5	5 \pm 0.00	16 \pm 4.9
<i>M. brassicae</i>	42 \pm 2.9	80 \pm 2.9	44 \pm 5.9	85 \pm 5.9
<i>O. nubilalis</i>	3 \pm 0.0	6 \pm 0.0	3 \pm 0.0	6 \pm 0.0
<i>S. exigua</i>	8 \pm 2.5	19 \pm 11.2	21 \pm 0.7	86 \pm 13.4
<i>S. frugiperda</i>	10 \pm 9.6	27 \pm 7.1	20 \pm 9.5	54 \pm 2.7
<i>S. littoralis</i>	19 \pm 2.9	73 \pm 2.9	21 \pm 5.9	88 \pm 5.9
<i>T. ni</i>	35 \pm 3.4	94 \pm 1.0	35 \pm 3.4	94 \pm 1.0

^a For *L. botrana* bioassays, the Vip3Ca toxin was incorporated into the diet at 10 and 100 μg protein/ml of diet.

^b Absolute mortality rates were corrected for the background mortality rate (control).

giperda, as reflected by the effective mortality rates. This outcome has already been reported for other *B. thuringiensis* toxins (1, 6, 10) but seems to be a particular response in certain host-toxin combinations and does not always occur. Actually, Vip3Ca3 had little or no effect on the growth (of surviving larvae) of 7 of the 10 species analyzed here.

Finally, two of the species tested showed very low susceptibility to Vip3Ca3. One of them was *Ostrinia nubilalis*, for which no toxic effects of any of the Vip3Aa proteins assayed so far have been reported (4, 12, 21). The other one was *L. botrana*, which has been challenged with Vip3 for the first time.

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