

Effects of Vip3AcAa+Cry1Ac Cotton on Midgut Tissue in *Helicoverpa armigera* (Lepidoptera: Noctuidae)

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Abstract

To determine cellular changes caused by the chimeric protein Vip3AcAa against *Helicoverpa armigera*, we used transmission electron microscopy to examine ultrastructural changes in midgut cells of third-instar larvae of Cry1Ac-susceptible *H. armigera* after feeding on an artificial diet containing the Vip3AcAa toxin. Midgut epithelial cells of Cry1Ac-resistant *H. armigera* larvae that had fed on an artificial diet containing Vip3AcAa or on Bt cotton expressing Vip3AcAa+Cry1Ac were also examined using optical microscopy and hematoxylin–eosin staining. In the midgut cells of *H. armigera* larvae fed with Vip3AcAa, microvilli were swollen and broken; inner cristae of the mitochondria were indistinct and vacuolated; endoplasmic reticulum was swollen, fractured, and disordered; boundaries of karyotheca in the nucleus were indistinct and chromatin underwent pyknosis and was pressed close to the karyotheca. Histopathological changes and the time of onset in midgut tissues of *H. armigera* larvae fed on Vip3AcAa or Cry1Ac were similar. Vip3AcAa and transgenic cotton expressing Vip3AcAa+Cry1Ac caused the goblet cell cavity and microvilli pathological changes in the midgut epithelial cells of the Cry1Ac-susceptible and Cry1Ac-resistant *H. armigera* larvae that eventually killed the larvae.

Key words: Cry1Ac-resistant, cotton bollworm, chimeric Vip3AcAa, transgenic Bt cotton, histopathological change

The cotton bollworm (*Helicoverpa armigera*), a polyphagous pest of various commercially important crops worldwide, has caused significant annual losses to farmers in China (Wu et al. 2008). Since 1997, the use of first-generation transgenic Bt cotton has facilitated the control of this pest in China (Lu et al. 2012). However, resistance alleles have been detected in the field, and field monitoring has revealed that *H. armigera* can develop resistance to Bt-Cry1Ac cotton (Zhang et al. 2011, 2012a). Alternative or complementary insecticidal toxins with a mode of action that differs from that of Cry1Ac toxin are urgently required to counter this resistance to Cry1Ac.

Vegetative insecticidal proteins (Vips), first isolated from *Bacillus thuringiensis* (Bt) in 1996, are secreted into the culture medium by the bacteria during vegetative growth (Estruch et al. 1996). These types of toxins exert their toxic action through apparently the same sequence of events as Cry1A proteins, including activation of protoxins by midgut proteases, crossing the peritrophic membrane, binding to specific proteins in the apical membrane of the epithelial midgut cells, and causing pore formation (Lee et al. 2003, 2006, Sena et al. 2009, Chakroun and Ferré 2014). However, specific receptors

of Vip3A differ from the known Cry toxin-binding receptors, and the receptors form ion channels that are distinct from those of Cry1A (Lee et al. 2003, 2006, Abdelkefi-Mesrati et al. 2011, Ben Hamadou-Charfi et al. 2013, Bergamasco et al. 2013). Vip3A proteins have great potential for controlling a wide variety of lepidopteran pests, particularly those resistant to Cry toxins. They share no sequence homology with the known Cry proteins and presumably have a distinct mode of action (Estruch et al. 1996, Yu et al. 1997, Chakroun et al. 2016, Chen et al. 2017a). Therefore, the Vip3A family proteins are considered an excellent alternative for developing new-generation transgenic cotton to achieve more efficient protection and delay the development of resistance.

Vip3AcAa is a chimeric protein, consisting of the N-terminal Vip3Ac1 with 600 amino acid residues and the C-terminal Vip3Aa1 with 189 amino acid residues (Fang et al. 2007). Our previous studies indicated that this protein is a valuable toxin because it has a broader insecticidal spectrum against lepidopteran agricultural pests, and we successfully introduced this gene into cotton to prevent and delay *H. armigera* resistance (Fang et al. 2007, Chen et al. 2017a).

