F_2 SCREEN FOR VIP3Aa RESISTANCE IN FIELD POPULATIONS OF *HELICOVERPA ZEA* (BODDIE) (LEPIDOPTERA: NOCTUIDAE) IN TEXAS, USA

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Abstract

The corn earworm, *Helicoverpa zea*, is a major target pest of the insecticidal Vip3Aa protein used in pyramided transgenic Bt corn and cotton with Cry1 and Cry2 proteins in the U.S. The widespread resistance to Cry1 and Cry2 proteins in *H. zea* will challenge the long-term efficacy of Vip3Aa technology. Determining the frequency of resistant alleles to Vip3Aa in field populations of *H. zea* is critically important for resistance management. Here, we provided the first F_2 screen study to estimate the resistance allele frequency for Vip3Aa in *H. zea* populations in Texas, U.S. In 2019, 128 *H. zea* neonates per isofamily for a total of 114 F_2 families were screened with a diagnostic concentration of 3.0 μg/cm^2 of Vip3Aa39 protein in diet-overlay bioassays. The F_2 screen detected two families carrying a major Vip3Aa resistance allele. The estimated frequency of major resistance alleles against Vip3Aa39 in *H. zea* in Texas from this study was 0.0065 with a 95% CI of 0.0014-0.0157. A Vip3Aa-resistant strain (RR) derived from the F_2 screen showed a high level of resistance to Vip3Aa39 protein, with a resistance ratio of >588.0-fold relative to a susceptible population (SS) based on diet-overlay bioassays. We provide the first documentation of a major resistance allele conferring high levels of Vip3Aa resistance in a field-derived strain of *H. zea* in the U.S. Data generated from this study contribute to development of management strategies for the sustainable use of the Vip3Aa technology to control *H. zea* in the U.S.

Introduction

Genetically engineered crops producing insecticidal Cry and Vip proteins from the bacterium *Bacillus thuringiensis* (Bt) have been planted for control of insect pests for more than two decades (James, 2018). Field efficacy of these Bt crops has been outstanding in controlling most target species, resulting in substantial economic, environmental and social gains (Carpenter, 2010; Hutchison et al., 2010; Kathage and Qaim, 2012; Wu et al., 2008). However, with large scale adoption comes intense selection pressure for development of resistance and challenges for long-term sustainability (Gould, 1998; Tabashnik et al., 2013). To date, field-evolved practical resistance to Bt crops has been globally reported in at least 21 cases (Tabashnik and Carrière, 2017; Grimi et al., 2018; Smith et al., 2017; Chandrasena et al., 2018; Gassmann et al., 2019; Yang et al., 2019). To delay insect resistance development, an insecticide resistance management (IRM) plan based on a "high-dose refuge" strategy has been implemented in the U.S. (US-EPA, 1998). Monitoring for evolution of resistance in field populations of the target insect species is an essential component of this IRM plan to maintain sustainability of Bt crop technologies.

The corn earworm/cotton bollworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), is a major target pest of both Bt cotton and Bt corn in North America. Control of lepidopteran pests is achieved by the adoption of corn hybrids producing combinations of Cry1Ab, Cry1F, Cry1A.105, Cry2Ab2 and Vip3Aa20 insecticidal Bt proteins, and Bt cotton varieties producing combinations of Cry1Ac, Cry1F, Cry1Ab, Cry2Ab, Cry2Ae, and Vip3Aa19. Currently, field-evolved resistance of *H. zea* to Cry1 and Cry2 proteins has been widely reported in the U.S., especially in the Southern states. For example, Yang et al. (2019) and Kaur et al. (2019) documented field-evolved practical resistance of *H. zea* populations in Texas and Louisiana to Cry1A.105/Cry2Ab2 corn, respectively. In 2018, Reisig et al. (2018) reported field-evolved practical resistance of *H. zea* to Bt cotton containing Cry1Ac/Cry1F and Cry1Ac/Cry2Ab proteins in North Carolina. Dively et al. (2016) documented field-evolved resistance of *H. zea* to Cry1A.105/Cry2Ab2 corn in Maryland.

