Bacillus thuringiensis Vip3Aa Toxin Resistance in *Heliothis virescens* (Lepidoptera: Noctuidae)

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ABSTRACT Laboratory selection with Vip3Aa of a field-derived population of *Heliothis virescens* produced >2,040-fold resistance in 12 generations of selection. The Vip3Aa-selected (Vip-Sel)-resistant population showed little cross-resistance to Cry1Ab and no cross-resistance to Cry1Ac. Resistance was unstable after 15 generations without exposure to the toxin. F₁ reciprocal crosses between Vip3Aa-unselected (Vip-Unsel) and Vip-Sel insects indicated a strong paternal influence on the inheritance of resistance. Resistance ranged from almost completely recessive (mean degree of dominance $[h] = 0.04$ if the resistant parent was female) to incompletely dominant (mean $h = 0.53$ if the resistant parent was male). Results from bioassays on the offspring from backcrosses of the F₁ progeny with Vip-Sel insects indicated that resistance was due to more than one locus. The results described in this article provide useful information for the insecticide resistance management strategies designed to overcome the evolution of resistance to Vip3Aa in insect pests.

IMPORTANCE *Heliothis virescens* is an important pest that has the ability to feed on many plant species. The extensive use of *Bacillus thuringiensis* (Bt) crops or spray has already led to the evolution of insect resistance in the field for some species of Lepidoptera and Coleoptera. The development of resistance in insect pests is the main threat to Bt crops. The effective resistance management strategies are very important to prolong the life of Bt plants. Lab selection is the key step to test the assumption and predictions of management strategies prior to field evaluation. Resistant insects offer useful information to determine the inheritance of resistance and the frequency of resistance alleles and to study the mechanism of resistance to insecticides.

KEYWORDS Bt toxins, insect resistance, selection for resistance

The tobacco budworm, *Heliothis virescens* (L.) (Lepidoptera: Noctuidae), is a polyphagous pest that has the ability to feed on more than 100 plant species (1). *Heliothis virescens* is considered one of the most important pests of cotton (*Gossypium hirsutum* L.), although it can feed on other crops, including chickpea, tobacco, tomato, soybean, and sunflower (2). The control of *H. virescens* on cotton is an important problem due to the development of resistance to many chemical insecticides (3). Genetically modified (GM) crops expressing genes from *Bacillus thuringiensis* (Bt crops) were introduced in 1996 for the control of this pest and other pests of cotton, maize, and potato (4–6).

Bt crops are the most extensively planted genetically modified crops after those transformed for herbicide tolerance. In 2014, 79 million ha were planted with GM crops expressing *B. thuringiensis* insecticidal proteins, either alone (27.4 million ha) or in combination with herbicide tolerance (51.4 million ha) (4). The extensive use of Bt crops

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TABLE 1 Summary of the selection experiment of a field-collected population of *Heliothis virescens*

Generation	No. of selection episodes	No. of larvae selected	Vip3Aa concn ($\mu\text{g/ml}$)	No. of larvae transferred to normal diet ^a	No. of healthy pupae	Survival (%) ^b
2	1	330	1.5	140 ^c	95	29
3	2	768	2	362	276	36
4	3	768	2	244	214	28
5	4	864	2	414	345	40
6	5	1,120	2	430 ^c	241	22
7	6	1,184	2	368 ^c	252	21
8	7	821	2	440	350	43
9	8	1,161	2	624	532	46
10	9	1,232	2.5	600	482	39
11	10	1,152	3	745	542	47
12	11	663	4	534	422	64
13	12	800	20	395	305	38
14	13	768	20	594	478	54
15				403	307	76
16				576	386	67
17	14	715	20	480	380	53

^aOnly larvae that had developed to 2nd instar or higher were transferred.^bSurvival rate of larvae up to pupation.^cThe number of larvae included 1st instar as there was poor larval development.

has already led to the evolution of insect resistance in the field for some species of Lepidoptera and Coleoptera (7, 8). So far, no case of field resistance to Bt crops has been reported for *H. virescens*.

