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Huang et al. Cry1A.105 resistance frequency in S. frugiperda

Frequency of *Bacillus thuringiensis* Cry1A.105 resistance alleles in field populations of the fall armyworm, *Spodoptera frugiperda*, in Louisiana and Florida

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ABSTRACT

Fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is a major pest of many crops and a cross-crop target of transgenic maize, cotton, and soybean containing *Bacillus thuringiensis* (Bt) genes. Some of the current Bt maize products for controlling lepidopteran species contain the Bt event MON89034. The objective of this study was to determine the frequency of resistance alleles in field populations of *S. frugiperda* collected from Louisiana and Florida, U.S. to Cry1A.105, one of the two Bt genes in MON89034. A total of 150 F₂ two-parent families of *S. frugiperda* were established using single-pair mating of field-collected individuals in 2011, which included 79 families from two locations in Louisiana and 71 families from one location in Florida. F₂ screen was conducted to detect resistance alleles in these families to Cry1A.105 protein in maize plants. Four out of the 79 Louisiana and 14 out of the 71 Florida families were identified to possess resistance alleles to the Cry1A.105 maize plants. Thus, the corresponding frequency of resistance alleles to Cry1A.105 maize was estimated to be 0.0158 with a 95% credibility interval (CI) of 0.0052 to 0.0323 for the Louisiana populations and 0.0559 with a 95% CI of 0.0319 to 0.0868 for the Florida populations. The resistant families survived on whole Cry1A.105 maize plants and demonstrated a significant level (> 116-fold) of resistance to the Cry1A.105 protein in a diet-incorporated bioassay. These findings suggest that resistance allele frequency in *S. frugiperda* to single-gene Cry1A.105 maize in the U.S. southeast region apparently is not rare.

Keywords: *Spodoptera frugiperda*, F₂ screen, *Bacillus thuringiensis*, Cry1A.105, resistance allele

1. Introduction

Transgenic maize, *Zea mays* L., expressing *Bacillus thuringiensis* (Bt) proteins has become a major tool for managing some maize insect pests including the fall armyworm, *Spodoptera frugiperda* (J.E. Smith). The effectiveness of Bt maize in controlling its target pests has encouraged maize producers to rapidly adopt this technology. In 2014, >77 Mha of Bt crops were planted in 25 countries of the world (James, 2014). In the same year, the U.S. growers alone planted ca. 29.7 Mha of Bt maize (NASS, 2014), which accounted for >61% of world's total Bt maize area. Development of resistance in target pest is a major threat to the long-term durability of Bt maize (Huang et al., 2011; Matten et al., 2012). To date, field resistance due to intensive use of Bt crops has been documented in at least six cases, which include the resistance of *S. frugiperda* to Cry1F maize in Puerto Rico (Storer et al., 2010), Brazil (Farias et al., 2014), and the U.S. mainland (Huang et al., 2014).

Spodoptera frugiperda is a major maize pest in both North and South America (Farias et al., 2014; Huang et al., 2014). To date, *S. frugiperda* is the first and the only target pest that has developed field resistance to Bt crops at multiple locations across different countries and continents, indicating a great challenge in managing the resistance, especially in Brazil and the U.S. south region where *S. frugiperda* is a cross-crop target of Bt maize and Bt cotton. It is also a target of Bt soybean in Brazil. The objective of this study was to conduct an F₂ screen to detect the Cry1A.105 resistance alleles in field populations of *S. frugiperda* collected from Louisiana and Florida in 2011. Cry1A.105 is a chimeric gene comprised of domains I and II which are identical with the respective domains from Cry1Ab or Cry1Ac and domain III of Cry1F, and the C-terminal domain from Cry1Ac (Biosafety Clearing-House, 2014). Cry1A.105 is one of the two Bt genes in MON89034, a Bt maize event that was incorporated into some pyramided Bt maize

products and was first commercially planted in 2010 in the U.S. for controlling above-ground lepidopteran pests including *S. frugiperda* (Buntin and Flanders, 2014). Here we report the detection and estimates of the frequency of Cry1A.105 resistance alleles in field populations of *S. frugiperda* collected from three locations in Louisiana and Florida in 2011.