The Vip3Aa protein is produced by Bt during its vegetative stage and shows very low sequence and structural homology with Cry proteins, resulting in recognition of unique binding sites in target host cells. Studies have shown that Vip3Aa is highly effective for control of *H. zea* in the field (Burkness et al., 2010; Yang et al., 2015) and Vip3Aa is currently used in combination with Cry1/Cry2 proteins in almost all Bt corn and Bt cotton products in the U.S. (DiFonzo et al., 2018). However, widespread field resistance to Cry1 and Cry2 proteins in *H. zea* populations in the U.S. makes Vip3Aa the only remaining effective protein against this pest in commercialized Bt crops. In addition, the

Vip3Aa proteins produced by transgenic Bt corn and cotton plants are >99% identical, which places strong selection pressure on *H. zea* that feed on both crops in the Southern U.S. All these factors greatly increase the risk of resistance to Vip3Aa in *H. zea*. One of the factors affecting evolution of resistance under the "high-dose refuge" IRM model is that resistance alleles must exist at low frequency in field populations (<0.001) (Gould, 1998). Consequently, it is important to determine current resistance allele frequency for Vip3Aa in field *H. zea* populations so that appropriate management strategies can be developed for the sustainable use of Bt crops including the Vip3Aa technology.

Screening with an F_2 approach has been widely used for estimating Bt resistance allele frequency in insect populations (Andow and Alstad, 1998; 1999). In *H. zea*, frequency of resistance alleles to Cry1Ac and Cry2Aa in North Carolina during 2003 was low (0.00043 and 0.00039, respectively) (Burd et al., 2003). Further studies to estimate resistance allele frequency have been hindered by extremely low mating frequency in single pair of male and female *H. zea* moths (Jones et al., 1979; Blanco et al., 2006). Consequently, it is very difficult to directly establish enough two-parent family-lines for F_2 screening by single-pairing of feral males and females of *H. zea*. In this work, we conducted the first F_2 screen study using light-trapped female moths to estimate the resistance allele frequency to Vip3Aa in *H. zea* in Texas. Furthermore, we provide the first documentation of a resistance allele conferring high levels of resistance to Vip3Aa in a field-derived *H. zea* strain in the U.S. Data generated from this study greatly contributes to improve IRM practices to increase sustainability of Bt crops producing Vip3Aa for management of *H. zea* in the U.S.

Materials and Methods

Insect collection and establishment of two-parent families

During May-September 2019, female moths of *H. zea* were collected from light-traps in Snook, Texas. Adult females were placed individually into a 32 oz. paper container (Choice Paper Company, Brooklyn, NY) with approximately 25g of vermiculite at the bottom and cotton gauze at the open end for oviposition. A 30-ml plastic cup containing paper towels saturated with 10% honey water solution was placed in the center of each container and the containers were placed into an insect rearing room maintained at 26 ± 1 °C, ~60% relative humidity (RH), and a photoperiod of 16:8 h (L:D). Progeny from each female was considered as a F_1 family. These F1 neonates were first reared on the meridic diet (Southland Product, Inc. Lake Village, AR) using 128-well bioassay trays (C-D International, Pitman, NJ). After 7 days, the larvae were individually transferred into 30-mL plastic cups containing meridic diet (WARD'S Stonefly Heliothis diet, Rochester, New York) until pupal stage. F_2 families were then generated by sib-mating approximately 60 viable adults of each F_1 family line in 3.8 L paper containers (Neptune Paper Products, Newark, New Jersey) with approximately 100g of vermiculite at the bottom. A 100-ml plastic cup containing paper towels saturated with 10% honey water solution was placed in the center of each container. These containers were maintained in the insect rearing room under the same conditions as mentioned above. Neonates of F_2 families were screened for Vip3Aa resistance as described below.