To avoid or delay the evolution of resistance to Bt crops, several strategies have been proposed, one of them being the combination of more than one *B. thuringiensis* gene coding for insecticidal proteins with different modes of action (gene pyramiding) (9, 10). Most Bt crops express one or more Cry proteins (insecticidal proteins that accumulate in a crystal or crystal-like structure during *B. thuringiensis* sporulation). Vip proteins are another family of insecticidal proteins produced during the vegetative phase of growth of *B. thuringiensis* and other bacteria (11). Vip3A proteins display broad insecticidal activity against many lepidopteran pests (11–13). Because Vip3A proteins do not share binding sites (14, 15) and have no sequence homology with Cry toxins (14, 16), their use in combination with Cry proteins in Bt crops will help to better preserve and extend the usefulness of this important insect control technology. In fact, Bt crops (cotton and maize) combining Cry1 and Vip3A proteins have already been registered and are being commercialized in the United States (17–19).

Resistant insects are important tools to validate resistance management practices and provide a means to identify resistance alleles with potential biological relevance to resistance evolution (20, 21). When selecting for resistance, it is preferable to start with samples derived from field populations because they exhibit potential resistance mechanisms that may evolve in the field (20, 21). Understanding the genetic basis of resistance to Bt toxins is important for developing and implementing strategies to delay and monitor pest resistance.

Very few studies have been carried out so far to select for resistance to Vip3 proteins, and the biochemical bases of resistance to these proteins are still unknown (22–24). In this article, we describe the result of the successful selection for Vip3Aa resistance of a field-derived population of *H. virescens* and the subsequent work carried out to characterize this population in terms of the genetic bases of resistance and whether Vip3Aa resistance confers cross-resistance to other *B. thuringiensis* insecticidal proteins.

RESULTS

Response to selection with Vip3Aa in an *H. virescens* population. A sample of the field-collected insects was subjected to selection with Vip3Aa. The average survival rate to pupation of Vip3Aa-selected larvae was 42% (Table 1). The Vip3Aa concentration

TABLE 2 Selection response to Vip3Aa toxin of a field-collected population of *H. virescens*

Population	No. of laboratory generations ^a	No. of selection episodes with Vip3Aa	LC ₅₀ (μg ml ⁻¹)	95% CI	Mean slope ± SE	No. of larvae tested ^b	RR ^c
Vip-Unsel	1		2.06	1.46–2.90	1.00 ± 0.11	384	
	7		0.73	0.43–1.24	0.80 ± 0.17	288	
	11		2.47	1.65–3.69	0.81 ± 0.16	288	
	12		2.63	1.87–3.70	0.82 ± 0.10	384	
	15		1.76	1.14–2.72	0.56 ± 0.07	384	
	18		1.13	0.72–1.75	0.66 ± 0.08	336	
Vip-Sel	5	3	2.44	0.92–6.48	0.26 ± 0.08	336	1
	7	5	2.02	1.19–3.43	0.47 ± 0.11	336	3
	9	7	38.1	7.39–197	0.48 ± 0.14	336	19
	11	9	516	111–2,400	0.33 ± 0.07	384	209
	12	10	246	102–595	0.29 ± 0.05	480	94
	14	12	>4,000 ^d				>2,000
	17	13	2,300	1,010–5,260	0.24 ± 0.05	432	2,040

^aVip-Unsel generations 15 and 18 were synchronous with Vip-Sel generations 14 and 17, respectively.

^bIncluding control.

^cRR, resistance ratio (LC₅₀ of Vip-Sel or Vip-SelREV divided by LC₅₀ of Vip-Unsel). The RRs for selections 3 and 7 were compared to that for Vip-Unsel generation 1.

