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Geographical Distribution, Allele Frequency, and Inheritance of CRY1A.105 and CRY2AB2 Resistance in Helicoverpa zea in the Southeastern Region of the United States

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GEOGRAPHICAL DISTRIBUTION, ALLELE FREQUENCY, AND INHERITANCE OF CRY1A.105 AND CRY2AB2 RESISTANCE IN *HELICOVERPA ZEA* IN THE SOUTHEASTERN REGION OF THE UNITED STATES

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ABSTRACT

The corn earworm, *Helicoverpa zea* (Boddie), is a target pest of *Bacillus thuringiensis* (Bt) maize and cotton in the U.S. Previous studies have reported that this pest has developed field resistance to pyramided Cry1A/Cry2A maize and cotton in certain areas of the southern United States. The objectives of this study were to 1) determine the current status and distribution of the resistance to Cry1A.105 and Cry2Ab2 in *H. zea* in the southeastern United States; 2) estimate the resistance allele frequencies to the two Bt proteins in *H. zea* field populations in the region, and 3) assess the inheritance of the Cry1A.105 and Cry2Ab2 resistance in the insect. Diet over-lay bioassays with 31 field populations collected from seven U.S. southeastern states during 2018 and 2019 showed that Cry1A.105 and Cry2Ab2 resistance in *H. zea* is widely distributed in the regions. The resistance to Cry1A.105 appeared to have plateaued, while selection for Cry2Ab2 resistance is likely still occurring. F2 screens with 103 isolines established using mass-mating of *H. zea* populations collected from the region in 2018 and 2019 exhibited that the resistance allele frequencies in *H. zea* populations were high for both Bt proteins, averaging 0.405 to Cry1A.105 and 0.330 to Cry2Ab2. Various genetic crosses and backcrosses revealed that the Cry1A.105 resistance in *H. zea* was inherited as a single, autosomal, and non-recessive gene. Meanwhile, the Cry2Ab2 resistance in the insect was more likely to be autosomal, non-recessive, and polygenic. The information generated from this study is helpful in resistance risk assessment, refining resistance modeling, and improving IRM programs to mitigate the great challenge of the Cry protein resistance in *H. zea* for the sustainable use of the Bt crop technology in the region.

CHAPTER 1. INTRODUCTION

1.1 *Helicoverpa zea*

The corn earworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), is one of the most damaging cross-crop pests of maize, cotton, and many other economic crops in the United States. The properties of polyphagy, high reproduction capacity, and long-distance migration of *H. zea* contribute to its positive adaptation to the modern agriculture systems (Cartwright, 1939; Buntin et al., 2001; 2004a; Reisig and Reay-Jones, 2015; Reay-Jones et al., 2016). In most conditions, *H. zea* larvae feed on the fruit, while the fertilized female moths are attracted to its wild host plants. Localized *H. zea* populations could occur after the emergence of diapausing pupae that survive through the winter. The

migratory *H. zea* populations usually originate from subtropical regions of North America. Both the localized and the migratory populations cause damage to crops across the continent, which falls in line with the availability of host plants and maturity in the plant growing season (Westbrook et al., 2010).

1.2 Damage of *H. zea* on Cotton and Corn

H. zea is polyphagous which gives the insect a broad host range, finishing its life cycle on cultivated and wild hosts (Sharma, 2005). In the southern United States, the feeding history of *H. zea* on maize may date back to 1939 (Cartwright, 1939; Buntin et al., 2001;2004a; Reay-Jones et al., 2009; Onstad et al., 2011; Reay-Jones and Wiatrak, 2011; Reay- Jones and Reisig, 2014). In the United States, evidence has shown that the first two generations of *H. zea* survive on the wild hosts or maizefields after over-wintering (Terry et

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al., 1987a; 1987b; Hardwick, 1965). The oviposition of *H. zea* on maize typically occurs in June-July in the southern United States (Bradley, 1993). Neonates feed on the silk and then extend to the silk channel, ear tip, and finally into the ear kernels (Wiseman et al., 1978). *H. zea* larvae can damage vegetative tissues, as well as ears. The ear kernel damage on timely planted maize has an insignificant impact on the yield in the southern United States (Reay-Jones and Reisig, 2014). When maize season passes and becomes unsuitable for larval feeding, *H. zea* moves to other hosts, notably cotton, grain sorghum, and soybean, for 2-3 additional generations (Head et al., 2010). However, the relationship between the injury (physical removal of plant tissues) of *H. zea* and its impact on field corn damage (yield loss) showed different results in many studies. Some studies indicated that yield damage is correlated to the kernel injury levels (DeLamar et al., 1999a; b; c; d; e; Buntin et al., 2004a), while other studies showed no relative (Buntin et al. 2004a; Buntin 2008; Reay-Jones and Wiatrak 2011; Bowen et al. 2014; Reay-Jones and Reisig 2014; Steckel and Stewart 2015; Bibb et al., 2018). It is worth noting that *H. zea* injury can result in the fungal infestation, which produces mycotoxin contamination of crops (Jones et al. 1981; Dorner et al. 1999; Koenning and Payne 1999).

H. zea is a crucial pest in cotton as its damage causes cotton yield loss. The development of cotton can be separated into five growth stages, including a) germination and emergence; b) seedling establishment; c) leaf area and canopy development; d) flowering and boll development; and e) maturation (Oosterhuis et al., 1990). Yield losses occur during the flowering and boll development stage if *H. zea* feeds on bolls developed for 15 to 18 days after flowering (Gore et al., 2000a). Significant yield loss (28%) occurs when 80% of bolls are injured during the fourth week of flowering (Gore et al., 2000b). If *H. zea* infests cotton before the peak squaring of cotton, yield damage will then increase (Wilson and Gutierrez, 1980). Significant yield losses were observed when *H. zea* injury happened to cotton bolls

during the maturation stage (Dunnam et al., 1943, McKinlay and Geering, 1957; Passlow and Trudgian, 1960; Evenson, 1969; Chilcutt et al., 2003). Since later instar larvae are likely to damage the fruiting structures in a way that the cotton plant cannot compensate, they tend to cause more yield losses (Wilson and Gutierrez, 1980).

1.3 *Bacillus thuringiensis* Bacterium and Bt Toxins

Bacillus thuringiensis (Bt) is a ubiquitous gram-positive, rod-shaped, sporulating, and soil-dwelling bacterium (Madigan and Martinko, 2005; du Rand, 2009). Bt bacterium belongs to the *Bacillus cereus* group, containing seven closely related species*: Bacillus cereus sensu stricto, Bacillus anthracis, Bacillus mycoides, Bacillus pseudomycoides, Bacillus cytotoxicus,* and Bt (Guinebretière et al., 2013). There is no consensus if *B. cereus sensu stricto, B. anthracis*, and *B. thuringiensis* should be considered one species or separate in classification (Vilas-Bôas et al., 2007). It is well-known that Bt proteins are a source of one class of biopesticides recognized as a selective compound (Whalon et al., 2003). The great majority of insecticidal proteins synthesized by Bt can be separated into two classes: δ-endotoxins (crystal and cytolytic toxins) and secreted toxins (Vip and Sip toxins). Cry1 targets Lepidoptera, Cry2 targets Lepidoptera and Diptera, Cry3 targets Coleoptera, and Cry4 targets Diptera (Höfte and Whiteley, 1989). In 1998, a new nomenclature was adopted, which is based on the degree of pairwise amino acid identity to the named proteins: proteins with < 45% pairwise amino acid identity have the different Arabic numbers (e.g., Vip1 and Vip2), ≥ 45 but <78% have a different capital letter (e.g., Vip3A and Vip3C), ≥ 78 but <95% have the different lowercase letter (e.g., Vip3Aa and Vip3Ab), and ≥ 95% have a different Arabic number at the last digit (e.g., Vip3Aa1 and Vip3Aa2) (Crickmore et al., 1998).

Crystal proteins (Cry and Cyt toxins) are synthesized at the onset of sporulation during the stationary growth phase as parasporal crystalline inclusions (Schnepf et al., 1998). The name Cry originates from the protein that forms the parasporal crystal. By 2014, over 700

insecticidal *cry* gene coding crystal (Cry) proteins had been discovered (Crickmore et al., 2018). Among these, 73 different types of Cry proteins have been identified, with some showing toxicity to insects. Some of these Cry toxins belong to the well-known three-domain Cry proteins (display toxic activity against lepidopteran, dipteran, coleopteran, hemipteran, and nematodes), and others belong to non-three-domain Cry toxins (mainly ETX-MTX2 family and Toxin-10 family). The three-domain Cry toxins are composed of a three-domain structure: a perforating domain, a central domain, and a galactose-binding domain. The perforating domain may be responsible for the toxin membrane insertion and pore formation (Schnepf et al., 1998; Ben-Dov, 2014; Xu et al., 2014). The central domain works in the toxin-receptor interactions (Xu et al., 2014; Jenkins and Dean, 2000). The galactose-binding domain involves binding with the receptor and forming the pore (Xu et al., 2014). Cry toxins in the ETX_MTX2 family have an extended beta-sheet structure related to aerolysin, a pore-forming toxin (Gonzalez et al., 2008; Knapp et al., 2010), and form beta-barrel pores in target cells (Popoff, 2011). Some of the Toxin_10 family Cry toxins also showed an aerolysin-like fold (Kelker et al., 2014; Srisucharitpanit et al., 2014).

Secreted toxins are produced by some Bt strains during the vegetative growth phase and are constituted by two classes: vegetative insecticidal proteins (Vip) (Crickmore et al., 2018; Estruch et al., 1996; Warren et al., 1998) and secreted insecticidal protein (Sip) (Donovan et al., 2006). According to the Bt Toxin Nomenclature Committee, four families are associated with the Vip proteins, such as Vip1, Vip2, Vip3, and Vip4 (Donovan et al., 2006). Vip1 and Vip2 have toxicity to coleopteran and *Aphis gossypii* (Hemiptera) (Warren et al., 1998; Sattar and Maiti, 2011); meanwhile, Vip3 is toxic to many lepidopteran species (Estruch et al., 1996). The secreted insecticidal protein (Sip) has insecticidal activity against coleopteran larvae. Up to now, both *cry* and *vip* Bt genes have been bioengineered into plant genomes to express the Cry and Vip proteins to control major agricultural insect pests (DiFonzo, 2021).

1.4 Mode of Action of Bt Toxins

Several models try to explain the mode of action of the three-domain Cry toxins: the classical model, the sequential binding model, the signaling-pathway model (Vachon et al., 2012), and the dual model (Tabashnik et al., 2015). The classical model indicates that lysis would occur on the midgut epithelial cells of susceptible insects with the following procedures (Vachon et al., 2012): (a) The Cry protein enters the digestive system and ingestion in the midgut; (b) the proteases in the midgut convert the protein to smaller active toxic fragments; (c) active toxic protein binds to receptors on the midgut epithelial surface causing the formation of the pores; (d) pores destroy the permeability to ions, amino acids, and other metabolic substances, leading to the lysis of epithelial cells. The sequential binding model (Bravo et al., 2007; 2011; Bravo and Soberón, 2008; Jiménez-Juárez et al., 2008; Pardo-López et al., 2009; Soberón et al., 2007; 2009; 2010) refers to the condition that the activation of protoxin by midgut protease releases toxic proteins that will bind to the cadherin-like proteins on the epithelial cells, causing a pre-pore structure formation. Then, binding to a secondary receptor promotes the extension of the pre-pore through the membrane. The signaling-pathway model (Zhang et al., 2005; 2006) suggests that activation of protoxin attributes to the binding with cadherin receptor, activating the Mg^{2+}-dependent and adenylyl cyclase/protein kinase A signaling pathway that results in cell death. In the dual model (Tabashnik et al., 2015), the primary pathway to kill the insect is just like the classical model; however, both the activitated protein and intact protoxin or part of the protoxin also play a role in the toxicity against the primary-pathway-resistant insects (Tabashnik et al., 2015). The protoxin also binds to the midgut receptor and the brush border membrane vesicles (Fabrick and Tabashnik, 2007; Gómez et al., 2014), leading to the post-binding events in the toxic pathway, like oligomerization and pore formation (Gómez et al., 2014).

1.5 Transgenic Bt Plants

Transgenic Bt plants are crops that produce toxins from the Bt bacterium to control some key pests, thus reducing the usage of insecticides (Shelton et al., 2002; Carrière et al., 2003). Up to now, commercial transgenic Bt crops can be categorized into two generations (Huang, 2015). The first generation of Bt crops produce single-toxin containing Cry1Ab or Cry1F in maize or Cry1Ac in cotton. The second generation Bt crops includes two or more pyramiding Bt genes, such as the combination of Cry1A.105 and Cry2Ab2 in Bt maize that was commercially planted in 2010. During the last 10+ years, at least 18 different combinations of 11 Bt toxins have been adapted for Bt crops against some species in Coleoptera and Lepidoptera. Many of the second generation transgenic Bt crops express several Bt proteins, including Cry and Vip3A (DiFonzo, 2021).

In 1996, transgenic Bt crops expressing a series of *cry* genes were first commercially released in the world crop market, particularly in the United States (de Maagd et al.,1999). By 2017, Bt crops planting area reached one billion hectares across 20 countries (ISAAA, 2018). The primary transgenic Bt crops are maize, cotton, and soybean. In the United States, Bt cotton planting area percentages increased from 14.6% in 1996 to 86% in 2020 (Fernandez-Cornejo and McBride, 2002; NASS, 2019), and the Bt maize area in the same period increased from <5% to 82% (NASS, 2019).

1.6 Bt Resistance

1.6.1. Overall Bt Resistance in Insect

The nature of resistant development can be attributed to the susceptibility change caused by frequent exposure in crops (Tabashnik et al., 2013; 2014). The war against Bt resistance happened before the commercial usage of transgenic Bt plants. Scientists predicted the devolvement of Bt resistance based on three main reasons: (a) insecticidal resistant development history; (b) the existence of laboratory-selected resistance of insects; (c) the

field-evolved Bt resistance in diamondback moth, *Plutella xylostella* (Linnaeus), due to microbial insecticide sprays (Lagunes-Tejeda, 1991; Tabashnik, 1994; Shelton et al., 2002; Ferre and van Rie, 2002; Heckel et al., 2001). Before 2003, there was no documented field resistance to Bt plants that resulted in field control problems. Thus, the focus of Bt resistance research was mainly based on laboratory selection of field populations for the detection of potential resistant pest targets and the frequency of resistance to Bt toxins. For the laboratory-selected resistant strains, their survivorship on a diet with Bt toxins may not give them a better chance to survive on the Bt crops but indicate the potential risk of Bt-resistant evolution of major pests (Tabashnik et al., 2003). For instance, *Ostrinia nubilalis* on Cry1Ab or Cry1Ac in Bt maize, *Heliothis virescens* on Cry1Ac in Bt cotton, and *Leptinotarsa decemlineata* on Cry3A on Bt potato have shown some levels of resistance relative to susceptible populations, but the laboratory-selected populations have 0% survival on Bt crops (Huang et al., 2002; Gould et al., 1995; Wierenga et al., 1996). Until recently, there was no evidence showing the increasing frequency of resistance to the Bt toxins in field populations in the three major pests, *H. virescens*, *Pectinophora gossypiella*, and *O. nubilalis* in the United States (Tabashnik et al., 2003; Huang et al., 2011). Three major pests, *H. zea*, *Pectinophora gossypiella*, and *Helicoverpa armigera* were considered the critical threats to the sustainable usage of Bt cotton (Tabashnik et al., 2003).

Many factors can be associated with resistant development. In a closed population, allele frequency, selection pressure, dominance, and the relative fitness of the various genotypes are the four main factors that influence the rate change of resistant alleles. In nature, a fifth factor, the population structure, such as the subdivision of population from area to area, would impact resistance development (Roush, 1986). In 2008, an announcement of a field-evolved resistance (decreased susceptibility) to first-generation Bt crop (Cry1Ac in Bt cotton and Cry1Ab in Bt maize) in *H. zea* was reported by Tabashnik et al. (2008). In this case, the

documentation of the field-evolved resistance when most *H. zea* populations were still susceptible was based on evidence that indicates the increased level of survival of resistant populations on Bt cotton in the field compared to susceptible populations (Tabashnik et al., 2008): (a) a Mississippi field-derived strain demonstrated 52% survival in the laboratory bioassay with cotton leaves compared to a susceptible strain showing 0% survival (Luttrell et al., 2004; Ali et al., 2006); (b) greenhouse experiments showing 40% survival of laboratory-selected strain on Cry1Ac Bt cotton compared to a 10% survival of field strain (Jackson et al., 2004). In the same year, an argument about the claim of the field-evolved resistance by Tabashnik et al. (2008) was also published because of (a) no change in Bt cotton efficacy in *H. zea* control; (b) larval sampling adopted on Bt crops; (c) LC_{50} introduced artifacts into the analysis; (d) the baseline may not represent the initial field susceptibility (Luttrell and Ali, 2007; Moar et al., 2008). In Moar et al. (2008), field resistance was defined as "a genetically mediated increase in the ability of a target pest to feed and complete development on one or more commercial lines of Bt cotton under field conditions."

The finding of major resistance alleles in field insect populations may have no causality with the occurrence of field resistance (Huang et al., 2011), which means that the laboratory bioassay itself may only reveal the existence of major resistance alleles and the frequency of the resistance in the field. In a recent publication, the term 'major resistance allele to Bt plants' is defined as insect individuals (populations, colonies, strains) possessing homozygous resistance alleles should exhibit a significantly greater rate to survive and complete their life cycles on the Bt plants, relative to their susceptible counterparts (Huang, 2021a). In contrast, insects containing minor resistance alleles could show some level of resistance to the Bt protein or Bt plant tissue, but on the Bt plants, they could not complete their life cycle and cannot produce offspring (Huang et al., 2007a). Up to date, major resistance alleles have been found in almost all major pest species targeted by Bt maize and Bt cotton, including *H.*

virescens, *P. gossypiella*, *O. nubilalis*, *Diatraea saccharalis*, and so on. (Gould et al., 1995; Jurat-Fuentes et al., 2000; Heckel et al., 2007; Liu et al., 2001; Tabashnik et al., 2000; Huang et al. 2007a; 2007b; Huang 2021a). Field resistance happens when "the field control problem of Bt plants occurs due to resistance" (Huang et al., 2011). Another term, 'practical resistance,' is defined as "field-evolved resistance that reduces the efficacy of a pesticide and has practical consequences for pest control" (Tabashnik et al., 2014). Thus, the two concepts 'field resistance' and 'practical resistance' are very similar.' However, practical resistance to Bt crops is a conception of an event with two characteristics: one or more populations with >50% resistant individuals and significant decreasing crop production (Tabashnik et al., 2014).

