A Beet Armyworm (Lepidoptera: Noctuidae) Ryanodine Receptor Mutation Associated with Diamide Resistance Is Present in a Chlorantraniliprole-Resistant Population from South Georgia, USA[1]

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Abstract A population of beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), larvae was collected from cowpeas (*Vigna unguiculata* [L.] Walpers) from Scooterville, GA (Tift Co.) in the summer of 2022, following a report of control failure after chlorantraniliprole application. The collected larvae were transported to Tifton, GA for study at the Coastal Plains Experiment Station. The 72-h maximum dose leaf-dip bioassays of the field-population determined percent mortality to be 26, 49, and 87% for the insecticide active ingredients chlorantraniliprole, cyantraniliprole, and spinetoram, respectively. In comparison, the percent mortality of a susceptible lab strain exposed to the same insecticides was 76, 88, and 98%, respectively. Previous studies of a ryanodine receptor target site mutation, I4743M, identified in Chinese populations of beet armyworm, demonstrated an association with resistance to diamide insecticides. All screened samples from the diamide-resistant field population were shown to possess this mutation, while samples from the diamide-susceptible lab colony were shown to be wild-type. A second mutation, G4900E, associated with diamide resistance in other lepidopteran species, was not identified in any of the sequenced samples from either population. This is the second report of diamide insecticide resistance in beet armyworm populations from the southeastern United States, and the first report of the I4743M target site mutation in Georgia.

Key Words Beet armyworm, chlorantraniliprole, cyantraniliprole, I4743M, G4900E

The beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), is a global, polyphagous pest of various vegetables, row crops, and ornamentals (Burris et al. 1994, Capinera 2020, Greenberg et al. 2001, Rabelo et al. 2020, Richardson et al. 2020, Taylor and Riley 2008, Yoshida and Parrella 1987). Beet armyworm larvae are particularly voracious, and may cause damage via defoliation or by feeding on fruit (Capinera 2020). Insecticide applications are a major component of beet armyworm control and are often dependent on action thresholds, however, calendar sprays are also common. Unfortunately, the polyphagous nature of this

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pest can complicate control efforts, particularly for action thresholds as they may vary depending on the host plant (Cartwright et al. 1987, Taylor and Riley 2008). This variability due to different host plant sensitivities to beet armyworm infestations highlights an important challenge faced in beet armyworm control, which is only further complicated when facing insecticide resistance.

Pyrethroids (IRAC Class 3A), carbamates (IRAC Class 1A), organophosphates (IRAC Class 1B), and spinosyns (IRAC Class 5) are some of the more commonly applied insecticides for beet armyworm control (Ahmad and Arif 2010; Che et al. 2013; Rabelo et al. 2020; Richardson et al. 2020; Saeed et al. 2017; Taylor and Riley 2007, 2008). However, insecticide resistance to active ingredients in all of these classes has been well documented worldwide (Ahmad and Arif 2010, Che et al. 2013, Kerns et al. 1998, Moulton et al. 2000, Rabelo et al. 2020, Richardson et al. 2020, Wang et al. 2021b). Another class of insecticides, referred to as diamides (IRAC Class 28), has been used extensively against lepidopteran pests (Rabelo et al. 2020, Richardson et al. 2020, Riley et al. 2020). This class includes several active ingredients, such as the anthranilic diamides chlorantraniliprole, cyantraniliprole, and cyclaniliprole, which all target the ryanodine receptor (RyR). The RyR is an ion channel that regulates Ca^{2+} ion flow from the sarcoplasmic reticulum to the muscle cells (Cordova et al. 2006; Ebbinghaus-Kintscher et al. 2006; Lahm et al. 2005, 2009). Once these insecticides have bound to the RyR, the channel becomes stabilized in an open conformation, resulting in the loss of calcium homeostasis that eventually leads to paralysis and death (Cordova et al. 2006; Ebbinghaus-Kintscher et al. 2006; Lahm et al. 2005, 2009; Teixeira and Andaloro 2013). Due to favorable properties such as low mammalian toxicity, low phytotoxicity, and a long residual period, these insecticides have been used extensively for lepidopteran control in multiple cropping systems worldwide.