^dLC₅₀ undetermined as mortality at the highest concentration of 4,000 μg ml⁻¹ was only 21%.

applied remained constant at 2 μg ml⁻¹ from the 3rd to the 9th generation, but it increased from the 10th generation (selection 9) onwards as resistance increased rapidly. No selection was applied at generations 15 and 16, as bioassay results indicated that 50% lethal concentrations (LC₅₀s) were unattainable as even high concentrations of Vip3A failed to kill sufficient larvae. A relaxation in selection aimed to reduce the level of resistance to allow the calculation of the LC₅₀. After 13 selections, the LC₅₀ of the selected population (Vip-Sel) was 2,300 μg ml⁻¹ with a resistance ratio of 2,040 relative to the unselected population (Vip-Unsel) (Table 2).

Stability of resistance in the Vip3Aa-selected population. A subpopulation of Vip-Sel was maintained continuously without selection and designated Vip-SelREV. After five generations without exposure to Vip3Aa (from generations 13 to 17), the Vip3Aa LC₅₀ for Vip-SelREV was 709 μg ml⁻¹. This value was significantly different (630-fold greater) from that with Vip-Unsel, which was 1.13 μg ml⁻¹ ($P < 0.01$) (Table 3). After 15 generations without exposure to Vip3Aa, the Vip3Aa LC₅₀ for Vip-SelREV was not significantly different from that for Vip-Unsel ($P > 0.01$) (Table 3).

Cross-resistance to Cry1Ab and Cry1Ac in the Vip3Aa-selected population. The LC₅₀ value of Cry1Ab for Vip-Sel was 7-fold greater than that for Vip-Unsel, a significant increase ($P < 0.01$). There was no significant difference in the Cry1Ac LC₅₀ for Vip-Sel compared with Vip-Unsel ($P > 0.01$) (Table 4).

Degree of dominance of resistance. Bioassays of F₁ progeny from single-pair crosses with two concentrations of Vip3Aa showed that dominance of resistance depended upon the F₁ reciprocal cross and the concentration of Vip3Aa (Table 5). The mean dominance values of F₁ progeny from Vip-Sel males × Vip-Unsel females showed that the degree of dominance (h) slightly increased with an increase in Vip3Aa concentration. Resistance was incompletely dominant (mean $h = 0.47$ to 0.58) based on

TABLE 3 Stability of resistance to Vip3Aa toxin in the Vip-SelREV population of *H. virescens* after 5 and 15 generations without selection

Population	No. of laboratory generations ^a	LC ₅₀ (μg ml ⁻¹)	95% CI	Mean slope ± SE	No. of larvae tested ^b	RR ^c
Vip-Unsel	19	1.13	0.72–1.75	0.66 ± 0.08	336	
Vip-SelREV	18	709	246–2,040	0.27 ± 0.04	480	627
Vip-Unsel	28	1.78	1.20–2.63	0.73 ± 0.10	240	
Vip-SelREV	29	1.96	1.45–2.65	1.76 ± 0.30	240	1.10

^aVip-Unsel generations 19 and 28 were synchronized with Vip-SelREV generations 18 and 29.

^bIncluding control.

^cRR, resistance ratio (LC₅₀ of Vip-SelREV divided by LC₅₀ of Vip-Unsel).

TABLE 4 Cross-resistance of Vip3Aa-selected *H. virescens* to Cry1Ac and Cry1Ab

Population	Toxin	No. of laboratory generations ^a	No. of generations of selection with Vip3Aa	LC ₅₀ (μg ml ⁻¹)	95% CI	Slope ± SE	No. of larvae tested ^b	RR ^c
Vip-Unsel	Cry1Ab	16		1.46	0.92–2.31	0.76 ± 0.10	288	
Vip-Sel	Cry1Ab	15	13	4.63	1.89–11.3	0.40 ± 0.22	288	3.2
Vip-Unsel	Cry1Ab	19		2.49	1.84–3.38	1.17 ± 0.15	288	
Vip-Sel	Cry1Ab	18	14	16.7	4.06–68.5	0.37 ± 0.09	288	6.7
Vip-Unsel	Cry1Ac	16		0.41	0.27–0.61	1.16 ± 0.16	240	
Vip-Sel	Cry1Ac	15	13	2.95	0.91–9.58	0.59 ± 0.17	336	7.1
Vip-Unsel	Cry1Ac	19		1.67	1.06–2.63	0.87 ± 0.14	288	
Vip-Sel	Cry1Ac	18	14	1.67	1.14–2.43	0.85 ± 0.10	384	1.0