Histopathological changes caused by Vip3A in the midgut cells of several susceptible insects showed extensive damage in the midgut, with disrupted, swollen, and lysed epithelial cells and leakage of cellular material into the lumen (Yu et al. 1997, Abdelkefi-Mesrati et al. 2011, Zhang et al. 2012b, Ben Hamadou-Charfi et al. 2013, Boukedi et al. 2015, Sellami et al. 2015). However, the histopathological changes in the midgut cells of Cry1Ac-resistant insect pests caused by Vip3A and the protein in transgenic plants have not been well studied. In the present study, to better understand the action of the chimeric protein, we investigated histopathological changes in the midgut cells of Cry1Ac-susceptible and Cry1Ac-resistant *H. armigera* larvae fed with an artificial diet containing Vip3AcAa or Bt cotton expressing the Vip3AcAa+Cry1Ac protein. The results of this study will be useful in developing strategies to prevent and delay *H. armigera* resistance.

Materials and Methods

Test Insects

Susceptible strain LF of *H. armigera* used in this study was collected from Langfang, Hebei Province (China), in 1998, and has since been reared in the laboratory on an artificial diet without exposure to any Bt toxin (Liang et al. 1999). Cry1Ac-resistant strains LF5 and LF60, derived from the LF strain, were reared on an artificial diet containing 5 and 60 µg/g of Cry1Ac protoxin, respectively (Cao et al. 2013, Chen et al. 2015). These populations were maintained on an artificial diet containing incorporated Cry1Ac protoxin and had developed resistance to Cry1Ac protoxin, as they demonstrated LC₅₀ values 64 and 896 times higher than those obtained with the LF susceptible strain (Chen et al. 2017a). All insects were cultured in our insectary at 27 ± 2°C and 75 ± 10% relative humidity, 14-h light/10-h dark.

Bt Protein

The Cry1Ac protoxin was provided by the Biotechnology Research Laboratory, Institute of Plant Protection, Chinese Academy of Agricultural Sciences (CAAS). The Vip3AcAa protoxin was extracted and purified from *Escherichia coli* strain BL21 Star that had been transformed with *vip3AcAa* gene using protocols reported previously (Chen et al. 2017b). Protein concentrations were determined using the Bradford (1976) method and bovine serum albumin as the standard.

Cotton Material

Bt cotton event CV163, and its nontransformed parental line Coker312, were obtained from the Biotechnology Research Institute, CAAS. Transgenic cotton CV163 variety was developed by *Agrobacterium*-mediated transformation; it carries the genes for *vip3AcAa* and *cry1Ac* linked in tandem with another gene encoding neomycin phosphotransferase, which confers resistance to kanamycin. NuCOTN33B is a transgenic cotton variety carrying the gene for the δ-endotoxin Cry1Ac from *B. thuringiensis* (Monsanto, St. Louis, MO). Cotton varieties CV163, Coker 312, and NuCOTN33B were planted at the Langfang Experimental Station of the CAAS, Hebei Province, China in 2017. At the 2–3-true-leaf stage, CV163 plants were sprayed with 2 g/kg kanamycin to cull negative-segregating plants. The remaining plants were grown using standard practices without insecticides. Newly unfurled leaves collected from field-grown cotton were used to feed *H. armigera* larvae at the insectary.

Preparation and Sectioning of Insect Tissues for Transmission Electron Microscopy Observations

Third instar of larvae *H. armigera* strain LF were starved for 24 h, then fed with 1.2 µg/g of Vip3AcAa, 1.2 µg/g of Cry1Ac toxin, or 0.01

M phosphate-buffered saline (PBS; as control) in an artificial diet for 3, 6, 12, 24, 36, and 48 h. The larvae were kept on ice for 15 min to immobilize them. Subsequently, the larvae were killed, and the midguts were excised and fixed in 2.5% w/v glutaraldehyde in 0.1 M phosphate buffer (PB) for 1 h at room temperature. Samples were washed three times with PB and post-fixed with 1% v/v osmic tetroxide in PB. Samples were dehydrated in a graded ethanol series, then rinsed in 100% acetone and embedded in epoxy resin 618 (Hitachi). Embedded tissues were sectioned using a Leica EM UC6 ultramicrotome, and double-stained with uranyl acetate and lead citrate. Images were captured via transmission electron microscopy (TEM) at an accelerating voltage of 80 kV (Hitachi, H-500) (Zhang et al. 2012b).

