1 **For:** Crop Protection (*Ms. Ref. No.: CROPRO-D-17-00075 revised March 8, 2017*)

2 **Running head:** Yang *et al.*: Susceptibility of *S. frugiperda* to Bt proteins

3
4 **Susceptibility of Cry1F-maize resistant, heterozygous, and**
5 **susceptible *Spodoptera frugiperda* to Bt proteins used in the**
6 **transgenic cotton**

7 Fei Yang^{a‡}, David L. Kerns^{a‡§}, Graham Head^b, Sebe Brown^a & Fangneng Huang^a

8
9 ^a Department of Entomology, Louisiana State University Agricultural Center, Baton Rouge, LA
10 70803, USA. ^b Monsanto Company, St. Louis, MO 63167, USA.

11 [‡] Correspondence: Fei Yang, 404 Life Sciences Building, Department of Entomology, Louisiana
12 State University Agricultural Center, Baton Rouge, LA 70803, USA. Tel: +10 225 578 1839; fax:
13 +10 578 225 1632; E-mail: FYang@agcenter.lsu.edu

14 David L. Kerns, 2475 TAMU, Department of Entomology, Texas A&M University, College
15 Station, TX 77843, USA. Tel: +10 979 458 0366; E-mail: DLKerns@tamu.edu

16
17 [§] Current address: Department of Entomology, Texas A&M University, College Station, TX
18 77843, USA.

Abstract

Fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is a major target species of Bt maize and Bt cotton in the United States. Field resistance to Cry1F maize in *S. frugiperda* has occurred in Puerto Rico, Brazil, and the southeast region of the United States. There is a great concern that the Cry1F-maize resistant *S. frugiperda* may move to the cotton fields and cause control problems to Bt cotton where both crops are planted. In this study, larval mortality and growth inhibition of Cry1F maize-susceptible (SS), -heterozygous (RS), and -resistant (RR) genotypes of *S. frugiperda* were evaluated using diet-incorporated bioassays with six Bt proteins that have been used in commercialized and future Bt cotton plants: Cry1F, Cry1Ab, Cry1Ac, Cry2Ab2, Cry2Ae, and Vip3A. Compared to the mortality data, the measuring of growth inhibition was more sensitive in determining Bt susceptibility of the insect to all six Bt proteins. Overall, RS had a similar performance as SS for the six Bt proteins with a resistance ratio of < 5.5-fold. The resistance ratio of RR calculated based on larval growth inhibition was 930-fold for Cry1F, 50.5-fold for Cry1Ab, and >172-fold for Cry1Ac. In contrast, all three insect genotypes were susceptible to Cry2Ab2, Cry2Ae, or Vip3A. The results showed that the Cry1F-maize resistant *S. frugiperda* was cross-resistant to Cry1Ab or Cry1Ac, but not cross-resistant to Cry2Ab2, Cry2Ae, or Vip3A. The strong cross-resistance among Cry1 proteins suggest that it is necessary to plant pyramided cotton traits that contain at least two active Bt genes for Cry1F-resistant *S. frugiperda* to ensure the continued success of the Bt cotton technology, especially in the areas where Bt resistance has already occurred.

Key words: *Bacillus thuringiensis*, fall armyworm, resistance management, cross-resistance, Cry, Vip3A

1. Introduction

In 2015, transgenic crops expressing *Bacillus thuringiensis* (Bt) proteins were planted on approximately 84 million hectares worldwide for insect pest control (James, 2016). During the same year, approximately 80% of the maize and 84% of the cotton in the United States were planted to Bt crops (NASS, 2016). These Bt crops are highly efficacious against the target insect pests (Carpenter, 2010; Wu et al., 2008; Yang et al., 2014; 2015). Other benefits associated with planting Bt crops include reduction in chemical insecticide use and decrease in overall target pest populations (Hutchison et al., 2010; Edgerton et al., 2012; Lu et al., 2012; Kathage & Qaim, 2012; Shi et al., 2013). However, evolution of resistance in target pest populations is a great threat to the sustainable use of the Bt crops (Huang et al., 2011; Tabashnik et al., 2013; Carrière et al., 2016). Thus, implementing effective insect resistance management (IRM) strategies is essential to ensure the continued success of the Bt crop technology. Currently, the high dose/refuge and gene-pyramiding are the two main IRM strategies that are implemented for planting Bt crops in the world (Ostlie et al., 1997; Gould, 1998; Matten et al., 2012; US-EPA, 2001; Monsanto, 2012).

Gene-pyramiding makes transgenic plants express two or more Bt proteins that are dissimilar in mode of action, but are effective against a same target pest (Ghimire et al., 2011; Monsanto, 2012). Relative to the single-gene Bt plants, pyramided Bt crops are expected to be more effective in delaying evolution of resistance because if one of the Bt proteins in the pyramids is ineffective for a pest, the remaining proteins can still kill the insect (Zhao et al., 2003). However, efficacy of gene-pyramiding can be reduced by cross-resistance, which occurs when insects possessing resistance to one Bt protein are also resistant to other proteins (Brévault et al., 2013; Carrière et al., 2015; 2016). It is generally agreed that cross-resistance is unlikely to

occur between Bt proteins that are considerably distinct in structures and target sites (e.g. Cry and Vip3A). However, numerous studies have documented that cross-resistance frequently occurs among closely-related Bt proteins (Tabashnik et al., 2009; Brévault et al., 2013; Bernardi et al., 2015; Carrière et al., 2016). The patterns of cross-resistance can be varied in different Bt proteins and insect species. Therefore, information on cross-resistance of an insect among Bt proteins is critical for understanding the mechanisms of resistance and assisting in pyramid-designs for Bt crop IRM.