Vip3Aa39 protein for F_2 screen

The Vip3Aa39 protein was produced in a recombinant *Escherichia coli* strain transformed with the pET-21b plasmid (EMD Millipore) containing the full length Vip3Aa39 insecticidal protein (GenBank accession AEH31410.1), which was a generous gift from Dr. Rongmei Liu (Northeast Agricultural University of Harbin, P. R. China). Production and purification of the Vip3Aa39 protein was as described elsewhere (Liu et al., 2007). Briefly, an overnight preculture was used to inoculate a 1 L culture of LB media containing 100 µg/ml of ampicillin, which was incubated at 37°C and 160 rpm, Once OD_{600} reached 0.6-0.8, Vip3Aa39 production was induced by addition of 1 M isopropyl- β-d-thiogalactopyranoside (IPTG) and overnight incubation. Bacterial cells were then collected by centrifugation (15,300 x *g*, 4°C, 8 min) and the pellet resuspended by shaking in 100 ml of lysis buffer (20 mM phosphate buffer, pH 7.4, 500 mM NaCl, 3 mg/ml lysozyme and 10 µg/ml of DNAse I). The solution was sonicated on ice for 7 cycles of 5 seconds on/off, and then incubated overnight at 37°C with constant shaking. Cellular debris was pelleted by centrifugation as before and the supernatant was applied to an anion exchange column (HiTrap Q HP) equilibrated in 20 mM Tris-HCl (pH 9) buffer connected to an AKTA Pure chromatography system (GE Healthcare). The Vip3Aa39 protein was recovered in the flow through, and then the pH of the solution was adjusted to 4.5 to precipitate contaminant proteins, which were collected by centrifugation (15,300 x g, 4°C, 20 min). The concentration of Vip3Aa39 in the supernatant was estimated using SDS-10%PAGE combined with densitometry using BSA as standard. The Vip3Aa39 protein shows 94.93% and 94.80% homology compared to the Vip3Aa19 and Vip3Aa20 proteins, respectively.

Screening of F_2 neonates

Before the F_2 screen bioassays, the toxicity of Vip3Aa39 protein was evaluated against a known susceptible population of *H. zea*. The results showed that the median lethal concentration (LC_{50}) that caused 50% mortality of the susceptible population was 0.17 μg/cm^2 with a 95% CL of 0.14-0.21 μg/cm^2, and 100% susceptible insects were killed on a 3.0 μg/cm^2 concentration (see Results). Therefore, we used 3.0 μg/cm^2 Vip3Aa39 as the diagnostic concentration for the F_2 screens. Susceptibility to Vip3Aa39 of the F_2 families of *H. zea* was evaluated using a diet-overlay bioassay as described in Yang et al. (2019). Briefly, 0.8 ml of liquid diet (Southland Product, Inc. Lake Village, AR) was dispensed using repeater pipets into each well of 128-well bioassay trays (C-D International, Pitman, NJ). Once the diet cooled and solidified, repeater pipets were used to overlay 40 μl of a Vip3Aa39 protein solution suspended in 0.1% Triton-X100 onto the diet surface of each well. Once the protein solution was air-dried, one neonate (< 24 h) was released on the diet surface in each well. Wells were covered with vented lids (C-D International, Pitman, NJ). For each F_2 family, 128 neonates were screened against Vip3Aa39 protein. Larval survival and development of the F_2 neonates were also examined on the control diet, with four replications and 32 insects per replication. The control diet was prepared by overlaying the same amount of buffer solution and 0.1% Triton-X100 as the 3.0 μg/cm^2 Vip3Aa39 solution. The bioassay trays were maintained in an insect rearing room under the conditions of 26 ± 1 ˚C, 60% RH, and a 16:8 (L:D) h photoperiod. Larval survival and their instar were recorded after 7 days. In addition, larval survival and development of a susceptible strain (SS) of *H. zea* was also evaluated on both control and 3.0 μg/cm^2 Vip3Aa39 using the same methods as described above. In each bioassay for SS, there were four replications with 32 larvae in each replication. The SS strain was originally collected from LSU AgCenter Macon Ridge Research Station in Franklin Parish in May 2016, and has been documented to be susceptible to Cry1Ac, Cry2Ab2, and Vip3Aa proteins.