Preparation and Sectioning of Insect Tissues for Hematoxylin–Eosin Staining Observations

Third-instar larvae of *H. armigera* strains LF, LF5, and LF60 were starved for 24 h, then allowed to feed on an artificial diet with 1.2 µg/g of Vip3AcAa, 1.2 µg/g of Cry1Ac toxin, or 0.01 M PBS buffer (control) or on non-Bt cotton Coker312, NuCOTN33B, or CV163 for 96 h. Excised midguts were fixed in 4% v/v paraformaldehyde in PBS buffer, dehydrated in a graded ethanol series, and then rinsed in toluene and embedded in paraffin wax. Four-micrometer sections were cut using a Thermo Scientific Finesse ME+ microtome and placed on microscope slides coated with a mixture of 1.5% egg albumin and 3% glycerol in distilled water. Sections were deparaffinized in 100% toluene, then stained with hematoxylin–eosin according to the protocol of Ruiz et al. (2004). The stained tissues were observed using an optical microscope (Optika, XDS-3FL4) and photographed.

Results

Ultrastructural Changes in Midgut Cells of Larvae of *H. armigera* Strain LF Fed With Vip3AcAa Toxin in Artificial Diet

In TEM observations of the time-course of histopathological changes caused by Vip3AcAa or Cry1Ac on the midgut epithelial cells of *H. armigera*, the control strain LF fed on the normal artificial diet after 48 h had numerous, slender microvilli in an ordered arrangement (Fig. 1A). The endoplasmic reticulum was dense and ordered (Fig. 1E). Inner cristae of mitochondria were distinct (Fig. 1H). Boundaries of the karyotheca were clear and intact, and the chromatin was evenly distributed (Fig. 1K). In midgut cross-sections of the larvae of *H. armigera* strain LF fed with Vip3AcAa or Cry1Ac, epithelial cells were distinctly damaged compared with those in larvae fed on the normal artificial diet. The histopathological changes caused by Vip3AcAa were consistent with those caused by Cry1Ac. In larvae on the diet with Vip3AcAa, after 6 h, the microvilli began to swell and break off (Fig. 1B), and the endoplasmic reticulum had begun to swell and fracture, become disordered and reduced in numbers (Fig. 1F). By 12 h, nearly all microvilli had broken off (Fig. 1C), and the inner cristae of mitochondria were becoming indistinct and vacuolated (Fig. 1I). By 36 h, the boundaries of karyotheca in the nucleus were blurred, and chromatin had undergone pyknosis and were pressed close to karyotheca (Fig. 1L). In larvae that fed on the artificial diet containing Cry1Ac, after 6 h, most microvilli had begun to break off (Fig. 1D), and mitochondria were obviously vacuolated (Fig. 1J). By 12 h, endoplasmic reticulum had begun to swell and break (Fig. 1G), and by 36 h, the chromatin had undergone pyknosis and were pressed close to karyotheca (Fig. 1M). The time of the histopathological changes caused by Vip3AcAa or Cry1Ac