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is a major target species for Bt maize and Bt cotton in North and South America. Field resistance to Cry1F maize that has resulted in control problems has occurred in Puerto Rico, Brazil, and the southeast region of the U.S. mainland (Storer et al., 2010; Farias et al., 2014; Huang et al., 2014; Li et al., 2016). Our previous study has shown that the Cry1F-maize resistant *S. frugiperda* can cause cross-crop resistance to WideStrike[®] cotton expressing Cry1Ac and Cry1F (Yang et al., 2016a; Horikoshi et al., 2016). There is a great concern that the Cry1F resistant *S. frugiperda* may also be less susceptible to other Bt proteins used in the pyramided Bt cotton. The most common Bt cotton traits that have been commercialized in the United States and other countries since 1996 are Bollgard[®] I expressing Cry1Ac, Bollgard[®] II containing Cry1Ac and Cry2Ab2, WideStrike[®] expressing Cry1Ac and Cry1F, TwinLink[™] producing Cry1Ab and Cry2Ae, and WideStrike[®] 3 possessing Cry1Ac, Cry1F, and Vip3A. In addition, two new Bt cotton traits, Bollard[®] III containing Cry1Ac, Cry2Ab2, and Vip3A and TwinLink[™] Plus expressing Cry1Ab, Cry2Ae, and Vip3A are expected to be commercially available shortly. The objective of this study was to evaluate the relative susceptibility of Cry1F-maize resistant *S. frugiperda* to the Bt proteins expressed in these commercialized and future Bt cotton products.

2. Materials and methods

2.1. Source of insect strains

Two previously described laboratory strains of *S. frugiperda* were used as the original insect sources in this study: a susceptible strain (SS) that was collected from non-Bt maize fields near Weslaco, TX in 2013 and a Cry1F-maize resistant strain (RR) that was established using F₂ screening with a two-parent family derived from feral individuals collected from non-Bt maize fields in south Florida in 2011 (Huang et al., 2014; Dangal & Huang, 2015; Yang et al., 2016a). SS has never been exposed to any Bt proteins or insecticides since it was established in the laboratory. It has been documented to be susceptible to Cry1F, Cry2Ab2, Cry1A.105 proteins in the diet as well as maize plants expressing each of the three Cry and Vip3A proteins (Huang et al., 2014; Dangal & Huang, 2015). RR has been confirmed to possess a major resistance allele, allowing it to survive and complete normal development on commercial Cry1F maize plants (Huang et al., 2014). RR is also highly resistant to purified Cry1F protein (>270-fold) in diet-incorporated bioassays (Huang et al., 2014). Prior to the current study, RR had been backcrossed with SS twice and re-selected for resistance with Cry1F maize leaf tissue for two generations. In addition, two F₁ heterozygous genotypes were developed from the reciprocal crosses between SS and RR. Because the inheritance of Cry1F resistance in RR was autosomal (Huang et al., 2014), the two F₁ heterozygous genotypes were pooled (RS) before they were used in this study.

2.2. Sources of Bt proteins

Susceptibility of SS, RS and RR genotypes of *S. frugiperda* was determined against all the six Bt proteins used in the commercialized and future Bt cotton traits mentioned above: Cry1Ab, Cry1Ac, Cry1F, Cry2Ab2, Cry2Ae, and Vip3A. Among these, Cry1Ac and Cry1F were provided

by Dow AgroSciences (Indianapolis, IN) as lyophilized powders with 14.4% and 53.0% AI, respectively. Cry2Ab2 protein was provided by Monsanto Company (St. Louis, MO) as a liquid formulation with a concentration of 0.31 mg/ml. Bayer CropScience (Morrisville, NC) provided the Cry1Ab and Cry2Ae proteins as liquid formulations with a concentration of 3.1 mg/ml and 1.2 mg/ml, respectively. Syngenta Biotechnology Inc. (Research Triangle Park, NC) provided the Vip3A protein with a purity of 77.8%.

2.3. Dose-response bioassay

Larval susceptibility of SS, RS and RR of *S. frugiperda* to the six Bt proteins were determined using a diet-incorporated bioassay as described in [Niu et al. \(2013\)](#). Each individual Bt protein was first suspended in distilled water or the corresponding buffer solution at room temperature and stirred completely to ensure that the protein was uniformly distributed in the solution. The protein was then incorporated into a meridic diet prepared for rearing *S. frugiperda* (WARD'S Stonefly Heliothis diet). Each bioassay consisted of 5-8 protein concentrations plus 1-2 untreated controls (water only and buffer only). The protein concentrations used in each bioassay ranged from 0.01 to 100 µg/g for assaying Cry1F, 0.0316 to 31.6 µg/g for assaying Vip3A, 0.1 to 100 µg/g for assaying Cry1Ab and Cry1Ac, and 1 to 100 µg/g for assaying Cry2Ab2 and Cry2Ae. For each bioassay, approximately 0.7 g of treated or control diet was placed into each cell of the 128-cell trays (Bio-Ba-128, C-D International, Pitman, NJ). One neonate (<24-h old) was placed on the diet surface of each cell. Each combination of insect genotype by Bt protein concentration was replicated four times with 16-32 larvae in each replication. Infested bioassay trays were placed in environmental chambers maintained at 28 °C, 50% RH, and a photoperiod of 16:8 (L:D)

h. Larval weight and number of dead larvae plus surviving larvae that were still in the first instar were recorded on the 7th day after inoculation.

2.4. Data analysis

Larval mortality in the dose response bioassays was calculated using the equation: mortality (%) = $100 * (\text{number of dead larvae} + \text{number of surviving larvae still in the first instar}) / \text{total number of insects assayed}$ (Zhang et al., 2013). Larval mortality at each concentration was corrected based on the control mortality using the method of Abbott (1925), followed by probit analysis (SAS Institute, 2010) to determine the median lethal concentration (LC₅₀) that caused 50% mortality and the corresponding 95% confidence limit (CL). Larval growth inhibition on a Bt protein-treated diet was calculated using the formula: growth inhibition (%) = $100 * (\text{body weight of larvae feeding on control diet} - \text{body weight of larvae feeding on Cry protein-treated diet}) / (\text{body weight of larvae feeding on control diet})$ (Zhang et al., 2013). The log-transformed concentration and probit-transformed growth inhibition data were processed using probit model to estimate the Bt protein concentration that reduces larval weight by 50% (GI₅₀) and the corresponding 95% CL (Christensen et al., 2009). In some cases, the LC₅₀ or GI₅₀ value of an insect genotype was considered to be greater than the highest Bt protein concentration used in the bioassay if its larval mortality or growth inhibition was <50% at the highest concentration. Resistance ratio of an insect genotype for a Bt protein was calculated using the LC₅₀ or GI₅₀ value of that genotype divided by the LC₅₀ or GI₅₀ value of SS. In some cases, if the LC₅₀ or GI₅₀ of an insect genotype was smaller than that of SS, the ratio was computed using the LC₅₀ or GI₅₀ of SS divided by the corresponding value of that genotypes and a negative sign was assigned to the ratio.