The first field control problems of Bt crops were observed in Puerto Rico in 2006, where Cry1F maize hybrids were first commercially planted in 2003 to control a major maize pest, the fall armyworm *Spodoptera frugiperda*. Only three years later, field control problems of the Cry1F maize were reported in several locations on the island (Storer et al., 2010). Laboratory bioassays showed no mortality at the highest Cry1F concentration assayed, and the first field resistance to Bt crops was thus documented (US-EPA, 2009; Storer et al., 2010). In 2007, after a few years of the use of Cry1Ab maize against the African stem borers, *Busseola fusca,* and *Chilo partellus*, unexpected damage from *B. fusca* was observed in South Africa (van Rensburg, 2007). The unexpected field control problem of the Cry1Ab maize in South Africa was also documented to be due to the resistance development of the insect to the Cry1Ab protein in the maize plant (van Rensburg, 2007). The resistance of *B. fusca* to Cry1Ab maize was the second case of field resistance to Bt crops globally. The third case of field resistance to Bt crops occurred in Gujarat, India, in 2008 when Bt cotton containing Cry1Ac experienced unexpected failure against *P. gossypiella* (Dhurua and Gujar, 2011). Currently, at least 21 cases of field resistance to Bt crops (maize and cotton) have been

documented in the world, which involves eight insect species and all types of Cry proteins that are used in commercial transgenic Bt maize and cotton (Table 1.1).

1.6.2 Resistance of *H. zea* to Cry1A.105/Cry2Ab2 Crops in the United States

Cry1A.105 and Cry2Ab2 are two pyramided Bt proteins expressed in the maize event MON 89034 (US-EPA, 2010). Cry1A.105 is not a single natural Bt protein but a bioengineered chimeric insecticidal protein consisting of domains I and II from Cry1Ab or Cry1Ac, domain III from Cry1F, and the C-terminal domain from Cry1Ac (Biosafety Cleaning-House, 2020). MON 89034 is among the first commercialized pyramided Bt maize traits targeting above-ground lepidopteran pests, including *H. zea* (Ghimire et al., 2011; DiFonzo, 2021). Compared to the earlier transgenic plants that contained only a single Bt gene, the use of pyramided traits expressing more than one Bt protein targeting the same insect can enhance pest control efficacy, broaden the target pest spectrum, and delay resistance evolution (Roush, 1998; Zhao et al., 2003; Storer et al., 2012).

As mentioned above, *H. zea* is an economically crucial polyphagous pest found in the Western Hemisphere (Jackson et al., 2008; Luttrell and Jackson, 2012; Reay-Jones, 2019). This insect is rarely considered an economic pest in early-planted field maize, but it can cause damage in late-planted fields in the southern United States (Reay-Jones, 2019). Earlier single-gene Cry1 (e.g., Cry1Ab and Cry1F) maize was not sufficiently effective for this species (Buntin et al., 2004a; 2004b; 2008), and thus, *H. zea* was not considered the main species targeted by Bt maize until 2010 when MON 89034 maize became commercially available. In the southern region of the United States, *H. zea* is a primary cotton pest (FIFRA Scientific Advisory Panel, 1998; Jackson et al., 2008; Reay-Jones, 2019). Similarly, earlier commercialized single-gene Bt cotton traits (e.g., Bollgard® cotton containing Cry1Ac) were only marginally effective against this pest (Gore et al., 2001; Jackson et al., 2004; Luttrell and Jackson, 2012). In 2002, pyramided Bt cotton traits (e.g., Bollgard® II) containing both

Table 1.1 Practical Resistance (= Field Resistance) to Bt Maize and Cotton: 21 Cases Involving Seven Pest Species, Nine Cry Toxins, and Six Countries, Updated from Tabashnik (2019).

Insect	Crop	Toxin	Country/ terrortory	Year occurred	References
B. fusca	Maize	Cry1Ab	S. Africa	2006	Strydom et al. (2019)
D.saccharalis	Maize	Cry1A.105	Argentina	2012	Grimi et al. (2017)
D.saccharalis	Maize	Cry1Fa	Argentina	2012	Grimi et al. (2017)
D. v. virgifera	Maize	Cry3Bb	United States	2009	Gassmann et al. (2011)
D. v. virgifera	Maize	Cry34/35Ab	United States	2013	Ludwick et al. (2017)
D. v. virgifera	Maize	eCry3.1Ab	United States	2014	Andow et al. (2016)
D. v. virgifera	Maize	mCry3A	United States	2011	Andow et al. (2016)
H. zea	Maize	Cry1Ab	United States	2004	Dively et al. (2016)
H. zea	Maize	Cry1A.105	United States	2016	Dively et al. (2016), Kaur et al. (2019)
H. zea	Cotton	Cry1Ac	United States	2002	Little et al. (2019)
H. zea	Both	Cry2Ab	United States	2005	Dively et al. (2016), Kaur et al. (2019)
P. gossypiella	Cotton	Cry1Ac	India	2011	Dhurua and Gujar (2011)
P. gossypiella	Cotton	Cry2Ab	India	2014	Naik et al. (2018)
S. albicosta	Maize	Cry1Fa	Canada	2012	Smith et al. (2017)
S. albicosta	Maize	Cry1Fa	United States	2013	Ostrem et al. (2016)
S. frugiperda	Maize	Cry1F	Puerto Rico	2006	Storer et al. (2010)
S. frugiperda	Maize	Cry1Ab	Brazil	2010	Omoto et al. (2016)

(table cont'd.)

Insect	Crop	Toxin	Country/ terrortory	Year occurred	References
S. frugiperda	Maize	Cry1Fa	Argentina	2013	Chandrasena et al. (2018)
S. frugiperda	Maize	Cry1Fa	Brazil	2011	Farias et al. (2014)
S. frugiperda	Maize	Cry1Fa	United States	2007	Huang et al. (2019)
O. nubilalis	Maize	Cry1F	Canada	2018	Smith et al. (2019)

*First year of commercial planting of a Bt crop in the region monitored.
*Years from the first commercial planting of a Bt crop in the region to the first sampling of field populations in the region yielding evidence of resistance.
*Cross-resistance suspected or known as a factor contributing to resistance.

Cry1Ac and Cry2Ab2 became available, and these traits initially were highly effective against *H. zea* (Gore et al., 2001; Jackson et al., 2003; 2004; Luttrell and Jackson, 2012).

Across the Cotton Belt of the southern United States, maize and cotton are often cultivated adjacently. In the crop growing seasons, *H. zea* larvae usually feed on maize first. When maize becomes unsuitable for oviposition, moths transfer to cotton and other alternative host crops. Larvae feed on these alternative crops and continue their life cycle for 2 to 3 more generations during the season (US-EPA, 2001). Thus, in the Cotton Belt, *H. zea* is a cross-crop insect species targeted by both Bt cotton and Bt maize (Yang et al., 2016; Reay-Jones, 2019). Currently, Bt maize and cotton plantings in the United States account for >80 and >90% of the total areas of the two crops, respectively (NASS, 2019). In addition, transgenic maize and cotton crops express the same or similar Bt proteins for lepidopteran control (US-EPA, 2018; Huang, 2020b). In the cropping landscape, the similarities in Bt genes in maize and cotton coupled with the phenology of *H. zea* in the Cotton Belt create a high risk for the evolution of Bt resistance.

Until recently, MON 89034 maize had effectively controlled *H. zea* (Siebert et al., 2012; Rule et al., 2014; Yang et al., 2014a; Kaur et al., 2019). However, in recent years, problems in controlling *H. zea* with MON 89034 have frequently been observed in the United States (US-EPA, 2018). For example, a study by Dively et al. (2016) reported that *H. zea* in Maryland, a Mid-Atlantic state of the United States, developed resistance that resulted in field control problems (or called field resistance) to transgenic sweet maize containing the *cry*1A.105/*cry*2Ab2 genes. Two recent studies (Kaur et al., 2019; Yang et al., 2019a) have also documented field resistance to MON 89034 in *H. zea* has occurred in parts of the southern United States. Furthermore, Bilbo et al. (2019) observed that, relative to laboratory strains, some *H. zea* populations sampled in North and South Carolina maize fields during

2017 and 2018 were significantly more tolerant to the Cry1A.105 protein. Additionally, Reisig et al. (2018) reported that field resistance in *H. zea* to Cry1A/Cry2A cotton has also occurred in North Carolina. The occurrence of the Cry protein resistance in *H. zea* represents a significant challenge to the continued success of Bt crop technology (FIFRA Scientific Advisory Panel, 1998; US-EPA, 2001; Jackson et al., 2008; Reay-Jones, 2019; Niu et al., 2021). Objective 1 of this project was an extended investigation to determine the current status and distribution of the Cry1A.105 and Cry2Ab2 resistance in *H. zea* across the southeastern United States.

1.6.3 Bt Resistance Allele Frequencies in *H. zea*

A sound resistance monitoring system should detect the early changes in the susceptibility to Bt toxins and resistance allele frequency in the field pest populations (Huang, 2006; Reisig et al., 2018). Tabashnik et al. (2013; 2014) defined field-evolved resistance into five categories based on the frequency of resistant individuals in the insect populations: 1) practical resistance: >50% resistant individuals and cases of reduced control efficacy in the field; 2) >50% resistant individuals with expected reduced efficacy, but no control failure in the field; 3) 6-50% resistant individuals, 4) early warning of resistance: 1-6% resistant individuals; 5) Incipient resistance: <1% resistant individuals; 6) no statistically significant decrease in susceptibility. The resistant individuals can be the homozygotes with functional recessive resistant genes or heterozygotes and homozygotes with dominant resistant genes. The judgment standard of >50% resistant individuals used in Tabashnik et al. (2013; 2014) is based on the LC_{50}s and resistance ratios in the field-selected populations in laboratory bioassays.

As mentioned before, field resistance (practical resistance) to Bt maize and Bt cotton expressing Cry1A and Cry2A proteins has been documented in several locations in the United States. In sweet corn, the conclusion of the field resistance to Cry1A/Cry2A proteins in *H.*

zea was made by monitoring data from an in-field screen using Cry1Ab (1996-2016) and Cry1A.105+Cry2Ab2 (2010-2016) sweet corn hybrids (Dively et al., 2016). The confirmation of Cry2A practical resistance to Bt cotton in *H. zea* was based on the damaged boll survey in the field and dose-response bioassay in the laboratory (Reisig et al., 2018). In addition, using diet over-laying bioassays, Bilbo et al. (2019) reported that *H. zea* collected during 2017 and 2018 in North and South Carolina showed that all field populations collected in the areas were resistant to Cry1A.105 with a resistance ratio from 13.5- to >4,000-fold, while susceptibility of these populations to Cry2Ab2 varied very much. Field resistance to Cry1A.105 and Cry2Ab2 in *H. zea* has also been documented in Louisiana by using a combination approach of field survey, diet over-laying bioassay, and field plot trials (Kaur et al., 2019).

Ideally, resistance allele frequency in field insect populations should be determined before or right after releasing a newly commercialized Bt toxin to acquire the initial resistant allele frequency (Carrière and Tabashnik, 2001). Because of the difficulty in using single-pairing to establish iso-line families of *H. zea* for F2 screen, Bt resistance allele frequency in field populations of *H. zea* has rarely been investigated. An early F1 screen reported that the non-recessive resistance allele frequency to Cry1Ac was 0.00043 and to Cry2Ab2 was 0.00039 (Burd et al., 2003). However, the F1 screen used in Burd et al. (2003) could not detect recessive resistance alleles and thus could underestimate the actual resistance allele frequency in the field pest populations. Recently, Yang et al. (2020a) used light- traps to collect mated feral females of *H. zea* in Texas and established 114 families for F2 screen against Vip3Aa39 protein, a Vip protein that is close to Vip3Aa19 used in Bt cotton and Vip3Aa20 expressed in Bt maize. The F2 screen showed a resistance allele frequency of 0.0065 with a 95% CI of 0.0014-0.0157 to Vip3Aa39 in this Texas population. Other than these two studies, Bt resistance allele frequency in *H. zea* had not been investigated for any

other Bt proteins, including Cry1A.105 and Cry2Ab2. Objective 2 of this project used a mass-mating method to establish F2 iso-line families for an F2 screen to determine the Cry1A.105 and Cry2Ab2 resistance allele frequencies in field populations of *H. zea* mainly collected from Louisiana. The information generated from this study should provide further evidence to categorize the status of the Cry1A.105 and Cry2Ab2 resistance in *H. zea* in the United States.

1.7 Cross-resistance among Bt Proteins

With different insecticide classes sharing the same mode of action, alterations in the target site can reduce the insecticide binding efficiency and lead to resistance to two or more classes of insecticide (Mitchell, 2012), which is called cross-resistance. In insecticide resistance studies, the common assumption is that the occurrence of cross-resistance in the population results from the target-site mutations, such as pyrethroid and DDT cross-resistance in *Aedes aegypti*; meanwhile, very little is known of cross-resistance induced by "metabolic resistance" (Ranson et al., 2001; Brengues et al., 2003; Dabiré et al., 2008). In Bt resistance studies, the occurrence of cross-resistance refers to the selection of one Bt toxin that produces resistance to other Bt toxin (Tabashnik et al., 2014), and the susceptibility often reflects on the LC_{50} (Tabashnik et al., 1987; Wei et al. 2015; Yang et al., 2017a; 2017b) or survival rate on certain Bt toxins or Bt plant tissue (Santos-Amaya et al., 2015). Under the current Bt resistance management system, pyramided Bt plants play an important role in delaying the dominance process of the initial rare, recessive, fitness cost associated resistant gene carriers in the pest population. In theory, with different toxin binding sites in a specific target pest, the possibility of producing an individual containing two resistant genes separately against two toxins with a different mode of action ought to be rare. Thus, cross-resistance in pyramiding confounds Bt resistance-management strategies, especially cross-resistance from different classes of Bt toxins, such as Cry1A and Cry2A families.

Results from previous studies have documented that cross-resistance among the Cry1 family (Cry1Ab, Cry1Ac, Cry1F), between Cry2A proteins (Cry2Ab and Cry2Ae), or within Cry3 toxins (Cry3Bb1, mCry3A, eCry3A) are common in insect pests targeted by Bt crops (e.g., *S. frugiperda* and the western corn rootworm, *Diabrotica virgifera virgifera*) (Huang et al., 2014; Santos-Amaya et al., 2015; Rodrigues-Silva et al., 2019; Yang et al., 2017a; 2017b; Jakka et al., 2016). These Bt proteins within the same family usually have similar protein structures and often show similar binding sites (Hernández-Rodríguez et al., 2013). However, cross-resistance among different Bt toxin families (groups) (e.g., Cry1, Cry2, and Vip3A) in *S. frugiperda* or *H. zea* appeared to be very rare. An explanation of this phenomenon is because of the same or similar binding sites shared among Bt toxins within the same family, while Bt toxins in different families have different binding sites in the insect midguts (Estruch et al., 1996; Lee et al., 2003; Hernández-Rodríguez et al., 2008; 2013). There are 7 cases of field resistance that have been suspected to be caused or at least partially caused by cross-resistance, which involved three Bt toxins (Cry1A, Cry2A, and Cry3A) in at least four insect species in Lepidoptera (*D. saccharalis, H. zea, S. frugiperda, and O. nubilalis*) and one species of Coleoptera (*D. v. virgifera*), and across three countries: Argentina, the United States, and Brazil.

1.8 Inheritance of Bt Resistance

1.8.1 An Overview of Inheritance of Bt Resistance

The knowledge of the mode of inheritance of resistance is critical in both understanding resistance development and resistance management. Learning about the inheritance of Bt resistance includes the dominance level, distinguishing between monogenic and polygenic, and the sex linkage (maternal effects) of a certain resistant insect population in most of the studies. These inheritance traits of the Bt-resistant genes are highly associated with

appropriating preventative management strategies, such as the use of 'high dose/refuge,' in theory (Alstad and Andow, 1995).

The definition of maternal effects is the causal influence of the maternal genotype on the offspring's phenotypes (Wolf and Wade, 2009). The discussion of maternal effects concerns more genetically but environmentally. The sex linkage (maternal effects) is often detected by comparing if the two F1 reciprocal genotypes have the insecticide concentrations at the same mortality level (e.g., LC_{50}) or mortality. Based on the published information, predominant Bt resistance, including these field resistance cases to Bt crops listed in Table 1.1, has been reported to be autosomal and not sex-linked, with few exceptions. For example, the commonly occurring Cry1F resistance in *S. frugiperda* in the Americas has been identified to be autosomal and not sex-linked (Santos-Amaya et al., 2016; Camargo et al., 2017). A Vip3A-resistant population of *H. zea* from Louisiana was also found to be autosomal and not sex-linked (Yang et al., 2018).

The level of dominance of resistance is a measure of the relative position of the phenotype of the heterozygotes relative to the phenotypes of the two corresponding susceptible and resistant homozygotes, indicating completely recessive, relative recessive, relative dominant, or completely dominant (Bourguet et al., 2000). The dominance of resistance is not an intrinsic property (Bourguet, 1999), which means the change of environmental parameters (for example, the shift in insecticide concentration) may change the outcome of dominance level calculation (Bourguet et al., 1996). The dominance level may convert from dominant to recessive when the insecticide dose reaches a particular high point leading to the so-called functionally recessive. The functionally recessive refers to a phenomenon that genetically non-recessive heterozygote (RS) could be suppressed at high levels of Bt proteins expressed in transgenic Bt plants (Carrière et al., 2015). The measure of the phenomenon in resistance could rely on insecticide concentration of different genotypes

(RR, RS, SS) at the same mortality level (D_{LC}), survival of different genotypes at a given insecticide dose (D_{ML}), and the fitness cost (larval development, female fecundity, offspring survival) (D_{WT}) at a given dose (Bourguet et al., 2000). Among these three measurements, D_{WT} provides the most helpful information for resistance management. Because of the relatively more difficulty in measuring D_{WT}, most studies use D_{ML} or D_{LC} as the parameters to value the dominance of resistance. It was believed that high-level resistant genes to Bt toxins were usually recessive (Tabashnik, 1994; Alstad and Andow, 1995; Gould, 1998; Bourguet et al., 2000). However, a recent analysis showed that the dominance levels of 13 cases of major resistance to single-protein Bt crops, only 5 out of the 13 cases were functionally recessive, while 61.5% of these cases were functionally non-recessive (Huang, 2021a). More importantly, all six cases with field control problems that have been evaluated were functionally non-recessive. In addition, Huang (2021a) also showed that dual/multiple-protein resistance has a greater tendency to be functionally recessive than single-protein resistance by comparing the D_{FL} (functionally dominance level) between the 13 cases of single-gene resistance and 4 cases of dual-gene resistance that had been evaluated.

1.8.2 Inheritance of Bt Resistance in *H. zea*

Before the proposed study of the current project, the inheritance of Cry1A.105 in *H. zea* had not been investigated, and there was only one study that characterized the genetic basis of Cry2Ab2 resistance in a Texas resistant population. Yang et al. (2020b) reported that a Texas Cry2Ab2-resistant population of *H. zea* was autosomally inherited and controlled by more than one locus. The resistance was completely dominant on leaf tissue of TweinLink cotton expressing Cry1Ab and Cry2Ae proteins.