^aVip-Unsel generations 16 and 19 were synchronized with Vip-Sel generations 15 and 18, respectively.^bIncluding control.^cRR, resistance ratio (LC₅₀ of Vip-Sel divided by LC₅₀ of Vip-Unsel).

larval mortality at 100 and 500 μg ml⁻¹. In comparison, the mean dominance values of F₁ progeny from Vip-Sel females × Vip-Unsel males showed that resistance was almost completely recessive both at 100 and 500 μg ml⁻¹ (Table 5).

Evaluation of genetic variation within the populations by single-pair crosses.

The mortality with Vip3Aa of the progeny of F₁ families from crosses between Vip-Sel and Vip-Unsel (Table 5) indicated that there were significant differences at three levels. There were significant differences in mortality within the seven single-pair families at 100 μg ml⁻¹ (F_{6, 17} = 44.51; *P* < 0.001) and within the 11 single-pair families at 500 μg ml⁻¹ (F_{11, 32} = 5.98; *P* < 0.001). There was a significant difference in mortality between the reciprocal crosses (Vip-Sel female × Vip-Unsel male and Vip-Sel male × Vip-Unsel female) at 100 μg ml⁻¹ (F_{1, 22} = 13.55; *P* < 0.01) and 500 μg ml⁻¹ (F_{1, 42} = 24.67; *P* < 0.001). There were significant differences in mortality within the Vip-Sel female × Vip-Unsel

TABLE 5 Dominance of resistance to Vip3Aa in the Vip-Sel *H. virescens* population using mortality values as a function of the Vip3Aa concentration for single-pair F₁ families

Population or family ^a	Characteristic of larvae at Vip3Aa concn of ^b :					
	100 μg ml ⁻¹			500 μg ml ⁻¹		
	Mortality (%)	Fitness	<i>h</i>	Mortality (%)	Fitness	<i>h</i>
Vip-Sel	38	1.00		27	1.00	
Vip-Unsel	90	0.16		89	0.16	
Single-pair F ₁ families						
Vip-Sel ♀ × Vip-Unsel ♂						
A	100	0.00	0.00	81	0.26	0.12
B	65	0.56	0.48	65	0.48	0.38
C				94	0.08	0.00
D				91	0.13	0.00
E	100	0.00	0.00	96	0.06	0.00
F				98	0.20	0.05
G	77	0.37	0.25	67	0.46	0.36
Mean	88	0.19	0.03	86	0.20	0.05
Vip-Unsel ♀ × Vip-Sel ♂						
H				68	0.45	0.34
I	65	0.56	0.47	28	0.99	0.98
J	60	0.63	0.56	47	0.73	0.69
K				67	0.46	0.36
L	87	0.21	0.06	65	0.48	0.38
Mean	66	0.55	0.47	53	0.65	0.58

^a♀, female; ♂, male.

^bMortality was adjusted for control mortality by Abbott's method (58). Fitness is the survival rate of the larvae divided by the survival rate of the Vip-Sel larvae. (The survival rate is estimated as 100 – % mortality.) *h*, degree of dominance. Estimates of dominance range from 0 (completely recessive resistance) to 1 (completely dominant). Mean values are highlighted in boldface.

TABLE 6 Direct test of monogenic inheritance for resistance to Vip3Aa by comparing expected and observed mortalities of the backcross of F_1 (Vip-Sel $\times$ Vip-Unsel) and Vip-Sel populations of *H. virescens* at a Vip3Aa concentration of 100 $\mu\text{g ml}^{-1}$