While initially effective, high levels of diamide resistance have been consistently reported for multiple lepidopteran pest species in recent years (Richardson et al. 2020), including the diamondback moth, *Plutella xylostella* (L.) (Riley et al. 2020, Wang et al. 2021a), the fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Boaventura et al. 2020, Gutiérrez-Moreno et al. 2019), and the tomato leafminer, *Tuta absoluta* (Meyrick) (Roditakis et al. 2017). Following these reports, target-site mutations of the RyR which are associated with resistance to diamides were discovered in several of these pest species. In *P. xylostella*, where the most intensive studies have occurred, three RyR mutations are of particular significance: G4946E (Troczka et al. 2012, 2015), I4790M (Guo et al. 2014), and I4790K (Jouraku et al. 2020). Following the identification of these mutations in *P. xylostella*, essentially identical mutations were also identified in the RyR of other diamide-resistant lepidopteran species, including *T. absoluta*, (G4903E/V and I4790M/T) (Roditakis et al. 2017), the Asiatic rice borer, *Chilo suppressalis* (Walker) (G4915E, I4758M, and Y4667C) (Huang et al. 2020), the rice leaffolder, *Cnaphalocrocis medinalis* (Guenee) (I4712M) (Sun et al. 2023), *S. frugiperda*, (I4743M/K) (Boaventura et al. 2020, Okuma et al. 2022), and the southern armyworm, *Spodoptera litura* (F.) (I4728M) (Mei et al. 2023).

In the case of the beet armyworm, reports of diamide resistance occurred in populations from China as early as 2008, where 18 different populations were shown to range from 2.8 to 17.1-fold resistant to chlorantraniliprole in comparison to a susceptible lab colony (Lai et al. 2011). Continued sampling from China in 2018 revealed beet armyworm populations with resistance ratios ranging from 220.58 to 2,597.39-fold resistant to chlorantraniliprole, which suggested that resistance had increased significantly (Wang et al. 2021b). Investigation into the molecular mechanisms of resistance discovered a target site mutation of the beet armyworm RyR, I4743M, in a diamide-resistant population from the Shandong province in China (Zuo et al. 2019). Huang et al. (2021) later demonstrated varying levels of resistance to chlorantraniliprole and tetraniliprole in beet armyworm populations across 8 field sites in China, in which the two populations that exhibited the highest levels of diamide resistance were shown to possess the I4743M mutation. In contrast to other lepidopterans, a mutation at the G4900 site (corresponding to G4946E in *P. xylostella*) has not been reported in diamide-resistant beet armyworm field populations. However, a CRISPR/Cas9 mediated knock-in of the G4900E mutation resulted in a $>$1,000-fold, 223-fold, and 336-fold increase in resistance to flubendiamide, chlorantraniliprole, and cyantraniliprole, respectively (Zuo et al. 2017). Further study indicated little fitness cost, suggesting the G4900E may be a potential RyR target site mutation to monitor in diamide-resistant populations (Zuo et al. 2017).

Recently, a chlorantraniliprole-resistant beet armyworm population was reported in the United States, where a population from the Florida panhandle was 629-fold resistant in comparison to a susceptible strain (Rabelo et al. 2022). While this was the first report of chlorantraniliprole-resistant beet armyworm in the southeastern United States, the mechanism of resistance was not identified. Han et al. (2023) reported the presence of the I4743M mutation in beet armyworm museum specimens collected from the United States dating back to the 1960s, however, no toxicological data could be collected for those samples. In the summer of 2022, we were alerted to a beet armyworm population from South Georgia that did not respond to control efforts using chlorantraniliprole. The objectives of this study were to toxicologically characterize a beet armyworm population after control failure following the application of chlorantraniliprole. Additionally, we aimed to determine the presence or absence of two potential RyR mutations in this field population while comparing to a susceptible lab strain.

