Cross-Resistance Responses of Cry1Ac-Selected *Heliothis virescens* (Lepidoptera: Noctuidae) to the *Bacillus thuringiensis* Protein Vip3A

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ABSTRACT One susceptible and three Cry1Ac-resistant strains of tobacco budworm, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), were used in laboratory studies to determine the level of cross-resistance between the *Bacillus thuringiensis* (Berliner) toxins Cry1Ac and Vip3A by using concentration–mortality and leaf tissue experiments. Concentration–mortality data demonstrated that the three Cry1Ac-resistant *H. virescens* strains, YHD2, KCBhyb, and CxC, were at least 215- to 316-fold resistant to Cry1Ac compared with the susceptible strain, YDK. Results from Vip3A concentration–mortality tests indicated that mortality was similar among all four *H. virescens* strains. Relative larval growth on Cry1Ac reflected concentration–mortality test results, because YHD2 larval growth was mostly unaffected by the Cry1Ac concentrations tested. Growth ratios for KCBhyb and CXC indicated that they had a more moderate level of resistance to Cry1Ac than did YHD2. Relative larval growth on Vip3A was highly variable at lower concentrations, but it was more consistent on concentrations of Vip3A above 25 μg/ml. Differences in larval growth among strains on Vip3A were not as pronounced as seen in Cry1Ac experiments. Mortality and larval growth also was assessed in leaf tissue bioassays in which YDK, CxC, and KCBhyb neonates were placed onto leaf disks from non-Bt and Bt cotton, *Gossypium hirsutum* L., for 5 d. Three Bt lines were used in an initial bioassay and consisted of two Vip3A-containing lines, COT203 and COT102, and a Cry1Ac-producing line. Mortality of KCBhyb and CXC was lower than that of YDK larvae in the presence of leaf tissue from the Cry1Ac-producing line. Additionally, increased larval growth and leaf tissue consumption on Cry1Ac-containing leaf disks was observed for KCBhyb and CXC. Mortality and larval weights were similar among strains when larvae were fed leaf tissue of either non-Bt, COT203, or COT102. A subsequent leaf tissue bioassay was conducted that evaluated four cotton lines: non-Bt, Cry1Ab-expressing, Vip3A-expressing, and pyramided-toxin plants that produced both Cry1Ab and Vip3A. Mortality levels were similar among strains when fed non-Bt, Vip3A-expressing, or pyramided-toxin leaf tissues. Mortality was higher for YDK than for KCBhyb or CXC on Cry1Ab-expressing leaf tissues. No differences in larval weights were observed among strains for any genotype tested. Results of these experiments demonstrate that cross-resistance is nonexistent between Cry1Ac and Vip3A in *H. virescens*. Thus, the introduction of Vip3A-producing lines could delay Cry1Ac-resistance evolution in *H. virescens*, if these lines gain a significant share of the market.

KEY WORDS *Bacillus thuringiensis*, Vip3A, *Heliothis virescens*, cross-resistance, resistance management

Transgenic cotton, *Gossypium hirsutum* L., that contains genes from *Bacillus thuringiensis* Berliner (Bt) that code for production of proteins toxic to two major caterpillar pests, *Helicoverpa zea* (Boddie) and *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), has been an important insect pest management tool since commercial release in 1996. Bollgard (Monsanto Company, St. Louis, MO) cotton varieties that produce a single endotoxin, Cry1Ac, have provided absolute control of *H. virescens*, but control problems with *H. zea* have arisen occasionally (Mahaffey et al. 1995, Jackson et al. 2003). In recent years, two other transgenic cottons were introduced commercially under the trade names of Bollgard II (Monsanto Company) and WideStrike (Dow AgroSciences). Bollgard II contains Bt genes that code for production of the Cry1Ac and Cry2Ab endotoxins, both of which are active against caterpillar pests (Greenplate et al. 2003). WideStrike also produces two Bt endotoxins, Cry1Ac and Cry1 F (Pellow et al. 2002). Both of these pyramided-gene cottons, particularly Bollgard II, have

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been shown to provide excellent control of *H. zea* in field tests across the cotton belt (Jackson et al. 2003, 2005). Thus, superior management tools for caterpillar pests of cotton are now available. However, because both Bollgard II and WideStrike cotton genotypes produce the Cry1Ac endotoxin, resistance evolution in *H. zea* and *H. virescens* remains a concern.