	No. of larvae tested	Observed mortality (%)	Autosomal			Sex linked		
			Expected mortality (%) ^b	χ^2 (df = 1)	P	Expected mortality (%) ^c	χ^2 (df = 1)	P
Single-pair mating ^a								
Vip-Sel ♀ × ♂	96	44						
Vip-Unsel ♀ × ♂	240	90						
F ₁ A = Vip-Sel ♀ × Vip-Unsel ♂	168	89						
F ₁ B = Vip-Unsel ♀ × Vip-Sel ♂	120	70						
F ₁ A ♀ × Vip-Sel ♂	733	80	67	64.41	<0.001	70	1.42	<0.05
F ₁ B ♀ × Vip-Sel ♂	635	86	57	210.61	<0.001	44	40.09	<0.05
F ₁ A ♂ × Vip-Sel ♀	834	79	67	62.07	<0.001	67	2.14	<0.05
F ₁ B ♂ × Vip-Sel ♀	507	69	57	31.95	<0.001	67	16.75	<0.05

^a♀, female; ♂, male.^bExpected number of larvae dead at 100 $\mu\text{g ml}^{-1}$ = 0.5 $\times$ (observed mortality of F_1 larvae + observed mortality of Vip-Sel).^cExpected number of larvae dead at 100 $\mu\text{g ml}^{-1}$ according to the sex-linked hypothesis and the observed mortality of the parental lines and the two F_1 crosses. The first backcross would produce the same offspring as F_1 B, the second backcross the same as Vip-Sel ♀ $\times$ ♂, and the third and fourth backcrosses a mixture of the offspring produced by F_1 A and Vip-Sel ♀ $\times$ ♂.

male cross at 100 $\mu\text{g ml}^{-1}$ for the four single-pair crosses ($F_{3,10} = 52.67$; $P < 0.001$) and at 500 $\mu\text{g ml}^{-1}$ for the seven single-pair crosses ($F_{6,19} = 4.58$; $P < 0.05$). Likewise, there were significant differences in mortality within the Vip-Sel male $\times$ Vip-Unsel female cross at 100 $\mu\text{g ml}^{-1}$ for the three single-pair crosses ($F_{2,7} = 8.19$; $P < 0.05$). However, at 500 $\mu\text{g ml}^{-1}$, there was no significant difference in the mortality within the five single-pair crosses ($F_{4,13} = 1.85$; $P > 0.05$).

Mode of inheritance in the Vip-Sel population. The direct test for a monogenic (single-gene) mode of inheritance of Vip3Aa resistance showed significantly greater mortality ($P < 0.001$) than expected values at 100 $\mu\text{g ml}^{-1}$ and 500 $\mu\text{g ml}^{-1}$ Vip3Aa for the backcross progeny of F_1 (Vip-Sel $\times$ Vip-Unsel) and Vip-Sel (Table 6), indicating that more than one locus is involved in conferring resistance.

DISCUSSION

The present study reports the laboratory selection of a *H. virescens* population for resistance to Vip3Aa. To our knowledge, resistance to Vip3Aa had only been obtained in *Spodoptera litura* (23), *Spodoptera frugiperda* (24), *Helicoverpa armigera*, and *Helicoverpa punctigera* (22). Our results show that the development of Vip3Aa resistance in the *H. virescens* population was rapid, reaching a level of 200-fold resistance after 9 selection episodes and over 2,000-fold resistance after 13 episodes of selection. A similar fast development of Cry1Ac resistance was observed in a *Helicoverpa zea* population that attained 123-fold resistance after 11 selection episodes (25). In the present study, the rapid development of resistance may have also been helped by the procedure of only selecting larvae that had moulted to at least 2nd instar after the 7-day bioassay period, thus removing more susceptible individuals, a procedure also followed for the selection of *H. zea* with Cry1Ac (25). The two big increases in resistance observed from generations 9 to 11 and 12 to 14 may be due to the sequential accumulation of resistance alleles at different loci.