Materials and Methods

Insects. *Spodoptera exigua* larvae were collected from a cowpea (*Vigna unguiculata* [L.] Walpers) field located in Scooterville, Tift Co., Georgia (31.382358, −83.707661) in August of 2022, following a report of control failure after chlorantraniliprole application (SCV population). Collected samples were transported to the Coastal Plains Experiment Station for further toxicological characterization via leaf-dip bioassays. Individuals from field collections were also frozen for future molecular analysis. For comparison, a commercially available susceptible strain (Frontier Agricultural Sciences, Newark, DE) was used as a control (FRT colony).

Toxicological bioassays. In order to assess the level of resistance to chlorantraniliprole, cyantraniliprole, and spinetoram, maximum dose bioassays were conducted using third-instar larvae from the FRT colony and SCV population. Leaf-dip bioassays were used to expose beet armyworm larvae to the maximum dose of chlorantraniliprole (Coragen® 1.67SC, FMC Corporation), cyantraniliprole (Exirel® 0.83SC, FMC Corporation), or spinetoram (Radiant® 1SC, Corteva AgriScience) according to their specific labels (Table 1). Larvae from the SCV

Table 1. The doses of each insecticide used in this bioassay, as well as their Insecticide Resistance Action Committee (IRAC) group number and molecular target site.

Active Ingredient (commercial product, company)	IRAC no.	ml_product/L[a]	Target Site
Chlorantraniliprole (Coragen 1.67SC, FMC Corporation)	28	0.59 ml/L	Ryanodine receptor
Cyantraniliprole (Exirel 0.83SC, FMC Corporation)	28	1.6 ml/L	Ryanodine receptor
Spinetoram (Radiant 1SC, Corteva AgriScience)	5	0.62 ml/L	Nicotinic acetylcholine receptor

Each dose was calculated using the maximum labeled rate for beet armyworm in cowpeas. Since beet armyworm was not on the cyantraniliprole label for control in cowpeas, the maximum rate for cowpeas was used in its place.

[a] Milliliter of product per Liter. Dose that is equivalent to the maximum labeled rate.

population and FRT colony were moved to untreated cowpea (*V. unguiculata*) leaves upon their arrival. This was done for the FRT colony to acclimate the larvae to the bioassay substrate, preventing mortality that may have been caused by switching directly from diet. For the SCV population, this was done to ensure larvae from that population would also consume our untreated *V. unguiculata* leaves. Both populations were acclimatized to the ambient laboratory temperature, which ranged from 21–24°C, and untreated, full-grown *V. unguiculata* leaves for at least 24 h before the preparation of the experiment. We acknowledge that we did not establish an SCV colony, but that the SCV population was assayed during the same generation as the field collection post-acclimatization. Third-instar larvae were selected for bioassay from the SCV population and FRT colony subsequent to acclimatization. Third-instar larvae were differentiated from other instars not only by size, but by other accompanying features described in Capinera (2020).

Bioassays were prepared in Petri dishes (90 mm) with 38 mm diameter holes cut into the lids for ventilation. The hole was covered with hot-glued nylon chiffon to ensure the larvae could not escape. Circular Whatman filter papers (90 mm) were placed into the bottom of the Petri dish and water was applied until damp to prevent leaf desiccation. Three top leaves from full-grown, untreated cowpea plants were dipped into a 0.25-L aqueous solution consisting of chlorantraniliprole, cyantraniliprole, or spinetoram at the maximum labeled dose, with 0.25 ml of Kinetic adjuvant (Helena Agri-Enterprises, Collierville, TN) to ensure even spreading and adherence. The treated leaf discs were placed into the Petri dish on top of the damp filter paper and allowed to air dry for 30 m. Bioassays were completed on benchtops in the same laboratory as the acclimatization and laboratory conditions were consistent with those in which the larvae were initially acclimatized. Once dried, 10 third-instar beet armyworm larvae were placed on the leaves, and the top of the Petri dish was secured with rubber bands to prevent escape. A minimum of 3 replicates were established for each treatment, and each replicate was checked every 24 h for live, down,

Table 2. The sequences of the primers used and how they were used in the study.

