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F_2 screen for resistance to *Bacillus thuringiensis* Cry2Ab2-maize in field populations of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) from the southern United States ☆

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ABSTRACT

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is a target pest of transgenic maize and cotton expressing *Bacillus thuringiensis* (Bt) proteins in both North and South America. In 2013 and 2014, a total of 215 F_2 two-parent families of *S. frugiperda* were established using single-pair mating of field individuals collected from seven locations in four states of the southern U.S.: Texas, Louisiana, Georgia, and Florida. The objective of the investigation was to detect resistance alleles in field populations to Cry2Ab2, a common Bt protein produced in transgenic maize and cotton. For each F_2 family, 128 F_2 neonates were screened on leaf tissue of Cry2Ab2 maize plants in the laboratory. A conservative estimate of the frequency of major Cry2Ab2 resistance alleles in *S. frugiperda* from the four states was 0.0023 with a 95% credibility interval of 0.0003–0.0064. In addition, six families were considered to likely possess minor resistance alleles at a frequency of 0.0082 with a 95% credibility interval of 0.0033–0.0152. One F_2 family from Georgia (GA-15) was confirmed to possess a major resistance allele to the Cry2Ab2 protein. Larvae from this family survived well on whole maize plants expressing Cry2Ab2 protein and demonstrated a significant level (>15-fold) of resistance when fed with the same protein incorporated in a meridic diet. The detection of the major resistance allele along with the relatively abundant minor resistance alleles revealed in this study may have important implications for resistance management.

1. Introduction

Transgenic crops expressing *Bacillus thuringiensis* (Bt) proteins have become a major tool for managing maize and cotton insect pests in many countries (James, 2014). Evolution of resistance to Bt proteins in target pests is a threat to the sustainable use of Bt crop technology (Huang et al., 2011; Tabashnik et al., 2013). Due to the intensive use of Bt crops during the last 20 years, pest resistance to transgenic Bt maize and cotton crops resulting in control problems has occurred for several target species and in several countries (van Rensburg, 2007; Storer et al., 2010; Dhurua and Gujar, 2011; Gassmann et al., 2011; Farias et al., 2014; Huang et al., 2014).

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is a target pest of Bt maize and Bt cotton in North and South America (Farias et al., 2014; Huang et al., 2014). In recent years, field resistance to Cry1F maize in *S. frugiperda* has been documented in Puerto Rico (Storer et al., 2010), Brazil (Farias et al., 2014), and the U.S. mainland (Huang et al., 2014). Up to date, *S. frugiperda* is the only target pest that has developed field resistance to Bt crops in multiple locations across different countries and continents (Dangal and Huang, 2015). Cry2Ab2 is one of the two pyramided Bt genes in

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the event MON 89034, which has been incorporated into some pyramided Bt maize hybrids. In 2010, maize hybrids containing MON 89034 became commercially available for controlling above-ground lepidopteran pests including *S. frugiperda* (Flanders, 2014). The wide occurrence of the Cry1F resistance in *S. frugiperda* makes it even more important to preserve the Cry2Ab2 susceptibility in the target pest populations to ensure the sustainable use of the Bt maize technology. However, because Cry2Ab2 is a relatively new Bt gene used in transgenic maize, information about Cry2Ab2 resistance in target pests of Bt maize is still very limited.

Several methods have been used in detection of resistance alleles to Bt crops in field insect populations (Huang, 2006). Among these, the F_2 screening method is believed to be more sensitive and accurate in detecting rare recessive alleles, compared to the traditional dose-response or discriminating dose bioassay (Andow and Alstad, 1998). For this reason, in the last two decades, F_2 screen has been widely used in detecting Bt resistance, which includes several recent studies with *S. frugiperda* (Vélez et al., 2013; Farias et al., 2014; Huang et al., 2014, 2016; Bernardi et al., 2015a; Li et al., 2016). Taking the advantage of the well-established procedures of the F_2 screen from previous studies, we also used the F_2 screen in the current study to detect resistance alleles in field populations of *S. frugiperda* to Cry2Ab2 maize. Here we report the first documentation of a major resistance allele detected using the F_2 screen to Cry2Ab2-containing maize plants in *S. frugiperda* and estimate the allele frequency in field populations collected from four states of the southern U.S. Information generated from this study should be useful in monitoring and management of Cry2Ab2 resistance in the insect.