Transgenic cottons engineered to produce a novel Bt protein, Vip3A, have recently received a nonregulated status for planting in the United States. The Vip3A protein is unique in that it is active in the vegetative phase as well as during the sporulation phase, whereas Cry1 and Cry2 protein activities are restricted to the sporulation phase (Estruch et al. 1996, Schnepf et al. 1998). Preliminary field data suggest that cotton producing the Vip3A protein, COT202 and COT203, exhibit levels of *H. zea* control similar to that reported for Bollgard II and WideStrike varieties (Bradley et al. 2004), but control of *H. virescens* by Vip3A-producing lines is less than that observed in Bt lines expressing the Cry1Ac protein. The Vip3A protein not only provides a new control measure for caterpillar pests but also may be considered as a potentially useful second-generation Bt toxin for resistance management of *H. zea* and *H. virescens* (Lee et al. 2003).

Commercialization will likely be sought for varieties expressing both Vip3A and Cry1Ab. Because cross-resistance has been demonstrated between Cry1Ac and Cry1Ab, insect resistance management will remain a concern with VipCot (Syngenta Crop Protection, Greensboro, NC) varieties. However, this introduction will provide a resistance management tool in addition to Bollgard II and WideStrike.

Here, we report on the impact of Vip3A toxin on mortality and growth of *H. virescens* strains that have different mechanisms and levels of resistance to Cry1Ac as well as other toxins.

Materials and Methods

Insects. Four strains of *H. virescens* were used in these experiments. The control strain, YDK, originated from *H. virescens* egg collections from tobacco (*Nicotiana* spp.) fields near Carpenter, NC, in July 1988 (Gould et al. 1992). The YHD2 strain was derived from the same collection of eggs but has been selected for adaptation to the Cry1Ac toxin. The YHD2 strain is highly resistant (20,000- to 40,000-fold) to Cry1Ac. In addition to developing cross-resistance to Cry1A and Cry1Fa toxins, this strain is also slightly cross-resistant to Cry2A (Gould et al. 1995, Jurat-Fuentes et al. 2002). The CXC strain was derived from the field at the same time as the YDK strain and was initially selected on Cry1Ac (Gould et al. 1992), and then it was selected on Cry2Aa2-containing artificial diet. CXC is a Cry1Ac- and Cry2Aa2-resistant strain that is ≈200–300-fold resistant to Cry1Ac and 393-fold resistant to Cry2Aa2 (Kota et al. 1999, Jurat-Fuentes et al. 2003). The KCBhyb strain was generated by crossing adults of the resistant KCB strain with susceptible moths (Jurat-Fuentes et al. 2003). The parental strain, KCB, which was collected from fields in Johnston and eastern Wake Counties, NC, also was selected with Cry1Ac. This strain developed cross-resistance to Cry2Aa (Gould et al. 1992, Forcada et al. 1999), and the resulting backcross, KCBhyb, was selected with Cry2Aa to elevate resistance. The KCBhyb strain was demonstrated to be 200–300-fold resistant to Cry1Ac and >250-fold resistant to Cry2Aa (Jurat-Fuentes et al. 2003). In addition to Cry1Ac and Cry2Aa, both KCBhyb and CXC are resistant to Cry1Aa, Cry1Ab, and Cry1Fa (Jurat-Fuentes et al. 2003) as well as other cry toxins (F.G., unpublished data).