Purpose	Primer Name	Primer Sequence
Amplification	BAW_RyR_F1	5′-CCAGCTCCATCAGCGTTATC-3′
Amplification	BAW_RyR_RV1	5′-CCAGCAAGATGACGATGATG-3′
Sequencing	BAW_RyR_F2	5′-GTTGGACGCGGAAGGTGG-3′
Sequencing	BAW_RyR_RV2	5′-CCTTTGTATAGGTTGAATACGAAGCA-3′

Primers spanning both beet armyworm ryanodine receptor mutation sites were designed using Primer3Plus.

pupated, and dead larvae until 72 h had passed. The response of each larva was recorded using a straight tip teasing probe (Bioquip Products, Rancho Dominguez, CA). Larvae that reacted rapidly to stimuli were categorized as live, larvae that reacted lethargically were categorized as down, and larvae with no response were categorized as dead. Mortality under each treatment was analyzed via Proc-GLM analysis in SAS Enterprise Guide (SAS Institute, Raleigh, NC) with mean separation using Tukey HSD tests ($P < 0.05$). The percent mortality was calculated by dividing the total number of dead larvae by the total number of larvae within each replicate, and the mean percent mortality was analyzed alongside the other responses for each treatment. Percent mortality of both populations in response to insecticide exposure were graphed using Sigma Plot 15.0 (Systat Software, Inc., San Jose, CA).

Genetic analysis. Each population was analyzed for the presence or absence of two potential diamide-related RyR target site mutations, G4900E and I4743M. At the time of the SCV collection, late third-instar beet armyworm larvae were preserved in 1.0 ml of RNAlater (Thermo Fisher Scientific, Waltham, MA) and stored at −40°C. mRNA was extracted from 10 pooled individuals per replicate using a Dynabeads mRNA DIRECT Kit (Invitrogen, Thermo Fisher Scientific), while cDNA was synthesized with SuperScript IV VILO MasterMix (Invitrogen) following the manufacturer's protocol. Three independent cDNA replicates were synthesized for each population, and cDNA samples were stored at −40°C until needed for polymerase chain reactions (PCR).

A BIO-RAD C1000 Touch (Life Science, Hercules, CA) thermal cycler was used to amplify cDNA samples. Primers used to generate an amplicon spanning both potential mutation sites of the beet armyworm RyR were designed using Primer3Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The first pair of primers (BAW_RyR_F1 × BAW_RyR_RV1) were used to generate an amplicon, while the second pair of primers (BAW_RyR_F2 × BAW_RyR_RV2) were used for sequencing reactions (Table 2). PCR samples were amplified using high-fidelity Phusion Taq polymerase (Promega, Madison, WI) and 5X HF Reaction Buffer (Promega) while following the manufacturer's protocol. Parameters for PCR reactions were: melting temperature 95°C for 10 s, annealing temperature 59°C for 15 s, extension at 72°C for 90 s (final extension at 72°C for 120 s), and a product size of 1,162 bp. The PCR products were analyzed in a 1.5% agarose gel to ensure a single product of the expected size had amplified. Purification of PCR products was accomplished via Monarch PCR and DNA Cleanup kit (Promega). The purified PCR products were sent to Eurofins Genomics in Louisville, Kentucky for Sanger sequencing. Sequencing chromatograms generated using the sequencing primer BAW_RyR_F2 were inspected for evidence of the RyR mutations within each sequenced sample.

Results

Toxicological bioassays. Maximum dose bioassays indicated resistance to both chlorantraniliprole and cyantraniliprole in the SCV population. The mean separation analysis of the FRT larvae categorized as dead indicated a significant difference between the untreated control and the 3 insecticidal treatments (Table 3). However, the analysis of maximum dose bioassay data for the SCV larvae categorized as dead only showed a significant difference in mortality between spinetoram and the untreated control, whereas cyantraniliprole was marginally different and chlorantraniliprole did not differ statistically from the control (Table 4). Recorded percent mortality in the SCV bioassays were 14, 26, 49, and 87% for the untreated control, chlorantraniliprole, cyantraniliprole, and spinetoram, respectively (Fig. 1). Recorded percent mortality in the FRT bioassays were 9, 76, 88, and 98% for the untreated control, chlorantraniliprole, cyantraniliprole, and spinetoram, respectively, suggesting significantly higher susceptibility to diamide insecticides in that population. Additionally, statistical differences for both populations were consistent for the total live, dead, and percent mortality categories (Tables 3 and 4). Significant bioassay effects were seen for the SCV population with larvae categorized as dead ($F = 5.49$; df = 8, 14; $P = 0.0262$), live ($F = 7.41$; df = 8, 14; $P = 0.012$), and percent mortality ($F = 7.73$; df = 8, 14; $P = 0.011$). Significant bioassay effects were seen for the FRT population with larvae categorized as dead ($F = 7.42$; df = 13, 28; $P = 0.0002$), live ($F = 9.94$; df = 15, 28; $P < 0.0001$), and percent mortality ($F = 11.75$; df = 13, 28; $P < 0.0001$).