Establishment of potential resistant family and confirmation test

In a total of 114 F_2 families tested, the F_2 screen identified five families having survivorship (≥ 2nd instar) on the discriminatory 3.0 μg/cm^2 Vip3Aa39 protein concentration after 7 days (Table 1). These survivors were reared on the control meridic diet and used to establish potential resistant families. Survivors from each family were first crossed with SS to create potential RS families. Due to the limited number of F_2 survivors, only three potential RS families (LT#16, LT#70, and LT#116) were successfully established (see Results). These potential RS insects were then sib-mated and neonates from these sib-mated colonies were evaluated again using diet-overlay bioassay as described above. For each sib-mated potential RS family, 512 insects were assayed at the concentration of 3.0 μg/cm^2 Vip3Aa39. According to Mendelian genetics, if resistance is controlled by one locus with two alleles; S (susceptible) and R (resistant), the F_2 strains from sib-mating RS are expected to consist of 25% RR, 50% RS and 25% SS genotypes. The confirmation test showed that survivors were only derived from LT#70 (see Results), suggesting that the LT#70 family possessed major resistance alleles against Vip3Aa39 protein. Survivors of LT#70 from the confirmation test were used to establish a resistant colony (renamed as RR). To further verify if the survival of RR in the F_2 screen was due to resistance to the Vip3Aa39 protein, susceptibility of RR, along with SS to Vip3Aa39 protein was determined using the full range dose response bioassays as described below.

Dose response bioassays

Susceptibility to Vip3Aa39 in RR and SS strains of *H. zea* was evaluated using a diet-overlay bioassay as described in Yang et al. (2019). In the full range bioassay, concentrations of Vip3Aa39 ranged from 0, 0.0316, 0.1, 0.316, 1, 3.16, 10, 31.6 to100.0 μg/cm^2. Each combination of insect population by Vip3Aa39 protein concentration was replicated four times with 16 larvae in each replication. Bioassay trays were placed in an environmental chamber maintained at 27 ± 1 ˚C, 50% RH, and a 16:8 (L:D) h photoperiod. Larval mortality and instar were recorded on the 7th day after inoculation.

Data analysis

In the diet bioassays, larval mortality was calculated as mortality % = 100 * (number of dead larvae + number of surviving larvae that were still in the first instar) / total number of insects assayed, and larval mortality at each concentration was corrected based on the control mortality. For the dose response bioassay, probit analysis was conducted to determine the median lethal concentration (LC_{50}) that caused 50% mortality and the corresponding 95% confidence limit (CL). The LC_{50} value of an insect was considered greater than the highest Bt protein concentration used in the bioassay if larval mortality was < 50% at the highest concentration. Resistance ratio for RR was calculated as its LC_{50} value divided by the LC_{50} of SS. Moreover, larval mortality and instar data were analyzed using a two-way ANOVA with insect genotype and protein concentration as the two main factors. To meet the normality assumptions for an ANOVA test, original data on the percentage of larval mortality and larval instar were transformed using arcsine ($\chi^{0.5}$) and log (x + 1) scale, respectively. Treatment means were separated using Tukey's HSD test at $\alpha = 0.05$ level.

The estimated resistance allele frequency and its corresponding 95% confidence intervals were estimated using the method described in Andow and Alstad (1999). The probability (detection power) that a resistance allele can be detected in a family if existing was estimated according to Stodola and Andow (2004).

Results and Discussion

Survival of F_2 families in the F_2 screen bioassay

After 7 days, survival of SS on control diet was 100.0 ± 0.0% with 1.6% 3rd and 98.4% 4th instar larvae. In contrast, there were no SS survivors on diet with 3.0 µg/cm^2 Vip3Aa39 protein. Based on these results, the concentration of 3.0 µg/cm^2 Vip3Aa39 as discriminating dose was sufficient to kill all susceptible *H. zea* in the F_2 screening, and thus appropriate to detect Vip3Aa39 resistant individuals. The 7-day larval survival of the F_2 families on control diet was 96.1 ± 1.6%, which was not significantly different ($P > 0.05$) compared to the survival of SS.

A total of 14,592 insects from 114 F_2 families were screened using the Vip3Aa39 discriminating dose in this study. After 7 days, five F_2 families had survivors (≥ 2nd instar) on the 3.0 µg/cm^2 Vip3Aa39 protein (Table 1). Three (LT#14, LT#16, and LT#116) of them contained only 2nd instar larvae. The LT#18 family contained three 2nd instar and one 3rd instar larvae. The LT#70 family had two 3rd and five 4th instar larvae.