However, the inheritance of resistance to Cry1A.105 and Cry2Ab2 proteins has been analyzed for several other insect pests targeted by Bt maize or Bt cotton. For example, Huang et al. (2015) reported that the Cry2Ab2 resistance in *D. saccharalis* was likely

inherited as a single or a few tightly linked autosomal non-recessive genes. Acharya et al. (2017) conducted laboratory bioassays and found that Cry2Ab2 resistance in a field population of *S. frugiperda* collected in Georgia in 2013 was controlled by a single autosomal recessive or incompletely recessive gene. For Cry1A.105 resistance, the inheritance in a Florida population of *S. frugiperda* was reported to be controlled by a single, autosomal non-recessive gene (Niu et al., 2018).

In addition, the dominance levels of dual-protein resistance to Cry1A.105/Cry2Ab2 have been evaluated in survival populations of *S. frugiperda*. For example, a Brazilian resistant population of *S. frugiperda* that was selected on MON 89034 maize leaf tissue exposing Cry1A.105/Cry2Ab2 showed completely recessive on the Bt maize leaf tissue (Santos-Amaya et al., 2015; Horikoshi et al., 2016). The dominance level of the Cry1A.105/Cry2Ab2 dual-protein resistance in *S. frugiperda* was also studied in a USA population, and the results showed that the dominance level varied from incompletely recessive to incompletely dominant on leaf tissue and whole Bt maize plants (Zhu et al., 2019). By using an F2 screening method mentioned in section 3.2 of this project, a Cry1A.105-single-protein resistant strain and a Cry2Ab2-single-protein resistant strain of *H. zea* have been established from the survivors in the F2 screen with isolines derived from the field in Louisiana. Both resistant strains have been documented to possess major resistance alleles that allowed the homozygous resistant individuals to survive and complete the larval life cycle (neonate-to-pupa) on the corresponding Bt maize ears. The availability of these Bt-resistant strains of *H. zea* provided an opportunity to characterize the genetic basis of the Cry1A.105 and Cry2Ab2 resistance in the insect. In this study, various crosses and backcrosses between a Bt-susceptible population and the Cry1A.105- resistant or Cry2Ab2-resistant population of *H. zea* were performed; and the susceptibility of these original, crossed, and backcrossed populations were assayed using a diet over-lay bioassay to analyze the inheritance of the

Cry1A.105 and Cry2Ab2 resistance in the populations collected from Louisiana (Objective 3).

1.9 Fitness Costs of Bt Resistance

In resistance studies, fitness costs refer to a negative influence on fitness (e.g., developmental delay, increased mortality, decreased adult lifetime) in resistant populations that associate with the existence of resistance alleles (RR and RS) compared to the susceptible populations (SS) (Huang, 2021a). A non-recessive fitness cost is established when both RR and RS populations show a phenotype of reduced fitness in the absence of selection pressure. Otherwise, if only RR populations show reduced fitness, but the fitness parameters of RS and SS populations are similar, the fitness cost is considered recessive (Carrière and Tabashnik, 2001; Gould et al., 2006; Gassmann et al., 2009). It was believed that the populations that survived under purified Bt proteins or Bt crops tended to possess fitness costs, especially in populations acquiring high resistance levels (Tabashnik, 1994; Ferré and van Rie, 2002; Gassmann et al., 2009). Because of the resistant genes combined with the fitness costs, the susceptible individuals dominate the wild populations when there is no selection pressure from Bt toxins, which means that the resistance could be reversed in theory. In this situation, the refuge strategy could be more meaningful in Bt crop IRM (Gassmann et al., 2009). However, in an analysis of 15 cases of single-protein major resistance to Bt plants in 28 studies in which their fitness costs on plants have been evaluated, only six of the 15 cases were associated with fitness costs. Four cases showed no fitness costs or advantages, and five cases exhibited some levels of fitness advantage (Huang, 2021a). In addition, fitness costs in RS showed in only one case, which was the resistance of *P. gossypiella* to Cry1Ac cotton. The analysis also showed that the patterns of fitness costs were similar between the cases of single-protein resistance and cases of dual/multiple-protein resistance (Huang, 2021a).

1.10 Resistance Management Strategies for Bt Crops

The original primary targets for Bt cotton were *H. virescens* and *P. gossypiella* (US-EPA, 2018), while the original primary targets of Bt maize were *O. nubilalis* and *Diatraea grandiosella* (Storer et al., 2001). Currently, insecticide resistance management (IRM) strategies to delay resistance to Bt crops in the United States and several other countries rely on three tactics: 1) availability of non-Bt refuge hosts, 2) high-dose/refuge strategy, and 3) pyramiding toxins. In addition, resistance monitoring should be included in all IRM programs for Bt crop planting in the United States (US-EPA, 2001).

1.10.1 Availability of Non-Bt Refugee Hosts

The application of non-Bt crop refuge as a favorable food source for susceptible insect populations in the field is the most widely used IRM method for planting Bt crops in the world. In the United States, before 2010, the planting of non-Bt structured maize refuge was required in the entire country (Matten et al., 2012). Until 2010, along with the popularization of pyramided Bt plants, the structured refuge requirement became one of two options in refuge plantings in planting pyramided Bt maize hybrids in the U.S. Maize Belt and parts of Texas. Another opinion of refuge planting for pyramided Bt maize is a seed blend (also called refuge-in-the-bag or RIB) of 5% non-Bt seeds, and 95% pyramided Bt seeds (Matten et al., 2012). The primary reason for using different refuge strategies in the southern region of the U.S. is that, in the growing seasons in the southern U.S., where cotton is planted, both maize and cotton contain similar Bt toxins. With an increased preference for maize, *H. zea* larvae occur on the maize with a higher probability than in other crops (Stadelbacher et al., 1986). Also, maize is the most common crop in the early planting seasons during July and August available for *H. zea* (Jackson et al., 2008; Head et al., 2010). It is believed that Bt maize is presumably the initial driver of *H. zea* resistance selection, threatening both maize and cotton in the field (Head et al., 2010). In addition, maize is cross-pollinated in the open

field, and *H. zea* larvae are ear-feeders. Studies have shown that the cross-pollination in seed blend of non-Bt and Bt maize will cause expressions of Bt proteins in 'refuge ears,' and the Bt contamination in refuge ears will significantly affect the susceptible insects that feed on the 'refuge ears' (Yang et al., 2014a; Dimase et al., 2020; Guo et al., 2021). Thus, it is essential to apply structured refuge to manage the Bt resistance in the southern region, even with the use of pyramided Bt maize traits (Head et al., 2010). This decision considers that the support of refuge cotton is insignificant compared to maize, sorghum, soybean, and wild hosts (Head et al., 2010). However, more selective pressure on field *H. zea* has been added after the extension of Bt maize. Also, in some conditions, farmers may not follow the maize structured refuge regulations, which have been documented in a report in 2017 (Reisig, 2017). In 2015, a phone survey indicated the problem with implementing required structured refuge planting, including 36% no Bt refuge planting, 38% without reaching the regulated refuge size, and 26% partially getting the regulated refuge size (ABSTC, 2016).

1.10.2. High Dose/Refuge Strategy

The high dose plus refuge strategy (high dose/refuge strategy) is of great importance in managing Bt resistance in the United States and several other countries. The term "high dose" describes toxin titers that are reaching a quantity that can kill ≥95% of the heterozygous resistance allele carriers (Gould, 1998). In the 'high dose/refuge' planting, susceptible insect populations (SS) from non-Bt refuge plants are expected to be able to mate with the rare homozygous resistant individuals (RR) from the Bt plants so that their offspring that carry resistance alleles should be heterozygous (RS) (Ostlie et al., 1997). The heterozygous-resistant individuals, by design, should be killed by the 'high does' Bt plants. As a result, the resistance allele frequency in the pest populations should be maintained at a low level for a long period of time (Ostlie et al., 1997; Huang et al., 2011). There are three fundamental requests for the success of the 'high dose/refuge' strategy: the plant must

produce a ‘high does’ against the pest (= resistance is functionally recessive); the initial resistance allele frequency in the pest population must be very low (e.g., <0.001); and there is random mating between susceptible insects from the refuge and resistant insects from the Bt plants (Huang et al., 1999). Studies have shown that the ‘high does/refuge’ strategy works as long as the three fundamental conditions are met (Huang et al., 2011). The ‘high dose/refuge’ strategy was originally designed for resistance management of single-protein Bt plants (Ostlie et al., 1997). The concept may be helpful for IRM of pyramided Bt crop traits. For example, in pyramiding Bt maize containing Cry1A and Vip3A, the protein expression nearly reaches the high dose requirement for *H. zea* (Yang et al., 2015). The particular pyramiding toxins are called an effective high dose if each toxin expression does not reach the high dose standard, but the combination of the toxins does (US-EPA 2009). However, after confirming an effective high dose of Bt maize, damage containing Cry1A, Cry1F, and Vip3A was more significant than expected in a multi-year and state replicated experiment (Reay-Jones et al., 2016). In Mississippi, across five sites, some *H. zea* larvae could survive in the terminals, squares, and bolls of Cry1Ac + Cry1F + Vip3Aa19 cotton (Little et al., 2017). Nevertheless, field resistance to Bt cotton and Bt maize that contains the Vip3A gene has not been documented yet.

1.10.3 Pyramiding Toxins to Delay Development of Bt Resistance

Pyramiding is also important in Bt crop IRM, which expresses more than one Bt toxin with distinct modes of action in the same event, with the Bt toxins aimed at the same target pest (Carrière et al., 2015). Pyramiding works well because the toxins involved have a great efficiency separately and can be superimposed to kill the pest (Carrière et al., 2016). In Bt maize, Vip3Aa20 toxin coupled with Cry1Ab, Cry1Ab + Cry1F, and Cry1A.105 + Cry2Ab2 are available for controlling above-ground moth pests in the current Bt maize market. In Bt cotton, Vip3Aa19 toxin coupled with Cry1Ac + Cry1F, Cry1Ac + Cry2Ab2, and Cry1Ab +

Cry2Ae are available for managing lepidopteran pests in the Bt cotton market. The Vip3A is a novel Bt toxin with different acting sites from Cry1A and Cry2A Bt toxins (Estruch et al., 1996; Lee et al., 2003), which has effective control to the Cry1A/2A resistance *H. zea* populations (Reay-Jones et al., 2016; Little et al., 2017; Bilbo et al., 2018; Kaur et al., 2019). The use of Cry1A and Cry2A in both Bt cotton and maize has a relatively long history. As mentioned above, *H. zea* has developed field resistance to Cry1A and Cry2A in the United States (Carrière et al., 2016; Dively et al., 2016; Reisig et al., 2018b; Kaur et al., 2019; Yang et al., 2019a; Yu et al., 2021). The existence of resistance to Cry1A and Cry2A toxins in *H. zea* in the field could accelerate the evolution of resistance to the Vip3A. Although the use of Vip3A maize and cotton has been minimal until recently, Bt cotton containing Cry1Ac, Cry1F, and Vip3Aa19 has been planted since 2015; and Bt maize containing Cry1Ab and Vip3Aa20 has been planted since 2011(Reisig and Kurtz, 2018). Thus, the overlapping use of the similar Bt proteins in both pyramiding cotton and maize should have created a favorable condition for Bt resistance development in *H. zea* populations in the southern region of the United States (Reisig and Kurtz, 2018).

1.10.4 Bt Resistance Monitoring

In the United States, resistance monitoring is an essential component of the IRM program for planting Bt crops (US-EPA 2001). The aim of monitoring Bt resistance could be to document field control failures and, more importantly, to detect the early increase in resistance allele frequency (a warning sign of resistance development) in the field (US-EPA, 2001; Huang, 2006; Tabashnik et al., 2008). Several methods have been used in Bt resistance monitoring. The most common methods include dose-response bioassays, dose discriminative bioassays, field inspections of unexpected larval survival or unexpected plant damage of Bt crops in grower's fields or sentinel plots, F1 screen, F2 screen, and the use of DNA markers (Huang, 2006). Each of these methods has different advantages and

disadvantages, and thus, effective resistance monitoring often consists of the combined use of two or more methods. In general, the traditional methods such as dose-response bioassay and dose discriminative bioassay are sample and easy to be conducted. Both methods are suitable to be used to establish baseline susceptibility and confirm if unexpected control problem in the field is due to resistance development, but they are not sensitive enough to monitor the changes in resistance allele frequency in the early stages of resistance development (Andow and Alstad, 1998; Huang, 2006). F1 screen is suitable to detect non-recessive resistance alleles but ineffective for recessive alleles unless it uses a known-resistant population to cross the field-collected populations (Yue et al., 2008). Compared to the dose-response or dose-discriminative bioassays, the F2 screen is much more sensitive to detecting rare resistance alleles in field populations, especially for recessive resistance alleles, but its cost is a significant limitation of the method (Andow and Alstad, 1998; Huang, 2006). In recent years, scientists have been able to develop DNA markers to detect Bt resistance alleles, but the molecular method has not been widely used in the field because of the difficulty of finding the effective markers, as well as the polymorphism of the gene mutations conferring Bt resistance (Zhang et al., 2012; Wu, 2014; Huang 2021b).

Insect sampling is an important process in detecting Bt resistance in primary and secondary target pests of Bt crops (US-EPA, 2001). Insect sampling for resistance monitoring should be conducted in both Bt crops and non-Bt crops (Tabashnik et al., 2008). Sampling size and sampling region are two other vital factors making the monitoring result strictly close to the facts (Huang, 2006). In the Bt crop system, a resistance allele frequency of 0.1 in a pest population can indicate control failures in just a few generations (Roush and Miller, 1986). Thus, an effective Bt monitoring program requires to have the ability to detect the resistance alleles when the resistance allele frequency is still rare (e.g., <0.001) (Andow and Alstad, 1998). Therefore, to increase detection accuracy, it is better to have a large

sample size per location (e.g., 500-1000) (US-EPA, 2001). It should distribute across the Bt crop regions; meanwhile, the areas with heavier selection pressure, with cross-resistant selection pressure, and with regular pest overwinter behavior require more frequency of samplings than other locations (US-EPA, 2001). The sampling of Bt crops gives a better chance to collect resistant individuals to feed with Bt protein existence (Tabashnik et al., 2008). The bioassays will be conducted on the progeny of wild-collected populations in the laboratory. The Bt toxin source to document the susceptibility could be Bt plant tissues or purified Bt protein. Generally, the susceptibility would be revealed by the mortality of the target pest exposed to the Bt protein. Bt resistance monitoring is usually conducted by screening/bioassays with populations of F0 (field populations), F1, and F2 populations, which are described below:

Observation of unexpected pest survival or plant damage on Bt plants in the field is a common method used in Bt resistance monitoring. In the field, the Bt plants act as a discriminative dose to detect phenotypic resistant individuals of the field populations (F0). In addition, the F0 screen also can be performed with the use of DAN markers. However, it is often challenging to conduct laboratory bioassays with F0 populations collected from the field. The rapid and relatively convenient F0 screen in the field could offer researchers a glance at substantial changes in field resistance populations, such as developing dominant resistance alleles. The advantage of the F0 screen compared to the F1 screen and F2 screen is the lower time and workforce cost. However, the F0 screen usually cannot provide statistically significant results for the recessive alleles, and thus, additional laboratory bioassays are needed to confirm the resistance detected in the field screen (Huang, 2006).

The F1 screen considers the field-collected population as F0 generation, which contains genotypes to be tested. The individuals in F0 populations would go through mating with homozygous resistant insects. If the field insect was a heterozygous genotype individual,

50% of the offspring must be heterozygous for the resistant allele, while another 50% homozygous for resistant offspring (Yue et al., 2009). In addition, the F1 screen can also be used to directly detect non-recessive resistance alleles in F1 generations of field-collected populations without crossing with a known-resistant strain (Burd et al., 2003; Li et al., 2016).

The F2 screen is an adequate method for detecting rare recessive alleles—a proportion of homozygous progeny in the F2 isoline family would be 1/16 if a mated female collected from the field (Andow and Alstad, 1998) or from single-paring of field-collected individuals (Huang et al., 2007c; 2012) carries a resistance allele. Because of its high level of sensitivity in detecting rare recessive alleles in field insect populations, the F2 screen has been widely used by researchers to detect Bt resistance alleles in many insect species targeted by Bt maize and/or Bt cotton, such as *O. nubilalis* (Andow and Alstad, 1998; Andow et al., 1998; 2000; Bourguet et al., 2003; Farinós et al., 2004; Stodola et al., 2006; Engels et al., 2010)., *D. grandiosella* (Huang et al., 2007b), *D. saccharalis* (Huang et al., 2007a; 2007c; 2012) *S. frugiperda* (Huang et al., 2014; Niu et al., 2016; Yang et al., 2018; 2019b), *H. zea* (Yang et al., 2021), and *H. armigera* (Liu et al. 2010). However, because of its relatively high costs and the complexity of the procedures, the F2 screen method has not been widely adopted by biotech industries for Bt resistance monitoring (Huang 2021b).

1.11 Objectives

The specific objectives of this study contain:

1. To determine the current status and distribution of the Cry1A.105/Cry2Ab2-resistance in *Helicoverpa zea* in the southeastern United States;

2. To estimate resistance allele frequency to Cry1A.105 and Cry2Ab2 proteins in *Helicoverpa zea* populations with F2 isolines generated from a mass-mating method; and

3. To characterize the inheritance of the Cry1A.105 and Cry2Ab2 resistance in in *Helicoverpa zea*.

CHAPTER 2. EXTENDED INVESTIGATION OF FIELD-EVOLVED RESISTANCE OF THE CORN EARWORM *HELICOVERPA ZEA* (LEPIDOPTERA: NOCTUIDAE) TO *BACILLUS THURINGIENSIS* CRY1A.105 AND CRY2AB2 PROTEINS IN THE SOUTHEASTERN UNITED STATES

2.1 Introduction

Cry1A.105 and Cry2Ab2 are two pyramided *Bacillus thuringiensis* (Bt) insecticidal proteins expressed in the maize event MON 89034 (US-EPA, 2010). Cry1A.105 is not a single natural Bt protein but a bioengineered chimeric insecticidal protein that consists of domains I and II from Cry1Ab or Cry1Ac, domain III from Cry1F, and the C-terminal domain from Cry1Ac (Biosafety Clearing-House, 2020). MON 89034 is among the first commercialized pyramided Bt maize traits targeting above-ground lepidopteran pests (Ghimire et al., 2011; DiFonzo, 2020). Compared to the earlier transgenic plants that contained only a single Bt gene, the use of pyramided traits expressing more than one Bt protein targeting the same insect can enhance pest control efficacy, broaden the target pest spectrum, and delay resistance evolution (Roush, 1998; Zhao et al., 2003; Storer et al., 2012).