2. Materials and Methods

2.1. Field collection of *S. frugiperda* and establishment of two-parent families

During 2011, third to fifth instars of *S. frugiperda* were collected from sorghum plants in Franklin (32°08' N; 91°41'W) and Rapides (32°08' N; 91°41'W) parishes, Louisiana and maize fields in Collier County (26° 28'N, 81° 26'W), Florida (Huang et al., 2014). Field collected larvae were individually reared in a meridic diet (WARD'S Natural Science, Rochester, NY) until the pupal stage (Niu et al., 2013). F₂ two-parent families were established using single-pair mating of the adults derived from the field-collected larvae as described in Huang et al. (2014).

2.2. Source of Bt and non-Bt maize plants

Seeds of a transgenic maize line containing Cry1A.105 gene (thereafter referred as Cry1A.105 maize) and a near iso-line of non-Bt maize (thereafter referred as non-Bt maize) were provided by Monsanto Company (St Louis, MO, USA). Two seeds of a maize line were planted in each 18.9-liter pot containing ~5 kg of a standard potting soil mixture (Perfect MixTM, Expert Gardener Products, St. Louis, MO) as described in Wangila et al. (2012). Pots were placed in the greenhouses at Louisiana State University Agricultural Center in Baton Rouge, LA. Maize plants were irrigated and fertilized for optimum growth during the experimental periods. Expression/not

expression of Cry1A.105 in plants was confirmed with the Cry1A Lateral Flow Strip Kits (Portland, ME).

2.3. *F₂ screen for Cry1A.105 resistance*

Detailed description of the *F₂* screening procedures has been reported in Huang et al. (2014). Briefly, leaf tissues dissected from V4-V9 stages of greenhouse grown Cry1A.105 maize plants were provided to *S. frugiperda* in 32-well trays (Bio-Smart-32, C-D International, Pitman, NJ). A group of four neonates (<24 h) of *S. frugiperda* were screened in each well. For each *F₂* two-parent family, 96 *F₂* neonates were screened in 24 wells (4 neonates/well) in environmental chambers maintained at 28°C, ~50% and a 16:8 (L:D) h photoperiod. Larval survival and growth were checked after 7 days. Live larvae were separated into two groups based on their growth, small (1st and 2nd instars) and large ($\geq$ 3rd instars) as described in Huang et al. (2014).

In addition, baseline survival of a known Bt-susceptible strain (SS-FL) on leaf tissue of non-Bt and Bt maize was determined using the same method as in *F₂ screen*. SS-FL was initiated from 96 larvae sampled from non-Bt maize fields in Hendry County in Florida in 2011 (Niu et al., 2013). SS-FL has been documented to be susceptible to Cry1A.105 and Cry1F proteins as well as to most Bt maize traits including Herculex[®]I, Genuity[®]VT Double Pro[™], Genuity[®]VT Triple Pro[™], Genuity[®] SmartStax[™], and Agrisure[®] Viptera[™] 3111 (Niu et al., 2013, Huang et al., 2014). Viability of the SS-FL and the field collected populations has been documented in Huang et al. (2014).

2.4. *Defining potentially positive families possessing resistance alleles*

The F₂ screen described above was used to identify potentially positive families carrying major resistance alleles to Cry1A.105. Andow (2008) suggested that a major resistance allele to a Bt crop is present when homozygous-resistant individuals can grow and mature on the Bt crop and can mate and produce viable offspring. Theoretically, if one of the two parents of a family possessed a major recessive resistance allele, 6.25% of F₂ larvae are expected to be homozygous for Bt resistance and able to survive in the F₂ screen (Andow and Alstad, 1998). Criteria for defining a potentially positive family in this study were based on the baseline susceptibility of SS-FL on non-Bt maize leaf tissue. With the same experimental design used in F₂ screen, baseline survival bioassays showed that 42.6% neonates of SS-FL survived after 7 days on the non-Bt maize leaf tissue and predominant live larvae were $\geq 3^{\text{rd}}$ instars. Therefore, we considered that a homozygous resistant larva should develop to $\geq 3^{\text{rd}}$ instar after 7 days in the F₂ screen. For each family, the number of neonates screened was 96 individuals. Based on χ^2 -tests, a survivorship of one individual ($\geq 3^{\text{rd}}$ instar) out of the 96 neonates screened for a family was not significantly different from the expected survivorship rate of 6.25% if one of the two parents in the family possessed a resistance allele. Thus it was defined that a potentially positive family should have ≥ 1 large live larvae ($\geq 3^{\text{rd}}$ instars) after 7 days in the F₂ screen (Huang et al., 2014).