The CXC strain possessed a mechanism conferring resistance to both Cry1Ac and Cry2Aa that was not related to an alteration of toxin binding (Forcada et al. 1999, Kota et al. 1999). It was hypothesized that differential midgut protease activity was the resistance mechanism in CXC (Forcada et al. 1999). Resistance to Cry1Ac and Cry2Aa in KCBhyb was caused by two separate resistance mechanisms (Forcada et al. 1999). The first resistance mechanism in KCBhyb was hypothesized to be the absence of a cadherin-like protein as observed in YHD2 (Gahan et al. 2001), whereas the second would be similar to the altered protease activity observed in CXC (Jurat-Fuentes et al. 2003).

Toxins and Diet. The Vip3A toxin used in the bioassays was obtained as a lyophilized powder (66.8% [AI] powder) provided by Syngenta Biotechnology, Inc. (Greensboro, NC). After the first of three runs, additional lyophilized powder containing the Vip3A toxin (0.892857 μg [AI]/μg powder) was supplied for use in runs 2 and 3. The Vip3A-containing powder was reconstituted in 200 mM ammonium carbonate buffer, pH 9.5, before use in these bioassays. The Cry1Ac toxin was obtained from a commercial Bt formulation, MVP II (Mycogen Corp., San Diego, CA), which contains only a single toxin with no other toxic ingredients.

Assessing Cross-Resistance. Artificial diet was prepared as described by Burton (1970), and multiple-concentration serial dilution tests were performed for each toxin. A two-fold increment serial dilution was used to obtain concentrations of 3.125 to 400 μg/ml of Vip3A toxin. For Cry1Ac, concentrations of 0.32 to 1000 μg/ml were obtained from a five-fold increment serial dilution. Then, 0.2 ml of either toxic or nontoxic artificial diet was placed into each of well of a 128-well bioassay tray (Bio-Serv, Frenchtown, NJ).

Forty-eight neonates from each *H. virescens* strain were tested on each dose of Vip3A-containing diet, whereas only 32 from each strain were tested per concentration of Cry1Ac. Larvae were placed onto diet using a fine camel's-hair paintbrush. Once neonates were in place, bioassay tray lids (Bio-Serv) with prepunched holes for air exchange were used to cover the wells. Trays of insects were maintained in a rearing room at 27 ± 2°C with a photoperiod of 14:10 (L:D) h. Mortality assessments were made, and larval weights were obtained using a XE-100 balance (Denver Instrument, Denver, CO) 6 d after neonates were placed on diet.

Survival on Vip3A-Expressing Cotton. For the initial assay, field plots were established near Jamesville, NC, that included Vip3A-expressing, Cry1Ac-expressing, and non-Bt cotton genotypes. Cotton lines that produced the Vip3A toxin included COT203 and COT102. Both transgenic lines were derived from Coker 312, which was the nontransgenic line used in the experiment. The Cry1Ac-expressing cotton line was event 531, also derived from Coker 312, and was used in the experiment as a check for the Cry1A-resistant *H. virescens* strains. All cotton genotypes were planted on either 19 or 20 May 2004. Terminal leaves were collected on 25 August for testing.

For the second assay, field plots were established near Jamesville, NC, and Winterville, MS, that included Vip3A-expressing, Cry1Ab-expressing, and non-Bt cotton genotypes. COT102 produced the Vip3A toxin, whereas 67B produced the Cry1Ab toxin. A pyramided-toxin cotton, 'COT102/67B', expressed both the Cry1Ab and Vip3A toxins. All transgenic lines were derived from Coker 312, which was the nontransgenic line used in the experiment. Cotton genotypes were planted on 18 May 2006 in North Carolina and 16 May 2006 in Mississippi. Terminal leaves were collected on 10 July in North Carolina and 19 July in Mississippi for testing.