In addition, because the probit analysis mentioned above was not used to analyze the dose-response data for some Bt proteins due to low mortalities and growth inhibition, and some of the bioassay data didn't fit the probit model well, larval mortality and growth inhibition data for each Bt protein were also analyzed using a two-way analysis of variance (ANOVA) with insect genotype and protein concentration as the two main factors (SAS Institute, 2010). For ANOVA, original data on the percentage of larval mortality and growth inhibition were transformed using $\arcsin(\chi^{0.5})$ to meet the assumptions of normality. Treatment means were separated using Tukey's HSD test at $\alpha = 0.05$ level (SAS Institute, 2010).

3. Results

3.1. Mortality response of *S. frugiperda* to Bt proteins

The main effect of insect genotype and protein concentration on larval mortality was significant for all six Bt proteins ($F \geq 4.7$; $df = 2$, 45-66; $P \leq 0.0125$ for insect genotype and $F \geq 8.51$; $df = 4-8$, 45-66; $P < 0.0001$ for protein concentration). The effect of the interaction of insect genotype and protein concentration was also significant ($F \geq 3.03$; $df = 8-12$, 45-66; $P \leq 0.0003$).

SS was susceptible to Cry1F with a mortality of 72% at 3.16 $\mu\text{g/g}$ and reached 100% at 31.6 $\mu\text{g/g}$ (Fig.1). The LC_{50} value of Cry1F against SS was estimated to be 1.62 $\mu\text{g/g}$ with a 95% CL of 0.67-5.71 $\mu\text{g/g}$ (Table 1). RS showed a lower ($P < 0.05$) mortality than that of SS at 3.16 and 10 $\mu\text{g/g}$, but its mortality increased significantly as the Cry1F concentrations increased, reached 95% at 31.6 $\mu\text{g/g}$, which was not significantly different compared to the mortality of SS at the same concentration (Fig.1). The estimated LC_{50} of Cry1F for RS was 5.99 $\mu\text{g/g}$ with a 95% CL of 4.67-7.86 $\mu\text{g/g}$. With the mortality of SS as a standard, the resistance ratio of RS was 3.7-fold,

which was not significant based on the overlapped 95% CLs (Table 1). In contrast, RR did not show significant mortality (0-10%) across all the Cry1F concentrations assayed, indicating a resistance ratio of much greater than 61.7-fold (Fig. 1 and Table 1).

Compared to the Cry1F protein, Cry1Ab and Cry1Ac were less toxic to *S. frugiperda*. In the bioassays with Cry1Ab, mortality at ≤ 31.6 $\mu\text{g/g}$ was low, $\leq 44.1\%$, and not significantly ($P > 0.05$) different among the three insect genotypes. At 100 $\mu\text{g/g}$, the mortality (67.8%) of SS was greater ($P < 0.05$) than the mortality of RR (8.9%) and RS (28.1%) (Fig. 1). LC_{50} of Cry1Ab for SS was 49.0 $\mu\text{g/g}$ with a 95% CL of 24.9-140, while for RS and RR, the LC_{50} should be >100 $\mu\text{g/g}$, which represents a resistance ratio of > 2 -fold for both insect genotypes (Table 1). For Cry1Ac, mortality of all three insect genotypes was low, $\leq 28.4\%$, across all the Bt concentrations assayed and thus, resistance ratio based on the larval mortality data could not be estimated (Fig. 1).

Compared to the three Cry1 proteins, the two Cry2A proteins, Cry2Ab2 and Cry2Ae, were effective against all the three genotypes. In general, dose-response of the two Cry2 proteins was similar. In the bioassays with Cry2Ab2, RR was somewhat more susceptible to the Bt protein compared to SS and RS. For example, at 31.6 $\mu\text{g/g}$, RR exhibited a mortality of 85.3%, while the mortality of SS was only 53.0% (Fig. 1). The calculated LC_{50} was 21.2 $\mu\text{g/g}$ with a 95% CL of 16.0 to 30.0 $\mu\text{g/g}$ for SS, 20.6 $\mu\text{g/g}$ with a 95% CL of 10.8 to 53.5 $\mu\text{g/g}$ for RS, and 4.3 $\mu\text{g/g}$ with a 95% CL of 2.86 to 6.21 for RR (Table 1). The LC_{50} of RS was not significantly different compared to the value of SS based on their overlapped 95 CLs, while the 4.9-fold greater sensitivity for RR to Cry2Ab2 was significant based on the non-overlapped 95% CLs. For Cry2Ae, the dose-response curve was similar between SS and RR. At ≤ 10 $\mu\text{g/g}$, mortality of the two genotypes was low, $\leq 26.6\%$; it reached $\geq 96.2\%$ at 100 $\mu\text{g/g}$. Mortality of RS at ≤ 31.6 $\mu\text{g/g}$

was $\leq 15.4\%$, but reached 96.9% at 100 $\mu\text{g/g}$ (Fig. 1). The estimated LC_{50} of Cry2Ae was 16.3 $\mu\text{g/g}$ with a 95% CL of 10.0-32.4 $\mu\text{g/g}$ for SS, 42.8 $\mu\text{g/g}$ with a 95% CL of 25.0-101 $\mu\text{g/g}$ for RS, and 17.5 with a 95% CL of 12.4-26.8 $\mu\text{g/g}$ for RR. Compared to the LC_{50} of SS, the 1.1-fold (for RR) and 2.6-fold (for RS) differences were not significant based on the overlapped 95% CLs of the LC_{50} values (Table 1).