2.5. Resistance confirmation and estimate of resistance allele frequency

In the F₂ screen, a total of 18 families were identified to be potentially positive families carrying resistance alleles to Cry1A.105 (see results). Survivors of the 18 families after the F₂ screen were transferred into the meridic diet and reared in the laboratory as described in Yang et al. (2013). To confirm if a potentially positive family actually possessed resistance alleles, four laboratory populations were established from the survivors (both large and small larvae) of four

potentially positive families. The four families were one family (family 37) of the Rapides, LA, population, thereafter referred as LA-DL-37 and three families (families 30, 32, and 67) of the Florida population, thereafter referred as FL-30, FL-32, and FL-67, respectively. The attempt to establish laboratory populations for other potentially positive families was unsuccessful because no or not enough progeny were produced from the F₂ survivors. In addition, another population was developed from 14 survivors in the F₂ screen with the family 74 of the Florida population, thereafter referred as FL-74. This family had a total of 14 survivors and all survivors were $\leq 2^{\text{nd}}$ instars. Thus FL-74 did not qualify as a potentially positive family possessing major resistance alleles. All of the five families were then tested for larval survival and plant injury on whole Cry1A.105 maize plants in the greenhouse. For the greenhouse confirmation tests, 5-8 neonates of a family along with SS-FL were placed into the whorl of each plant at V7-VT stages (Table 3). For each family or population, larval survival was evaluated on a total of 5-10 plants in five pots (1-2 plants/pot). In addition, survival of two families (FL-32 and FL-67) on the non-Bt maize plants was also evaluated in the greenhouse using the same method described in the tests with the Bt maize plants. Bt protein expression/non-expression in the plants was confirmed with the ELISA-based assays mentioned above. Larval survival, body weight, and plant injury were recorded after 13-15 days. Plant injury was measured using Davis' 1-9 scale (Davis et al., 1992). A potentially positive family that survived on the whole Bt plants and developed to $\geq 4^{\text{th}}$ instars was considered to actually possess major resistance alleles (Huang et al., 2014). Expected allele frequencies to Cry1A.105 maize plants and the corresponding 95% credibility intervals (CI) were calculated using a Bayesian analysis as described in Andow and Alstad (1998; 1999).

2.6. Susceptibility of Cry1A.105-maize-resistant strains of *S. frugiperda* to Cry1A.105 protein

A diet-incorporated bioassay was performed to determine if the survival of the Cry1A.105-maize-resistant strains of *S. frugiperda* detected from the F₂ screen was due to resistance to the Cry1A.105 protein in the plants. Cry1A.105 protein was provided by Monsanto Company (St. Louis, MO). The Cry1A.105 was produced in a culture of *Escherichia coli* containing the pMON96851 expression plasmid and had a molecular weight of 58.1 - 131.5 kDa. This protein was 80% pure and was provided at a concentration of 1.2 mg/mL in a buffer consisting of 25 mM CAPS, pH 10.3, 1 mM benzamidine-HCl, 0.1 mM EDTA, and 0.2 mM DTT.