The corn earworm, *Helicoverpa zea* (Boddie), is an economically important polyphagous pest in the Western Hemisphere (Jackson et al., 2008; Luttrell and Jackson, 2012; Reay-Jones, 2019). *H. zea* is rarely considered an economic pest in early planted field maize, but it can cause damage in late-planted fields in the southern United States (Reay-Jones, 2019). Earlier single-gene Cry1 (e.g., Cry1Ab and Cry1F) maize was not sufficiently effective for this species (Buntin et al., 2004a; 2004b; 2008), and thus, *H. zea* was not considered the main species targeted by Bt maize until 2010 when MON 89034 maize became commercially available. In the southern region of the United States, *H. zea* is a primary cotton pest (FIFRA

Scientific Advisory Panel, 1998; Jackson et al., 2008; Reay-Jones, 2019). Similarly, earlier commercialized single-gene Bt cotton traits (e.g., Bollgard® containing Cry1Ac) were only marginally effective against this pest (Gore et al., 2001; Jackson et al., 2004; Luttrell and Jackson, 2012). In 2002, pyramided Bt cotton traits (e.g., Bollgard® II containing both Cry1Ac and Cry2Ab2) became available, and these traits initially were highly effective against *H. zea* (Gore et al., 2001; Jackson et al., 2003; 2004; Luttrell and Jackson, 2012).

Across the Cotton Belt of the southern United States, maize and cotton are often adjacently cultivated. During the crop growing seasons, *H. zea* larvae usually feed on maize first. After maize becomes unsuitable for oviposition, the moth switches to cotton and other alternative host crops. Larvae feed on these alternative crops and continue their life cycle for 2 to 3 more generations during the season (US-EPA, 2001). Thus, in the Cotton Belt, *H. zea* is a cross-crop insect species targeted by both Bt cotton and Bt maize (Reay-Jones, 2019). Currently, Bt maize and cotton plantings in the United States account for >80 and >90% of the total areas of the two crops, respectively (NASS, 2019). In addition, transgenic maize and cotton crops express the same or similar Bt proteins for lepidopteran control (US-EPA, 2018; Huang, 2020). In the cropping landscape, the similarity of Bt genes in maize and cotton and the phenology of *H. zea* in the Cotton Belt creates a high risk of Bt resistance evolution.

Until recently, MON 89034 maize had been effective in controlling *H. zea* (Siebert et al., 2012; Rule et al., 2014; Yang et al., 2014a; Kaur et al., 2019). In recent years, problems in controlling *H. zea* with MON 89034 have frequently been observed in the United States (US-EPA, 2018). A study by Dively et al. (2016) reported that *H. zea* in Maryland, a northeastern state of the United States, developed resistance that resulted in field control problems (or called field resistance) to transgenic sweet corn containing the Cry1A.105/Cry2Ab2 genes. Two recent studies (Kaur et al., 2019; Yang et al., 2019) have documented that field resistance to MON 89034 in *H. zea* has occurred in parts of the southern United States.

Furthermore, Bilbo et al. (2019) observed that, relative to laboratory strains, some *H. zea* populations sampled in North and South Carolina maize fields during 2017 and 2018 were significantly more tolerant to the Cry1A.105 protein. Additionally, Reisig et al. (2018) reported that field resistance in *H. zea* to Cry1A/Cry2A cotton has occurred in North Carolina. The occurrence of Cry resistance in *H. zea* represents a significant challenge to the continued success of Bt crop technology (FIFRA Scientific Advisory Panel, 1998; US-EPA, 2001; Jackson et al., 2008; Reay-Jones, 2019). The present study was an extended investigation to determine the current status and distribution of the Cry1A.105 and Cry2Ab2 resistance in *H. zea* across the southeastern United States. During 2018 and 2019, a total of 31 field populations of *H. zea* were collected from the major maize areas of seven southeastern states of the United States. These populations were assayed against the Cry1A.105 and Cry2Ab2 proteins. The results revealed that resistance to both Cry1A.105 and Cry2Ab2 is widely distributed in the region. Implications of these findings to insect resistance management are also discussed.

2.2 Materials and Methods

2.2.1 Insect Collection and Laboratory Rearing

During the 2018 and 2019 crop growing seasons, a total of 31 populations of *H. zea* were collected across the major maize areas in seven southeastern states of the United States (Table 2.1). Among these, 20 populations were collected during 2018, and 11 were sampled in 2019; 12 of the totals were from Louisiana (LA), six from Mississippi (MS), one from Arkansas (AR), two from Georgia (GA), six from South Carolina (SC), two from North Carolina (NC), and two from Florida (FL) (Table 2.1). Among the 20 populations collected in 2018, 11 populations were collected from non-Bt maize, and nine were from Bt maize or Bt cotton. For the 11 populations sampled in 2019, seven were from non-Bt maize fields, and four were from Bt maize. Of the 13 populations sampled from Bt crops in the two years, nine

were collected from Genuity VT Double Pro® maize (VT2P), two from Genuity SmartStax® maize (SMT), one from Intrasect maize (ITR), and one from WideStrike® 3 cotton (WS3) (Table 2.1). VT2P maize contains the event MON 89034 trait expressing pyramided Cry1A.105/Cry2Ab2 proteins; SMT possesses MON 89034 and TC1507 (Cry1F protein); ITR produces Cry1Ab and Cry1F proteins; and WS3 cotton expresses three pyramided Bt proteins: Cry1Ac, Cry1F, and Vip3A. These Cry and Vip proteins target lepidopteran moth pests, including *H. zea* (DiFonzo, 2020).

Most *H. zea* populations originated from a collection of ≥50 individuals of 3rd to 6th instar larvae, with a majority of 3rd to 4th instars (Table 2.1). Larvae collected from fields were reared in 30-ml cups (1 larva/cup) containing Ward's *Heliothis* meridic diet (Rochester, NY, USA) as described in Kaur et al. (2019). Pupae that developed from each population were placed into a Seville Classic 20-L cage (Torrance, CA, USA) containing vermiculite and 10% honey water as adult supplementary nutrition. The cages with pupae were placed in an environmentally controlled insect rearing room with 14:10 h (L:D) at 26 ˚C and >70% relative humidity for oviposition (Kaur et al. 2019). Eggs from each population were harvested daily and placed in plastic bags. F1 or F2 neonates derived from the parental populations sampled from fields were used in the laboratory bioassays described below.

2.2.2 Sources of Bt Proteins

Both the Cry1A.105 and Cry2Ab2 proteins were obtained from Bayer Crop Science in St. Louis, MO, USA. Two forms of the same purified Cry1A.105 protein, which was produced from an *E. coli* culture expressing the pMON96851 plasmid (Wu et al., 2009; Kaur et al., 2019), were provided by the company. The first form was provided in 2018, in which the Cry1A.105 protein was solubilized in a buffer at a protein concentration of 1.3 mg/mL with 95% purity. The second form was provided in 2019, in which the same Cry1A.105

protein was lyophilized. The lyophilized Cry1A.105 protein provided by the company was solubilized in the same buffer at a concentration of 1.2 mg/mL prior to being used in the

Table 2.1 Information on *H. zea* Populations Collected from the Southeastern United States during 2018 and 2019

H. zea Population*	Location Collected: City, State	Host Plants	No. Individuals Collected	*H. zea* Stages Collected/Received	Generation Assayed
Populations Collected in 2018					
LA-BR-NBt	Baton Rouge, LA	Non-Bt maize	155	4^{th}-5^{th}	F2
LA-WB-NBt-1	Winnsboro, LA	Non-Bt maize	283	3^{rd}-5^{th}	F2
LA-WB-NBt-2	Winnsboro, LA	Non-Bt maize	120	3^{rd}-5^{th}	F2
MS-ST-NBt-1	Stoneville, MS	Non-Bt maize	129	Pupae (R)	F2
MS-LL-NBt	Leland, MS	Non-Bt maize	98	Pupae (R)	F2
GA-TF-NBt-1	Tifton, GA	Non-Bt maize	192	5^{th}	F1
GA-TF-NBt-2	Tifton, GA	Non-Bt maize	>100	3^{rd}-5^{th}	F1
SC-FR-NBt-1	Florence, SC	Non-Bt maize	89	3^{rd}-5^{th}	F1
SC-FR-NBt-2	Florence, SC	Non-Bt maize	81	4^{th}-5^{th}	F1
SC-DL-NBt-1	Darlington, SC	Non-Bt maize	68	4^{th}	F1
NC-LR-NBt	Lenoir, NC	Non-Bt maize	N/A	F1 neonates (R)	F2
LA-WB-VT2P-1	Winnsboro, LA	VT-2P maize	> 60	3^{rd}-5^{th}	F1
LA-ST-VT2P	St. Joseph, LA	VT-2P maize	65	3^{rd}-5^{th}	F1
LA-WB-SMT	Winnsboro, LA	SmartStax maize	85	3^{rd}-5^{th}	F1
LA-SJ-SMT	St. Joseph, LA	SmartStax maize	> 60	3^{rd} -5^{th}	F1
LA-CF-WS3	Colfax, LA	WideStrike-3 cotton	60	3^{rd}-4^{th}	F1
MS-ST-VT2P-1	Stoneville, MS	VT-2P maize	152	Pupae (R)	F1
MS-LL-ITR	Leland, MS	Intrasect maize	139	Pupae (R)	F1
SC-DL-VT2P-1	Darlington, SC	VT-2P maize	75	5^{th}	F1
FL-SR-VT2P	Santa Rosa, FL	VT-2P maize	148	5^{th}-6^{th}	F2
Populations Collected in 2019					
LA-WB-NBt-3	Winnsboro, LA	Non-Bt maize	950	3^{rd}-5^{th}	F1
LA-AX-NBt	Alexandria, LA	Non-Bt maize	675	3^{rd}-6^{th}	F1

(table cont'd.)

H. zea Population*	Location Collected: City, State	Host Plants	No. Individuals Collected	*H. zea* Stages Collected/Received	Generation Assayed
Populations Collected in 2019					
MS-ST-NBt-2	Stoneville, MS	Non-Bt maize	97	Pupae (R)	F2
AR-TR-NBt	Tillar, AR	Non-Bt maize	95	4^{th}-6^{th}	F1
SC-DL-NBt-2	Darlington, SC	Non-Bt maize	96	4^{th}-6^{th}	F1
NC-LT-NBt	Lillington, NC	Non-Bt maize	52	5^{th}-6^{th}	F1
FL-SR-NBt	Santa Rosa, FL	Non-Bt maize	N/A	F1 neonates (R)	F2
LA-WB-VT2P-2	Winnsboro, LA	VT-2P maize	≈100	3^{rd}-6^{th}	F2
LA-AX-VT2P	Alexandria, LA	VT-2P maize	≈100	3^{rd}-5^{th}	F2
SC-DL-VT2P-2	Darlington, SC	VT-2P maize	65	3^{rd}-5^{th}	F1
MS-ST-VT2P-2	Stoneville, MS	VT-2P maize	98	Pupae (R)	F1

* The first two letters in the name of a field-collected insect population represent the state where the population was collected. The third and fourth letters denote the nearest city to where the population was collected. A population name containing the letters 'NBt' means that the population was collected from non-Bt maize fields, while a population name having the letters 'VT2P', 'SMT', 'ITR', or 'WS3' indicates that the population was sampled from Genuity® VT Double® PRO Bt maize, Genuity®SmartStax® Bt maize, Intrasect® Bt maize, or WideStrike® 3 Bt cotton fields, respectively. The VT2P maize trait contains the event MON 89034 expressing the pyramided Cry1A.105/Cry2Ab2 proteins; SMT contains MON 89034 and TC1507 (Cry1F); ITR produces Cry1Ab and Cry1F; and WS3 cotton contains three pyramided.
*(R): Received.

bioassays. The first form of the Cry1A.105 protein was used in the 2018 bioassays, while the second form was used in the 2019 bioassays. Solubilized Cry2Ab2 that was 87% pure with a total protein concentration of 0.36 mg/mL in buffer was used in the bioassays for populations collected in both 2018 and 2019. Detailed information on the buffers and the procedures in the determination of total protein concentration and Bt protein molecular weight and purity have been described in Wu et al. (2009) and Kaur et al. (2019).

2.2.3 Diet Bioassays

Concentration-responses of the field-collected insect populations, together with a known Bt-susceptible laboratory strain (BZ) of *H. zea,* to the Cry1A.105 and Cry2Ab2 proteins, were determined with a diet-overlay method (Marçon et al., 1999; Kaur et al., 2019). The BZ strain, obtained from Benzon Research Inc. (Carlisle, PA, USA), had been reared with *Heliothis* meridic diet in the laboratory for many generations without exposure to any insecticides or Bt proteins and was susceptible to Cry1A.105 and Cry2Ab2 (Bilbo et al., 2019; Kaur et al., 2019). Two field-collected populations (LA-SJ-SMT and SC-DL-NBt-2) were assayed against Cry1A.105 only, while one population (MS-ST-VT2P-2) was tested against Cry2Ab2 only, and the remaining 28 field-collected populations were assayed against both proteins. Bioassays of the field-collected populations consisted of five to eight concentrations that ranged from 0.1 to 10 μg/cm^2 for Cry1A.105 and 0.01 to 10 μg/cm^2 for Cry2Ab2. Because low larval mortality (< 50%) at 10 μg/cm^2 was observed with many field-collected populations, bioassays with several populations collected later in 2018 included an additional higher Bt concentration, 21.6 or 31.6 μg/cm^2 (Tables 2.2 and 2.3), to obtain more accurate information on the resistance levels. Concentration-responses of population SC-FR-NBt-2 in the bioassay against Cry1A.105 were irregular, and thus data from that bioassay were not included in Table 2. The bioassays with BZ consisted of six to ten concentrations,

ranging from 0.0013 to 10 µg/cm^2. Three independent bioassays for each of the two Bt proteins with BZ were conducted during the study, two bioassays in 2018 and one in 2019.

Protein concentrations were prepared in distilled water containing 0.1% Triton X-100. In the diet-overlay bioassays, 0.8 ml of Southland meridic diet (Lake Village, AR) was dispensed into each well of 16-well plates with syringes. The 16-well plates were made by cutting a 128-well CD-International tray (Pitman, NJ, USA) into eight plates, each representing a replicate. After the diet in wells cooled and solidified, 50 µl of a Cry1A.105 protein solution or 100 or 200 µl of a Cry2Ab2 solution was overlaid on a diet in each well using an Eppendorf Repeater® M4 pipette (Pipett.com, San Diego, CA, USA). A solution of 100 µl of Cry2Ab2 was dispensed into wells for the 10 µg/cm^2 (highest concentration), while a 200 µl volume of Cry2Ab2 solution was used in bioassays with 21.6 µg/cm^2 as the highest concentration. In addition, a blank control with only 0.1% Triton X-100 and a negative control containing the corresponding buffer and 0.1% Triton X-100 were included in the bioassays. After a solution was overlaid, the plates were manually shaken to ensure uniform coverage of the diet surface. Once the solution on the diet surface dried, one newly emerged larva (<24 h old) was released on a diet in each well. Each treatment consisted of four replications, and each replication contained 16 wells in a plate. The assay plates were placed in environmentally controlled incubators with 16:8h (L:D) at 26°C and ~50% relative humidity. The status of larval survival in the bioassays was recorded after 7 d. A measurement of 'practical mortality' as described in Huang et al. (2006), was used to determine the protein toxicity. The practical mortality for replication was computed by dividing the sum of the dead individuals and live larvae with severe growth inhibition (still at the 1st or 2nd instars after 7 d) by the total larvae tested in the replication.

2.2.4 Data Analysis

The observed practical mortality for each replication in a bioassay was adjusted for control mortality (Abbott, 1925). Probit model (PROC PROBIT, SAS Institute, 2010) was used to calculate the LC_{50} values and the associated 95% confidence limits (95% CLs) (Finney, 1971). Concentration responses of BZ in the three bioassays were consistent; thus, data for each protein were pooled in the probit analysis. The Bt resistance ratio for a field population was determined by dividing the LC_{50} of the field-collected population by that of the BZ strain. As previously mentioned, in some bioassays with the field-collected populations, larval mortalities were low (e.g., < 50%) even at the highest Bt concentrations tested (e.g., 10 µg/cm^2); therefore, the corresponding LC_{50} values could not be calculated with the probit analysis and were estimated to be greater than the highest concentrations assayed.

In addition, the mean practical larval mortality of a field-collected population at 10 µg/cm^2 was adjusted based on the control mortality (Abbott, 1925). For each protein, Student t-tests (PROC TTEST, SAS Institute, 2010) were employed to compare the differences between mean mortalities of the populations collected from Bt and non-Bt crop fields or between populations collected during 2018 and 2019. Mortality at 10 µg/cm^2 was chosen for t-tests because of two reasons: 1) this concentration was included in all bioassays, and 2) in most cases, it was the highest concentration used in the bioassays. Based on the bioassay data (see results), it is apparent that the mortality values at 10 µg/cm^2 for the populations that showed <50% mortality at the highest concentration assayed provided more meaningful information about the actual resistance levels than the LC_{50} values (e.g.,>10 µg/cm^2) estimated based on the highest concentration.

2.3 Results

2.3.1 Resistance to Cry1A.105 in *H. zea* Populations from the Southeastern United States

Based on the diet-overlay bioassays, the calculated LC_{50} for BZ was 0.015 µg/cm^{2,} and the related 95% CLs were 0.013 and 0.017. Relative to BZ, two of the 19 field populations sampled in 2018 exhibited a resistance ratio ≤18, which were MS-LL-NBt with a resistance ratio of 4-fold (statistically similar to BZ based on the 95% confidence intervals) and FL-SR-VT2P with a resistance ratio of 18-fold (significantly different from BZ) (Table 2.2). Seven populations (five from non-Bt maize and two from Bt maize) collected during 2018 showed a resistance ratio between 120- and 339-fold (Table 2.2). The remaining ten populations collected during 2018 had resistance ratios of ≥549-fold, with seven of the ten populations having resistance ratios of > 667-fold and all seven populations exhibiting <50% mortality at 10 µg/cm^2. The only population collected from Bt cotton (LA-CF-WS3) had a resistance ratio of 549-fold to Cry1A.105. In the assays where the 31.6 µg/cm^2 were included, four populations, LA-WB-VT2P-1, LA-SJ-VT2P, LA-WB-SMT, and LA-SJ-SMT, exhibited resistance ratios from 578- to >2,107-fold (Table 2.2). Among these, LA-SJ-SMT showed a mortality of 24.3% at 31.6 µg/cm^2, the highest concentration assayed with the population. Among the ten populations collected in 2019, the population MS-ST-NBt-2 had an 88-fold resistance ratio and two other populations, AR-TR-NBt and NC-LT-NBt, exhibited resistance ratios of 199- and 412-fold, respectively. The remaining seven populations had resistance ratios of >667-fold, with larval mortalities of <50% at the highest concentration tested (10 µg/cm^2).