Genetic analysis. Sequencing chromatograms were used to screen the populations for 2 potential target site mutations, I4743M and G4900E, of the beet armyworm RyR. Of the 2 mutations, only the I4743M mutation was identified in these samples. Generally, superimposed peaks in the chromatograms indicate a mixture of susceptible and resistant alleles in a sample [see Dunn et al. (2022) for examples]. The FRT chromatograms only depicted the susceptible ATA codon at the 4743 position for each sequenced sample (Fig. 2A). The opposite was seen with the SCV population, only depicting the resistant ATG codon at this position in each sequenced sample, with no evidence for the susceptible wild-type codon (Fig. 2A). The other potential target site mutation, G4900E, was not identified in any of the sequenced samples from either population. Chromatograms for each sample only depicted the susceptible wild-type GGT codon, regardless of the population (Fig. 2B).

Discussion

In the current study, we collected a beet armyworm population from cowpeas in South Georgia subsequent to control failure with chlorantraniliprole. We determined that this beet armyworm population was highly resistant (defined as $< 40\%$ mortality) (Riley et al. 2020) to the maximum labeled dose of chlorantraniliprole, while only being intermediately resistant (40–80% mortality) to cyantraniliprole. This same population was highly susceptible ($> 80\%$ mortality) to an unrelated active ingredient,

Table 3. Analysis of variance mean separation results from the analysis of FRT maximum dose bioassay based on 10 beet armyworm larvae per arena.

Population	Insecticide	Dead	Alive	Down	Pupae	Replicates	Percent Mortality
FRT	Untreated Control	1.091b	8.909a	0.500a	0.00a	11	8.34c
FRT	Chlorantraniliprole	7.167a	2.167b	0.500a	0.00a	6	76.1b
FRT	Cyantranilirpole	8.833a	1.167b	0.091a	0.00a	6	88.3ab
FRT	Spinetoram	9.333a	0.167b	0.00a	0.00a	6	98.3a

Averages of recorded response to insecticidal treatment during the 72-h leaf dip bioassay for the FRT population. Statistically significant differences for the mean values of each response category are established using different letters (a, b, c, etc.). Means within columns followed by different letters were significantly different, Tukey tests ($P < 0.05$). Statistical values for each category were: dead ($F = 7.42$; df = 13, 28; $P = 0.0002$), live ($F = 9.94$; df = 15, 28; $P < 0.0001$), down ($F = 0.36$; df = 13, 28; $P = 0.963$), and percent mortality ($F = 11.75$; df = 13, 28; $P < 0.0001$).

Table 4. Analysis of variance mean separation results from the analysis of SCV maximum dose bioassay based on 10 beet armyworm larvae per arena.

Population	Insecticide	Dead	Alive	Down	Pupae	Replicates	Percent Mortality
SCV	Untreated Control	1.333b	7.667b	0.333a	0.00a	6	14.1b
SCV	Chlorantraniliprole	2.667b	6.667b	0.667a	0.00a	3	25.9b
SCV	Cyantranilirpole	4.333ab	2.667a	1.667a	0.00a	3	48.3b
SCV	Spinetoram	8.667a	1.000a	0.333a	0.00a	3	86.7a

Averages of recorded response to insecticidal treatment during the 72-h leaf dip bioassay for the SCV population. Statistically significant differences for the mean values of each response category are established using different letters (a, b, c, etc.). Means within columns followed by different letters were significantly different, Tukey tests ($P < 0.05$). Statistical values for each category were: dead ($F = 5.49$; df = 8, 14; $P = 0.0262$), live ($F = 7.41$; df = 8, 14; $P = 0.012$), down ($F = 0.58$; df = 8, 14; $P = 0.769$), and percent mortality ($F = 7.73$; df = 8, 14; $P = 0.011$).