Two laboratory strains, FL-32 and FL-67, were established from the survivors of the two families on Cry1A.105 plants in the greenhouse confirmation tests. To establish the laboratory strains, the greenhouse survivors were transferred into the meridic diet and reared in the laboratory as described above. Susceptibility of FL-32 and FL-67, along with a known Bt-susceptible strain (SS-TX), to Cry1A.105 protein was individually assayed using a diet incorporated bioassay method as described in Niu et al. (2013). SS-TX was collected in a non-Bt corn field near Weslaco in southeast Texas in 2013. SS-TX has demonstrated to be susceptible to purified proteins of Cry1A.105, Cry2Ab2, and Cry1F, as well as to corn leaf tissue and whole plants expressing Cry1A.105, Cry2Ab2, and/or Cry1F proteins (Huang et al., 2014; Dangal and Huang, 2015). Larval mortality in the diet-incorporated bioassay was recorded on the 7th day after inoculation. Larvae were considered dead if they did not respond after being touched with a camel hair brush. In each concentration, there were four replications with 16-32 larvae in each replication.

In the diet-incorporated bioassay, larval mortality at a respective Bt concentration was corrected with mortality on the negative control diet (buffer treated only). The corrected concentration/mortality data were then subjected to a probit analysis to determine the Cry1A.105

concentration that produced a 50% mortality value (LC_{50}) and the corresponding 95% confidence interval (CI). Resistance ratios were calculated by dividing the LC_{50} value of FL-32 or FL-67 by the LC_{50} of SS-FL.

3. Results

3.1. Baseline survivorship

Larval survival rate of SS-FL was $42.6 \pm 8.2\%$ on non-Bt maize leaf tissue after 7 days, and zero on Cry1A.105 maize leaf tissues. The larval survival rate of SS-FL on non-Bt corn leaf tissue was comparable to the published data (Niu et al., 2013; Yang et al., 2013). The baseline survival data suggested that leaf tissue of the Cry1A.105 maize plants expressed a sufficient level of the Cry1A.105 protein to kill susceptible *S. frugiperda* and thus could be used to identify individuals that were resistant to the Cry1A.105 maize plants.

3.2. Survival of F_2 two-parent families of Louisiana populations of *S. frugiperda* in the F_2 screen for resistance to Cry1A.105 maize leaf tissue

A total of 79 F_2 two-parent families were established from 158 feral individuals of *S. frugiperda* collected from Louisiana (Table 1). Among these, 50 and 29 families were developed from insects collected from Rapides and Franklin parishes, respectively. The F_2 screen showed that nine of the 50 F_2 families from Rapides and three of 29 families from Franklin had a proportion of larvae that survived after 7 days on Cry1A.105 leaf tissue (Table 1). Among these Rapides families, three families (families 26, 27, and 37) exceeded the minimum criteria for a potentially positive family carrying resistance alleles (Table 2). For the 29 Franklin families,

three families survived in the F₂ screen and one (family 3) met the minimum criteria for a potentially positive family.

3.3. Survival of F₂ two-parent families of Florida populations of S. frugiperda in the F₂ screen for resistance to Cry1A.105 maize leaf tissue

A total of 71 F₂ two-parent families were established from 142 feral individuals collected from Collier County, Florida. Among these, 26 families had a proportion of larvae that survived after 7 days on Cry1A.105 leaf tissue in the F₂ screen (Table 1). Among these 26 families that survived in the F₂ screen, 14 families (no. 7, 18, 30, 31, 32, 34, 42, 45, 46, 48, 52, 66, 67, and 72) qualified as potentially positive families carrying resistance alleles (Table 2).

3.4. Larval survival and plant injury of potentially positive families of S. frugiperda on whole plants of Cry1A.105 maize

Larvae of SS-FL survived well on non-Bt maize plants and caused considerable leaf injury (Table 3). A total of 16 live larvae were recovered from non-Bt plants after 14 days and 9 out of the survivors reached $\geq 4^{\text{th}}$ instars, with an average body weight of 116.6 ± 23.0 mg/larva. The SS-FL on the non-Bt maize plants caused an average leaf injury rating of 7.8 ± 0.2 after 14 days. In contrast, no live larvae of SS-FL were found on the Cry1A.105 plants after 14 days, suggesting that the greenhouse-grown plants could be used to identify Cry1A.105-resistant individuals. Like the SS-FL, larvae of FL-32 and FL-67 also survived well and caused considerable amount of leaf injury to the non-Bt plants in the greenhouse (Table 3).