The alleles at different loci responsible for resistance to Vip3Aa appeared to be unstable as resistance in Vip-Sel declined significantly from generations G13 to G28. Little or no cross-resistance was apparent between Vip3Aa and Cry1Ab or Cry1Ac. There was 7-fold resistance to Cry1Ab based on mortality data. Only resistance ratios that are more than 10-fold will generally reflect heritable decreases in susceptibility (26, 27); thus, no significant cross-resistance can be assumed. The same lack of cross-resistance against Cry proteins (Cry1Ac and Cry2Ab) was found in the Vip3Aa-resistant *H. armigera* and *H. punctigera* populations (22). The lack of observed cross-resistance is also consistent with the results of Jackson et al. (28), who found no cross-resistance to Vip3Aa in three *H. virescens* populations selected for resistance to Cry1 toxins and

Cry2A. A Cry1Ac-resistant *H. zea* population also demonstrated a lack of cross-resistance to Vip3Aa (25). A Cry1Ac-selected population of *H. armigera* showed 1.7-fold resistance to Vip3Aa (29). In another study, cross-resistance between Cry1Ac and Vip3Aa was low in Cry1Ac-Sel *H. armigera* (30). All of these findings are supported indirectly by previous work demonstrating the lack of sequence homology and differing modes of action between Vip3Aa and Cry toxins (14–16, 31), thus reducing the likelihood of cross-resistance mechanisms based on altered target site, the most commonly observed resistance mechanism (20, 21).

The significantly lower mortality of larvae from the Vip-Sel male $\times$ Vip-Unsel female cross compared with the Vip-Sel female $\times$ Vip-Unsel male cross suggested a paternal influence on Vip3Aa resistance. While maternal influences have been suggested in Cry1Ac- and Cry1Ab-resistant *Plutella xylostella* populations (32–34), sex linkage was rejected in a previous study as no significant difference in the number of male and female survivors was found (33). Reduced mating success observed in resistant males may help to limit an increase in the frequency of the resistant allele and, with the possible paternal influence on Vip3A resistance, may contribute to delays in the evolution of resistance in the field with the involvement of current management strategies involving the use of refuges.

The degree of dominance of Vip3Aa depended on the F_1 reciprocal cross and the Vip3Aa concentration. Inheritance of resistance in the Vip-Sel female $\times$ Vip-Unsel male cross was almost completely recessive using mortality data at two toxin concentrations (100 and 500 $\mu\text{g ml}^{-1}$), whereas it was incompletely dominant (mean $h = 0.53$) for the Vip-Sel male $\times$ Vip-Unsel female cross. This apparent split mode of dominance gives further evidence of a possible paternal influence on Vip3Aa resistance. A similar split, but reversed, was found in a *P. xylostella* population with incomplete dominance in resistant females crossed with susceptible males but incomplete recessively in resistant males crossed with susceptible females (34). Dominance of resistance in other *H. virescens* populations against Cry1Ac and Cry1Ab has been reported to be either incompletely recessive or incompletely dominant (35–37). This variation in degree of dominance of resistance to *B. thuringiensis* toxins has also been found in *P. xylostella* (38, 39), *H. armigera* (40, 41), and *Pectinophora gossypiella* populations (42, 43). Dominance of resistance in other selected populations against Cry toxins has revealed both recessive and incompletely dominant resistance that can vary depending on the concentration of the toxin used. However, the general pattern frequently found shows that the degree of dominance decreases with increasing toxin concentration (34, 44, 45), the opposite trend to that found in the present study.

In the present study, analysis involving the backcross experiments suggested that resistance to Vip3Aa in Vip-Sel was due to more than one locus (polygenic) at both concentrations tested for mortality data. Other populations resistant to Cry toxins have also been shown to exhibit polygenic resistance: for example, populations of *H. virescens* (46) and *H. armigera* (47) resistant to Cry2A and populations of *P. xylostella* (32) and *P. gossypiella* (48) resistant to Cry1Ac. Nevertheless, monogenic resistance has been found in other populations of *H. virescens* (37), *H. armigera* (41), *P. xylostella* (49), and *Ostrinia nubilalis* (50).

Our data regarding the degree of dominance and the number of genes involved in resistance to Vip3Aa are in contrast to the type of inheritance found in the Vip3Aa-resistant populations of *H. armigera* and *H. punctigera* from Australia. For these populations, resistance was found to be completely or almost completely recessive and most likely due to a single locus (22). This difference in the genetic bases of resistance is most likely a consequence of the different approaches followed to obtain the resistant populations. While we used a classical selection protocol, Mahon et al. (22) used the F_2 screen method. It is well known that classical selection regimes tend to select for additive genes, in contrast to the F_2 screen method, which is based on inbreeding and selects for homozygotes at the same locus (21).