2. Materials and methods

2.1. Insect collection, rearing, and development of F_2 two-parent families

During 2013–14, 3rd to 5th instars of *S. frugiperda* were collected from non-Bt maize fields at seven geographical locations across four states of the southern U.S.: Texas (TX), Louisiana (LA), Georgia (GA) and Florida (FL). The seven locations included one site in Hidalgo County, TX; two sites in LA, one each in Franklin and Rapides parishes; one site in Tift County, GA; and three sites in FL, one each in Miami-Dade, Hendry, and Collier counties. Field collected larvae were reared on meridic diet until the pupal stage as described in Niu et al. (2013). F_2 two-parent families were developed by single-pair mating of the individuals derived from the field collections as described in Yang et al. (2013). For each two-parent family, ≈55 viable F_1 pupae were used in sib-mating to generate the F_2 generation of the family.

2.2. Screening of F_2 neonates to identify potentially positive families

To determine if a family possessed Cry2Ab2 resistance alleles, 128 F_2 neonates of each family were screened on leaf tissue removed from greenhouse grown Cry2Ab2 maize plants at V5–V10 stages, using the method described in Huang et al. (2016). The Cry2Ab2 maize product used in the study was an experimental line provided by Monsanto Company (St. Louis). The expression of the Cry2Ab2 protein in the greenhouse grown plants was confirmed with an ELISA-based qualitative assay (EnviroLogix, Quantiplate™ kits, Portland, ME). Larval survival was checked at the 4th and 7th days after insect inoculation, and growth stages of the live larvae were recorded after 7 days only. Live larvae at the 7th day were separated into two groups based on their growth, small (⩽2nd instar) and large (⩾3rd instar) as described in Huang et al. (2014, 2016). Larval survival of a Bt-susceptible strain (TX-SS) of *S. frugiperda* on the Cry2Ab2 maize line and an isogenic line of non-Bt maize (Monsanto Company) was also determined using the same methods as in the F_2 screen. Plants of the non-Bt maize isoline were confirmed for non-expression of Bt proteins with the ELISA-based assay mentioned above. TX-SS was obtained from larvae collected from maize fields near Weslaco, TX in 2013 and it has been documented to be susceptible to the Cry2Ab2, Cry1A.105 and Cry1F proteins, as well as to maize plants expressing these proteins (Huang et al., 2014; Dangal and Huang, 2015). Criteria for a potentially positive family (PPF) possessing major Cry2Ab2 resistance alleles in this study were the same as used for defining the Cry1F and Cry1A.105 resistance as described in Huang et al. (2014, 2016); a PPF means that the family had ⩾1 large live larvae (⩾3rd instar) after 7 days in the F_2 screen.

2.3. Confirmation of resistance on whole plants of Cry2Ab2 maize

Based on the survival in the F_2 screen, seven families qualified as PPF (see results). To confirm if a PPF actually possessed a major resistance allele, laboratory strains of the seven PPFs were established from the survivors of the F_2 screen as described in Huang et al. (2014). Due to the varied number of F_2 survivors among families and to preserve resistance alleles, the 7-day survivors including all small (⩽2nd instar) and large (⩾3rd instar) larvae in the F_2 screen were transferred and individually reared on a non-Bt meridic diet for 3–4 generations as described in Huang et al. (2014). For each family that had ⩾2 large live larvae, a laboratory strain was established from both large and small larvae. There were two families (GA-15 from Tift Co, GA and FL-CL-14 from Collier Co, FL) that had ⩾2 large live larvae after the F_2 screen (see results) and thus two corresponding laboratory strains, GA-15 and FL-CL-14, were developed from their survivors. For the families that had a small number of survivors (e.g. <5 total survivors and one or no large live larvae), their survivors were combined for progeny production. Two combined groups were formed, named Mixed-A and Mixed-B. Mixed-A was established from one PPF plus the F_2 survivors of the seven non-PPFs collected in 2013. Mixed-B was established from two PPFs plus the F_2 survivors of the five non-PPFs collected in 2014. In addition, a laboratory strain (FL-CL-21) was established from 8 small live larvae in the F_2 screen of a family collected from Collier Co., FL in 2014.