Resistance confirmation

On the confirmation test, insects derived from sib-mating of potential RS families of LT#16 and LT#116 were all killed on 3.0 µg/cm^2 Vip3Aa39 at the 7-day. However, insects derived from sib-mating of potential RS family of LT#70 had a survival of 24.8% on the discriminating Vip3Aa39 dose after 7 days, which was not significantly (P >0.05) different from the expected survivorship of 25% for homozygous resistant insects according to Mendelian genetic transmission for monogenic recessive resistance. These survivors included eight 2nd, eight 3rd, and one hundred and eleven 4th instar larvae, suggesting that only LT#70 family (renamed as RR) probably carries a major resistance allele conferring to Vip3Aa39 protein.

Susceptibility of SS and RR populations of *H. zea* to Vip3Aa39 protein

Larvae of the SS strain were highly susceptible to Vip3Aa39, with 75.8% mortality observed at 0.316 µg/cm^2 and 100% mortality at 1-31.6 µg/cm^2 (Fig.1). The LC_{50} value of SS against Vip3Aa39 protein was estimated as 0.17 µg/cm^2 with a 95% CL of 0.14-0.21 µg/cm^2 (Table 2). In contrast, larvae from the RR strain was highly resistant to Vip3Aa39 protein, and showed no differences ($P > 0.05$) in mortality (0-2.5%) across all the tested concentrations (Fig.1). Consequently, we were unable to estimate the LC_{50} for strain RR, as the mortality at the highest tested concentration of 100.0 µg/cm^2 was only 2.5% (Fig. 1). Based on this observation, the LC_{50} value for RR was considered >100.0 µg/cm^2, with an estimated resistance ratio >588.0-fold relative to SS (Table 2).

The main effect of insect population and protein concentration on larval mortality was significant for the Vip3Aa39 protein ($F = 3401.95$; df = 1, 42; $P < 0.0001$ for insect population and $F = 162.93$; df = 7, 42; $P < 0.0001$ for protein concentration). The effect of the interaction of insect population and protein concentration was also significant ($F = 67.20$; df = 5, 42; $P < 0.0001$).

The effects of insect population, protein concentration and their interactions on larval instar were all significant for the Vip3Aa39 protein ($F = 226.72$; df = 1, 36; $P < 0.0001$ for insect population; $F = 15.44$; df = 8, 36; $P < 0.0001$ for protein concentration; and $F = 48.74$; df = 2, 36; $P < 0.0001$ for the interactions). Larval growth on control diet was similar ($P > 0.05$) between SS and RR after 7 days, with an average instar of 3.80 and 3.90, respectively (Fig. 2). Larval development of SS at 0.0316-0.316 µg/cm^2 of Vip3Aa39 protein was significantly ($P < 0.05$) slower than that on control diet (Fig. 2). RR showed no differences ($P > 0.05$) in larval growth across all the concentrations from 0-100 µg/cm^2 with an average instar of 3.84 (Fig.2).

Major resistance allele frequency for Vip3Aa39

Based on the results of F_2 screening, resistance confirmation and dose response bioassays, two out of 114 families collected from Snook (Texas) were presumed to carry a major resistance allele against Vip3Aa39 protein. Thus, the expected resistance allele frequency of the Snook populations of *H. zea* to Vip3Aa39 protein is estimated to be 0.0065 with a 95% CI of 0.0014-0.0157. The F_2 screen had a detection power of 98.2%.

Table 1. Families containing survivors of *Helicoverpa zea* from the F_2 screen on 3.0 µg/cm^2 of Vip3Aa39 protein.

Family No.	No. insects screened	No. survivor	No. insect within instar		
			2nd	3rd	4th
LT#14	128	1	1	0	0
LT#16	128	5	5	0	0
LT#18	128	4	3	1	0
LT#70	128	7	0	2	5
LT#116	128	4	4	0	0

Table 2. Mortality response (LC_{50}) of different populations of *Helicoverpa zea* to Vip3Aa39 protein in diet-overlay bioassays.

Insect	N*	LC_{50} (95% CL) (µg/cm^2)#	Slope ± SE£	X^2	df	Resistance ratio§
SS	512	0.17 (0.14, 0.21)	2.84 ± 0.29	10.5	26	-
RR	512	> 100	/	/	/	> 588.0

*Total number of neonates assayed.
#Median lethal concentration (LC_{50}) that caused 50% mortality and the corresponding 95% confidence limit (CL). The LC_{50} value of an insect population was considered to be greater than the highest Bt protein concentration used in the bioassay if its larval mortality was <50% at the highest concentration. Larval mortality was calculated based on the number of dead larvae plus survivors that were still in the first instar divided by the total number of insects assayed.
£SE, standard error.
§Resistance ratio was calculated using the LC_{50} value of RR divided by the LC_{50} of SS.