Young leaves ≈7–10 cm in diameter were collected from the terminal region of each cotton genotype. Leaves were immediately placed in a cooler with blue ice and transported to the laboratory where a set of leaf disks was punched from each leaf by using a 1.5-cm cork borer. Four leaf disks from the appropriate cotton line were placed into a Fisherbrand Media-Miser petri dish (Fisher International Inc., Hampton, NH) that contained a single filter paper moistened with 0.5 ml of distilled water to prevent desiccation. A single neonate was placed onto the leaf disks in each dish for a total of 50 larvae from each strain (YDK, KCBhyb, and CXC) on leaf disks from each of four cotton lines. YHD2 was not used in the leaf tissue bioassays because of its poor performance on non-Bt cotton. Petri dishes were bound by rubber bands and allowed to incubate at 27°C under a photoperiod of 14:10 (L:D) h for 5 d.

Statistical Analyses. Concentration–mortality data were analyzed using the probit procedure (SAS Institute 1982) to estimate the LC_{50} and fiducial limits for each strain on each toxin. Because of variability among strains in mean larval weights on the control diet in each experiment, raw weight data from sublethal concentrations of each toxin were used to calculate growth ratios and standard errors of larvae on toxic diet relative to larval performance on nontoxic diet.

Larval mortality means from the first leaf tissue experiment were separated by testing the equality of percentages as described by Sokal and Rohlf (1995). For the second experiment, mortality and larval weight data were subjected to analysis of variance (ANOVA), and means were separated ($P \leq 0.05$) by using Fisher Protected least significant difference (LSD) test. For each experiment, percent mortality and larval weights are presented for each of three *H. virescens* strains (YDK, KCBhyb, and CXC) on each cotton line.

Table 1. LC_{50} PROBIT values for Cry1Ac-resistant and susceptible tobacco budworm (TBW) strains on artificial diet containing Cry1Ac or Vip3A averaged across three runs

TBW strain	Toxin	Slope ± SE	LC_{50}	Fiducial limits	χ^2
YDK	Cry1Ac	0.470 ± 0.178	1.62	0.63–5.31	0.008
YHD2	Cry1Ac	−0.001 ± 0.001	NC	NC	0.368
KCBhyb	Cry1Ac	0.003 ± 0.001	347.62	219.90–604.36	<0.001
CXC	Cry1Ac	0.002 ± 0.001	512.50	304.68–1,036.76	<0.001
YDK	Vip3A	0.009 ± 0.001	179.03	158.82–204.22	<0.001
YHD2	Vip3A	0.009 ± 0.001	209.60	183.25–244.11	<0.001
KCBhyb	Vip3A	0.012 ± 0.001	185.18	168.43–205.88	<0.001
CXC	Vip3A	0.010 ± 0.001	207.50	181.30–243.97	<0.001

NC, not calculated by SAS probit program (SAS Institute 1982) because of poor fit to log/probit model.

Results

Cross-Resistance Response. Comparisons of larval strains from multiple concentration–mortality tests with Cry1Ac (Table 1) demonstrated that the three Cry1Ac-resistant *H. virescens* strains were 215- to 316-fold resistant compared with the YDK strain. LC_{50} values of the KCBhyb and CXC strains ranged from 347.6 to 512.5 μg/ml, whereas that of YDK was 1.62 μg/ml. No LC_{50} values or fiducial limits were calculated for the YHD2 strain on Cry1Ac-containing diet because none of the doses tested (up to 1,000 μg/ml) increased the level of mortality above that of the control.

Multiple concentration–mortality tests with Vip3A (Table 1) indicated that Cry1Ac-resistant and susceptible *H. virescens* strains were equally susceptible to this toxin. Strains YHD2, KCBhyb, and CXC had LC_{50} values ranging from 185.18 to 209.6 μg/ml, whereas that of YDK was 179.0 μg/ml. Within-run concentration–mortality data revealed that 62–207 times more Vip3A toxin was required to kill 50% of the YDK strain compared with Cry1Ac. However, in most instances, less Vip3A toxin was required to reach 50% mortality for the Cry1Ac-resistant strains compared with Cry1Ac.