Thus, a total of five laboratory strains (GA-15, FL-CL-14, Mixed-A, Mixed-B, and FL-CL-21) were tested for larval survival on whole Cry2Ab2 plants in the greenhouse. For the greenhouse tests, 25 neonates of the 2nd or 3rd generations of each family/group were inoculated into the whorl of each plant at V5–V9 growth stages. For each family/group, a total of 100–125 neonates were infested on 4–5 plants in 4 or 5 pots (1 plant/pot). Larval survival and leaf injury ratings (Davis' 1–9 scale, Davis et al., 1992) were recorded 11 days after the larval release. At the same time, baseline survival of TX-SS at the 14th day after larval release was evaluated on both Bt and non-Bt maize plants. Cry2Ab2 and non-Bt plants were confirmed for Cry2Ab2 protein expression/non-expression with the ELISA-based assay mentioned above. Andow (2008) suggested that a major resistance allele to a Bt crop is present when resistant individuals can grow and mature on the Bt crop and can mate and produce viable offspring. In this study, we used the criteria described in Huang et al. (2014, 2016), a PPF that survived on the whole Bt plants after 11 days was confirmed to possess a major resistance allele, otherwise it was considered to carry minor resistance alleles to Cry2Ab2.

2.4. Estimation of Cry2Ab2 resistance allele frequency

Expected allele frequencies to Cry2Ab2 maize plants and the corresponding 95% credibility intervals (CI) were calculated using

the Bayesian analysis method described in Andow and Alstad (1998, 1999). The probability (detection power) that a resistance allele was detected in a family if one had actually existed in the family was computed using the methods described in Stodola and Andow (2004). Based on our observations, a 1:1 sex ratio and 100% F_1 pupal emergence were used in computing the detection power (Dangal and Huang, 2015).

2.5. Susceptibility of Cry2Ab2-maize-selected families to Cry2Ab2 protein incorporated in meridic diet

The F_2 screen and greenhouse confirmation tests showed that one family from Georgia (GA-15) possessed a major resistance allele to Cry2Ab2 maize plants (see results). To further verify if the survival of GA-15 in the F_2 screen and whole plant tests was due to resistance to the Cry2Ab2 protein in the plants, susceptibility of GA-15, along with TX-SS, to Cry2Ab2 protein was determined using the diet-incorporated bioassay method described in Niu et al. (2013). Cry2Ab2 protein and its buffer solution were provided by Monsanto Company (St. Louis, MO). Six Cry2Ab2 protein concentrations (0.1, 0.316, 1, 3.16, 10, and 31.6 μg/g) plus two controls (blank and negative) were used in assaying TX-SS and six higher concentrations (1, 3.16, 10, 31.6, 100, and 316 μg/g) plus the two controls were used in assaying GA-15. Distilled water was used for the blank control, while the buffer only was used for the negative control. Larval mortality was recorded on the 7th day after neonate inoculation (Niu et al., 2013). Each Cry2Ab2 concentration and control were replicated four times with 16–32 larvae in each replicate.

Corrected concentration/mortality data were subjected to probit analysis to calculate the Cry2Ab2 concentration that produced 50% mortality (LC_{50}) and the corresponding 95% confidence interval (CI). The resistance ratio for the Cry2Ab2-maize-resistant strain was determined by dividing the LC_{50} value of the resistant strain by that of TX-SS.