On Cry1A.105 maize plants, larvae of all four potentially positive families survived well with varied levels of leaf injury depending on the infested plant stages (Table 3). Most live larvae

were recovered from the whorls of the plants, while some larvae were found on young ears in the plants that were infested at late stages (e.g. plants infested with FL-32 at the early tussling stage). From the ten Cry1A.105 maize plants infested with five neonates of LA-DL-37 per plant, a total of 6 larvae (one 3rd and five $\geq 4^{\text{th}}$ instars) were found after 13 days with an average body weight of 124.3 mg/larva. For FL-30, a total of 7 live larvae (one 3rd and six $\geq 4^{\text{th}}$ instars) were recovered after 15 days from the eight infested plants with an average body weight of 229.6 mg/larva. From the five plants that were infested with eight FL-32 neonates per plant, a total of 22 live larvae (seven 3rd and fifteen $\geq 4^{\text{th}}$ instars) were recovered after 13 days with an average body weight of 153.7 mg/larva. Similarly, a total of nine larvae (three 3rd and six $\geq 4^{\text{th}}$ instars) survived on the ten plants infested with five neonates of FL-67 per plant after 13 days. These FL-67 survivors had an average body weight of 158.3 mg/larva. Results of the whole plant tests in the greenhouse confirmed that all four potentially positive families identified in the F₂ screen actually possessed major resistance alleles to Cry1A.105 maize plants. In contrast, no live larvae were recovered from the ten Cry1A.105 maize plants that were infested with five FL-74 neonates per plant. The results validated that FL-74 did not carry major resistance alleles to Cry1A.105.

3.5. Resistance allele frequency in the three populations of *S. frugiperda* to Cry1A.105 maize

Based on the results of F₂ screen with maize leaf tissue, a total of 18 families were considered to be potentially positive families possessing major resistance alleles to Cry1A.105 maize in the three populations of *S. frugiperda* (Table 2). Whole plant tests confirmed that all of the four potentially positive families that were evaluated in the greenhouse possessed major resistance alleles to Cry1A.105 maize plants (Table 3). Due to the unsuccessful establishment of progeny populations for the other 14 potentially positive families, resistance confirmation tests

were not performed for these families. However, based on their similar performance of the 18 families in the F₂ screen (Table 2), the 14 potentially positive families that were not retested in the greenhouse **most likely possessed** major resistance alleles to the Cry1A.105 maize plants. Therefore, we considered all of the 18 potentially positive families identified in the F₂ screen possessing major resistance alleles to Cry1A.105 maize plants in calculating resistance allele frequency.

In summary, the leaf-tissue F₂ screen and whole plant confirmation tests suggested that three of the 50 F₂ two-parent families of the Rapides population and one of the 29 families of the Franklin population, Louisiana possessed major resistance alleles to Cry1A.105 maize (Table 4). By using Bayesian estimation, the expected frequency of resistance alleles to Cry1A.105 maize was 0.0198 with a 95% CI of 0.0055 to 0.0433 for the Rapides population and 0.0165 with a 95% CI of 0.0021 to 0.0461 for the Franklin population. The difference in the resistance allele frequency between the two Louisiana populations was considered not significant because of their overlapped 95% CIs. Thus, the frequency of major resistance alleles for the combined Louisiana populations was 0.0158 with a 95% CI of 0.0052 to 0.0323. For the Florida population, the results of the F₂ screen and confirmation tests showed that 14 out of the 71 families possessed major resistance alleles to Cry1A.105 maize. Thus, the frequency of major resistance alleles to Cry1A.105 maize in the population collected from Collier County, FL was estimated to be 0.0559 with a 95% CI of 0.0319 to 0.0868 (Table 4).