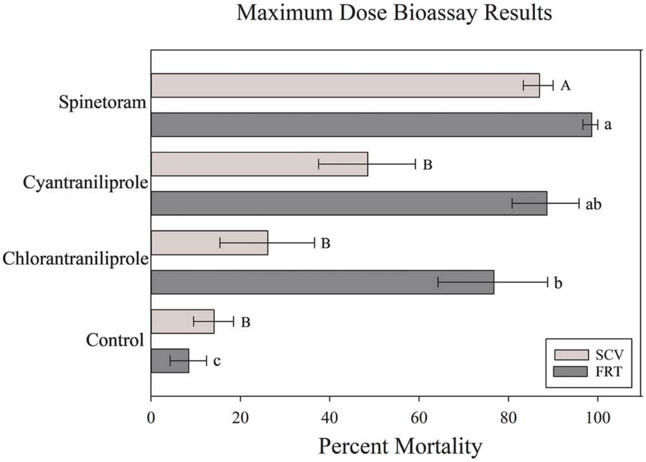

Fig. 1. The figure depicts maximum dose responses to insecticides from the FRT and SCV populations. The responses for each population are based on the doses shown in Table 1. Statistically significant differences for the mean FRT percent mortality are established using different lowercased letters (a, b, c, etc.) ($F = 11.75$; df = 13, 28; $P < 0.0001$), while uppercased letters are used for SCV (A, B, C, etc.) ($F = 7.73$; df = 8, 14; $P = 0.011$). Error bars are representative of 1 standard error. Statistical differences were determined via Tukey tests ($P < 0.05$).

spinetoram. Chromatograms generated from Sanger sequencing PCR amplicons revealed the susceptible colony only possessed the wild-type (ATA) codon at the location corresponding to I4743. In marked contrast, we saw only sequence indicative of an ATG codon (M4743) in each of our samples from the field population. While CRISPR/Cas9 mediated knock-in of a G4900E mutation has been shown to significantly increase diamide resistance with little evidence of any fitness costs (Zuo et al. 2017), we found no evidence for this mutation in either population.

Diamide insecticides have been registered for use since 2006, starting with the registration of the phthalic acid diamide, flubendiamide (Lahm et al. 2009). This was soon followed by the registration of the anthranilic diamides, chlorantraniliprole in 2007 (Lahm et al. 2009) and cyantraniliprole in 2014. As previously mentioned, the favorable qualities of these insecticides seem to have resulted in over reliance in the field, eventually leading to resistance outbreaks in multiple pest species, such as *P. xylostella* (Dunn et al. 2022, Guo et al. 2014, Riley et al. 2020, Troczka et al. 2012, Wang et al. 2021a), *T. absoluta* (Silva et al. 2016), *S. frugiperda* (Chen et al. 2023), and the neotropical coffee leaf miner, *Leucoptera coffeella* (Guérin-Méneville) (Leite et al. 2020), among others. This pattern has been recapitulated by reports of chlorantraniliprole-resistant beet armyworm in China from 2008 to 2018 (Lai et al. 2011, Wang et al. 2021b). The appearance of similar mutations at two sites (I4790 and G4946) in the RyR of multiple lepidopteran species post-diamide selection suggests their importance to the diamide mechanism of action. Diamide insecticides bind to a pocket in the hinge region of the RyR, a region that is critical for opening and