3. Results

3.1. Development of two-parent families

In 2013 and 2014, a total of 253 single pairs of *S. frugiperda* were established from the moths derived from the field-collected larvae in the four states (Table 1). From these single-pair matings, a total of 215 F_2 two-parent families were successfully established and produced sufficient F_2 ($\geqslant$120 neonates) progeny for F_2 screening. Among the 215 F_2 families, 32, 101, 17, and 65 were derived from collections in TX, LA, GA and FL, respectively. Of the 101 Louisiana families, 57 were established from Franklin Parish and 44 were from Rapides Parish. Of the 65 Florida families, 14 were developed from Miami-Dade Co., 16 were from Hendry Co., and 35 were from Collier Co.

3.2. Baseline performance of TX-SS on leaf tissue of non-Bt and Cry2Ab2 maize lines

Larval survival of TX-SS on leaf tissue of the non-Bt maize line averaged 57.8 ± 4.7% (mean ± sem) with a larval body weight of 52.8 ± 2.8 mg after 7 days, and all survivors except one were 3rd instars or greater. Survival on the non-Bt maize leaf tissue recorded in this study was similar to previous studies (Niu et al., 2013; Yang et al., 2013; Huang et al., 2014). In contrast, none of the TX-SS larvae survived on Cry2Ab2 leaf tissue after 7 days. The results of the baseline bioassay showed that the expression level of the Cry2Ab2 protein in the leaf tissue was high enough to kill the susceptible larvae and thus it is suitable for use as a discriminating concentration to detect Cry2Ab2 resistant individuals.

3.3. Survival of F_2 families in F_2 screen

All of the 215 F_2 families mentioned above were screened on Cry2Ab2 maize leaf tissue (Table 1). Among the 32 Texas-Hidalgo families, a total of 47 larvae from 9 families survived on Cry2Ab2 leaf tissue after 4 days (Table 1). After 7 days, 16 larvae from 6 families survived, which included 14 small larvae ($\leqslant$2nd instar) and 2 large larvae ($\geqslant$3rd instars). The two large larvae were from the same family, TX-29 (Table 2).

In the 57 families from Louisiana-Franklin, 21 families had some larvae surviving with a total of 79 individuals after 4 days (Table 1). After 7 days, 10 larvae from two families were still alive. All of the 10 survivors were small, $\leqslant$2nd instar. From 44 Louisiana-Rapides families, 20 had some larvae surviving to 4 days with a total of 50 survivors. After 7 days, 6 larvae from 3 families were surviving and all were 2nd instar or smaller (Table 1).

From 17 Georgia-Tift families, 63 larvae from 12 families survived through 4 days on the Cry2Ab2 leaf tissue (Table 1). At 7 days, 12 larvae from 4 families were still alive. The survivors included six large larvae ($\geqslant$3rd instars) from a single family, GA-15 (Table 2). Other survivors were 2nd instars or smaller.

In the 14 Florida-Miami/Dade families, a total of 20 larvae from 5 families survived through 4 days, while four families still had 12 live larvae after 7 days, which included 11 small larvae and one large larva (Table 1). The large larva belonged to the family FL-MD-8 (Table 2). Of the 16 Florida-Hendry families, eight families had live larvae after 4 days with a total of 16 survivors, which was reduced to one family and one small larva on the 7th day. For the 35 Florida-Collier families, 21 families had 231 live larvae at 4 days, which reduced to 11 families with 64 larvae at 7 days. These survivors consisted of 42 small larvae and 22 large larvae

Table 1
Performance of F_2 two-parent families of seven populations of *Spodoptera frugiperda* collected from Texas (TX), Louisiana (LA), Georgia (GA), and Florida (FL) in F_2 screen for resistance to Cry2Ab2 maize.[a]

State	Parish/County	No. single pairs	No. F_2 families screened	Survival after 4 days		Survival after 7 days			
				No. families	No. larvae	No. families	No. larvae $\leqslant$2nd instar	No. larvae $\geqslant$3rd instar	No. total larvae
TX	Hidalgo	36	32	9	47	6	14	2	16
LA	Franklin	66	57	21	79	2	10	0	10
	Rapides	53	44	20	50	3	6	0	6
GA	Tift	20	17	12	63	4	6	6	12
FL	Miami/Dade	20	14	5	20	4	11	1	12
	Hendry	20	16	8	16	1	1	0	1
	Collier	38	35	21	231	11	42	22	64
Overall		253	215	96	506	31	90	31	121

[a] For each F_2 family, 128 neonates were screened on Cry2Ab2 maize leaf tissue.