3.6. Susceptibility of Cry1A.105 maize resistant strains of S. frugiperda to Cry1A.105 protein

The LC₅₀ value of Cry1A.105 protein for the SS-TX was 0.86 µg/g with a 95% CL of 0.45 to 1.49 µg/g (Table 5). Compared to SS-TX, both FL-32 and FL-67 that were identified to possess

major resistance alleles to Cry1A.105 maize were considerably less susceptible to the Cry1A.105 protein (Table 5). The LC₅₀ values of Cry1A.105 for FL-32 and FL-67 couldn't be determined with the probit analysis because the highest concentration, 100 µg/g, used in the bioassays resulted in only 31.1% mortality for FL-32 and 46.2% for FL-67. In contrast, mortality of SS-TX was 100% at 10 µg/g (data not shown). Thus, resistance ratios to Cry1A.105 based on the highest Bt concentration tested in the bioassays for FL-32 and FL-67 were >116-fold. The results of the diet incorporated bioassays validated that the survival of the Cry1A.105 maize resistant families on maize leaf tissue in the F₂ screen and whole plant in the greenhouse was a result of resistance to the Cry1A.105 protein in the plants.

4. Discussion

F₂ screen is more sensitive in detecting rare recessive resistance alleles in field insect populations compared to some other traditional methods such as dose-response and discriminating dose bioassays (Andow et al., 2000). The resistance alleles identified in this study represents the first case of Cry1A.105 resistance alleles in a target pest of Bt crops. The successful identification of the Cry1A.105 resistant alleles in the field populations of *S. frugiperda* in the current study also demonstrates the effectiveness of the F₂ screening method. The Cry1A.105 resistance allele frequency in *S. frugiperda* was estimated to be 0.0158 with a 95% CI of 0.0052 to 0.0323 for the Louisiana populations and 0.0559 with a 95% CI of 0.0319 to 0.0868 for the Florida population. It appeared that the resistance allele frequency in the Florida population was somewhat greater than that of the Louisiana populations, but the difference was not significant based on the overlap of 95% CIs. Thus we also estimated the allele frequency for the combined populations of Louisiana and Florida to be 0.0328 with a 95% CI of

0.0200 to 0.0489. Nevertheless, the results of this study suggest that resistance allele frequency to the single-gene Cry1A.105 maize in field populations of *S. frugiperda* in Louisiana and Florida apparently is greater than those estimated to Cry1Ab maize in maize stalk borers including the European corn borer, *Ostrinia nubilalis* (Hübner) (Andow et al., 2000; Bourguet et al., 2003; Farinós et al., 2004; Siegfried et al., 2007; Engels et al., 2010), southwestern corn borer, *Diatraea grandiosella* Dyer (Trisyono and Chippendale, 2002; Huang et al., 2007), and sugarcane borer, *Diatraea saccharalis* (F.) (Huang et al., 2012).

In addition, survivors of the 150 two-parent families of *S. frugiperda* developed in the current study were also screened against a commercial Cry1F maize hybrid (TC1507) (Huang et al., 2014). The results showed that 17 out of the 18 Cry1A.105-resistant families identified in the current study were also resistant to Cry1F maize plants (Huang et al., 2014). These findings suggest that there is a highly positive correlation between the Cry1A.105 and Cry1F resistance in *S. frugiperda*. As pointed out in Huang et al. (2014), it is not surprising that **there is a certain level of cross-resistance** in *S. frugiperda* between Cry1A.105 and Cry1F because of the similarity in their gene structures and the tolerance of the insect to both Cry1Ab/Cry1Ac (US-EPA, 2001; Chilcutt et al., 2007; Hardke et al., 2011). The cross-resistance between Cry1A.105 and Cry1F suggests that these two Bt genes are not ideal proteins for pyramiding to manage *S. frugiperda*.

In addition, the cross-resistance between Cry1A.105 and Cry1F could create a great challenge in managing the already widespread resistance of *S. frugiperda* to Bt crops (Huang et al., 2014; Bernardi et al., 2015). In the current transgenic crop market, Bt genes targeting lepidopteran pests in the three major commercial Bt crops (maize, cotton and soybean) can be classified into three groups: Cry1 which includes Cry1Ab, Cry1Ac, Cry1F, and Cry1A.105; Cry2 which includes Cry2Ab and Cry2Ae; and Vip which includes only the Vip3A. As mentioned

above, *S. frugiperda* is naturally tolerant to both Cry1Ab and Cry1Ac (Huang et al. 2014). Thus, once resistance to either Cry1F or Cry1A.105 occurs, the cross-resistance between the two proteins virtually eliminate all the Cry1A proteins for *S. frugiperda* and leaves only Cry2A and Vip3A being active for the insect.