Student t-tests showed that the mean mortality (58.6% ± 8.2, mean ± SEM) at 10 µg/cm^2 of the 10 populations collected from non-Bt maize in 2018 was not significantly different than the mortality (53.1% ± 7.4) of the nine populations from Bt crop fields collected during the same year (equality of variance analysis: $F_{9,8} = 1.37$, $P = 0.6688$ and t-test: $t_{df = 17} = 0.50$,

P = 0.6265). In 2019, the mean mortality for the seven populations collected from non-Bt maize (47.7% ± 9.1) also was not significantly different from that (34.8% ± 10.8) of the three populations collected from Bt maize (equality of variance analysis: $F_{6,2}$ = 1.66, P = 0.8452 and t-test: $t_{df=8}$ = 0.81, P = 0.4392). The overall mean mortality (54.1% ± 6.1) of the 17 populations from non-Bt maize was not significantly different from that (48.5% ± 6.4) of the 12 populations collected from Bt crop fields (equality of variance analysis: $F_{16, 11}$ = 1.28, P = 0.6879 and t-test: $t_{df=27}$ = 0.62, P = 0.5401). Larvae from the 19 populations collected during 2018 and the ten populations from 2019 at10 µg/cm^2 had mean mortalities of 56.0% ± 5.4 and 43.8% ± 7.1, respectively. The 12.2% difference between the two years also was not significant (equality of variance analysis: $F_{18,9}$ = 1.12, P = 0.9051 and t-test: $t_{df=27}$ = 1.33, P = 0.1933).

2.3.2 Resistance to Cry2Ab2 in *H. zea* Populations from the Southeastern United States

The estimated LC_{50} of BZ was 0.069 µg/cm^2 and the associated 95% CLs were 0.06 and 0.08 µg/cm^2 (Table 2.3). Of the 19 populations sampled in 2018, the populations with the lowest LC_{50}s values were MS-ST-NBt-1, MS-LL-NBt, and FL-SR-VT2P, with LC_{50}s of 0.58, 0.22, and 0.14 µg/cm^2, respectively (Table 2.3). Of the remaining 16 field-collected populations, seven exhibited resistance ratios between 17- to 50-fold, four had resistance ratios between 53- to 87-fold, and five exhibited resistance ratios >100-fold, with three of the five (LA-WB-NBt-1, SC-DL-NBt-1, and MS-ST-VT2P-1) exhibiting < 50% larval mortality at the highest Cry2Ab2 concentration (10 µg/cm^2). Therefore, the LC_{50}s of these three populations were >10 µg/cm^2 and equivalent to >145-fold resistance ratio. Among the 10 populations collected in 2019, population MS-ST-VT2P-2 exhibited an LC_{50} of 5.67 µg/cm^2, which is equivalent to an 82-fold resistance ratio. The larval mortality of the remaining nine

Table 2.2 Concentration-Responses and Larval Mortalities (mean ± SEM) for 2018-2019 Field Populations of *H. zea* from the Southeastern United States in Diet-Overlay Bioassays with Cry1A.105.

H. zea Population*	No. Larvae Assayed	Slope ± SE	LC_{50} (95%CL) ‡ (µg/cm^2)	χ^2	*P*-value	Resistance Ratio §	Mortality (%)† at 10 µg/cm^2
SS-BZ	1918	2.76 ± 0.27	0.015 (0.013, 0.017)	46.02	0.0309	N/A	100 ± 0.0
			2018 Field Populations				
LA-BR-NBt	407	0.92 ± 0.15	1.99 (1.17,3.90)	28.73	0.0517	133	79.1 ± 6.1
LA-WB-NBt-1	411	N/A	>10	N/A	N/A	> 667	21.0 ± 9.0
LA-WB-NBt-2	425	0.90 ± 0.12	3.68 (2.46,5.61)	22.30	0.2178	245	68.4 ± 8.9
MS-ST-NBt-1	426	0.82 ± 0.17	2.12 (1.07,5.56)	40.16	0.0020	141	74.9 ± 7.6
MS-LL-NBt	437	1.58 ± 0.46	0.062 (0.006,0.120)	18.45	0.0478	4	100 ± 0.0
GA-TF-NBt-1	426	1.03 ± 0.32	5.08 (2.58,22.10)	17.56	0.0628	339	58.3 ± 10.9
GA-TF-NBt-2	506	N/A	>10	N/A	N/A	>667	35.0 ± 9.1
SC-FR-NBt-1	425	N/A	>10	N/A	N/A	>667	25.3 ± 4.1
SC-DL-NBt-1	389	N/A	>10	N/A	N/A	>667	46.9 ± 3.1
NC-LR-NBt	425	0.94 ± 0.15	1.80 (1.08,3.31)	28.57	0.0539	120	77.2 ± 10.5
LA-WB-VT2P-1	441	1.33 ± 0.15	13.9 (9.9,20.8)	24.75	0.1319	927	35.9 ± 2.7
LA-SJ-VT2P	463	1.47 ± 0.20	19.8 (14.5,30.2)	19.76	0.1380	1320	35.7 ± 3.2
LA-WB-SMT	442	0.64 ± 0.11	8.67 (3.98,31.40)	26.59	0.0871	578	49.9 ± 6.6
LA-SJ-SMT	377	N/A	>31.6	N/A	N/A	>2107	21.5 ± 4.3
LA-CF-WS3	439	1.15 ± 0.19	8.23 (5.39,16.00)	12.06	0.6012	549	51.6 ± 6.7
MS-ST-VT2P-1	413	1.49 ± 0.22	9.41 (5.31,31.30)	35.60	0.0052	627	47.3 ± 5.4
MS-LL-ITR	432	1.28 ± 0.20	2.85 (1.81,5.07)	35.38	0.0085	190	85.6 ± 6.9
SC-DL-VT2P-1	417	1.01 ± 0.13	4.34 (2.89,7.50)	15.19	0.6490	289	63.7 ± 5.0

(table cont'd.)

H. zea Population*	No. Larvae Assayed	Slope ± SE	LC_{50} (95%CL) ‡ (µg/cm^2)	χ^2	*P*-value	Resistance Ratio §	Mortality (%)† at 10 µg/cm^2
2019 Field Populations							
LA-WB-NBt-3	504	N/A	>10	N/A	N/A	>667	46.0 ± 6.6
FL-SR-VT2P	412	1.87 ± 0.21	0.266 (0.202,0.338)	22.16	0.2251	18	86.6 ± 4.6
LA-AX-NBt	506	N/A	>10	N/A	N/A	>667	25.5 ± 3.9
MS-ST-NBt-2	506	1.12 ± 0.16	1.32 (0.90,1.85)	15.5	0.3518	88	85.4 ± 3.0
AR-TR-NBt	493	0.91 ± 0.16	2.99 (1.96,5.19)	12.1	0.6025	199	67.9 ± 2.3
SC-DL-NBt-2	569	N/A	>10	N/A	N/A	>667	17.7 ± 5.2
NC-LT-NBt	505	0.88 ± 0.12	6.18 (3.82, 2.58)	21.9	0.2363	412	57.0 ± 8.9
FL-SR-NBt	243	N/A	>10	N/A	N/A	>667	34.6 ± 4.9
LA-WB-VT2P-2	508	N/A	>10	N/A	N/A	>667	13.2 ± 5.5
LA-AX-VT2P	512	N/A	>10	N/A	N/A	>667	46.0 ± 8.0
SC-DL-VT2P-2	211	N/A	>10	N/A	N/A	>667	45.3 ± 7.8

*BZ was a known Bt-susceptible laboratory strain. Notations for field-collected populations are the same as in Table 1. Bioassays with the population SC-FR-NBt-2 was also performed, but the concentration-responses of SC-FR-NBt-2 in the bioassay against Cry1A.105 were irregular, and thus data from that bioassay were not included in the Table.

† The highest Bt concentrations used in the bioassays was 21.6 µg/cm^2 for LA-WB-NBt-2, 31.6 µg/cm^2 for LA-WB-VT2P-1, LA-SJ-VT2P, LA-WB-SMT, and LA-SJ-SMT. LA-SJ-SMT exhibited a mortality of 23.4 ± 6.1% (mean ± SEM) at 31.6 µg/cm^2, the highest concentration assayed. The highest Bt concentration for all other populations was 10 µg/cm^2.

‡ Larval mortalities for some field-collected populations were < 50% at the highest Bt concentration assayed. The LC_{50}s for these populations were considered to be greater than the highest concentrations used in the bioassay.

§ Resistance ratio of a field population to Cry1A.105 was computed by dividing the LC_{50} value of the population by the LC_{50} of BZ.

Results of the probit analysis have been reported in Kaur et al. (2019).

N/A: not available

populations at the highest Cry2Ab2 concentration tested (10 µg/cm^2) was < 50%, suggesting >145-fold resistance ratios.

Student t-tests showed that the mean mortality (72.2% ± 6.7) at 10 µg/cm^2 of the 11 populations sampled from non-Bt fields during 2018 was similar to that (66.7% ± 8.0) of the eight populations collected from Bt crop fields in the same year (equality of variance analysis: $F_{10,7} = 1.05$, $P = 0.9135$ and *t*-test: $t_{df=17} = 0.53$, $P = 0.6015$). There were no significant differences in mortality between the six populations collected from non-Bt maize fields (13.9% ± 5.4) and the four populations (15.5% ± 12.3) from Bt maize plants during 2019 (equality of variance analysis: $F_{5,3} = 3.45$, $P = 0.2159$ and *t*-test: $t_{df=8} = -0.13$, $P = 0.9014$). The overall mean mortality of the 17 populations collected from non-Bt fields during the two years was 51.6% ± 8.4, which was also similar to the mortality (49.6% ± 9.7) of the 12 populations from Bt crops (equality of variance analysis: $F_{16,11} = 1.05$, $P = 0.9591$ and *t*-test: $t_{df=27} = 0.16$, $P = 0.8756$). However, mortality of the ten populations collected during 2019 was 14.5% ± 5.5, which was significantly less than the mortality (69.0 %± 5.0) of the 19 populations collected during 2018 (Equality of variance analysis $F_{18,9} = 1.59$, $P = 0.4844$ and *t*-test $t_{df=27} = 6.96$, $P < 0.0001$).

2.4 Discussion

A previous study stated that the BZ strain had been reared in the laboratory for many generations, and the results obtained from the associated bioassays may only represent one end of the susceptibility spectrum of *H. zea* (Kaur et al., 2019). However, several recent bioassays have indicated that BZ is still a suitable reference to compare with Bt susceptibilities of field populations. For example, Bilbo et al. (2019) reported that, relative to BZ, several populations collected from maize fields in South and North Carolina during 2017-2018 still exhibited similar or lower susceptibility to Cry1A.105 and Cry2Ab2. In

addition, Guo et al. (2020) observed that ten out of 11 *H. zea* populations collected from seed blend refuges in 2018-2019 were similar or less susceptible to Vip3A than the BZ strain. In this study, BZ exhibited a similar Cry1A.105 LC_{50} to the field population MS-LL-NBt, and a similar Cry2Ab2 susceptibility to the field population FL-SR-VT2P. It should be noted that the field populations assayed in the three studies mentioned here were all sampled from the southeastern region of the U. S. where Cry1A/Cry2A field resistance in *H. zea* has already occurred (Kaur et al., 2019) and the results do not provide evidence of any unusual responses of BZ to either Cry1A.105 or Cry2Ab2.

The bioassay results from this study demonstrate that *H. zea* populations sampled during 2018 and 2019 predominantly exhibited a significant level of Cry1A.105 resistance, and all populations collected during 2019 were resistant to Cry2Ab2. For each of the two Bt proteins, the overall resistance levels were similar between populations collected from non-Bt and Bt fields. A previous study from North and South Carolina reported that *H. zea* populations sampled from maize fields in 2018 were resistant to Cry1A.105 (Bilbo et al., 2019). These results from the current study and Bilbo et al. (2019) indicate that resistance to both Cry1A.105 and Cry2Ab2 in *H. zea* is widely distributed across the southeastern United States.

Kaur et al. (2019) reported that the Cry1A.105/Cry2Ab2 susceptibility levels in *H. zea* populations collected during 2017 from LA, GA, and FL varied greatly, strongly suggesting an uneven distribution of resistance in the region at that time. Similarly, considerable variation in Cry1A.105 susceptibility was also observed among *H. zea* populations sampled in maize fields in North and South Carolina in 2017 (Bilbo et al., 2019). In contrast, most field populations collected during 2018 and 2019 in this study, as well as populations collected during 2018 in Bilbo et al. (2019), demonstrated a significant level of resistance to

Table 2.3 Concentration-Rresponses and Larval Mortalities (mean ± SEM) for 2018-2019 Field Populations of *H. zea* from the Southeastern United States in Diet-Overlay Bioassays with Cry2Ab2

H. zea Population*	No. Larvae Assayed	Slope ± SE	LC_{50} (95%CL) ‡ (µg/cm^2)	χ^2	*P*-value	Resistance Ratio§	% Mortality† at 10 µg/cm^2 §
SS-BZ	1768	2.58 ± 0.24	0.069 (0.060,0.080)	78.63	0.0005	N/A	100 ± 0.0
2018 Field Populations							
LA-BR-NBt	446	1.21± 0.17	3.67 (2.65,5.50)	20.68	0.1102	53	67.2 ± 7.8
LA-WB-NBt-1	398	N/A	>10	N/A	N/A	>145	46.7 ± 4.4
LA-WB-NBt-2#	533	1.16 ± 0.18	3.44 (2.03,6.32)	62.30	<0.0001	50	47.8 ± 5.0
MS-ST-NBt-1	429	1.91 ± 0.18	0.58 (0.46,0.73)	12.42	0.8250	8	100 ± 0.0
MS-LL-NBt	424	2.55 ± 0.30	0.22 (0.18,0.27)	8.99	0.9598	3	100 ± 0.0
GA-TF-NBt-1	480	1.89 ± 0.38	4.74 (2.50,5.87)	22.22	0.0140	69	67.8 ± 2.3
GA-TF-NBt-2	506	1.08 ± 0.24	2.21 (1.20,4.54)	31.80	0.0043	32	80.3 ± 5.3
SC-FR-NBt-1	422	1.19 ± 0.29	8.88 (4.70,38.10)	31.23	0.0051	129	51.8 ± 10.3
SC-FR-NBt-2	425	2.79 ± 0.45	2.84 (2.07,3.99)	31.38	0.0049	41	96.9 ± 1.8
SC-DL-NBt-1	369	N/A	>10	N/A	N/A	>145	46.4 ± 5.5
NC-LR-NBt	445	1.09 ± 0.12	1.16 (0.84,1.63)	13.74	0.7453	17	88.9 ± 4.0
LA-WB-VT2P-1	458	2.01 ± 0.32	6.03 (4.24,8.55)	27.20	0.0182	87	48.2 ± 2.2
LA-SJ-VT2P #	445	2.18 ± 0.42	6.99 (4.64,10.75)	61.60	<0.0001	101	41.2 ± 13.6
LA-WB-SMT	495	1.14 ± 0.10	2.04 (1.51,2.77)	24.09	0.3472	30	76.6 ± 5.3
LA-CF-WS3	532	1.74 ± 0.17	2.15 (1.68,2.77)	11.88	0.8533	31	87.5 ± 2.6
MS-ST-VT2P-1	397	N/A	>10	N/A	N/A	>145	41.6 ± 11.1
MS-LL-ITR	435	1.21 ± 0.13	1.57 (1.16,2.18)	20.47	0.3069	23	81.4 ± 3.2
SC-DL-VT2P-1	364	0.71 ± 0.13	4.43 (2.32,13.60)	17.41	0.3598	64	56.7 ± 8.8
FL-SR-VT2P	441	1.20 ± 0.21	0.14 (0.06,0.24)	32.84	0.0174	2	100 ± 0.0
2019 Field Populations							
LA-WB-NBt-3	538	N/A	>10	N/A	N/A	>145	19.1 ± 2.1
LA-AX-NBt	444	N/A	>10	N/A	N/A	>145	6.7 ± 3.7

(table cont'd.)

H. zea Population*	No. Larvae assayed	Slope ± SE	LC_{50} (95%CL) ‡ (µg/cm^2)	χ^2	*P*-value	Resistance Ratio§	% Mortality† at 10 µg/cm^2 §
SS-BZ	1768	2.58 ± 0.24	0.069 (0.060,0.080)	78.63	0.0005	N/A	100 ± 0.0
MS-ST-NBt-2	574	N/A	>10	N/A	N/A	>145	20.4 ± 9.7
AR-TR-NBt	557	N/A	>10	N/A	N/A	>145	34.8 ± 4/0
NC-LT-NBt	521	N/A	>10	N/A	N/A	>145	0.0 ± 0.0
FL-SR-NBt	532	N/A	>10	N/A	N/A	>145	2.6 ± 2.6
LA-WB-VT2P-2	519	N/A	>10	N/A	N/A	>145	3.5 ± 1.9
MS-ST-VT2P-2	448	1.03 ± 0.16	5.67 (3.77,10.30)	19.76	0.1378	82	52.5 ± 2.8
SC-DL-VT2P-2	567	N/A	>10	N/A	N/A	>145	6.1 ± 2.7
LA-AX-VT2P	512	N/A	>10	N/A	N/A	>145	0.0 ± 0.0

* BZ was a known Bt-susceptible laboratory strain. Notations for field-collected populations are the same as in Table 1.

† The highest Bt concentrations used in the bioassays was 21.6 µg/cm^2 for LA-WB-NBt-2, LA-WB-VT2P-1, LA-SJ-VT2P, and LA-WB-SMT. Highest Bt concentration for all other populations was 10 µg/cm^2.

‡ Larval mortalities for some field-collected populations were < 50% at the highest Bt concentration assayed. The LC_{50}s for these populations were considered to be greater than the highest concentrations used in the bioassay.

§ Resistance ratio of a field population to Cry2Ab2 was computed by dividing the LC_{50} value of the population by the LC_{50} of BZ.

Results of the probit analysis have been reported in Kaur et al. (2019).

N/A: not available.

Cry1A.105. The changes in *H. zea* susceptibility to Cry1A.105 between collections from 2017 and those from 2018 and 2019 suggest that field populations were undergoing significant selection for Cry1A.105 resistance before 2018. On the other hand, the similar resistance levels to Cry1A.105 in the populations collected during 2018 and 2019 observed in this study suggest the resistance to Cry1A.105 now is widely distributed and has likely plateaued in the southeastern United States since 2018.