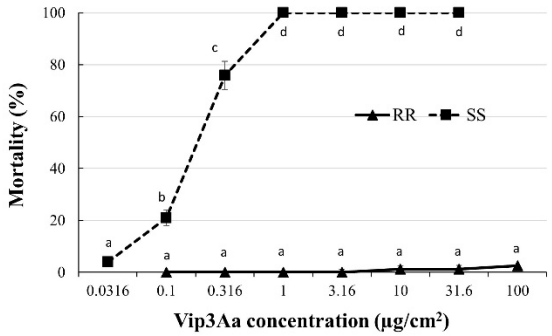

Fig. 1. Concentration-larval mortality response of the SS and RR strains of *Helicoverpa zea* to Vip3Aa39 protein. Mean percentage mortality values followed by a different letter are significantly different (Tukey's HSD test, $\alpha = 0.05$).

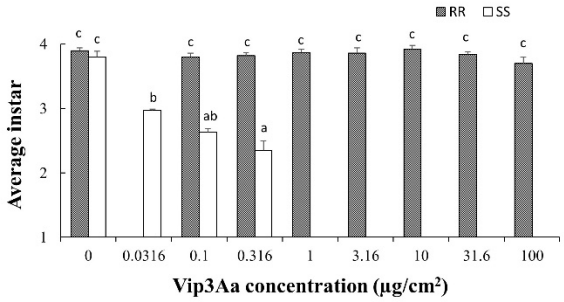

Fig. 2. Average larval instar detected for SS and RR populations of *Helicoverpa zea* on different concentrations of Vip3Aa39 protein, as indicated. Mean values followed by a different letter are significantly different (Tukey's HSD test, $\alpha = 0.05$).

References

Andow DA, and Alstad DN, Credibility interval for rare resistance allele frequencies. *J Econ Entomol* **92**: 755-758 (1999).

Andow DA, and Alstad DN, The F_2 screen for rare resistance alleles. *J Econ Entomol* **91**: 572-578 (1998).

Blanco CA, Sumerford DV, Lopez JD Jr, and Hernandez G, Mating incidence of feral *Heliothis virescens* (Lepidoptera: Noctuidae) males confined with laboratory females. *J Cotton Sci* **10**: 105-113 (2006).

Burd AD, Gould F, Bradley JR, Van Duyn JW, and Moar WJ, Estimated frequency of nonrecessive Bt resistance genes in bollworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) in eastern North Carolina. *J Econ Entomol* **96**: 137-142. (2003).

Burkness EC, Dively G, Patton T, Morey AC, and Hutchison WD, Novel Vip3A *Bacillus thuringiensis* (Bt) maize approaches high-dose efficacy against *Helicoverpa zea* (Lepidoptera: Noctuidae) under field conditions. *GM Crops* **1**: 1-7 (2010).

Carpenter JE, Peer-reviewed surveys indicate positive impact of commercialized GM crops. *Nat Biotechnol* **28:** 319-321 (2010).

Chandrasena DI, Signorini AM, Abratti G, Storer NP, Olaciregui ML, Alves AP, and Pilcher CD, Characterization of field-evolved resistance to *Bacillus thuringiensis*-derived Cry1F δ-endotoxin in *Spodoptera frugiperda* populations from Argentina. *Pest Manag Sci* **74**: 746-754 (2018).

DiFonzo C, Porter P, and Tilmon K, The Handy Bt trait table (2018). https://lubbock.tamu.edu/files/2018/01/BtTraitTableJan2018.pdf

Dively GP, Venugopal PD, and Finkenbinder C, Field-evolved resistance in corn earworm to Cry proteins expressed by transgenic sweet corn. *PloS One* **11:** e0169115 (2016).

Gassmann AJ, Shrestha RB, Kropf AL, St Clair CR, and Brenizer BD, Field-Evolved Resistance by Western Corn Rootworm to Cry34/35Ab1 and other *Bacillus thuringiensis* Traits in Transgenic Maize. *Pest Manag Sci* **DOI**: 10.1002/ps.5510 (2019).