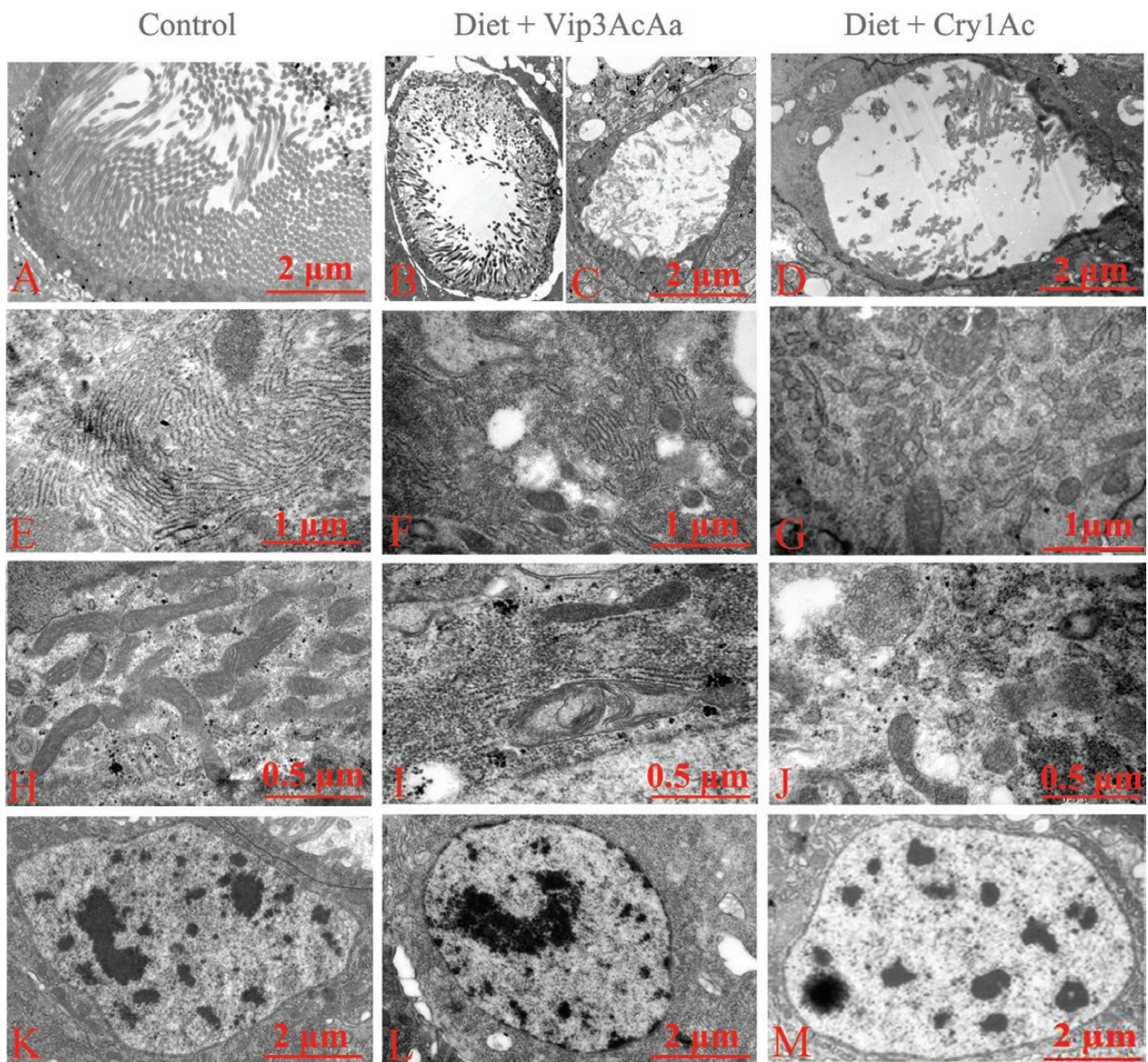


Fig. 1. Ultrastructural changes in midgut cells of larvae of *H. armigera* strain LF fed with an artificial diet containing Vip3AcAa or Cry1Ac toxins.

toxins in midgut epithelial cells of *H. armigera* larvae was the same, and the most obvious symptoms were the breaking of the microvilli and vacuolization of mitochondria.

Histopathological Changes in the Midgut of Cry1Ac-Resistant *H. armigera* Larvae Fed With Vip3AcAa Toxin or Bt-Transgenic Cotton Expressing Vip3AcAa+Cry1Ac

When larvae of the Cry1Ac-susceptible LF strain of *H. armigera* were fed with normal artificial diet (control) or non-Bt cotton (Coker312), the midgut basement membrane and columnar cells were found to be closely connected, columnar cells were arranged in an orderly fashion and had dense microvilli, and goblet cells contained abundant microvilli. In contrast, when larvae of the LF strain were fed with the artificial diet containing Vip3AcAa or Cry1Ac toxins (Fig. 2) or with Bt cotton NuCOTN33B or CV163 cotton expressing Vip3AcAa+Cry1Ac (Fig. 3), their midgut basement membrane and columnar cells began to split, the goblet cell cavity became enlarged,

and microvilli broke off. No significant histopathological differences were found between the midgut tissues of the larvae of Cry1Ac-resistant strains LF5 and LF60 fed with a diet containing Cry1Ac toxin or NuCOTN33B expressing Cry1Ac and the midgut tissues of larvae fed with a normal diet or non-Bt cotton. While the midgut tissues of the larvae of Cry1Ac-resistant strains LF5 and LF60 fed with a diet containing Vip3AcAa toxin or CV163 cotton expressing Vip3AcAa+Cry1Ac exhibited distinct pathological changes, columnar cells were also found to be separated from the midgut basement membrane, the goblet cell cavity was enlarged, and microvilli had broken off (Figs. 2 and 3).