Several studies have shown that Cry1A and Vip3A proteins do not compete for the same binding sites (reviewed in reference 11). The observation that Vip3Aa-

resistant *H. virescens* insects are not cross-resistant to Cry1A proteins might suggest a change in the Vip3Aa binding site, as opposed to the more unspecific mechanisms, such as altered proteolysis, increased cell repair, sequestration by esterases, and elevated immune response. Studies to investigate the biochemical mechanism of resistance in the Vip3Aa-resistant population are in progress.

MATERIALS AND METHODS

Insects and selection. A field population of *H. virescens* (WF06) (51) was collected from velvetleaf, *Abutilon theophrasti*, on Wildy Farms, Leachville, Mississippi County, AR, USA, in September 2006. The WF06 population was divided into two subpopulations at the larval stage of the 2nd generation of laboratory culture. One subpopulation was left unselected (Vip-Unsel), and the other was selected with Vip3Aa (Vip-Sel) at the 1st instar larval stage from the 2nd generation onwards. The initial concentration of Vip3Aa used for selection was 1.5 $\mu\text{g/ml}$, and this was increased during the selection process up to 20 $\mu\text{g/ml}$ at generation 13 (Table 1). Only larvae that had moulted to at least 2nd instar after the 7 days of exposure to the Vip3Aa toxin were transferred to untreated diet and selected to give rise to the adults that will become the parents to produce the next generation. The number of larvae selected per generation ranged from approximately 600 to 1,200, with the exception of the initial selection, when the number of larvae available (330) was low. Insects were reared in the laboratory on artificial diet at $25 \pm 5^\circ\text{C}$ and $70\% \pm 5\%$ rH under a 16-h-light/8-h-dark cycle.

Bacillus thuringiensis toxins. Vip3Aa19 was obtained from Syngenta (Research Triangle Park, NC, USA) and stored at -80°C . The Vip3Aa protoxin was overexpressed in *Escherichia coli* and purified as described by Yu et al. (52). Cry1Ab and Cry1Ac were obtained from Neil Crickmore and Ali Sayyed (University of Sussex, United Kingdom) and stored at -80°C . Cry proteins (protoxins) were expressed as inclusion bodies in *E. coli*. Cells were broken by sonication, and the inclusion bodies were subjected to successive washes with 0.5 M NaCl and water as described by Sayyed et al. (38).

Bioassays. The diet incorporation method as described by Dulmage et al. (53) was used to determine the susceptibility of neonate to Vip3Aa toxin. Five to nine toxin concentrations, plus a control of distilled water only, were used in the bioassay, with 48 larvae per concentration split into 4 replicates. Bioassays were performed in 24-well plates. Approximately 3 ml of diet with toxin was dispensed into each well and allowed to solidify. One 1st instar larva (<24 h) was transferred with a fine brush to each well. Breathable polyester film was used to cover the wells. The bioassay plates were placed in a controlled environment room at $25 \pm 2^\circ\text{C}$, $65\% \pm 10\%$ rH, and a 16-h-light/8-h-dark cycle. Mortality was determined after 7 days, with mortality recorded as larvae that failed to respond to gentle contact with a fine brush.

Stability of resistance. A subpopulation of Vip-Sel was designated Vip-SelREV and maintained continuously without selection for 15 generations. Bioassays were conducted at generations 18 and 28, after 5 and 15 generations, respectively, without exposure to Vip3Aa.