Relative larval growth on Cry1Ac reflected concentration–mortality test results (Fig. 1). Most larval growth ratios for YHD2 remained near 1, which indicates no difference from larval growth on the control diet. Growth ratios for KCBhyb and CXC suggested a more moderate level of resistance to Cry1Ac than that of YHD2.

Growth ratios of larvae on Vip3A were highly variable at the lower concentrations (Fig. 2). On average, CXC grew larger on Vip3A concentrations up to 25 μg/ml than it did on regular diet. There was, however, a clear pattern of decreasing growth with increasing toxin concentration. Relative growth rates were most consistent on concentrations of Vip3A from 50 μg/ml and higher, where growth rates were much lower than

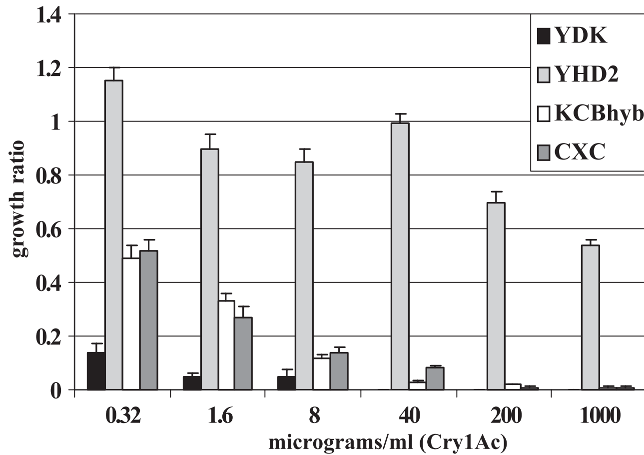

Fig. 1. Comparative growth ratios of Cry1Ac-resistant and susceptible *H. virescens* strains on Cry1Ac serial dilutions averaged across three runs.

on the control diet. In no cases were the differences in growth rates of the strains as pronounced as seen in the Cry1Ac experiments.

Survival on Vip3A-Expressing Cotton. The initial leaf tissue bioassay indicated that mortality was similar among *H. virescens* strains YDK, KCBhyb, and CXC on leaf tissue from all cotton lines, with the exception of the Cry1Ac-expressing line (Table 2). Levels of mortality for KCBhyb and CXC were much lower than that of YDK on Cry1Ac-expressing leaf tissues (Table 2). Mortality among *H. virescens* strains varied little on COT203 or COT102. Larval weights of these strains varied when fed Cry1Ac-expressing leaf tissue, but weights on leaf tissue from each Vip3A-expressing genotype showed that larval growth was similar (Table 2).

In the second leaf tissue bioassay, mortality was again similar among *H. virescens* strains on leaf tissue from the non-Bt line ($F = 9.25$; df = 2, 2; $P = 0.0975$), COT102 ($F = 0.89$; df = 2, 2; $P = 0.5293$), and COT102/67B ($F = 0.73$; df = 2, 2; $P = 0.5765$) (Table 3). KCBhyb and CXC mortality levels were lower than that of YDK on Cry1Ab-expressing leaf tissues ($F = 33.99$; df = 2, 2; $P = 0.0286$). Larval weights were similar among the *H. virescens* strains for each of the genotypes tested, including the non-Bt genotype ($F = 1.71$; df = 2, 2; $P = 0.1845$), 67B ($F = 1.34$; df = 2, 2; $P = 0.2672$), COT102 ($F = 2.62$; df = 2, 2; $P = 0.0800$), and COT102/67B ($F = 0.30$; df = 2, 2; $P = 0.7481$).