Vip3A was toxic to all three genotypes of *S. frugiperda*, larval mortality of the three insect genotypes at $\leq 1 \mu\text{g/g}$ was low, $\leq 11.3\%$, but it reached $\geq 92.1\%$ at 10 $\mu\text{g/g}$ and 100% at 31.6 $\mu\text{g/g}$ (Fig. 1). The calculated LC_{50} values of Vip3A were 2.03 $\mu\text{g/g}$ with a 95% CL of 1.29-3.30 $\mu\text{g/g}$ for SS, 3.54 $\mu\text{g/g}$ with a 95% CL of 1.72-7.98 $\mu\text{g/g}$ for RS, and 3.01 $\mu\text{g/g}$ with a 95% CL of 1.10-11.0 $\mu\text{g/g}$ for RR (Table 1). The corresponding resistance ratios for RS and RR were 1.7- and 1.5-fold, respectively, which was not significant based on the overlapped 95% CLs of the LC_{50} s (Table 1).

3.2. Growth inhibition response of *S. frugiperda* to Bt proteins

The main effect of insect genotype and protein concentration on growth inhibition was also significant for all six Bt proteins ($F \geq 4.10$; $\text{df} = 2, 42-66$; $P \leq 0.0237$ for insect genotype and $F \geq 13.83$; $\text{df} = 4-8, 42-66$; $P < 0.0001$ for protein concentration). The effect of the interaction of the two main factors for Cry2Ab2, Cry2Ae and Vip3a was significant ($F \geq 2.23$; $\text{df} = 7-12, 42-62$; $P \leq 0.0207$), but it was not significant for the three Cry1 proteins ($F \leq 1.62$; $\text{df} = 11-12, 42-62$; $P \geq 0.1079$).

The overall patterns of the concentration-growth responses were similar among the three Cry1 proteins. In general, growth inhibition of SS, RS, and RR increased as protein concentrations increased for all three Cry1 proteins, but the inhibition for RR was significantly

less ($P < 0.05$) than for SS across all the Bt concentrations assayed with few exceptions (Fig. 2). The growth inhibition at each Bt concentration was similar ($P > 0.05$) between SS and RS across all the Bt concentrations assayed and for all three Cry1 proteins (Fig. 2). Probit analysis showed that the GI_{50} values of SS were 0.02 $\mu\text{g/g}$ with a 95% CL of 0.01-0.03 $\mu\text{g/g}$ for Cry1F, 1.21 $\mu\text{g/g}$ with a 95% CL of 0.92-1.56 $\mu\text{g/g}$ for Cry1Ab, and 0.58 $\mu\text{g/g}$ for with a 95% CL of 0.25-1.06 $\mu\text{g/g}$ for Cry1Ac (Table 1). The GI_{50} s for RS were 0.11 $\mu\text{g/g}$ with a 95% CL of 0.07-0.16 $\mu\text{g/g}$ for Cry1F, 1.24 with a 95% CL of 0.76-1.89 $\mu\text{g/g}$ for Cry1Ab, and 1.32 $\mu\text{g/g}$ with a 95% CL of 0.85-1.97 $\mu\text{g/g}$ for Cry1Ac. Based on their overlapped 95%, the 1.0- to 5.5-fold differences in the GI_{50} s between SS and RS was not significant for all three Cry1 protein (Table 1). In contrast, GI_{50} s for RR were considerably greater than the values of SS and RS, 18.6 $\mu\text{g/g}$ with a 95% CL of 8.21-63.5 $\mu\text{g/g}$ for Cry1F, 61.1 $\mu\text{g/g}$ with a 95% CL of 27.9-217 for Cry1Ab, and > 100 $\mu\text{g/g}$ for Cry1Ac. The resistance ratio of RR based on the inhibition data were 930-fold for Cry1F, 50.5-fold for Cry1Ab, and > 172 -fold for Cry1Ac (Table 1).

The two Cry2 proteins showed a high efficacy of growth inhibition to all three insect genotypes. The general patterns of concentration-growth inhibition responses were similar between Cry2Ab2 and Cry2Ae. The percentage of the growth inhibition of the three insect genotypes at each Bt concentration was not significantly different ($P > 0.05$) across all concentrations assayed and for both Cry2 proteins with only one exception (Fig. 2). The Cry2 proteins caused 39.5-72.9% larval growth inhibition to the three insect genotypes at the relatively low concentration of 1 $\mu\text{g/g}$, reached $> 91\%$ inhibition at 31.6 $\mu\text{g/g}$. The calculated GI_{50} for SS was 0.34 $\mu\text{g/g}$ with a 95% CL of 0.16-0.56 $\mu\text{g/g}$ for Cry2Ab2 and 0.86 $\mu\text{g/g}$ with a 95% CL of 0.57-1.18 $\mu\text{g/g}$ for Cry2Ae. GI_{50} for RS was 1.26 $\mu\text{g/g}$ with a 95% CL of 0.96-1.56 $\mu\text{g/g}$ for Cry2Ab2 and 1.65 $\mu\text{g/g}$ with a 95% CL of 1.23-2.11 $\mu\text{g/g}$ for Cry2Ae. Compared to SS, the 3.7-

fold (for Cry2Ab2) and 1.9-fold (for Cry2Ae) greater inhibitions against RS were significant based on the non-overlapped 95% CLs of the GI₅₀s. GI₅₀ value for RR was 0.26 µg/g with a 95% CL of 0.09-0.50 µg/g for Cry2Ab2 and 0.32 µg/g with a 95% CL of 0.14-0.56 µg/g. Relative to SS, the 1.3-fold difference in GI₅₀ of Cry2Ab2 against RR was not significant based on the overlapped 95% CLs of GI₅₀s, while the 2.7-fold greater inhibition of Cry2Ae against RR was significant based on the non-overlapped 95% CLs (Table 1).

Vip3A was highly potent in inhibiting larval growth for all three genotypes of *S. frugiperda*. ANOVA result showed that the percentage of growth inhibition at each Vip3A concentration was similar ($P > 0.05$) among the three insect genotypes across all the concentrations assayed with only two exceptions (Fig. 2). Vip3A caused > 57% larval growth inhibition at 1 µg/g and > 78% inhibition at 3.16 µg/g to the three insect genotypes. The calculated GI₅₀ value was 0.66 µg/g with a 95% CL of 0.55-0.79 µg/g for SS, 0.48 µg/g with a 95% CL of 0.38-0.60 µg/g for RS, and 0.36 µg/g with a 95% CL of 0.27-0.49 µg/g for RR. Compared to SS, the 1.4-fold greater sensitivity for RS was not significant based on the overlapped 95% CLs, while the 1.8-fold sensitivity for RR was significant based on the non-overlapped 95% of CLs (Table 1).