Table 2
Potentially positive families that might possess Cry2Ab2 resistance alleles in *Spodoptera frugiperda* collected from Texas, Louisiana, Georgia and Florida.[a]

Family	No. live small larvae after 7 days (⩽2nd instar)	No. live large larvae after 7 days (⩾3rd instar)	Total surviving larvae after 7 days
Texas-Hidalgo Co.			
29	8	2	10
Georgia-Tift Co.			
15	0	6	6
Florida-Miami/Dade Co.			
8	5	1	6
Florida-Collier Co.			
7	0	1	1
10	1	1	2
13	2	1	3
14	18	19	37

[a] No potentially positive families were identified from the 101 F_2 families collected from Louisiana.

(Table 1). The large larvae were recovered from families of FL-CL-7 (1 larva), FL-CL-10 (1 larva), FL-CL-13 (1 larva), and FL-CL-14 (19 larvae) (Table 2).

Thus, based on the criteria mentioned above, the F_2 screen showed that seven families qualified as PPFs, which included one from the Texas-Hidalgo population (TX-29); one from the Georgia-Tift population (GA-15), one from the Florida-Miami/Dade population (FL-MD-8) and four from the Florida-Collier population (FL-CL-7, FL-CL-10, FL-CL-13, and FL-CL-14) (Table 2).

3.4. Performance of PPFs on whole plants of Cry2Ab2 maize in the greenhouse confirmation tests

Survivors of two PPFs (TX-29, FL-CL-10) in the F_2 screen did not develop to the adult stage. Thus, resistance confirmation for these two families was not performed and both families were judged as without major resistance alleles. Mixed-A was derived from the survivors of FL-MD-8 plus the F_2 survivors from seven non-PPFs collected in 2013. Mixed-B was established from F_2 survivors of two PPFs (FL-CL-7 and FL-CL-13) plus five non-PPFs collected in 2014. The greenhouse tests showed that TX-SS did not cause any leaf injury (leaf injury rating of 1 on a scale of 1–9 with 1 being the least) and after 14 days of larval release, no live larvae were found from the Cry2Ab2 maize plants. On non-Bt maize plants, leaf injury by TX-SS was rated 7.4 and 50% plants contained large live larvae (⩾4th instars) (data not presented). Therefore the Cry2Ab2 maize expressed a high level of Bt protein to kill the susceptible *S. frugiperda* larvae and can be used to identify Cry2Ab2 resistant individuals.

On whole plants of Cry2Ab2 maize in the greenhouse, GA-15 caused severe plant injury after 11 days with a leaf injury rating of 9 on the 1–9 scale (Table 3) and two large live larvae (4th instars) were recovered from two of the four plants infested with GA-15 neonates. The other two plants were also severely damaged, indicating that larvae had moved away from these two Cry2Ab2 plants. In contrast, all Cry2Ab2 plants infested with *S. frugiperda* larvae from other PPFs/groups (FL-CL-14, FL-CL-21, Mixed-A, and Mixed-B) were not injured and no live larvae were found (Table 3). Thus, only GA-15 family was confirmed to carry a major resistance allele against Cry2Ab2 maize plants.

3.5. Major resistance allele frequency

The F_2 screen and whole plant confirmation tests showed that of the 17 families collected from Georgia-Tift Co., one F_2 family, GA-15, possessed a major resistance allele to Cry2Ab2 maize plants (Table 4), while major resistance alleles, based on the criteria mentioned above, were not evident in the other 214 families. Based on the Bayesian analysis, the expected Cry2Ab2 resistance allele frequency associated with a major allele for the Georgia population was 0.0274 with a 95% CI of 0.0035–0.0766. The corresponding frequency with 95% possibility was <0.0224 for the Texas population, <0.0073 for the Louisiana populations, and <0.0113 for the Florida populations (Table 4). The expected frequencies in the four states were not significantly different based on their overlapping 95% CIs. Thus, the Cry2Ab2 major resistance allele frequency for the combined populations collected from the four states was estimated

Table 3
Performance of potentially positive families/groups and a negative family of *Spodoptera frugiperda* on whole plants of Cry2Ab2 maize in the greenhouse.