Maternal/paternal effects, genetic variation, and mode of inheritance in the Vip-Sel population. F_1 progeny from 12 single-pair crosses between the Vip-Unsel and Vip-Sel populations were obtained. Single pairs consisted of a Vip-Unsel virgin male and a Vip-Sel virgin female or vice versa. The F_1 progeny from each family were reared on artificial diet. F_1 larvae were tested in a diet incorporation bioassay with 0 (control), 100, and 500 $\mu\text{g ml}^{-1}$ of Vip3Aa. To obtain the F_2 progeny, single-pair crosses were made between the F_1 progeny and Vip-Sel (73 for all the backcrosses). The F_2 progeny from single-pair setups (also 73 crosses) were tested with 0 (control), 100, and 500 $\mu\text{g ml}^{-1}$ of Vip3Aa. Mortality was determined after 7 days and used to determine the genetic variation within the populations and the mode of inheritance (monogenic or polygenic). Tests of F_1 and F_2 progeny from single-pair crosses enabled detection of genetic variation within parental strains, which is not possible with mass crosses (54).

Estimation of degree of dominance. The degree of dominance (h) was estimated using the single-concentration method, based on Hartl's definition of dominance and on survival at any single concentration (44).

Statistical analysis. Statistical package R version 2.8.1 (55) was used for analysis of LC bioassay data. The data were analyzed by specifying a generalized linear model with binomial errors (or quasibinomial if data were overdispersed) to estimate the slope and its standard error, with significance tested at the 5% level. Pairwise comparisons of LC_{50} s were significant at the 1% level if their respective 95% confidence intervals (CIs) did not overlap (56).

The degree of dominance (h) was estimated using the single-concentration method, based on Hartl's (57) definition of dominance and based on survival at any single concentration (42). The calculation is $h = (w_{12} - w_{22}) / (w_{11} - w_{22})$, where w_{11} , w_{12} , and w_{22} are the fitness values at a particular concentration for resistant homozygotes, heterozygotes, and susceptible homozygotes, respectively. The fitness of treated resistant homozygotes (Vip-Sel) is defined as 1. The fitness for treated susceptible homozygotes (Vip-Unsel) was determined as the survival rate of treated Vip-Unsel larvae divided by the survival rate of treated Vip-Sel larvae. For treated heterozygotes (Vip-Sel $\times$ Vip-Unsel), the fitness was determined as the survival rate of treated F_1 larvae divided by the survival rate of treated Vip-Sel larvae. Mortality was corrected for control mortality using Abbott's method (58). The survival rate was estimated as $100 - \%$ mortality. Values of h range from 0 (completely recessive) to 1 (completely dominant) (44). The backcross data were used as a direct test of a monogenic model of resistance (59). The null hypothesis is that resistance is controlled by one locus with two alleles (monogenic resistance), S (susceptible) and R (resistant), with the parental resistant population RR, and the F_1 offspring RS. If so, then a backcross of F_1 (Vip-Sel $\times$ Vip-Unsel) RS $\times$ Vip-Sel RR will produce progeny that are 50% RR and 50% RS. This

hypothesis is tested through calculation of the expected mortality, followed by a χ^2 test for goodness of fit between the expected and observed mortality of the backcross data at each concentration. The expected mortality, $Y(x)$, for the backcross progeny at concentration x is calculated as $Y(x) = 0.50 (WRS + WRR)$, where WRS and WRR are the mortality values of the presumed RS (F_1) and RR (resistant parental line, Vip-Sel) genotypes at concentration x , respectively (59, 60). The χ^2 test for goodness of fit between the backcross and expected mortality was calculated as described by Sokal and Rohlf (61) as $\chi^2 = (F_1 - pn)^2 / pqn$, where F_1 is the observed number of dead larvae in the backcross generation at concentration x , p is the expected proportion of dead larvae calculated as $Y(x)$, n is the number of backcross progeny exposed to concentration x , and $q = 1 - p$. The χ^2 value is compared with the χ^2 distribution with 1 degree of freedom, and if P is <0.05 , the null hypothesis of monogenic resistance is rejected (59, 60). The genetic variation within Vip-Unsel, Vip-Sel, F_1 reciprocal crosses, backcrosses, and F_2 crosses was determined using the analysis of variance (ANOVA) to test for significant variation in mortality among families produced by the single-pair crosses. Percentage mortality data were arcsine transformed prior to ANOVA. Backcrosses were used as a direct test of a monogenic model of resistance (59).

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