4. Discussion

Insect mortality data have been the most commonly used in measuring toxicity of insecticides including Bt toxins. In this study, susceptibility of three genotypes, SS, RS, and RR of *S. frugiperda* to the six Bt proteins (Cry1F, Cry1Ab, Cry1Ac, Cry2Ab2, Cry2Ae, and Vip3A) expressed in Bt cotton traits was assayed based on both larval mortality and growth inhibition data. Both measurement methods showed that RR was highly resistant to Cry1F, but not cross-resistant to Cry2Ab2, Cry2Ae, and Vip3A. In addition, the concentration-growth inhibition data

also suggested a significant cross-resistance of RR to Cry1Ab (50.5-fold) and Cry1Ac (>172-fold). In contrast, LC₅₀s of Cry1Ab and Cry1Ac based on the larval mortality data could not be determined for five out of the six proteins due to the low mortality occurred. Overall, the current bioassays showed that the measurement of growth inhibition was more powerful to differentiate the susceptibility of the three insect genotypes for all six Bt proteins, especially at the lower Bt concentrations. For example, all six Bt proteins at 1 µg/g caused < 24% larval mortality to any of the three insect genotypes. In contrast, these proteins at the same concentration resulted in a wide range of growth inhibition, with Cry1F from 15.0 to 92.9%, with Cry1Ab and Cry1Ac from 11.1 to 50.8%, with the two Cry2 proteins from 39.5 to 72.9%, and for Vip3A from 56.6 to 64.7%. These results clearly showed that the growth inhibition was more sensitive in determining Bt susceptibility for *S. frugiperda* compared to the mortality data. Similar trends have also been observed in the bioassays with other major target pests of Bt crops, such as the European corn borer, *Ostrinia nubilalis* (Hübner) (Marçon et al., 1999), corn earworm, *Helicoverpa zea* (Boddie) (Siegfried et al., 2000), western corn rootworm *Diabrotica virgifera virgifera* LeConte (Siegfried et al., 2005), old world bollworm *Helicoverpa armigera* Hübner (Brévault et al., 2009), and sugarcane borer, *Diatraea saccharalis* (F.) (Zhang et al., 2013). The results of the current study, together with the published data, suggest that measuring insect growth inhibition is more cost-effective for Bt resistance monitoring, especially for the species that are naturally tolerant to Bt toxins, such as *S. frugiperda* and *H. zea*.

While larval growth inhibition data generated from the current study showed a significant cross-resistance of RR to Cry1Ab and Cry1Ac, the inhibition data also indicated that RR was slightly, but significantly in some cases, more susceptible to the Cry2 or Vip3A. Similar results were also observed in the mortality response of RR to Cry2Ab2. Additional studies are needed to

validate if the observed differences have any biological significances **because** the difference was, in general, small, only 1.3- to 4.9-fold. Thus, even **if** such low levels of inversed cross-resistance statistically exist, it should not be practically important in terms of IRM. Nevertheless, the results of the current study did not indicate any cross-resistance between Cry1F and Cry2Ab2, Cry2Ae, or Vip3A. Cross-resistance of Cry1F resistance in *S. frugiperda* have been evaluated in several previous studies (Vélez et al., 2013; Huang et al., 2014; Bernardi et al., 2015), but those studies were focused on the proteins in Bt maize. Yang et al. (2016a) reported the first study of the performance of a Cry1F-resistant population of *S. frugiperda* on cotton plants containing different Bt traits. The susceptibility data of Cry1F resistance to Cry2Ae reported in the current study is new, while, for other five proteins, the overall results of the current study were similar as reported in the past studies.

Based on the number of Bt genes expressed in the plants, the Bt cotton varieties that have been commercialized since 1996 can be categorized into three generations. The first generation Bt cotton contains only a single Bt gene, such as Bollgard[®] I. The second generation that was first released in 2002 in the United States contains two pyramided Bt genes. To date, a total of three traits of the second generation Bt cotton have been commercialized in the United States, which are Bollgrad II[®], Widestrike[®], and Twinlink[™]. The third generation Bt cotton that first became commercially available in 2016 in the United States contains three pyramided Bt genes. Up to now, Widestrike[®] 3 is the only third generation Bt cotton trait that has been commercialized. As mentioned above, it is highly expected that other two traits of the third generation Bt cotton, Bollgard[®] III and Twinlink[™] Plus, will become commercially available in the near future, very likely in the 2017 crop seasons. In the United States and several other

countries, the first generation single-gene Bt cotton was already phased out from the market several years ago, and the majority of the currently planted belongs to the second generation.

The significant cross-resistance between Cry1F and Cry1A proteins observed in the current study plus the natural tolerance of *S. frugiperda* to Cry1A proteins (Niu et al., 2013; 2014; Huang et al., 2014) suggests that none of the Bt proteins in Widestrike[®] will be active, and only a single Bt protein in Bollgard II[®] (e.g. Cry2Ab2) or Twinlink[™] (Cry2Ae) is still fully functioning against the Cry1F-resistant *S. frugiperda*. The ineffectiveness of the greenhouse-grown Widestrike[®] cotton plants against a Cry1F-resistant population of *S. frugiperda* observed in Yang et al. (2016a) is good evidence to show the cross-resistance property between Cry1F and Cry1A proteins. Therefore, all the three second generation Bt cotton traits, in terms of IRM, may not been considered as pyramided technologies for *S. frugiperda* once Cry1F resistance has already occurred. In the southern region of the United States where Bt maize and Bt cotton are planted in close proximity to one another (Huang et al., 2014), the risk of resistance development to these Bt cotton technologies could be high if the Cry1F-maize resistant populations of *S. frugiperda* move to the cotton fields.

In addition, other studies have shown that the susceptibility to Cry1A proteins in *H. zea* collected from some cotton fields in the southern region of the United States has decreased since the commercial use of Bt crops (Luttrell et al., 2004, Ali et al., 2006, Tabashnik et al., 2008, Yang et al., 2016b). In some occasions, field control problems of Bt cotton against *H. zea* could be observed in the region (Yang et al. unpublished data). While on-going investigations to determine if the field control problems are due to resistance development are being conducted, the results of the current and past studies support the use of the third generation Bt cotton to

ensure the continued success of the technology, especially in the areas where resistance to one of the Bt genes has already occurred, such as the southeast region of the United States and Brazil.