Compared to the Cry1A.105 resistance, the Cry2Ab2 resistance in *H. zea* appeared to be less pronounced. For instance, 11 of the 19 populations collected in 2018 in this study exhibited a relatively low resistance level (≤ 50-fold) to Cry2Ab2. However, the resistance levels were elevated to >145-fold for 9 of the 10 populations collected in 2019. In Kaur et al. (2019), more than half of the populations from LA, GA, and FL collected during 2017 had a < 20-fold resistance ratio to Cry2Ab2. Additionally, Bilbo et al. (2019) reported that all the *H. zea* populations sampled in 2017 from North and South Carolina were still relatively susceptible with resistance ratios of 0.26- to 11.0-fold; and of the 11 populations collected during 2018, eight populations showed < 20-fold resistance and none had > 50-fold resistance. These results indicated that significant selection for Cry2Ab2 resistance was likely still ongoing in the field but becoming more widely distributed in 2019.

Cry1A and Cry2A proteins have different binding sites (Hernández-Rodríguez et al., 2013); thus, cross-resistance between these two Cry protein groups is uncommon (Huang, 2020). MON 89034 was produced by insertion of a single functional copy of Cry1A.105/Cry2Ab2 at a single locus within the maize genome (USDA-APHIS, 2006). As a result, resistance selection in the fields with MON 89034 always occurs concurrently for the two Bt proteins. In addition, it is well-documented that there is significant cross-resistance among Cry1 proteins (e.g., Cry1A.105, Cry1Ab, Cry1Ac, and Cry1F), as well as between Cry2Ab2 and other Cry2Ae proteins (Hernández-Rodríguez et al., 2008; 2013; Welch et al.,

2015; Yang et al., 2017a). Selection for single gene Cry1 or Cry2 resistance in *H. zea* by previous use of the single-gene Cry crops could have occurred before MON 89034 was released in 2010 (Reisig et al. 2018). Prior selection due to the previous use of single-gene Cry crops and the use of similar Bt proteins in maize and cotton should have been important factors in the resistance development to both the Cry1A.105 and Cry2Ab2.

In conclusion, the resistance of *H. zea* to both Cry1A.105 and Cry2Ab2 is widely distributed across the southeastern United States. Cry1A.105 resistance may have plateaued, while selection for Cry2Ab2 resistance is still underway and could develop to higher levels of resistance in the near future. The documented widespread resistance to Cry1A/Cry2A in *H. zea* represents a great challenge for the sustainability of Bt crop technology. Effective measures to mitigate the Cry1A/Cry2A resistance in *H. zea* need to be developed and implemented to ensure the sustainable use of Bt crop biotechnology.

CHAPTER 3. ESTIMATION OF RESISTANCE ALLELE FREQUENCIES TO CRY1A.105 AND CRY2AB2 IN THE CORN EARWORM (LEPIDOPTERA: NOCTUIDAE) WITH F2 ISOLINES GENERATED FROM A MASS-MATING METHOD

3.1 Introduction

Cry1A.105 and Cry2Ab2 are two *Bacillus thuringiensis* (Bt) insecticidal proteins expressed in the transgenic maize event MON 89034 (US-EPA, 2010). Since their commercial release in 2010, maize hybrids with MON 89034 have been widely planted in the Americas to control above-ground lepidopteran pests, including the corn earworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) (Reay-Jones, 2019; DiFonzo, 2021). During the initial years after commercial release, Cry1A.105/Cry2Ab2 maize hybrids were effective in *H. zea* control (Siebert et al., 2012; Rule et al., 2014; Yang et al., 2014a). However, recent studies have demonstrated that field resistance to Cry1A.105/Cry2Ab2 maize in *H. zea* has occurred widely in the United States (Dively et al., 2016; 2020; Bilbo et al., 2019; Kaur et al., 2019; Yang et al., 2019a,b; Yu et al., 2021). In the southern U.S. Cotton Belt, *H. zea* is also a primary pest species targeted by Bt cotton. Due to the use of the same or similar Bt proteins expressed in transgenic cotton and maize (Huang, 2021), field resistance of *H. zea* to Cry1A/Cry2A cotton varieties has also occurred (Reisig et al., 2018; US-EPA, 2018). The broad occurrence of the Cry protein resistance in *H. zea* has become a major challenge for the U.S. cotton industry (US-EPA, 2018).

Resistance monitoring is a foundation of insect resistance management (IRM) programs (FIFRA Scientific Advisory Panel, 1998). Several methods have been used in Bt resistance monitoring (Huang, 2006). Among these, F2 screen is commonly used to detect heterozygous resistant individuals in field insect populations, especially when the resistance is recessive

(Andow and Alstad, 1998). During the last two decades, F2 screening method has been used in monitoring Bt resistance allele frequencies in field populations of several major pest species targeted by Bt crops (Huang et al., 2006). However, due to the difficulty in the establishment of *H. zea* F2 isoline via single-pair mating, Bt resistance allele frequencies in the insect have so far been investigated in only three studies. Burd et al. (2003) established F1 isolines derived from light-trapped mated *H. zea* females collected in North Carolina and screened the isolines for non-recessive resistance alleles to Cry1Ac and Cry2Ab. Yang et al. (2019b) examined F2 isolines originating from light traps in Texas for resistance alleles to Vip3A. More recently, González et al. (2021) performed an F2 screen with isolines established from light traps and mass mating between known-susceptible males and field-derived females for resistance to Cry1Ac and Cry2Ab2 in populations from Texas. In this study, 103 F2 isolines were successfully established via mass-mating of field-collected *H. zea* populations from four southeastern U.S. states during 2018 and 2019, and these isolines were then screened for resistance alleles to Cry1A.105 and Cry2Ab2, the two Bt proteins expressed in MON 89034. The applied mass-mating method and the results of the F2 screen are reported here, and the related IRM implications are also discussed.

3.2 Materials and Methods

3.2.1 Insect Collection and F2 Isoline Establishment

During 2018 and 2019, approximately 4,500 *H. zea* larvae (3rd to 6th stars) were collected from maize fields at eight sites in four southeastern U.S. states: Louisiana, Mississippi, Georgia, and South Carolina (Table 3.1). Most larvae (~80% of the total) were collected from Louisiana at four sites (Alexandria, Winnsboro, St. Joseph, and Baton Rouge), and the rest were collected near Leland and Stoneville in Mississippi (~10%), Tifton in Georgia (~5%), and Florence in South Carolina (~5%). Field-collected larvae were individually reared on a

meridic diet (Rochester, NY, USA) until pupation. Pupae of a population from the same sampling site were placed in plastic containers in a Seville Classic 20-L cage (Torrance, CA,

Table 3.1 F2 Isolines of *H. zea* Established from Four Southeastern U.S. States for Detection of Resistance Alleles to Cry1A.105 and Cry2Ab2 proteins

Year	State	Collection Site	Host Crops of the Larvae Collected		
			Non-Bt Maize	VT-2P Maize	Total
2018	Louisiana	Winnsboro	25	0	25
		Baton Rouge	2	0	2
		St. Joseph	1	0	1
		Sub-total-LA	28	0	28
	Mississippi	Stoneville	5	2	7
		Leland	3	1	4
		Sub-total-MS	8	3	11
	Georgia	Tifton	5	0	5
	South Carolina	Florence	2	0	2
	Total-2018		43	3	46
2019	Louisiana	Alexandria	57	0	57
Total			100	3	103

USA), and the cages were maintained in an insect rearing room with a photoperiod of 14:10 h (L:D) at 26 °C and >70% r.h. for adult emergence as described in Yu et al. (2021). To ensure similar adult emergence timing, the plastic containers with the remaining pupae were moved to a new cage after 1 to 2 d when a certain number (usually 30-60) of adults had emerged. The procedures were repeated until all adults emerged. After un-emerged pupae from a cage were removed, the adults in the cage were allowed to mass-mate for an additional 2 to 3 d.

To establish F2 isolines, females from the cages (which were assumed to have mated) were individually transferred into 3.8-L paper containers (Huhtamaki Foodservice, De Soto, KS) (1 female per container). The paper containers were then placed in the same insect rearing room used for oviposition. Eggs produced from each female were harvested daily and placed in plastic bags fully inflated with air. Progeny produced from each female was considered an isoline. To determine if multiple mating is common in *H. zea* in the mass-mating conditions used in this study, females that successfully produced F1 isoline progeny were dissected to record the number of spermatophores in the bursa copulatrix (Lum, 1979). F1 neonates hatched from each isoline were individually reared on a liquid diet (Lake Village, AR) in 128-well trays (Pitman, NJ, USA) (1 larva/well) for 7 d, and then 60 to 120 larvae were transferred and reared on a meridic diet (Rochester, NY, USA) in 30-mL plastic cups (Fill-Rite, Newark, NJ) (1 larva/cup) until the late pupal stage. F1 adults of each isoline were sib-mated in a 20-L cage maintained in the same conditions as above. F2 eggs produced from each isoline were collected daily and placed in air-inflated plastic bags. A total of 103 *H. zea* isolines were successfully established via mass-mating for an F2 screen described below.

3.2.2 Screening F2 Neonates of Each Isoline for Resistance Alleles to Cry1A.105 and Cry2Ab2

Cry1A.105 and Cry2Ab2 proteins used in the F2 screen were provided by Bayer Crop Science (St. Louis, MO) (Yu et al., 2021). Based on previous studies (Kaur et al., 2019; Yu et

al., 2021), a concentration of 10 μg/cm^2 of Cry1A.105 or Cry2Ab2 was used as a diagnostic concentration in the F2 screen to detect resistance alleles for each toxin. F2 neonates of 95 of the 103 isolines were screened against both Cry1A.105 and Cry2Ab2. The remaining isolines were screened against either Cry1A.105 (2 isolines) or Cry2Ab2 (6 isolines) only. Thus, there were a total of 198 screens across the two Bt proteins. In 185 screens, 128 F2 neonates were individually screened for each isoline in a 128-well tray (1 neonate/cell), with each cell containing ~0.8 ml of diet overlaid with 10 μg/cm^2 of Cry1A.105 or Cry2Ab2. For the remaining 13 screens, there were 48 to 256 neonates per screen, depending on availability. F2 screening trays were placed in growth chambers at 26 ºC, ~50% r.h, and a photoperiod of 8:16 h (D:L). After 7 d, larval survival and developmental stage (≥ 3rd instars) were recorded. Isolines that had live late-stage larvae (3rd instars or older) after the 7-d screen were considered potential positive lines (PPL) carrying resistance alleles to Cry1A.105 or Cry2Ab2. In addition, baseline larval survival of a population that was generated from F1 neonates of multiple isolines was assayed on a non-Bt diet under the same conditions as described for the F2 screen.

3.2.3 Relationships between Larval Survival Rates on Cry1A.105 and Cry2Ab2 in F2 Screen

As mentioned above, 95 of the 103 *H. zea* isolines were screened against both Cry1A.105 and Cry2Ab2. Linear regression analyses were used to determine if there were a significant relationship between larval survival rates of the 95 isolines on Cry1A.105 and Cry2Ab2 in the F2 screen (PROC REG, SAS Institute, 2016). The survival rate of an isoline against Cry1A.105 was considered as the independent variable (x) and the survival against Cry2Ab2 was treated as the dependent variable (Y). Regression analysis was performed for measurements of total larval survival, as well as for the survival based on the number of live late-stage larvae only.

3.2.4 Establishment of Laboratory Insect Strains from Survivors in F2 Screen for Resistance Confirmation

The F2 screen showed that most of the isolines survived in the resistance screen against either Cry1A.105 or Cry2Ab2 and might carry resistant alleles to at least one of the two Bt proteins (see results). For resistance confirmation, survivors (≥3rd instars) from the F2 screen with each of the two Bt proteins within a crop season were collected and divided into one or two groups (strains) and were individually reared on a diet in 30-ml plastic cups until pupation. Pupae of each strain were transferred to a 20-L cage, and the cage was placed in the insect rearing room maintained under the same conditions as described above. For isolines established during 2018, laboratory strains were generated from the isolines screened against either Cry1A.105 (Cry1A.105-PPL-2018) or Cry2Ab2 (Cry2Ab2-PPL-2018). For isolines established during 2019, two strains were developed from the survivors in the F2 screens against Cry1A.105 (Cry1A.105-PPL-2019-A and Cry1A.105-PPL-2019-B) and one for Cry2Ab2 survivors (Cry2Ab2-PPL-2019). Resistance confirmation of the PPL strains was performed using two methods: diet overlay bioassay and a detached maize ear test.

3.2.5 Resistance Confirmation of PPLs with Diet Overlay Bioassays

To confirm if the PPLs identified in the F2 screen possessed resistance alleles to Cry1A.105 or Cry2Ab2, susceptibility of PPL strains and a known Bt-susceptible laboratory strain (SS-BZ) were assayed using a diet overlay bioassay as described in Kaur et al. (2019). SS-BZ was provided by Benzon Research Inc. (Carlisle, PA, USA) and has been documented to be susceptible to Cry1A.105 and Cry2Ab2 (Kaur et al., 2019; Yu et al., 2021). The concentrations applied in bioassays ranged from 0.1 to 31.6 µg/cm^2 for Cry1A.105 and 0.01 to 31.6 µg/cm^2 for Cry2Ab2. In the bioassay, neonates of each insect strain were individually (1 larva/cell) assayed in 128-well trays in growth chambers at 26 ^{o}C, ~50% r.h., and a photoperiod of 8:16 h (D:L). Practical larval mortalities were recorded after 7 d, as described in Yu et al. (2021). For each Bt concentration and control, there were four replications with

16-32 neonates in each replication. The concentration-mortality data were corrected for control mortality before probit analysis to estimate LC_{50} values and associated 95% confidence limits (95% CLs) (Finney, 1971; PROC PROBIT, SAS Institute, 2016). Resistance ratios for a PPL strain to a Bt protein were determined by dividing the LC_{50} of the PPL by the LC_{50} of BZ-SS. Prior to the resistance confirmation assays, Cry1A.105-PPL-2018 had been selected against Cry1A.105 at 10 µg /cm^2 for two additional generations and Cry2Ab2-PPL-2018 had been selected against Cry2Ab2 at 10 µg /cm^2 for an additional generation. There were no additional selections for the other three PPL strains.

3.2.6 Resistance Reconfirmation on Bt Maize Ears

To determine if the PPLs identified in the F2 screen could survive on maize ears expressing Cry1A.105 or Cry2Ab2, the survival of PPL strains was evaluated on detached ears of Cry1A.105 or Cry2Ab2 maize in the laboratory as described in Guo et al. (2021). A Cry1A.105 single-gene Bt maize line and a Cry2Ab2 single-gene maize line provided by Bayer Crop Science (St. Louis, MO) (Huang et al., 2014) were planted in a greenhouse as described in Ghimire et al. (2011). An ELISA-based assay (EnviroLogix, Quantiplate™ kits, Portland, ME) was conducted to verify Bt protein expression in leaf tissues of the two lines. At the same time, a non-Bt line was planted in the same greenhouse as described in Lin (2021). Maize ears at the R2 growth stage were removed from the greenhouse-grown plants and placed (1 ear/container) in plastic containers (32 cm long × 19 cm wide × 12 cm high) (Guo et al., 2021). In the laboratory, 50 neonates of a PPL strain were manually infested on the silks of each ear. The containers containing ears infested with neonates were placed in an insect rearing room at 24-26 ºC, ~50% r.h., and 16:8 h (L:D) photoperiod. Maize ears were replaced with fresh ears every 7-10 d. Larval survival and development were monitored and recorded until ≥ 5^{th} instars or pupae were observed. A PPL identified in the F2 screens was

considered to carry functional resistance alleles to the Bt maize if its offspring survived and developed to ≥ 5th instars or pupae on the corresponding Bt maize ears (Huang et al., 2007a).

3.2.7 Determination of the Number of Resistance Alleles in Parents of Each Isoline and Estimation of Resistance Allele Frequencies

In the F2 screens, larvae of many *H zea* isolines survived when exposed to Cry1A.105 or Cry2Ab2 and some isolines had a larval survival rate much greater than the expected survival rate (6.25%) for parents possessing a single recessive allele (see results; Huang et al., 2014). To determine the number of resistance alleles carried in the parents of an isoline, larval survival of SS-BZ, a Cry1A.105-resistant genotype (RR-Cry1A.105), and a heterozygous Cry1A.105- resistant genotype (RS-Cry1A.105) were assayed on a diet overlaid with Cry1A.105 protein at 10 µg/cm^2 using the same procedures as described for the F2 screen. Similarly, larval survival of SS-BZ, a Cry2Ab2-resistant genotype (RR-Cry2Ab2), and a heterozygous Cry2Ab2-resistant genotype (RS-Cry2Ab2) was tested against Cry2Ab2 protein at 10 µg/cm^2. The expected number of survivors in the F2 screens for 0, 1, 2, 3, and 4 resistance alleles in the parents of an isoline was calculated based on the Mendelian inheritance model and the observed larval survivorship of the three insect genotypes at 10 µg/cm^2 of Cry1A.105 or 10 µg/cm^2 Cry2Ab2 (Table 3.2). Chi-square tests were utilized to compare the expected survivorship estimated based on the Mendelian model with the observed survivorship of an isoline in the F2 screen to determine the number of resistance alleles possessed in the parents of the isoline (Huang et al., 2014).

Table 3.2 Parent Genotypes and the Expected Genotype Frequency (*F*) in their F2 Progeny of an Isoline

Genotypes of the Two Parents of an Isoline	Expected Genotype Frequency (F) in the F2 Population of an Isoline*		
	F_{SS}	F_{rs}	F_{rr}
ss x ss	1	0	0
rs x ss	0.5625	0.375	0.0625
rr x ss or rs x rs	0.25	0.5	0.25
rr x rs	0.0625	0.375	0.5625
rr x rr	0	0	1

* F_{ss}, F_{rs}, and F_{rr} are the frequencies of homozygous susceptible (ss), heterozygous resistant (rs), and homozygous resistant genotypes in the F2 progeny of an isoline based on the Mendelian inheritance model.

After the number of alleles in the parents of each isoline was determined, a Bayesian statistical method described in Huang et al. (2014) was used to estimate the resistance allele frequencies and 95% credibility intervals (CIs) for *H. zea* populations collected from Louisiana and the three other states for each of the two years.

3.3 Results

3.3.1 Isoline Establishment

Over the two years, a total of 103 *H. zea* F2 isolines were successfully established (Table 3.1). Among these, 85 isolines were from Louisiana, 11 from Mississippi, 5 from Georgia, and 2 from South Carolina. Three of the 11 Mississippi isolines were derived from larvae collected from a Genuity VT Double Pro maize hybrid expressing Cry1A.105 and Cry2Ab2 protein, and the other 100 isolines were derived from larvae collected from non-Bt maize fields. For data presentation, the 103 isolines were divided into three groups: Group 1 consisted of 28 isolines that originated from field collections in 2018 in Louisiana; Group 2 included 18 isolines that were generated from field collections in 2018 in the other three states; and Group 3 contained the other 57 lines that were established from collections in 2019 in Louisiana (Table 3.1).