Discussion

Results of concentration–mortality tests and leaf tissue bioassays presented here demonstrate that *H. virescens* strains with variable levels of resistance to Cry1Ac (0- to >20,000-fold) are equally susceptible to the Vip3A toxin. The lack of cross-resistance between Cry1Ac and Vip3A is likely due to the dissimilarity in amino acid sequences (Estruch et al. 1996). Although lysis of midgut epithelium cells is the primary mode of

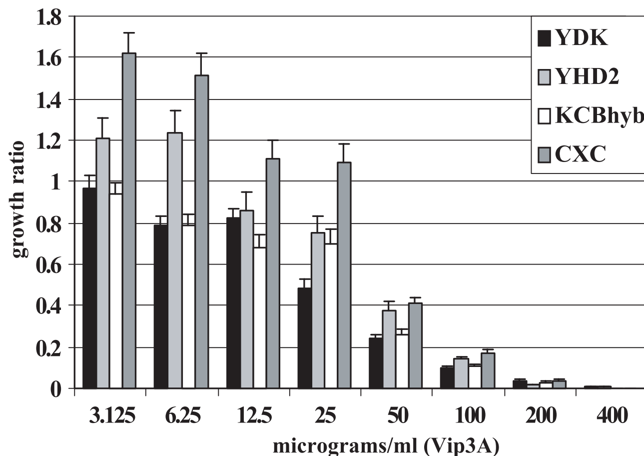

Fig. 2. Comparative growth ratios of Cry1Ac-resistant and susceptible *H. virescens* strains on Vip3A serial dilutions averaged across three runs.

Table 2. Effects of Cry1Ac- and Vip3A-expressing cotton lines, along with a non-Bt line, on mortality and growth of Cry1A-resistant and susceptible *H. virescens* larvae

Genotype	*H. virescens* strain	5-d Mortality (%)[a]	Mean ± SE 5-d wt (mg)
Non-Bt	YDK	16a	4.55 ± 0.51
Non-Bt	KCBhyb	16a	2.65 ± 0.25
Non-Bt	CXC	16a	4.14 ± 0.45
Event 531	YDK	92a	0.43 ± 0.08
Event 531	KCBhyb	36b	1.19 ± 0.13
Event 531	CXC	40b	0.97 ± 0.13
COT102	YDK	30a	1.63 ± 0.20
COT102	KCBhyb	34a	1.30 ± 0.12
COT102	CXC	24a	1.66 ± 0.17
COT203	YDK	48a	1.39 ± 0.19
COT203	KCBhyb	40a	1.83 ± 0.13
COT203	CXC	38a	1.69 ± 0.21

[a] For a given genotype, means within the same column and followed by the same letter are not significantly different ($P \leq 0.05$, testing the equality of percentages; Sokal and Rohlf 1995).

action of Vip3A (Yu et al. 1997), this toxin targets different receptors than Cry1A toxins (Nagamatsu et al. 1999, Dorsch et al. 2002, Lee et al. 2003) and forms distinct ion channels compared with the Cry1Ab toxin (Lee et al. 2003).

Two of the *H. virescens* strains tested had >100-fold resistance to Cry2A (Gould et al. 1995, Jurat-Fuentes et al. 2003), but they showed no cross-resistance to Vip3A. As with Cry1A toxins, the molecular targets of Cry2A differ from those of Vip3A, and the ion channels differ with regard to voltage dependence and cation selectivity (English et al. 1994, Lee et al. 2003).

The moderate toxicity of Vip3A compared with Cry1Ac could be caused by a lower saturation of functional binding sites, a difference in assembly of pores, and/or a difference in flux through pores (Lee et al. 2003). Heliothine larval mortality on single-gene Vip3A-expressing lines in field trials seems to be similar to that of current Bt cotton varieties (Bradley et al. 2004, Leonard et al. 2005). Because Cry1Ac is more active than Vip3A against *H. virescens*, either Vip3A is expressed at higher levels within the plant than Cry1Ac, or protein expression over time is less variable in Vip3A-producing plants than in Cry1Ac-expressing plants.