Family[a]	Plant stage at infestation	No. plants	No. larvae inoculated/plant	Days after inoculation	Leaf injury rating ± SE	No. total live larvae
GA-15	V6	4	25	11	9.0 ± 0.0	2 (4th instar)
FL-CL-14	V9	4	25	11	1.0 ± 0.0	0
FL-CL-21[b]	V5	5	25	11	1.0 ± 0.0	0
Mixed-A[c]	V6	4	25	11	1.5 ± 0.3	0
Mixed-B[d]	V6	4	25	11	1.0 ± 0.0	0

[a] Larval survival and plant injury of TX-SS on the Cry2Ab2 maize and non-Bt maize plants were evaluated after 14 days of neonate release. TX-SS didn't cause any leaf injury (leaf injury rating of 1 on a scale of 1–9 with 1 being the least) and no live larvae were recovered from the Cry2Ab2 plants, while on non-Bt maize plants, TX-SS caused significant leaf damage rated 7.4 and 50% plants contained large live larvae (⩾5th instars).
[b] FL-CL-21 was a negative family which was treated as a control to confirm the criteria for major resistance alleles.
[c] Mixed-A is the progeny of the mixture of the F_2 survivors of FL-MD-1, FL-MD-4, FL-MD-8, FL-MD-20, FL-HD-8, GA-2, GA-5, and GA-10 collected in 2013.
[d] Mixed B is the progeny of the F_2 survivors of FL-CL-1, 3, 5, 7, 12, 13, and 22 collected in 2014.

Table 4
Expected frequency and/or corresponding 95% credibility interval (CI) of resistance alleles to Cry2Ab2 in field populations of *Spodoptera frugiperda* from Texas, Louisiana, Georgia and Florida.

Location	No. of F_2 lines screened	No. of minor resistance alleles	Expected minor resistance allele frequency with 95% CI	No. of major resistance alleles	Expected major resistance allele frequency with 95% CI
Texas	32	1	0.015 (0.0019, 0.042)	0	<0.0224
Louisiana	101	0	<0.0073	0	<0.0073
Georgia	17	0	<0.0408	1	0.0274 (0.0035, 0.0766)
Florida	65	5	0.0232 (0.0086, 0.0449)	0	<0.0113
Total	215	6	0.0082 (0.0033, 0.0152)	1	0.0023 (0.0003, 0.0064)

Table 5
Susceptibility of a Cry2Ab2-susceptible strain (TX-SS) and family GA-15 of *Spodoptera frugiperda* on meridic diet containing Cry2Ab2 protein.

Strain	N[a]	Slope ± SE	LC_{50} (95%CI) (μg/g)[b]	χ^2	df	Resistance ratio[c]
TX-SS	558	1.71 ± 0.42	20.8 (12.9, 51.6)	24.4	10	–
GA-15	690	–	>316	–	–	>15

[a] Total number of neonates assayed.
[b] LC_{50} = 50% lethal concentration and 95%CI = 95% confidence intervals. The LC_{50} for GA-15 could not be calculated with the probit analysis because of the low mortality (42.2%) at the highest concentration of 316 μg/g in the bioassay. Therefore, the LC_{50} value for GA-15 was considered >316 μg/g.
[c] Resistance ratio of an insect population was calculated by dividing the LC_{50} value of the population by that of the Bt-SS strain.

to be 0.0023 with a 95% CI of 0.0003–0.0064 (Table 4). The F_2 screen had a detection power of 97.0% for identifying a resistance allele if one existed in a family.