Acknowledgments

This material is based upon work that is supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, Hatch project. This article is published with the approval of the Director of the Louisiana Agricultural Experiment Station as manuscript No. 2016-234-29575. We thank Dow Agrosciences, Monsanto Company, Bayer CropScience, and Syngenta Biotechnology Inc. for providing Bt proteins used in this study.

References

- Abbott, W.S., 1925. A method of computing the effectiveness of an insecticide. J. Econ. Entomol. 18, 265-267.
- Ali, M.I., Luttrell, R.G., Young, S.Y., 2006. Susceptibilities of *Helicoverpa zea* and *Heliothis virescens* (Lepidoptera: Noctuidae) populations to Cry1Ac insecticidal protein. J. Econ. Entomol. 99, 164-175.
- Bernardi, D., Salmeron, E., Horikoshi, R.J., Bernardi, O., Dourado, P.M., Carvalho, R.A., Martinelli, S., Head, G.P., Omoto, C., 2015. Cross-resistance between Cry1F proteins in fall armyworm (*Spodoptera frugiperda*) may affect the durability of current pyramided Bt maize hybrids in Brazil. PLoS One 10, e0140130.
- Brévault, T., Heuberger, S., Zhang, M., Ellers-Kirk, C., Ni, X., Masson, L., Li, X., Tabashnik, B.E., Carrière, Y., 2013. Potential shortfall of pyramided transgenic cotton for insect resistance management. Proc. Natl. Acad. Sci. USA 110, 5806-5811.

361 Brévault, T., Prudent, P., Vaissayre, M., Carrière, Y., 2009. Susceptibility of *Helicoverpa*
 362 *armigera* (Lepidoptera: Noctuidae) to Cry1Ac and Cry2Ab2 insecticidal proteins in four
 363 countries of the West African cotton belt. J. Econ. Entomol. 102, 2301-2309.
 364 Carpenter, J.E., 2010. Peer-reviewed surveys indicate positive impact of commercialized GM
 365 crops. Nat. Biotechnol. 28, 319-321.
 366 Carrière, Y., Crickmore, N., Tabashnik, B.E., 2015. Optimizing pyramided transgenic Bt crops
 367 for sustainable pest management. Nat. Biotechnol. 33, 161-168.
 368 Carrière, Y., Fabrick, J.A., Tabashnik, B.E., 2016. Can Pyramids and Seed Mixtures Delay
 369 Resistance to Bt Crops? Trends Biotechnol. 34, 291-302.
 370 Christensen, E.R., Kusk, K.O., Nyholm, N., 2009. Dose–response regressions for algal growth
 371 and similar continuous endpoints: calculation of effective concentrations. Environ. Toxicol.
 372 Chem. 28, 826-835.
 373 Dangal, V., Huang, F., 2015. Fitness costs of Cry1F resistance in two populations of fall
 374 armyworm, *Spodoptera frugiperda* (JE Smith), collected from Puerto Rico and Florida. J.
 375 Invertebr. Pathol. 127, 81-86.
 376 Edgerton, M.D., Fridgen, J., Anderson-Jr, J.R., Ahlgrim, J., Criswell, M., Dhungana, P., Gocken,
 377 T., Li, Z., Mariappan, S., Pilcher, C.D., Rosielle, A., Stark, S.B., 2012. Transgenic insect
 378 resistance traits increase corn yield and yield stability. Nat. Biotechnol. 30, 493-496.
 379 Farias, J.R., Andow, D.A., Horikoshi, R.J., Sorgatto, R.J., Fresia, P., dos Santos, A.C., Omoto,
 380 C., 2014. Field-evolved resistance to Cry1F maize by *Spodoptera frugiperda* (Lepidoptera:
 381 Noctuidae) in Brazil. Crop Prot. 64,150-158.
 382 Ghimire, M.N., Huang, F., Leonard, R.B., Head, G.P., Yang, Y., 2011. Susceptibility of Cry1Ab-
 383 susceptible and -resistant sugarcane borer to transgenic corn plants containing single or

pyramided *Bacillus thuringiensis* genes. Crop Prot. 30, 74-81.

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

Horikoshi, R.J., Bernardi, D., Bernardi, O., Malaquias, J.B., Okuma, D.M., Miraldo, L.L., e Amaral, F.S.D.A., Omoto, C., 2016. Effective dominance of resistance of *Spodoptera frugiperda* to Bt maize and cotton varieties: implications for resistance management. Sci. Rep. 6, 34864.

Huang, F., Andow, D.A., Buschman, L.L., 2011. Success of the high dose/refuge resistance management strategy after 15 years of Bt crop use in North America. Entomol. Exp. Appl. 140, 1-16.

Huang, F., Qureshi, J.A., Meagher Jr, R.L., Reisig, D.D., Head, G.P., Andow, D.A., Ni, X., Kerns, D., Buntin, G.D., Niu, Y., Yang, F., Dangal, V., 2014. Cry1F resistance in fall armyworm *Spodoptera frugiperda*: single gene versus pyramided Bt maize. PLoS One 9, e112958.

Hutchison, W.D., Burkness, E.C., Mitchell, P.D., Moon, R.D., Leslie, T.W., Fleischer, S.J., Abrahamson, M., Hamilton, K.L., Steffey, K.L., Gray, M.E., Hellmich, R.L., 2010. Areawide suppression of European corn borer with Bt maize reaps savings to non-Bt maize growers. Science 330, 222-225.

James, C., 2016. Global status of commercialized biotech/GM crops: 2015. Brief No. 52. ISAAA: Ithaca, NY, USA.

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

Li, G., Reisig, D., Miao, J., Gould, F., Huang, F., Feng, H., 2016. Frequency of Cry1F Non-Recessive Resistance Alleles in North Carolina Field Populations of *Spodoptera frugiperda*

407 (Lepidoptera: Noctuidae). PloS One 11, e0154492.

408 Lu, Y.H., Wu, K.M., Jiang, Y.Y., Guo, Y.Y., Desneux, N., 2012. Widespread adoption of Bt cotton
409 and insecticide decrease promotes biocontrol services. Nature 487, 362-365.