Discussion

The histopathological effects of the insecticidal protein Vip3A on target insects have been studied since its discovery in 1997. Yu et al. (1997) reported that the histopathology associated with the ingestion of Vip3Aa toxin resembles that associated with ingestion

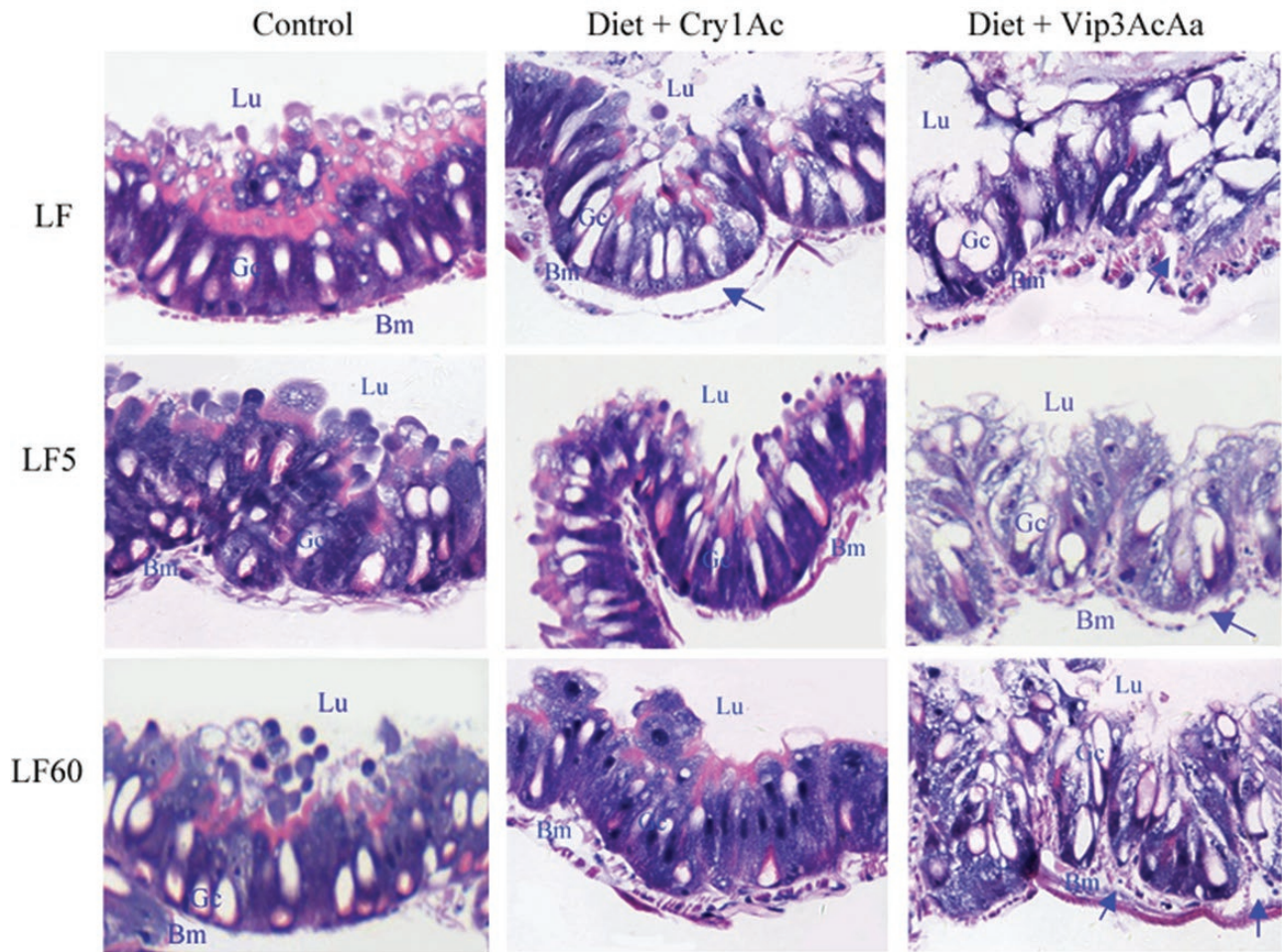


Fig. 2. Histopathological changes in the midgut of *H. armigera* larvae fed with an artificial diet containing Vip3AcAa or Cry1Ac toxins. Blue arrows indicate separation of columnar cells from basement membrane. Lu, lumen; Gc, goblet cell; Bm, basement membrane. Magnification $\times 40$.