3.6. Minor resistance allele frequency

Besides GA-15, results of the F_2 screen also showed that six other F_2 families had some survival on Cry2Ab2 maize leaf tissue and had at least one large survivor ($\geqslant$3rd instars). These six families were not confirmed to possess major resistance alleles because they could not develop to the adult stage in the laboratory or the progeny of the families could not survive on whole plants of the Cry2Ab2 maize in the greenhouse. Based on the criteria described above, these six families were considered to possess minor resistance alleles to the Cry2Ab2 maize. The six families were TX-29 from the Texas population, FL-MD-8 from the Florida-Miami/Dade population, and FL-CL-7, FL-CL-10, FL-CL-13, and FL-CL-14 from the Florida-Collier population. The estimated minor resistance allele frequency with 95% possibility was <0.0073 for the Louisiana populations and <0.0408 for the Georgia population (Table 4). It was 0.015 with a 95% CI of 0.0019–0.042 for the Texas population, 0.0232 with a 95% CI of 0.0086–0.0449 for the Florida populations, and 0.0082 with a 95% CI of 0.0033–0.0152 for the combined populations across the four states.

3.7. Susceptibility of TX-SS and GA-15 to Cry2Ab2 protein

The estimated LC_{50} value for TX-SS was 20.8 μg/g with a 95% CI of 12.9–51.6 μg/g (Table 5). The LC_{50} for GA-15 could not be calculated with the probit analysis because of the low mortality (42.2%) at the highest concentration of 316 μg/g in the bioassay. Therefore, the LC_{50} value for GA-15 was considered >316 μg/g. Using the LC_{50} value of TX-SS as a standard, the resistance ratio to Cry2Ab2 protein in GA-15 was >15-fold. The results of the bioassays further confirmed that the survival of GA-15 on leaf tissue and whole plants of the Cry2Ab2 maize was due to a resistance to the Cry2Ab2 protein in the plants.

4. Discussion

The F_2 screen in the current study identified that at least one (GA-15) out of the 215 two-parent families of *S. frugiperda* from populations collected in the southern U.S. possessed a major resistance allele to the Cry2Ab2 protein. It should be noted that the Cry2Ab2 resistance allele frequency calculated in this study for these populations is a conservative estimation. Due to the difficulty in establishing colonies from the very limited number of survivors after F_2 screen, resistance confirmation was not performed for all PPFs. Although we used the well-established procedures (Huang et al., 2014, 2016), the result of the current study still could not completely exclude the possibility that some PPFs (e.g. FL-MD-8; FL-CL-7, and FL-CL-13) might actually possess major alleles. The current study, as other published studies, did not provide any additional information to confirm the 'minor' resistance alleles. In addition, in the greenhouse confirmation tests, survival on non-Bt plants was evaluated only for the susceptible strain, but not for PPFs. However, based on our previous studies (Yang et al., 2013; Huang et al., 2014, 2016), the possibility that the observed mortality of the tested PPFs on Cry2Ab2 maize was not caused by the insecticidal Bt plants was extremely low. Our on-going studies also showed that a Cry2Ab2-resistant strain that was developed from GA-15 feeding on non-Bt maize leaves survived well, and developed to pupae and produced offspring normally (B.A. and F. H., unpublished data).

Several studies have shown that most Bt maize products do not produce a high dose against *S. frugiperda*, and even susceptible larvae could survival on some Bt maize plants (Niu et al., 2014; Yang et al., 2013). For this reason, in this study, the survivors of each *S. frugiperda* family after the F_2 screen were separated into two groups based on their developmental stages and the families that had only small survivors ($\leqslant$2nd instars) were not considered to carry resistance alleles. As mentioned above, the same method has been used in several previous studies (Niu et al., 2013; Yang et al., 2013; Huang et al., 2014, 2016). The greenhouse whole plant test for the family FL-CL-21 in the current study showed little plant injury and a 100% mortality on the Bt plants, also suggesting the chance that possesses resistance alleles to the Bt plants was very low.