Table 3. Effects of Cry1Ab- and Vip3A-expressing cotton lines, along with a non-Bt line, on mortality and growth of Cry1A-resistant and susceptible *H. virescens* larvae averaged across two runs

Genotype	*H. virescens* strain	Mean ± SE 5-d mortality (%)[a]	Mean ± SE 5-d wt (mg)[a]
Non-Bt	YDK	40 ± 23.7a	1.05 ± 0.25a
Non-Bt	KCBhyb	54 ± 25.2a	0.65 ± 0.04a
Non-Bt	CXC	35 ± 19.1a	0.73 ± 0.06a
67B	YDK	88 ± 5.5a	0.33 ± 0.06a
67B	KCBhyb	47 ± 13.4b	0.39 ± 0.03a
67B	CXC	48 ± 9.6b	0.45 ± 0.04a
COT102	YDK	72 ± 1.7a	0.22 ± 0.04a
COT102	KCBhyb	84 ± 6.3a	0.35 ± 0.05a
COT102	CXC	70 ± 17.9a	0.34 ± 0.02a
COT102/67B	YDK	92 ± 0.4a	0.24 ± 0.06a
COT102/67B	KCBhyb	96 ± 4.0a	0.18 ± 0.05a
COT102/67B	CXC	93 ± 0.6a	0.25 ± 0.07a

[a] For a given genotype, means within the same column and followed by the same letter are not significantly different ($P \leq 0.05$; Fisher's protected least-significant difference test).

The variability observed among growth ratios of the four *H. virescens* strains on Vip3A at lower concentrations (0–25 μg/ml) does not suggest any type of resistance to the toxin; however, it is an indication of the moderate level of toxicity of Vip3A against *H. virescens.* The CXC and YHD2 strains grew larger on concentrations of Vip3A up to 25 and 12.5 μg/ml, respectively, than on control diet when averaged across three runs. However, this was not observed at concentrations of 50 μg/ml and higher. When LC_{50} values are taken into account, it is obvious that the lower doses of Vip3A had no effects on larval growth of these *H. virescens* strains and that higher doses, such as those expressed in plant tissues, were very effective in increasing mortality or inhibiting growth.

Although the plant tissue bioassay demonstrated that the threat of cross-resistance between Cry1Ac and Vip3A in *H. virescens* does not exist, results from the initial study showed that mortality on Vip3A-expressing plant tissues was moderate. This outcome was likely due to degradation of the toxic protein in the excised plant tissues over the course of the experiment because field and laboratory data generated with COT102, COT202, and COT203 have shown that efficacy against heliothines is similar to that of commercially available Bt cottons (Bradley et al. 2004, Leonard et al. 2005, Bommireddy et al. 2006). However, it is also possible that a decline in toxin expression can be seen after a certain period as observed in Bollgard plant tissues (Greenplate 1999) because plant tissues used in this assay were ≈100 d old. The second plant tissue bioassay that included the pyramided toxin genotype demonstrated higher levels of mortality on Vip3A-expressing leaf tissues than on Cry1Ab-expressing tissues for KCBhyb and CXC. Higher protein expression in younger plants may explain the increase in mortality in the second assay compared with the moderate mortality in the first assay.

Studies have shown that cotton expressing only the Vip3A protein provides sufficient control of heliothines (Bradley et al. 2004, Burd et al. 2005, Leonard et al. 2005, Bommireddy et al. 2006). However, because varieties expressing two toxins generally provide better control of pests and afford greater durability from the standpoint of insect resistance management (Caprio 1998), registration will be sought for VipCot varieties containing both Vip3A and Cry1Ab proteins (COT102/67B). Assuming that inheritance of resistance to Vip3A is recessive, the frequency of resistance alleles should not increase within the next 15–20 yr, even when introduced as a single-gene construct (McCaffery et al. 2005, McCaffery et al. 2006). Effects of the stacked gene construct on insect resistance management are currently being investigated. These findings, along with those presented here, suggest that the introduction of Vip3A-expressing cotton lines to a significant proportion of the area planted to cotton should delay Cry1Ac resistance evolution in helioth-

ines, thus increasing the sustainability of commercially available Bt technologies.

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