3.3.2 Number of Spermatophores in Mated *H. zea* Females

During the study, a total of 110 females that were mass-mated and produced viable F1 eggs were dissected to record the number of spermatophores in the bursa copulatrix. The dissection showed that 92.7% (102 females) of these females contained a single spermatophore, 3.6% (4 females) had two, 2.7% (3 females) possessed 3, and 0.9% (1 female) had four spermatophores.

3.3.3 Larval Survival of *H. zea* Isolines in F2 Screen against Cry1A.105

Baseline susceptibility assays showed that the combined field-collected *H. zea* population survived well on the non-Bt diet with overall survivorship of 98.4 ± 1.2% (mean ±

sem), as also reported in Lin (2021). During the two years, a total of 12,168 larvae across 97 *H. zea* F2 isolines were screened for resistance alleles against Cry1A.105, with 46 and 51 lines screened in 2018 and 2019, respectively (Table 3.3). Twenty-eight lines screened in 2018 and the 51 lines screened in 2019 were from Louisiana, and the other 18 lines were from the other three states. Some larvae of all 97 lines screened against Cry1A.105 survived in F2 screens with an overall larval survival rate of 31.3% (Table 3.3). Twenty-six of the 28 lines from Louisiana in 2018 had live late-stage larvae ($\geq 3^{rd}$ instars) with an overall (late-stage) larval survival rate of 14.1%. For the 18 lines collected in 2018 from the other three states, 17 lines had live late-stage larvae with an overall survival rate of 13.2%. Live late-stage larvae were also observed in all 51 lines from Louisiana in 2019, with an average survival rate of 28.5% (Table 3.3).

3.3.4 Larval Survival of *H. zea* Isolines in F2 Screen against Cry2Ab2

During the two years, a total of 12,512 larvae of 101 *H. zea* F2 isolines were screened for resistance alleles against Cry2Ab2, with 46 and 55 lines screened in 2018 and 2019, respectively (Table 3.3). The 46 lines screened in 2018 were the same 46 lines that were screened against Cry1A.105 in 2018. The other 55 lines were all from Louisiana in 2019, in which 49 lines were the same lines that were screened against Cry1A.105 described above. Larvae of 91 out of the 101 isolines survived in F2 screens, with an overall survival rate of 34.4% (Table 3.3). The survival rate based on live late-stage larvae was less for isolines screened in 2018 than those screened in 2019. Twenty of the 28 lines from Louisiana in 2018 had live late-stage larvae with a survival rate of 8.8%. For the 18 lines collected in 2018 from the other three states, 13 lines had late-stage larvae with an overall survival rate of

Table 3.3 Overall Survival of *H. zea* Isolines in F2 Screen on Diet Over-laid with Cry1A.105 and Cry2Ab2 at 10 µg/cm^2

Year	State	Number of Isolines Screened	Total Larvae Screened	Number of Isolines Survived	Total Larval Survival Rate (%)	Number of Isolines Having Late-Stage Larvae	Late-Stage Larval Survival Rate (%)
			F2 screen against Cry1A.105				
2018	Louisiana	28	3,648	28	33.3 (1,179)	26	14.1 (514)
2018	Other three states	18	2,304	18	22.7 (524)	17	13.2 (305)
	2018-total	46	5,952	46	28.6 (1,703)	43	13.8 (819)
2019	Louisiana	51	6,216	51	33.8 (2,100)	51	28.5 (1,771)
Cry1A.105-total		97	12,168	97	31.3 (3,803)	94	21.3 (2,590)
			F2 screen against Cry2Ab2				
2018	Louisiana	28	3,520	25	16.8 (590)	20	8.8 (310)
2018	Other three states	18	2,304	13	9.9 (227)	13	5.8 (134)
	2018-total	46	5,824	38	14.0 (817)	33	7.6 (444)
2019	Louisiana	55	6,688	53	52.1 (3,486)	53	47.6 (3,181)
Cry2Ab2-total		101	12,512	91	34.4 (4,303)	88	29.0 (3,625)

5.8%. In contrast, live late-stage larvae were observed in 53 of the 55 lines screened in 2019, with an overall survival rate of 47.6% (Table 3.3).

3.3.5 Relationship between Larval Survival Rates of *H. zea* Isolines on Cry1A.105 and Cry2Ab2 in F2 Screens

Regression analysis showed that there were significantly positive relationships between the larval survival rates (X) of *H. zea* isolines on Cry1A.105 and the rates (Y) on Cry2Ab2 in F2 screens (Fig. 3.1). The relationship was described by equations Y = 0.167 + 0.540 X (n = 95; R^2 = 0.135; t = 3.18; df = 1; P = 0.0003) based on total larval survival, and Y = 0.120 + 0.775 X (n = 95; R^2 = 0.236; t = 5.36; df = 1; $P < 0.0001$) based on the number of live late-stage larvae.

3.3.6 Susceptibility of PPL Strains to Cry1A.105 and Cry2Ab2

In diet overlay bioassays with Cry1A.105, SS-BZ had an LC_{50} of 0.013 µg/cm^2 with a 95% CI of 0.010 to 0.017 (Table 3.4). All three PPL strains established from the survivors in the F2 screen against Cry1A.105 exhibited significant levels of resistance to the protein. Relative to BZ-SS, Cry1A.105-PPL-2018 showed 2,208-fold resistance. The other two PPL strains, Cry1A.105-PPL-2009-A and Cry1A.105-PPL-2009-B, had a resistance ratio of 228- and 92-fold, respectively. Similarly, in the bioassays with Cry2Ab2, BZ-SS had an LC_{50} of 0.079 µg/cm^2 with a 95% CI of 0.070 to 0.0089 (Table 3.4). Relative to BZ-SS, both PPL strains established from the F2 survivors exhibited significant levels of resistance to the protein, with 57-fold for Cry2Ab2-PPL-2008 and 62-fold for Cry2Ab2-PPL-2019.

3.3.7 Larval Survival of PPL Strains on Bt Maize Ears

On five detached non-Bt ears that were initially infested with 50 neonates of BZ-SS (10 neonates/ear) in the laboratory, one 5th instar, one 6th instar, and one pupa were observed after 22 d of infestation, as also reported in Lin (2021). From five Cry1A.105 ears that were

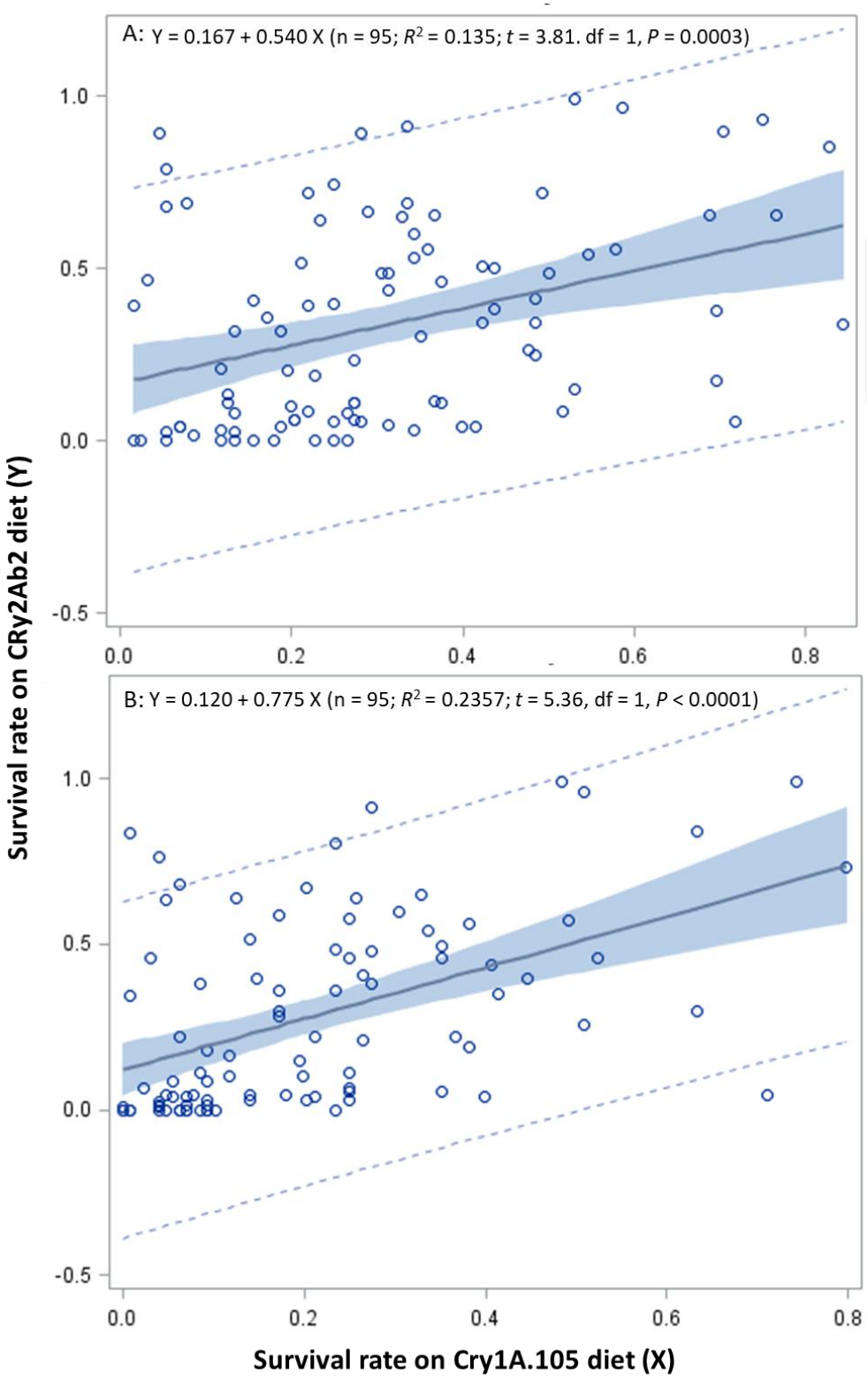

Figure 3.1 Regression Analysis of the Larval Survival Rates of 94 Iso-Line Families of *H. zea* Derived from Field Collections in Louisiana and Three Other Southeastern U.S. States in an F2 Screen on Cry1A.105 Diet at 10 µg/cm^2 (Independent Variable, X) and on Cry2Ab2 Diet at 10 µg/cm^2 (Dependent Variable, Y).

initially infested with 50 neonates of Cry1A.105- PPL-2018/ear, 12 live larvae (mainly 5^{th} instars) were recovered after 37 d. On five Cry1A105 ears that were infested with 50 neonates of Cry1A.105-PPL-2019-A per ear, three $5^{th}/6^{th}$ instars and four pupae were observed after 43 d. On five Cry1A.105 ears that were infested with 50 neonates of Cry1A.105-PPL-2019-B per ear, seven $5^{th}/6^{th}$ instars were found after 44 d. Similarly, from three Cry2Ab2 ears that were infested with 50 neonates of Cry2Ab2-PPL-2018 per ear, five $5^{th}/6^{th}$ instar larvae were recovered after 45 d. From three Cry2Ab2 ears that were infested with 50 neonates of Cry2Ab2-PPL-2019 per ear, two $5^{th}/6^{th}$ instars and five pupae were recovered after 29 d.

3.3.8 Resistance Allele Frequencies to Cry1A.105 and Cry2Ab2

On a diet overlaid with 10 µg/cm2 of Cry1A.105, BZ-SS had 100% mortality, while Cry1A.105-RR and Cry1A.105-RS had survivorship of 68.8 ± 6.8% and 20.3 ± 5.8%, respectively. In assays with 10 µg/cm^2 of Cry1A.105, BZ-SS had survivorship of 3.1 ± 3.1%, while Cry2Ab2-RR and Cry2Ab2-RS had survivorship of 88.9 ± 3.0% and 34.8 ± 2.7%, respectively. The expected number of survivors of an isoline in F2 screen (EXP) was estimated as: EXP = n (0 Fss + 0.203 Frs + 0.685 Frr) against Cry1A.105; and EXP = n (0.031 Fss + 0.348 Frs + 0.889 Frr) against Cry2Ab2. Here, n is the number of F2 neonates of an isoline screened, and Fss, Frs, and Frr are the frequencies of homozygous susceptible (ss), heterozygous resistant (rs), and homozygous resistant genotypes in the F2 progeny of an isoline based on the Mendelian inheritance model (Table 3.2).

Table 3.4 Dose-responses of *H. zea* Potential Positive Lines (PPL) Possessing Resistance Alleles to Cry1A.105 and Cry2Ab2

Insect Strain	Number of Larvae Assayed	Slope ± SE	LC_{50} (95% CI)	Chi-Square	*P*-value	Resistance Ratio[†]
BZ-SS[‡]	1401	2.00 ± 0.27	0.013 (0.010, 0.017)	80.34	<0.0001	----
Cry1A.105-PPL-2018	431	0.74±0.15	28.7 (15.1, 101.0)	5.80	0.9714	2,208
Cry1A.105-PPL-2019-A	409	0.65±0.15	2.97 (1.37, 12.26)	31.23	0.0271	228
Cry1A.105-PPL-2009-B	436	0.64±0.15	1.19 (0.54, 2.14)	20.13	0.1260	92
BZ-SS[‡]	1,258	2.40 ± 0.17	0.079 (0.070, 0.089)	43.60	0.1252	----
Cry2Ab2-PPL-2018	493	2.28±0.33	4.50 (3.25, 6.22)	26.80	0.0205	57
Cry2Ab2-PPL-2019	425	1.40±0.19	4.92(3.61, 7.26)	15.91	0.3191	62

[‡] For each Bt protein, two diet overlay bioassays were conducted, one in 2018 and one in 2019. The overall dose-responses of the two bioassays were similar and thus, data from the two bioassays were pooled in the probit analysis to estimate the LC and 95% CI.

[†] Resistance ratio was computed by dividing the LC_{50} of a PPL strain by the value of BZ-SS.

In F2 screens against Cry1A.105, among the 28 *H. zea* isolines collected in 2018 from Louisiana, 19 lines possessed at least one Cry1A.105 resistance allele, with a total of 37 resistance alleles based on Chi-square tests. The resistance allele frequency to Cry1A.105 was estimated to be 0.333 (Table 3.5). For the 18 isolines from the other three states, a total of 20 resistance alleles were detected with a resistance allele frequency of 0.284. The estimated resistance allele frequencies between the populations collected from Louisiana and the other three states were not significantly different based on overlapping 95% CIs. Thus, the resistance allele frequency to Cry1A.105 based on the combined 46 isolines established during 2018 from the four states was estimated to be 0.312 with a 95% CI of 0.247 to 0.380. For the 51 isolines collected during 2019 in Louisiana, a total of 159 resistance alleles were detected with a resistance allele frequency of 0.490 and a 95% CI of 0.442 to 0.558. The estimated frequencies for the populations collected in 2019 were significantly greater than the frequency estimated in 2018 based on non-overlapping 95% CIs (Table 3.5). For the combined populations collected over the two years, the Cry1A.105 resistance allele frequency was 0.405 with a 95% CI of 0.357 to 0.454.

In F2 screens against Cry2Ab2, among the 28 isolines collected in 2018 in Louisiana, 9 lines possessed at least one resistance allele to Cry2Ab2 with a total of 11 resistance alleles. The resistance allele frequency to Cry2Ab2 for this population was 0.105 (Table 5). For the 18 isolines from the other three states, only 3 lines were identified to carry one resistance allele, with an estimated resistance allele frequency was 0.054. The difference in resistance allele frequencies between the populations collected from Louisiana and the other three states in 2018 was not significant. Based on the combined 46 isolines collected in 2018 from the four states, the resistance allele frequency to Cry1A.105 was 0.081 with a 95% CI of 0.046 to 0.124. For the 55 isolines collected during 2019 in Louisiana, a total of 119 resistance alleles were detected with a resistance allele frequency of 0.541 and a 95% CI of 0.475 to 0.606.

Table 3.5 Frequencies of Resistance Alleles to Cry1A.105 and Cry2Ab2 in *H. zea* Collected from Four Southeastern U.S. States during 2018 and 2019

Year	State	Total Iso-Line Screened	Number of Resistance Alleles in Two Parents of an Isoline						Expected Resistance Allele Frequency (95% CI)
			0	1	2	3	4	Total	
F2 screen against Cry1A.105									
2018	Louisiana	28	2	19	4	2	1	37	0.333 (0.250, 0.422)
2018	Other three states	18	1	14	3	0	0	20	0.284 (0.188, 0.391)
	2018-total	46	3	33	7	2	1	57	0.312 (0.247, 0.380)
2019	Louisiana	51	0	18	31	8	4	100	0.490 (0.442, 0.558)
Cry1A.105-total		97	3	51	38	10	5	159	0.405 (0.357, 0.454)
F2 screen against Cry2Ab2									
2018	Louisiana	28	19	7	2	0	0	11	0.105 (0.056, 0.168)
2018	Other three states	18	15	3	0	0	0	3	0.054 (0.015, 0.115)
	2018-total	46	34	10	2	0	0	14	0.081 (0.046, 0.124)
2019	Louisiana	55	7	7	19	14	8	119	0.541 (0.475, 0.606)
Cry2Ab2-total		101	41	17	21	14	8	133	0.330 (0.285, 0.377)

Similarly, as observed in the F2 screen against Cry2Ab2, the estimated Cry2Ab2 resistance frequencies for the populations collected in 2018 were significantly greater than for the populations collected in 2019 (Table 3.5). For the combined populations collected in the two years, the Cry2Ab2 resistance allele frequency was 0.330 with a 95% CI of 0.285 to 0.377.

3.4 Discussion

An optimal assumption for an F2 screen is that the gametes of an isoline are from a single male and a single female (Andow and Alstad, 1998). As mentioned above, due to the difficulty in the use of single-pair mating to establish *H. zea* isolines (Lin, 2021), in the current study, we applied a mass-mating approach to generate the necessary isolines for the F2 screen. A potential weakness in this approach is the possibility of multiple matings by individuals (Lin, 2021). Previous studies have reported that *H zea* females usually mate only once under natural conditions (Hendricks et al., 1970; Burd et al., 2003). Dissection of the mated females that were used to generate the F2 isolines in the current study showed that 92.7% of females successfully mated only once. In addition, Carpenter (1992) reported that even for multiple mated *H. zea* females, progeny typically originated from a single male (i.e., the first male mated). A similar phenomenon was also observed in multiple-mated females of *Heliothis virescens*, a close relative of *H. zea* (Lamunyon, 2000). Furthermore, as discussed in Lin (2021), even with the use of the mass-mating method, only 5-10% of the females, after mass-mating, successfully produced viable F1 eggs for isoline establishment. Therefore, it is likely that almost all the *H. zea* isolines established in the current study originated from single matings, and thus the issue of multiple mating should not have caused significant errors in the calculation of Bt resistance allele frequencies. The documentation of rare multiple mating of *H. zea* suggests that the mass-mating method applied in the current study is a suitable approach to establish F2 isolines for screening Bt resistance alleles in natural populations of this species (Lin 2021).