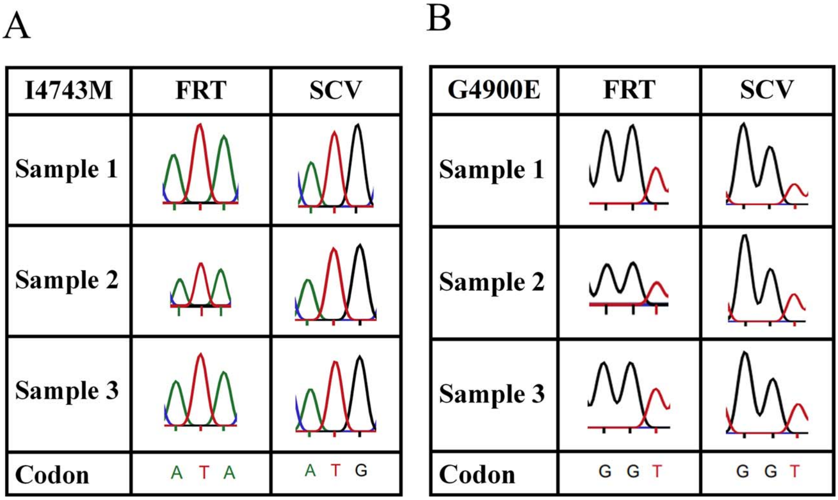

Fig. 2. The figure depicts sequencing chromatograms from Sanger sequencing of 3 individual PCR products from the FRT and SCV populations. (A) The FRT samples depict the wild-type ATA codon, resulting in isoleucine (I) at amino acid position 4,743 of the beet armyworm ryanodine receptor. The SCV samples depict the mutant ATG codon, resulting in a methionine (M) at the same position. (B) All sequenced FRT and SCV samples depict the wild-type GGT codon. This codon results in a glycine (G) at amino acid position 4,900 of the beet armyworm ryanodine receptor.

closing of the channel that regulates Ca^{2+} efflux (Ebbinghaus-Kintscher et al. 2006, Lin et al. 2020). In *P. xylostella*, mutations at G4946E and I4790M/K alter the geometry and hydrophobicity of the binding pocket, thereby reducing the ability of the diamides to induce toxicity (Lin et al. 2020). While the G4946E mutation primarily reduces sensitivity to flubendiamide, chlorantraniliprole, and tetraniliprole (Dunn et al. 2022, Jiang et al. 2021, Steinbach et al. 2015, Troczka et al. 2012), the I4790K mutation confers resistance to cyantraniliprole and cyclaniliprole, as well as to tetraniliprole, chlorantraniliprole, and flubendiamide (Jiang et al. 2021, Jouraku et al. 2020).

While we have no information regarding the possible history of exposure of the studied population to these insecticides, the observed resistance to cyantraniliprole alongside chlorantraniliprole suggests that cross-resistance may be a factor. Although published data on cross-resistance in beet armyworm is limited, Bolzan et al. (2019) characterized chlorantraniliprole resistance in a close relative of beet armyworm, *S. frugiperda.* The authors found that chlorantraniliprole resistance in *S. frugiperda* behaves as a monogenic trait, and is associated with an intermediate level of cross-resistance to cyantraniliprole (Bolzan et al. 2019). Additionally, high levels of resistance to multiple diamides, including chlorantraniliprole, cyantraniliprole, cyclaniliprole, and flubendiamide, have been reported in beet armyworm populations from South Korea (Cho et al. 2018). Our results, alongside these other studies, suggest that the I4743M mutation may contribute to cross-resistance among diamide active ingredients and should be considered in future management plans.