410 Luttrell, R.G., Ali, I., Allen, K.C., Young lii, S.Y., Szalanski, A., Williams, K., Lorenz, G., Parker
411 Jr, C.D., Blanco, C. 2004. Resistance to Bt in Arkansas populations of cotton bollworm. In
412 Proceedings of the 2004 Beltwide Cotton Conferences, San Antonio, TX, January 5-9, 2004.

413 Marçon, P.C., Young, L.J., Steffey, K.L., Siegfried, B.D., 1999. Baseline susceptibility of
414 European corn borer (Lepidoptera: Crambidae) to *Bacillus thuringiensis* toxins. J. Econ.
415 Entomol. 92, 279-285.

416 Matten, S.R., Frederick, R.J., Reynolds, A.H., 2012. United States Environmental Protection
417 Agency insect resistance management programs for plant-incorporated protectants and use
418 of simulation modeling. pp 175-267. In: C.A. Wozniak, A. McHughen (eds), "Regulation of
419 agricultural biotechnology: The United States and Canada". Springer.

420 Monsanto (2012) IRM grower guide: insect resistance management for U.S. corn and cotton-
421 growing areas. Available at: [http://www.monsanto.com/products/pages/insect-resistance-](http://www.monsanto.com/products/pages/insect-resistance-management.aspx)
422 [management.aspx](http://www.monsanto.com/products/pages/insect-resistance-management.aspx). (accessed 16.10.25).

423 NASS (National Agricultural Statistics Service), 2016. Acreage. USDA, Washington, DC, USA.
424 Available at: <http://usda.mannlib.cornell.edu/usda/current/Acre/Acre-06-30-2016.pdf>.
425 (accessed 16.10.25).

426 Niu, Y., Meagher Jr, R.L., Yang, F., Huang, F., 2013. Susceptibility of field populations of the fall
427 armyworm (Lepidoptera: Noctuidae) from Florida and Puerto Rico to purified Cry1F protein
428 and corn leaf tissue containing single and pyramided Bt genes. Fla. Entomol. 96, 701-713.

429 Niu, Y., Yang, F., Dangal, V., Huang, F., 2014. Larval survival and plant injury of Cry1F-

susceptible, -resistant, and -heterozygous fall armyworm (Lepidoptera: Noctuidae) on non-Bt and Bt corn containing single or pyramided genes. Crop Prot. 59, 22-28.

Ostlie, K.R., Hutchison, W.D., Hellmich, R.L., 1997. Bt corn & European corn borer: long term success through resistance management. North Central Regional Extension Publication NCR 602. University of Minnesota, St. Paul, MN, USA.

SAS Institute, 2010. *SAS/STAT User's Third Edition*, SAS Institute Inc, Cary, NC, USA.

Shi, G., Chavas, J.P., Lauer, J., 2013. Commercialized transgenic traits, maize productivity and yield risk. Nat. Biotechnol. 31, 111-114.

Siegfried, B.D., Spencer, T., Nearman, J., 2000. Baseline susceptibility of the corn earworm (Lepidoptera: Noctuidae) to the Cry1Ab toxin from *Bacillus thuringiensis*. J. Econ. Entomol. 93, 1265-1268.

Siegfried, B.D., Vaughn, T.T., Spencer, T., 2005. Baseline susceptibility of western corn rootworm (Coleoptera: Crysomelidae) to Cry3Bb1 *Bacillus thuringiensis* toxin. J. Econ. Entomol. 98, 1320-1324.

Storer, N.P., Babcock, J.M., Schlenz, M., Meade, T., Thompson, G.D., Bing, J.W., Huckaba, R.M., 2010. Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. J. Econ. Entomol. 103, 1031-1038.

Tabashnik, B.E., Brévault, T., Carrière, Y., 2013. Insect resistance to Bt crops: lessons from the first billion acres. Nat. Biotechnol. 31, 510-521.

Tabashnik, B.E., Gassmann, A.J., Crowder, D.W., Carrière, Y., 2008. Insect resistance to Bt crops: evidence versus theory. Nat. Biotechnol. 26, 199-202.

451 Tabashnik, B.E., Unnithan, G.C., Masson, L., Crowder, D.W., Li, X., Carrière, Y., 2009.
 452 Asymmetrical cross-resistance between *Bacillus thuringiensis* toxins Cry1Ac and Cry2Ab in
 453 pink bollworm. Proc. Natl. Acad. Sci. USA 29, 11889-11894.
 454 US-EPA, 2001. Biopesticide registration action document: *Bacillus thuringiensis* plant-
 455 incorporated protectants. [http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad2/1-](http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad2/1-overview.pdf)
 456 [overview.pdf](http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad2/1-overview.pdf). (accessed 13.10.01)
 457 Vélez, A.M., Spencer, T.A., Alves, A.P., Moellenbeck, D., Meagher, R.L., Chirakkal, H.,
 458 Siegfried, B.D., 2013. Inheritance of Cry1F resistance, cross-resistance and frequency of
 459 resistant alleles in *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Bull. Entomol. Res. 103,
 460 700-713.
 461 Wu, K.M., Lu, Y.H., Feng, H.Q., Jiang, Y.Y., Zhao, J.Z., 2008. Suppression of cotton bollworm
 462 in multiple crops in China in areas with Bt toxin-containing cotton. Science 321, 1676-1678.
 463 Yang, F., Kerns, D.L., Brown, S., Kurtz, R., Dennehy, T., Braxton, B., Head, G., Huang, F.,
 464 2016a. Performance and cross-crop resistance of Cry1F-maize selected *Spodoptera*
 465 *frugiperda* on transgenic Bt cotton: implications for resistance management. Sci. Rep. 6,
 466 28059.
 467 Yang, F., Kerns, D.L., Head, G.P., Leonard, B.R., Niu, Y., Huang, F., 2014. Occurrence,
 468 distribution, and ear damage of *Helicoverpa zea* (Lepidoptera: Noctuidae) in mixed
 469 plantings of non-Bt and Bt corn containing SmartStax traits. Crop Prot. 55, 127-132.
 470 Yang, F., Kerns, D.L., Leonard, B.R., Oyediran, I., Burd, T., Niu, Y., Huang, F., 2015.
 471 Performance of Agrisure[®]Viptera[™] 3111 corn against *Helicoverpa zea* (Lepidoptera:
 472 Noctuidae) in seed mixed plantings. Crop Prot. 69, 77-82.