The single-gene Cry1A.105 maize used in this study is not commercially available for controlling insect pests. As mentioned above, Cry1A.105 is one of the two pyramided genes in the event MON89034 that has been incorporated into several commonly used pyramided Bt maize technologies such as Genuity® VT Double Pro™, Genuity® VT Triple Pro™, and Genuity® SmartStax™ (Ghimire et al., 2011). Thus, resistance to the Cry1A.105 gene in the plants can be a concern for these technologies. Another Bt gene in MON89034 is Cry2Ab2, which is dissimilar in protein structures compared to Cry1A and has different binding sites in the midguts of target insects, indicating that Cry2Ab2 represents a distinct mode of action compared to Cry1A (Storer et al., 2012). Several studies have shown that a Cry1A resistant insect is usually not cross-resistant to Cry2Ab2 (Sivasupramaniam et al., 2008; Brévault et al., 2009; Wu et al., 2009; Vélez et al., 2013; Huang et al., 2014). In another independent study, 215 F₂ two-parent families of *S. frugiperda* that were also collected from the U.S. southern region (Texas, Louisiana, Georgia, and Florida) were screened for resistance to Cry2Ab2 maize plants and one of the 215 families was identified to possess major resistance alleles to the Cry2Ab2 plants (YN and FH, unpublished data). Thus, the resistance allele frequency of *S. frugiperda* in these populations was estimated to be 0.0023 with a 95% CI of 0.0003 to 0.0064. If it is assumed that the Cry1A.105 and Cry2Ab2 work independently in MON89034, the frequency of their joint resistance alleles to MON89034 in these populations in the U.S. southeast region should still be very low, ca. 0.00008.

To verify this assumption, survival of 149 out of the 150 F₂ two-parent families evaluated in the current study were assayed against a commercial maize hybrid containing Genuity® VT Double Pro™ traits using the similar methods (Yang et al., 2013). Genuity® VT Double Pro™ maize contains event MON89034. Five of the 149 families had some survivors in the 7-day leaf tissue bioassay with Genuity® VT Double Pro™, but larvae of these families could not survive on whole plants of Genuity® VT Double Pro™ in the greenhouse (Yang et al., 2013). The results validated that, although resistance alleles to the single gene Cry1A.105 was not rare, the joint frequency of the resistance alleles to both Cry1A.105 and Cry2Ab2 in MON89034 in *S. frugiperda* could still be rare in these populations. Thus pyramiding Cry1A.105 and Cry2Ab2 will be a good strategy for managing *S. frugiperda*.

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Table 1. F₂ screen for detecting Cry1A.105 resistance alleles in three populations of *Spodoptera frugiperda* collected from Louisiana (LA) and Florida (FL).^a

Table 2. Potentially positive families possessing resistance alleles to Cry1A.105 maize that were identified in the F₂ screen in three populations of *Spodoptera frugiperda* collected from Louisiana (LA) and Florida (FL).^a

Table 3. Larval survival and plant injury rating of the four positive and one negative families of *Spodoptera frugiperda* on whole Cry1A.105 maize plants in the greenhouse.^a

Table 4. Estimated allele frequency and/or corresponding 95% credibility interval (CI) of Cry1A.105-maize resistance in three populations of *Spodoptera frugiperda* collected from Louisiana (LA) and Florida (FL).

Table 5. LC₅₀s and 95% confidence intervals (CI) based on larval mortality of SS-TX and two Cry1A.105-maize resistant strains of *Spodoptera frugiperda* on meridic diet treated with Cry1A.105 protein.^a

485 **Table 1**

State	Parish/ County	No. F ₂ family screened	Live big larvae			Live small larvae			Total survival		
			No. family	No. larvae	Body weight (mg/larva)	No. family	No. larvae	Body weight (mg/larva)	No. family	No. larvae	Body weight (mg/larva)
LA	Rapides	50	3	9	8.1	9	38	1.7	9	47	2.9
	Franklin	29	1	1	6.0	3	4	2.3	3	5	3.0
	Subtotal	79	4	10	7.9	12	42	1.8	12	52	2.9
FL	Collier	71	14	64	11.7	23	79	2.3	26	143	6.5
Overall		150	18	74	11.2	35	121	2.1	38	195	5.5

^a For each F₂ family, 96 neonates were screened on Cry1A.105 maize leaf tissue and larval survival was checked after 7 days.