of Cry toxins by susceptible insects *Agrotis ipsilon* and *Spodoptera frugiperda*. In gut cross-sections, the midgut was extensively damaged but the foregut and hindgut had no discernible changes. In the midguts of susceptible insects *Spodoptera littoralis*, *Agrotis segetum*, *Tuta absoluta*, and *Ectomyelois ceratoniae* that fed on Vip3Aa16, epithelial cells were disrupted, swollen, or lysed and their contents had leaked into the lumen (Abdelkefi-Mesrati et al. 2011, Ben Hamadou-Charfi et al. 2013, Boukedi et al. 2015, Sellami et al. 2015). Such swelling of organelles and cells are consistent with those described for Bt and Bti in susceptible lepidopteran and mosquito larvae (Knowles and Ellar 1987, Yiallourous et al. 1999). *Spodoptera litura* larvae fed with Vip3Aa toxin from *B. thuringiensis* WB5 occurred histopathological modifications, including vacuolization of the cytoplasm, cellular swelling, brush border membrane destruction and mitochondria swelling and deformation with disintegration and lysis of cristae of midgut epithelial cells (Song et al. 2016). In a study of the histopathology of larval midguts of Cry1Ac-susceptible *H. armigera* strain 96S after feeding on a diet with 0.5 $\mu\text{g/g}$ of Cry1Ac or 50 $\mu\text{g/g}$ of Vip3Aa, Zhang et al. (2012b) found that Cry1Ac and Vip3Aa induced similar symptoms, but the changes induced by Vip3Aa occurred slower than those induced by Cry1Ac. In our study, the histopathological changes in midgut cells initially caused by Cry1Ac was pronounced to have been more serious than those caused by Vip3AcAa, but the time taken for these

histopathological symptoms to appear was approximately the same for the two toxins when administered at the same dose.

Here, we investigated, for the first time, the histopathological effects caused by the chimeric Vip3AcAa toxin and by Bt cotton that expresses Vip3AcAa+Cry1Ac on the midgut epithelial cells of Cry1Ac-resistant *H. armigera* larvae. Breakage of the microvilli and vacuolization of the mitochondria are similar to symptoms reported for Vip3A in susceptible target insects (Abdelkefi-Mesrati et al. 2011, Zhang et al. 2012b, Ben Hamadou-Charfi et al. 2013, Boukedi et al. 2015, Sellami et al. 2015). We observed no obvious histopathological effects of 1.2 $\mu\text{g/g}$ Cry1Ac in an artificial diet or NuCOTN33B cotton (expressing only Cry1Ac) on the midgut cells of third-instar larvae of the Cry1Ac-resistant *H. armigera* strains LF5 and LF60. Thus, the laboratory-reared Cry1Ac-resistant *H. armigera* larvae were resistant to the first-generation Bt cotton that produced only Cry1Ac, suggesting no cross-resistance between Vip3AcAa and Cry1Ac. The histopathological changes caused by Vip3AcAa and Cry1Ac were similar. The mechanism of action of Vip3AcAa is somewhat similar to that of Cry1Ac. Briefly, when susceptible larvae ingest Bt protoxin, it is solubilized and activated by gut proteases (Chakroun et al. 2016). The activated toxin then undergoes through a complex sequence of binding events with different insect gut toxin-binding receptors, leading to its insertion into the plasma membrane and pore formation. But the specific receptors of Vip3AcAa