473 Yang, F., Kerns, D.L., Gore, J., Cook, D., Seiter, N., 2016b. Susceptibility of field populations of
474 the cotton bollworm to purified Cry1Ac protein and cotton leaf tissues containing Cry1Ac
475 protein. In Proceedings of the 2016 Beltwide Cotton Conference, New Orleans, LA, Jan 5-7,
476 2016.

477 Zhang, L., Huang, F., Leonard, B.R., Chen, M., Clark, T., Zhu, Y.C., Wangila, D.S., Yang, F.,
478 Niu, Y., 2013. Susceptibility of Cry1Ab maize-resistant and-susceptible strains of sugarcane
479 borer (Lepidoptera: Crambidae) to four individual Cry proteins. J. Invertebr. Pathol. 112,
480 267-272.

481 Zhao, J.Z., Cao, J., Li, Y., Collins, H.L., Roush, R.T., Earle, E.D., Shelton, A.M., 2003.
482 Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance
483 evolution. Nat. Biotechnol. 21, 1493-1497.

484 **Figure Legend**

485 **Fig. 1.** Concentration-larval mortality response of Cry1F-susceptible (SS), -heterozygous (RS),
486 and –resistant (RR) genotypes of *Spodoptera frugiperda* to Bt proteins used in transgenic cotton.

487 **Fig. 2.** Concentration-larval growth inhibition response of Cry1F-susceptible (SS), -heterozygous
488 (RS), and –resistant (RR) genotypes of *Spodoptera frugiperda* to Bt proteins used in transgenic
489 cotton.

490 **Table 1.** Larval mortality (LC₅₀) and growth inhibition (GI₅₀) responses of Cry1F-susceptible (SS), -heterozygous (RS), and -resistant
491 (RR) genotypes of *Spodoptera frugiperda* to six Bt proteins used in transgenic cotton.

Bt protein	Insect genotype	N ^a	Mortality response			Growth inhibition response		
			Slope ± SE	LC ₅₀ (95% CL) (µg/g) ^b	Resistance ratio ^d	Slope ± SE	GI ₅₀ (95% CL) (µg/g) ^c	Resistance ratio ^d
Cry1F	SS	1016	1.2 ± 0.3	1.62 (0.67, 5.71)	---	0.82 ± 0.04	0.02 (0.01, 0.03)	---
	RS	1023	1.4 ± 0.1	5.99 (4.67, 7.86)	3.7	0.85 ± 0.06	0.11 (0.07, 0.16)	5.5
	RR	888	/	> 100	> 61.7	0.59 ± 0.10	18.6 (8.21, 63.5)	930
Cry1Ab	SS	896	0.8 ± 0.1	49.0 (24.9, 140)	---	0.89 ± 0.05	1.21 (0.92, 1.56)	---
	RS	896	/	> 100	> 2.0	0.68 ± 0.07	1.24 (0.76, 1.89)	1.0
	RR	896	/	> 100	> 2.0	0.66 ± 0.10	61.1(27.9, 216)	50.5
Cry1Ac	SS	887	/	> 100	---	0.60 ± 0.08	0.58 (0.25, 1.06)	---
	RS	896	/	> 100	/	0.58 ± 0.05	1.32 (0.85, 1.97)	2.3
	RR	885	/	> 100	/	/	> 100	> 172
Cry2Ab2	SS	765	1.7 ± 0.2	21.2 (16.0, 30.0)	---	0.83 ± 0.08	0.34 (0.16, 0.56)	---
	RS	767	2.3 ± 0.6	20.6 (10.8, 53.5)	-1.0	1.39 ± 0.10	1.26 (0.96, 1.56)	3.7
	RR	781	1.3 ± 0.2	4.30 (2.86, 6.21)	-4.9	0.96 ± 0.12	0.26 (0.09, 0.50)	-1.3
Cry2Ae	SS	768	1.5 ± 0.3	16.3 (10.0, 32.4)	---	1.05 ± 0.09	0.86 (0.57, 1.18)	---
	RS	768	3.1 ± 0.9	42.8 (25.0, 101)	2.6	1.12 ± 0.08	1.65 (1.23, 2.11)	1.9
	RR	800	1.6 ± 0.2	17.6 (12.4, 26.8)	1.1	0.73 ± 0.08	0.32 (0.14, 0.56)	-2.7
Vip3A	SS	576	2.5 ± 0.5	2.03 (1.29, 3.30)	---	2.06 ± 0.14	0.66 (0.55, 0.79)	---
	RS	557	2.2 ± 0.6	3.54 (1.72, 7.98)	1.7	1.25 ± 0.08	0.48 (0.38, 0.60)	-1.4
	RR	576	1.5 ± 0.4	3.01 (1.10, 11.0)	1.5	1.05 ± 0.08	0.36 (0.27, 0.49)	-1.8

492

493 ^a Total number of neonates assayed.

494 ^b Median lethal concentration (LC₅₀) that caused 50% mortality and the corresponding 95% confidence limit (CL). The LC₅₀ value of
495 an insect genotype was considered to be greater than the highest Bt protein concentration used in the bioassay if its larval mortality
496 was <50% at the highest concentration. Larval mortality were calculated based on the number of dead larvae plus survivors that were
497 still in the first instar divided by the total number of insects assayed.

498 ^c Growth inhibition concentration (GI₅₀) that reduced larval weight by 50% and the corresponding 95% confidence limit (CL). The
499 GI₅₀ value of an insect genotype was considered to be greater than the highest Bt protein concentration used in the bioassay if its larval
500 growth inhibition was <50% at the highest concentration.

501 ^d Resistance ratio of an insect genotype for a Bt protein was calculated using the LC₅₀ or GI₅₀ value of that genotype divided by the
502 LC₅₀ or GI₅₀ value of SS. In some cases, if the LC₅₀ or GI₅₀ of an insect genotype was smaller than that of SS, the ratio was computed
503 using the LC₅₀ or GI₅₀ of SS divided by the corresponding value of that genotypes and a negative sign was assigned to the ratio.

Fig. 1

Fig. 2