Recently, Han et al. (2023) questioned the contribution of the I4743M mutation to diamide resistance in beet armyworm populations. In this study, the authors identified the I4743M mutation in samples collected prior to diamide registration, as well as in beet armyworm populations with varying levels of diamide resistance. While it is interesting that this mutation was identified in some samples collected prior to diamide registration, the occurrence of this mutation at high frequencies in multiple Asian and North American diamide-resistant lepidopteran species suggests that the I4743M mutation is an important factor contributing to diamide resistance in these pest populations (Boaventura et al. 2020; Guo et al. 2014; Huang et al. 2020, 2021; Jiang et al. 2021; Jouraku et al. 2020; Mei et al. 2023; Okuma et al. 2022; Roditakis et al. 2017; Sun et al. 2023; Zuo et al. 2019). However, it must be recognized that the overall contribution of this mutation to diamide resistance is still unclear in the beet armyworm, especially in populations from the southeastern United States, as other mechanisms may also be contributing to the observed resistance in this studied population. In *P. xylostella*, where target-site mutations are well documented as resistance factors (Dunn et al. 2022, Guo et al. 2014, Jiang et al. 2021, Jouraku et al. 2020, Steinbach et al. 2015, Troczka et al. 2012), metabolic resistance has also been shown to contribute to chlorantraniliprole resistance (Gao et al. 2018, Li et al. 2018). Wang et al. (2018) demonstrated that field populations of beet armyworm from China, which were highly resistant to chlorantraniliprole, significantly increased the expression of several genes upon exposure to sublethal doses. RNAi knockdown of one gene in particular, *CYP9A21v3,* significantly increased susceptibility to chlorantraniliprole in resistant larvae exposed to a dose tolerated by larvae treated with an irrelevant dsRNA. We did not study *CYP9A21v3* or other metabolic resistance factors in our population, and we do not exclude them as a potential additional resistance factor in this population.

A primary concern is the stability of the resistance associated with this mutation. If the observed resistance is stable, it may reduce the efficacy of diamides in beet armyworm populations in this region for prolonged periods. Fortunately, Rabelo et al. (2022) reported that chlorantraniliprole resistance in a beet armyworm population collected from Florida was unstable, declining 160-fold when comparing the second generation to the 27th generation. Liu et al. (2021) selected on a chlorantraniliprole-resistant strain of beet armyworm from a field population in the Sichuan Province of China, and demonstrated several fitness costs for that population upon repetitive selection with chlorantraniliprole. These included increased development time and reduced fecundity in comparison to a susceptible strain that was derived from the same field population and maintained without selection for six generations. These observed reductions in fitness could reasonably result in the loss of resistance in the absence of insecticide selection pressure (Liu et al. 2021). For both Liu et al. (2021) and Rabelo et al. (2022), it was not determined if the I4743M mutation was present in either population. Still, the observed instability may imply an associated fitness cost in the absence of diamide selection pressure and may be relevant for future studies.

In contrast to beet armyworm, Padovez et al. (2022a) generated near-isogenic chlorantraniliprole-resistant (Iso-RR) and susceptible (SS) lines of *S. frugiperda* (Iso-RR 96.8-fold resistant compared to SS). These lines had nearly identical genetic backgrounds, except for a monogenic recessive resistance factor, and did not differ when several fitness parameters (egg and pupae survival, number of eggs laid per female, etc.) were compared. However, an unrelated, field-derived resistant strain (RR) (223.7-fold resistant) did show significant fitness costs, suggesting that the genetic background of the strains being compared is important. It is possible that the resistance associated with a single genetic factor may not have any associated fitness costs, but strains with different genetic backgrounds may have marked fitness differences. Further study of the closely related Iso strains also demonstrated that potential fitness costs of chlorantraniliprole resistance may be influenced by different host plants, establishing that the fitness of chlorantraniliprole-resistant *S. frugiperda* could be extremely complex (Padovez et al. 2022b). Importantly, the I4743M/K resistance alleles in chlorantraniliprole-resistant field populations of *S. frugiperda* have been shown to be stable, suggesting that it is possible to establish stable, chlorantraniliprole-resistant populations outside of laboratory settings (Okuma et al. 2022). As *S. frugiperda* is closely related to beet armyworm, these studies suggest future directions for examining the significance of the I4743M mutation in relation to diamide resistance in beet armyworm populations. This topic should be investigated further in beet armyworm from the southeastern United States, due to implications for diamide resistance management programs.

In summary, we have identified a population of beet armyworm that are highly resistant to chlorantraniliprole in the southeastern USA. This same population also exhibited intermediate levels of resistance to cyantraniliprole. This is the second report of a diamide-resistant population in the southeastern United States, and may indicate an emerging resistance problem. The population carries a target-site mutation, I4743M, in the ryanodine receptor, associated with resistance to chlorantraniliprole and cross-resistance to cyantraniliprole in other lepidopteran pest species. The presence of this mutation has implications for insecticide resistance management, which will require further study and monitoring.

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