Prior to the current study, there had been no information available about the frequency of Cry2Ab2 resistance alleles in *S. frugiperda*. In Australia, Cry2Ab2 resistance allele frequency has been investigated for field populations of two major target species of Bt cotton, *Helicoverpa armigera* (Hübner) and *H. punctigera* (Wallengren) (Mahon et al., 2007; Downes et al., 2009). The resistance allele frequency to Cry2Ab2 for the populations collected from multiple locations in 2002–2006 in Australia was reported to be 0.0033 with a 95% CI of 0.0017–0.0055 for *H. armigera,* and 0.0018 with a 95% CI of 0.0005–0.0040 for *H. punctigera*. Australia first commercially introduced pyramided Bt cotton expressing the Cry1Ac and Cry2Ab2 proteins in the 2004/2005 season. Cry2Ab2 resistance alleles were detected in both *Helicoverpa* species in the field before the introduction of Bt cotton containing this protein. The Cry2Ab2 resistance allele frequency in *S. frugiperda* estimated in the current study for the southern U.S. falls within the range estimated for *H. armigera* and *H. punctigera* in Australia. In addition, a major resistance allele to Cry2Ab2 maize was detected in a field population of the sugarcane borer, *Diatraea saccharalis* (F.), in Louisiana, USA (Huang et al., 2015). In both *H. armigera* and *H. punctigera,* a binding site alteration is responsible for the Cry2Ab2 resistance (Caccia et al., 2010), while the resistance mechanism in *D. saccharalis* and *S. frugiperda* is still unknown. The availability of the Cry2Ab2-resistant strain of *S. frugiperda* established in the current study warrants further studies to characterize the mechanisms of the resistance in the pest. The results of the current F_2 screen also suggest that the major Cry2Ab2 resistance allele frequency in *S. frugiperda* is apparently still relatively low in the southern U.S.

Besides the family that possessed a major resistance allele, six other families were considered to possess 'minor' resistance alleles

to the Cry2Ab2 protein. The detection of the major resistance allele coupled with the relatively more common 'minor' resistance alleles in the field populations of *S. frugiperda* may have important implications for resistance management. Single gene Cry2Ab2 maize is not commercially available for controlling insect pests, but Cry2Ab2 is one of the two Bt proteins in MON 89034 that has been incorporated into many pyramided Bt maize products (Ghimire et al., 2011). The other Bt protein in MON 89034 is Cry1A.105 that is also active against *S. frugiperda* (Huang et al., 2016). Cry2Ab2 is dissimilar in protein structure from Cry1 proteins (e.g. Cry1A and Cry1F) and has different binding sites in the midguts of target insects, indicating that Cry2Ab2 represents a distinct mode of action from Cry1A and Cry1F proteins (Storer et al., 2012). Several studies have shown that a Cry1 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), suggesting that pyramiding Cry2Ab2 and Cry1A.105 (e.g. MON 89034) should be a good strategy for resistance management (Wu et al., 2009; Ghimire et al., 2011; Wangila et al., 2012).

However, as mentioned above, field resistance of *S. frugiperda* to Cry1F maize has occurred in some regions of the world (Storer et al., 2010; Farias et al., 2014; Huang et al., 2014). Studies have demonstrated that Cry1A.105 and Cry1F are cross-resistant to each other in *S. frugiperda* (Huang et al., 2014, 2016; Bernardi et al., 2015b). The cross-resistance between Cry1F and Cry1A.105 could significantly reduce the activity of the Cry1A.105 protein in MON 89034, leaving the Cry2Ab2 protein only partially protected against Cry1F-resistant *S. frugiperda.* Thus, Cry1F-resistant individuals of *S. frugiperda* that possess resistance alleles (major or minor) to other Bt proteins could have an advantage in survival on pyramided Bt plants. Recently, a resistant strain of *S. frugiperda* to MON 89034 was selected from a Cry1F-resistant population in 10 generations in the laboratory (Santos-Amaya et al., 2015). More knowledge is needed to develop effective strategies to manage resistance evolution to pyramided Bt crops, especially when resistance/cross-resistance to one Bt protein has already occurred.

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