Gould F, Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. *Annu Rev Entomol* **43**: 701-726 (1998).

Grimi DA, Parody B, Ramos ML, Machado M, Ocampo F, Willse A, Martinelli S, and Head G, Field-evolved resistance to Bt maize in sugarcane borer (*Diatraea saccharalis*) in Argentina. *Pest Manag Sci* **74**: 905-913 (2018).

Hutchison WD, Burkness EC, Mitchell PD, Moon RD, Leslie TW, Fleischer SJ, *et al.*, Areawide suppression of European corn borer with Bt maize reaps savings to non-Bt maize growers. *Science* **330:** 222-225(2010).

James C, Global status of commercialized biotech/GM crops: 2017. Brief No. 53. ISAAA: Ithaca, NY, USA. (2018).
Jones RL, and Perkins WD, Sparks AN, Effects of sex ratios on reproduction by the corn earworm in the laboratory. *Ann Entomol Soc Am* **72**: 35-37 (1979).

Kathage J, and Qaim M, Economic impacts and impact dynamics of Bt (*Bacillus thuringiensis*) cotton in India. *Pro Nat Acad Sci USA* **109:** 11652-11656 (2012).

Kaur G, Guo J, Brown S, Head GP, Price PA, Paula-Moraes S, Ni X, Dimase M, and Huang F, Field-evolved resistance of *Helicoverpa zea* (Boddie) to transgenic maize expressing pyramided Cry1A. 105/Cry2Ab2 proteins in northeast Louisiana, the United States. *J Invertebr Patho* **163:** 11-20 (2019).

Liu J, Song F, Zhang J, Liu R, He K, Tan J and Huang D, Identification of vip3A-type genes from Bacillus thuringiensis strains and characterization of a novel vip3A-type gene. *Lett Appl Microbiol* **45**: 432-438 (2007).

Reisig DD, Huseth AS, Bacheler JS, Aghaee MA, Braswell L, Burrack HJ, Flanders K, Greene JK, Herbert DA, Jacobson A, and Paula-Moraes SV, Long-term empirical and observational evidence of practical *Helicoverpa zea* resistance to cotton with pyramided Bt toxins. *J Econ Entomol* **111**: 1824-1833 (2018).

Smith JL, Lepping MD, Rule DM, Farhan Y, and Schaafsma AW, Evidence for field-evolved resistance of *Striacosta albicosta* (Lepidoptera: Noctuidae) to Cry1F *Bacillus thuringiensis* protein and transgenic corn hybrids in Ontario, Canada. *J Econ Entomol* **110**: 2217-2228. (2017).

Stodola TJ, and Andow DA, F_2 screen variations and associated statistics. *J Econ Entomol* **97**: 1756-1764 (2004).

Tabashnik BE, and Carrière Y, Surge in insect resistance to transgenic crops and prospects for sustainability. *Nat Biotechnol* **35**: 926-935 (2017).

Tabashnik BE, Brévault T, and Carrière Y, Insect resistance to Bt crops: lessons from the first billion acres. *Nat Biotechnol* **31**: 510-521 (2013).

US-EPA (US Environmental Protection Agency), Report of subpanel on *Bacillus thuringiensis* (Bt) plant-pesticides and resistance management. Available at: https://archive.epa.gov/scipoly/sap/meetings/web/pdf/finalfeb.pdf (1998).

Wu KM, Lu YH, Feng HQ, Jiang YY, and Zhao JZ, Suppression of cotton bollworm in multiple crops in China in areas with Bt toxin-containing cotton. *Science* **321:** 1676-1678 (2008).

Yang F, González JCS, Williams J, Cook DC, Gilreath RT, Kerns D, Occurrence and ear damage of *Helicoverpa zea* on transgenic *Bacillus thuringiensis* maize in the field in Texas, US and its susceptibility to Vip3A protein. *Toxins* **11**: 102 (2019).

Yang F, Kerns DL, Leonard BR, Oyediran I, Burd T, Niu Y, and Huang F, Performance of Agrisure®Viptera™ 3111 corn against *Helicoverpa zea* (Lepidoptera: Noctuidae) in seed mixed plantings. *Crop Prot* **69**: 77-82 (2015).