Table 2.

Family	Live big larvae		Live small larvae		Total survival	
	No. larvae	Body weight (mg/larva)	No. larvae	Body weight (mg/larva)	No. larvae	Body weight (mg/larva)
Louisiana-Rapides						
26	3	7.0	5	1.6	8	3.6
27	2	7.5	4	1.8	6	2.2
37	4	9.3	1	3.0	5	8.0

Louisiana-Franklin						
3	1	6.0	2	3.0	3	4.0

Florida-Collier						
7	1	5.0	2	1.5	3	3.7
18	1	8.0	3	1.7	4	3.3
30	8	7.8	3	2.0	9	7.6
31	2	6	6	2.2	8	3.2
32	9	10.0	7	2.7	16	7.0
34	8	14.3	7	3.5	15	9.3
42	1	12.0	0	-	1	12.0
45	15	17.0	3	1.7	18	14.5
46	6	10.7	2	3.5	8	8.9
48	5	8.4	1	2.0	6	7.3

52	3	17.0	1	2.0	4	13.3
66	1	5.0	0	-	1	5.0
67	2	8.5	6	1.4	8	3.2
72	1	7.0	0	-	1	7.0

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Table 3

Family	Maize	Plant stages at infestation	No. plants	No. larvae infested /plant	Days after inoculation	Leaf injury ratings $\pm$ SE	No. total live larvae	No. $\geq 4^{\text{th}}$ instar larvae	Larval body weight (mg/larva $\pm$ SE)
SS-FL	Non-Bt	V6-V7	10	5	14	7.8 ± 0.2	16	9	116.6 ± 23.0
FL-32	Non-Bt	V6-V7	10	5	13	7.8 ± 0.4	17	7	91.9 ± 12.4
FL-67	Non-Bt	V6-V7	10	5	13	8.6 ± 0.2	15	11	143.9 ± 15.0
SS-FL	Bt maize	V6-V7	10	5	14	1.2 ± 0.1	0	0	---
LA-DL-37	Bt maize	V11-V12	10	5	13	2.4 ± 0.5	6	5	124.3 ± 15.8
FL-30	Bt maize	V7-V8	8	5	15	8.1 ± 0.2	7	6	229.6 ± 46.8
FL-32	Bt maize	Early tussling	5	8	13	3.2 ± 0.4	22	15	153.7 ± 19.8
FL-67	Bt maize	V11-V12	10	5	13	3.1 ± 0.4	9	6	158.3 ± 35.3
FL-74	Bt maize	V6-V7	10	5	7	1.3 ± 0.1	0	0	---

^a Plant injury was measured using Davis' 1-9 scale (Davis et al., 1992).

498 **Table 4**

499

State	Parish /County	No. families screened	No. major alleles	Expected major resistance allele frequency (95% CI)	500
					501
LA	Rapides	50	3	0.0198 (0.0055, 0.0433)	
	Franklin	29	1	0.0165 (0.0021, 0.0461)	
	Subtotal	79	4	0.0158 (0.0052, 0.0323)	
FL	Collier	71	14	0.0559 (0.0319, 0.0868)	
	Total	150	18	0.0328 (0.0200, 0.0489)	

502 **Table 5**

Insect strain	No. larvae assayed	Slope $\pm$ SE	LC ₅₀ (95% CI)	χ^2	df	Resistance ratio
SS-TX	576	1.27 $\pm$ 0.29	0.86(0.45, 1.49)	51.85	14	--
FL-32	992	---	> 100	---	---	>116
FL-67	981	---	>100	---	---	>116

503 ^a Corrected larval mortality at 100 μ g/g, the highest Cry1A.105 concentration tested, was 31.1% for FL-32 and 46.2 % for FL-67.