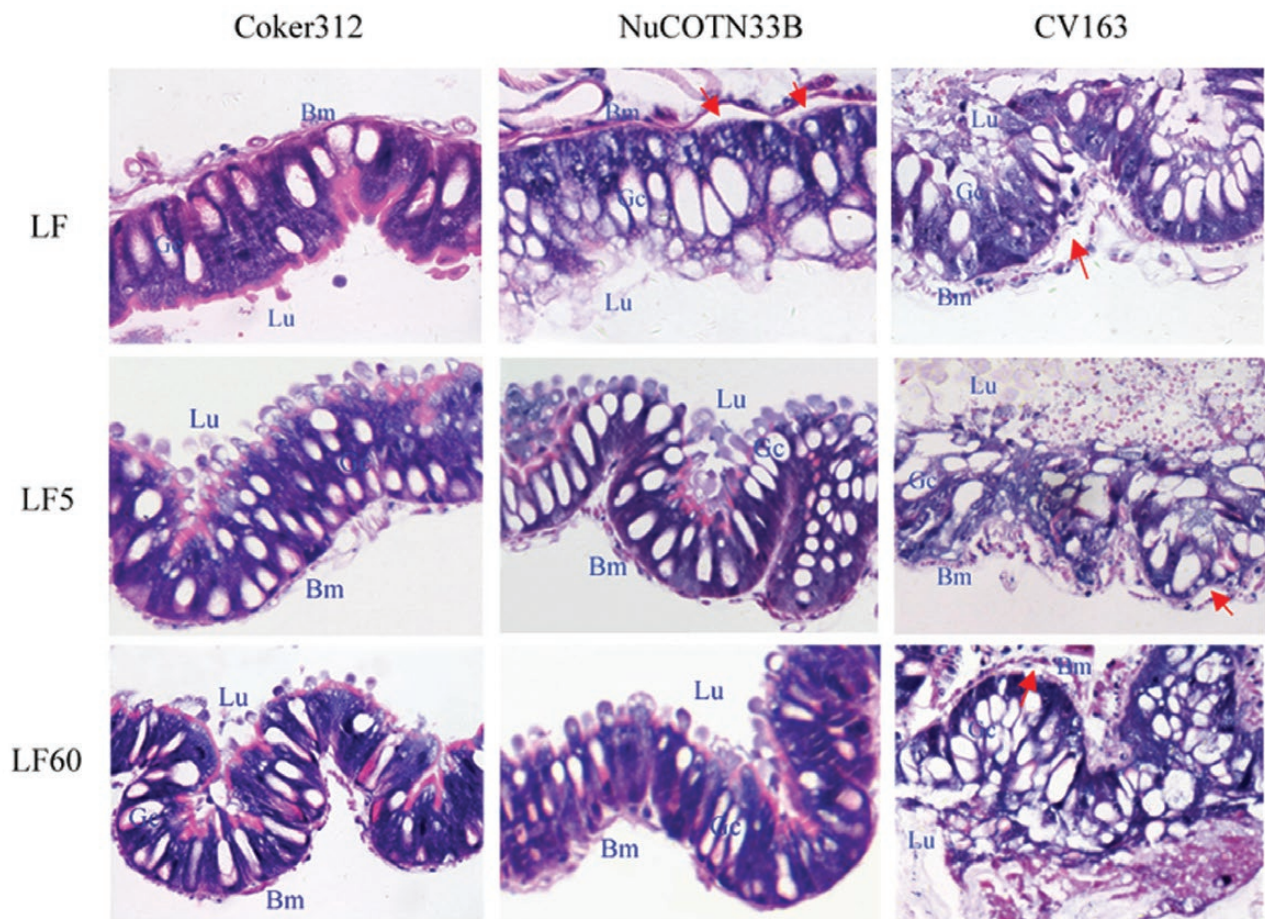


Fig. 3. Histopathological changes in the midgut of *H. armigera* larvae fed with leaves of Coker312, NuCOTN33B, or CV163 cotton plants. Coker312 is conventional cotton, NuCOTN33B cotton expressing only Cry1Ac toxin, CV163 expressing Vip3AcAc and Cry1Ac toxin. Red arrows indicate separation of columnar cells from basement membrane. Lu, lumen; Gc, goblet cell; Bm, basement membrane. Magnification $\times 40$.

differ from the known Cry1Ac toxin-binding receptors, and thus probably differ from that of Cry1Ac (Chen et al. 2017b). The probability of developing cross-resistance between these two types of insecticidal proteins is thus lower.

In conclusion, the data obtained in the present study on the histopathological effects of Vip3AcAa toxin in the midguts of Cry1Ac-resistant *H. armigera* larvae contribute to a better understanding of the mechanism of action of this toxin. The pathological changes caused by Vip3AcAa demonstrate that CV163 cotton expressing Vip3AcAa+Cry1Ac could serve as a future seed source to manage Cry1Ac resistance in *H. armigera* more efficiently.

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