Prior to the current study, resistance allele frequencies in *H. zea* for Cry1A.105 had not been evaluated and there was only one study that estimated the resistance allele frequency for Cry2Ab2. González et al. (2021) conducted an F2 screen and estimated that the Cry2Ab2 resistance allele frequency in *H. zea* collected from Texas during 2018 and 2019 ranged from 0.110 to 0.123. The Cry2Ab2 resistance allele frequency estimated in the *H. zea* populations collected during 2018 from the southeastern United States in the current study was similar to the results reported for the Texas populations. However, the resistance allele frequency for Cry2Ab2 for the populations collected during 2019 in Louisiana was greater. In a simultaneous study, the majority of the *H. zea* isolines evaluated in the current study were screened for resistance alleles to Cry1Ab and Vip3Aa20, two common Bt proteins expressed in transgenic maize plants (Lin, 2021). The results showed that the resistance allele frequency to Cry1Ab was high (0.256), while no functional major resistance alleles to Vip3Aa20 were detected in a total of 101 isolines screened (Lin, 2021). Cry1A.105 and Cry1Ab share midgut binding sites (Hernández-Rodríguez et al., 2013), and thus the similar resistance allele frequencies for Cry1A.105 and Cry1Ab observed in the current study and in Lin (2021) were likely due to cross-resistance between the two Bt proteins (Bernardi et al., 2015; Wehch et al., 2015; Niu et al., 2021). The high resistance allele frequencies to both Cry1A and Cry2A detected in the current study and in Lin (2021) further validate that Cry1A/Cry2A resistance in *H. zea* has widely occurred in the region. However, resistance alleles to Vip3Aa20 in *H. zea* were likely rare (Lin, 2012), and thus Bt crop traits containing Vip3Aa should still be effective in controlling Cry1A/Cry2A resistant *H. zea*.

Results of this study showed that resistance allele frequencies to both Cry1A.105 and Cry2Ab2 in the populations collected during 2019 were significantly greater than the frequencies in the populations collected during 2018. Larvae of *H. zea* used to establish F2 isolines in 2018 were collected from four southeastern U.S. states, while all isolines evaluated

in 2019 were sampled from a single site near Alexandria in central Louisiana, a sampling site that was not included in the collections in 2018. Thus, locations of insect sampling could be a factor that contributed to the observed differences in the frequencies. In addition, continued selection pressure could be an important factor in the observed increase in frequencies from 2018 to 2019. Chapter 2 conducted dose-response bioassays and evaluated the susceptibility of *H. zea* populations collected in 2018 and 2019 from similar locations to those sampled in the current study. The dose-response bioassays showed that resistance levels to Cry1A.105 were similar between populations collected in the two years, while resistance levels to Cry2Ab2 in the populations collected in 2019 were considerably greater than those sampled during 2018. Based on these results, Chapter 2 concluded that selection for resistance to Cry2Ab2 appeared to be ongoing in the field, while resistance to Cry1A.105 had likely plateaued. The F2 screening method is thought to be more sensitive than other resistance monitoring methods when resistance alleles are rare. Thus, the F2 screen was originally designed to detect rare recessive resistance alleles in insect populations (Andow and Alstad, 1998). If the observed increase in resistance allele frequencies from 2018 to 2019 was associated with ongoing field selections, the detected significant difference in the Cry1A.105 resistance frequency between the two years indicates that the F2 screening method is more sensitive than the dose-response bioassays even when resistance alleles are already abundant in the field.

The current study also showed that there was a significant correlation between larval survivorship of *H. zea* isolines in F2 screens against Cry1A.105 and Cry2Ab2. With the documented different binding sites in insect midguts between Cry1A and Cry2A (Hernández -Rodríguez et al., 2008; 2013), there is little chance of cross-resistance between Cry1A and Cry2A (Huang et al., 2014; Yang et al., 2017a; Rodrigues-Silva et al., 2019; Wei et al., 2019; Huang, 2021). In MON 89034 maize, the pyramided Cry1A.105 and Cry2Ab2 genes were

inserted into the maize genome just like a single functional gene copy at a single locus (USDA-APHIS, 2006). Thus, resistance selection to Cry1A.105 and Cry2Ab2 in MON 89034 fields takes place simultaneously (Yu et al., 2021). In addition, as shown in the current F2 screen, resistance allele frequencies to both Cry1A.105 and Cry2Ab2 in *H. zea* populations were high (e.g., 0.405 to Cry1A.105 and 0.330 to Cry2Ab2), and thus *H. zea* individuals possessing resistance alleles to both Cry1A.105 and Cry2Ab2 are common. Thus, the observed positive correlation between larval survivorship in F2 isolines against Cry1A.105 and Cry2Ab2 is likely due to multiple resistance rather than cross-resistance. However, low levels of possible cross-resistance between Cry1A and Cry2A in *H. zea* have been suggested in previous studies (Brévault et al., 2013; Wei et al., 2015; 2019; Welch et al., 2015).

In summary, by using a mass-mating method, 103 F2 isolines were established from field collection of *H. zea* in 2018 and 2019 in four southeastern U.S. states. Dissection of females showed that at least 92.7% of these isolines originated from single matings, suggesting that the mass-mating method applied in the study is a suitable approach to establish *H. zea* F2 isolines. F2 screen showed that resistance allele frequencies in these isolines were high for the two Bt proteins expressed in MON 89034 maize hybrids, averaging 0.405 for Cry1A.105 and 0.330 for Cry2Ab2, validating that resistance to Cry1A/Cry2A in the insect is common in the region. In addition, the estimated Bt resistance allele frequencies for populations collected during 2019 were significantly greater than the frequencies for populations from 2018, indicating that selection for the Cry resistance is likely still ongoing in the field. The information generated from this study should be useful in resistance risk assessment, refining resistance modeling, and improving IRM programs to mitigate the challenge of widespread Cry resistance in *H. zea* in the United States

CHAPTER 4. INHERITANCE OF CRY1A.105 AND CRY2AB2 RESISTANCE IN THE CORN EARWORM (LEPIDOPTERA: NOCTUIDAE)

4.1 Introduction

Cry1A.105 and Cry2Ab2 are two pyramided *Bacillus thuringiensis* (Bt) proteins expressed in transgenic maize hybrids containing the MON 89034 event. These Bt maize hybrids were first commercially planted in the Americas in 2010 to control some above-ground moth pests including the corn earworm, *Helicoverpa zea* (Boddie) (US-EPA, 2010). Studies have shown that the MON 89034 maize hybrids were effective against *H. zea* during the first few years of their commercial planting (Siebert et al., 2012; Rule et al., 2014; Yang et al., 2014a; 2014b). However, several recent studies have documented that resistance to the Cry1A/Cry2A maize hybrids has widely occurred in the United States (Dively et al., 2016; 2020; Bilbo et al., 2019; Kaur et al., 2019; Yang et al., 2019; Yu et al., 2021; 2022). In the southern region of the United States, *H. zea* is a cross-crop pest and a pest targeted by both Bt maize and Bt cotton (US-EPA, 2018; Reay-Jones, 2019). Similar to the observed resistance to Bt maize, field populations of *H. zea* have also developed resistance to Cry1A/Cry2A cotton in the region (Reisig et al., 2018). The widespread occurrence of Cry resistance has placed a great challenge to the continued success of the Bt crop biotechnology for *H. zea* control.

Knowledge of genetic bases of resistance is critically important in resistance monitoring, risk assessment, and IRM (Huang et al., 1999). Learning about the inheritance of resistance includes the information about the dominance level, distinguishing of monogenic or polygenic, and sex linkage aspects (maternal effects) of a certain resistant insect population. These inheritance traits of resistance are highly associated with the theories of appropriating preventative management programs for planting Bt crops, such as the use of the ‘high dose/refuge’ IRM strategy (Alstad and Andow, 1995). For these reasons, numerous studies

have been performed to investigate the inheritance of Bt resistance in many insect pest species targeted by Bt crops. However, due to the well-known difficulty in selection and maintenance of Bt-resistant *H. zea* colonies in the laboratory (Anilkumar et al., 2008; Lin et al., 2022), to date, inheritance of Bt resistance in *H. zea* has been evaluated in only two cases, one with a Vip3A resistance and another with Cry2Ab resistance in two Texas populations (Yang et al., 2020; 2021).

By using an F2 screening method described in Chapter 3, a Cry1A.105-single-protein resistant population and a Cry2Ab2-single-protein resistant population of *H. zea* were established from the field-collected individuals in Louisiana in 2018 and 2019 (Yu et al., 2022). Both resistant strains have been documented to possess major resistance alleles that allowed the homozygous resistant individuals to survive and complete larval life cycle (neonate-to-pupa) on the corresponding Bt maize ears (Yu et al., 2022). The availability of these well-documented Bt-resistant populations provided an opportunity to characterize the Cry1A.105 and Cry2Ab2 resistance in *H. zea*. In this study, various genetic crosses and backcrosses were performed between a known-susceptible population and the well-documented resistant populations. Bt susceptibilities of these original, crossed, and backcrossed populations were assayed using diet over-lay bioassays. The inheritance of the Cry1A.105 and Cry2Ab2 resistance was then characterized based on the data generated from the dose-response bioassays.

4.2 Materials and Methods

4.2.1 Insect Sources

By using the F2 screen described in Chapter 3 of this dissertation, a single-protein Cry1A.105-resistant population (Cry1A.105-RR) and a single-protein Cry2Ab2-resistant population (Cry2Ab2-RR) of *H. zea* were established from field collections in Louisiana in 2019 (Tables 4.1 and 4.2). Cry1A.105-RR was established from the iso-line families that

survived in the F2 screen against Cry1A.105, but no survival against Cry2Ab2; vice versa, Cry2Ab2-RR was generated from the iso-line families that survived in the F2 screen against Cry2Ab2 but no survival against Cry1A.105. These two Bt-resistant populations have been documented to carry major resistance alleles to Bt maize that allowed homozygous resistant individuals to survive and complete larval development on the corresponding maize ears expressing Cry1A.105 or Cry2Ab2 protein (Yu et al., 2022). The two Bt-resistant populations, along with a known Bt-susceptible population, BZ-SS, were used as the original insect sources to examine the inheritance of the Cry1A.105-resistance and Cry2Ab2-resistance in *H. zea*. As mentioned above, BZ-SS was obtained from Benzon Research Inc. (Carlisle, PA, USA). It has been documented to be susceptible to Cry1Ab, Cry1A.105, Cry2Ab2, and Vip3A, as well as maize ears expressing one or more of these Bt proteins (Bilbo et al., 2019; Kaur et al., 2019; Guo et al., 2021; Niu et al., 2021).

4.2.2 Genetic Crosses

In this study, a total of two tests (Test-I and Test-II) were performed to assess the inheritance of the Cry1A.105 resistance in *H. zea*. In Test-I, the original Cry1A.105-RR and BZ-SS mentioned above were reciprocally crossed to generate two F1 hybrid populations (Cry1A.105-$F1_a$ and Cry1A.105-$F1_b$) (Table 4.1). Cry1A.105-$F1_a$ was produced by crossing Cry1A.105-RR♂ and BZ-SS♀; and Cry1A.105-$F1_b$ was produced by crossing Cry1A.105-RR♀ and BZ-SS♂. Susceptibilities of these four insect populations to Cry1A.105 protein were examined using diet over-lay bioassays in Test-I to analyze the inheritance of the Cry1A.105 resistance in the Cry1A.105-RR population.

As mentioned above, SS-BZ had been maintained and reared on meridic diet in the laboratory for many years. There was a concern that the genetic background of SS-BZ could be different from that of the Bt-resistant populations that were recently established from the

field collections. Crosses between insect populations with different genetic backgrounds that are not related to the Bt resistance could cause hybrid vigor that could confound the

Table 4.1 Insect Populations used in Characterization of the Cry1A.105 Resistance in *H. zea*

Insect Population	Sources of Insect Populations
Test I with the original Cry1A.105-RR without backcrosses and reselections	
BZ-SS	A known Bt susceptible laboratory strain provided from Benzon Research Inc (Carlisle, PA, USA)
Cry1A.105-RR	A single-gene Cry1A.105-resistant strain isolated from F2 screen of isoline families collected in a maizefield in Louisiana in 2019
Cry1A.105-F1$_a$	A heterozygous Cry1A.105-resistant genotype by crossing Cry1A.105-RR♂ and BZ-SS♀
Cry1A.105-F1$_b$	A heterozygous Cry1A.105-resistant genotype by crossing Cry1A.105-RR♀ and BZ-SS♂
Test II with a backcrossed-and-reselected Cry1A.105-resistant strain	
BZ-SS	A known Bt susceptible laboratory strain provided from Benzon Research Inc (Carlisle, PA, USA)
Cry1A.105-RR'	A backcrossed and reselected Cry1A.105-resistant strain
Cry1A.105-F1'$_a$	A heterozygous Cry1A.105-resistant genotype by crossing Cry1A.105-RR'♂ and BZ-SS♀
Cry1A.105-F1'$_b$	A heterozygous Cry1A.105-resistant genotype by crossing Cry1A.105-RR'♀ and BZ-SS♂
Cry1A.105-F2'	A mixed F2 strain by sib-mating within Cry1A.105-F1'$_a$ and Cry1A.105-F1'$_b$
Cry1A.105-BC'	A mixed strain from reciprocal crosses between BZ-SS and (Cry1A.105-F1'$_a$ + Cry1A.105-F1'$_b$)

assessment of Bt resistance (Huang, 2021). Thus, in Test-II, to ensure a similar genetic background among the insect populations to be evaluated, the original Cry1A.105-RR was crossed and backcrossed with BZ-SS. A Cry1A.105 resistant strain (Cry1A.105-RR') was reselected in the F2 and F3 generations after the backcrosses on Cry1A.105 diet at 10 µg/cm^2. In each resistance reselection, 5-8 neonates were infested on the diet surface of each cell of the 128-cell international trays for 7 days in a total of 4 trays (or ~2,500 to 4,000 neonates). Approximately 300 well-grown larvae were then selected from each selection. The backcrossed-and-reselected resistant population, Cry1A.105-RR', along with BZ-SS was used as the insect sources for genetic crosses in Test-II. Three types of crosses in Test-II were

performed, which consisted of 1) reciprocal crosses between BZ-SS and Cry1A.105-RR' to produce two F1 heterozygous populations (Cry1A.105-F1'$_a$ and Cry1A.105-F1'$_b$); 2) backcrosses of Cry1A.105-F1'$_a$ to BZ-SS to produce a mixed backcrossed population (Cry1A.105-BC'), and 3) sib-mating within Cry1A.105-F1'$_a$ and Cry1A.105-F1'$_b$ to produce a mixed F2 population (Cry1A.105-F2') (Table 4.1). In Test-II, Cry1A.105 susceptibilities of the six populations (BZ-SS, Cry1A.105-RR', Cry1A.105-F1'$_a$, Cry1A.105-F1'$_b$, Cry1A.105-BC', and Cry1A.105-F2') were analyzed using a diet over-lay bioassay described below.

Genetic crosses performed in assessing the inheritance of Cry2Ab2 resistance in *H. zea* were similar as Test-II for the study with Cry1A.105 resistance. To ensure a similar genetic background, the original Cry2Ab2 resistant population established from survivors in the F2 screen (Yu et al., 2022) was crossed and backcrossed with BZ-SS as described in Test-II for Cry1A.105 resistance. A backcrossed-and-reselected resistant population (Cry2Ab2-RR') was established with selections at F2, F3, and F4 for three generations at concentrations of 3-5 µg/cm^2 in diet over-lay bioassays. The number of larvae selected and the selection pressure in each generation were also similar to those used in the Cry1A.105 resistance reselections mentioned above. The backcrossed-and-reselected Cry2Ab2 resistant population, Cry2Ab2-RR', was reciprocally crossed with BZ-SS to generate two F1 heterozygous populations (Cry2Ab2-F1'$_a$ and Cry2Ab2-F1'$_b$) (Table 4.2). Cry2Ab2-F1'$_a$ was the F1 hybrid of the cross between Cry2Ab2-RR'♂ and BZ-SS♀, and Cry2Ab2-F1'$_b$ was the F1 progeny of the cross between Cry2Ab2-RR'♀ and BZ-SS♂. In addition, two F2 populations (Cry2Ab2-F2$_a$ and Cry2Ab2-F2$_b$) were established by sib-mating within each of the two F1' genotypes, respectively. Cry2Ab2-F2$_a$ were generated from sib-mating within Cry1Ab2-F1'$_a$, while Cry2Ab2-F2$_b$ was produced by sib-mating within Cry2Ab2- F1'$_b$ (Table 4.2). Cry2Ab2 susceptibilities of the six populations (BZ-SS, Cry2Ab2-RR', Cry2Ab2-F1'$_a$, Cry2Ab2-F1'$_b$,

Cry2Ab2-F2$_a$ and Cry2Ab2-F2$_b$) were assessed using diet over-lay bioassays described below.

In each genetic cross mentioned above, approximately 50-60 males of an insect strains were mass-crossed with 50-60 females of another strain in a Seville Classic 20-L cage (Torrance, CA, USA) and the cages were placed in the insect rearing room with 14:10 h (L:D) at 26 °C and >70% r.h. for adult mating and oviposition (Yu et al. 2022). Eggs for each insect population were harvested daily and stored in air bags. Neonate larvae hatched from each of BZ-SS, Bt-resistant populations, and each of the crosses were used in diet over-lay bioassays as described below.

Table 4.2 Insect Populations used in Characterization of the Cry2Ab2 Resistance in *H. zea*

Insect Population	Sources of Insect Populations
BZ-SS	A known Bt susceptible laboratory strain provided from Benzon Research Inc (Carlisle, PA, USA)
Cry2Ab2-RR'	A Cry2Ab2-resistant strain isolated from F2 screen of isoline families collected in a maizefield in Louisiana in 2019
Cry2Ab2-F1'$_a$	A heterozygous Cry2Ab2-resistant genotype by crossing Cry2Ab2-RR'♂ and BZ-SS♀
Cry2Ab2-F1'$_b$	A heterozygous Cry2Ab2-resistant genotype by crossing Cry2Ab2-RR'♀ and BZ-SS♂
Cry2Ab2-F2'$_a$	An F2 strain by sib-mating within Cry2Ab2-F1'$_a$
Cry2Ab2-F2'$_b$	An F2 strain by sib-mating within Cry2Ab2-F1'$_b$

4.2.3 Diet Overlay Bioassays with Cry1A.105 or Cry2Ab2 Protein

Cry1A.105 and Cry2Ab2 proteins and the related buffer solutions were provided by Bayer Crop Science (St. Louis, MO) as described in Chapters 2 and 3. Detailed information of the buffers and the procedures in the determination of total protein concentration, Bt protein molecular weight, and purity have been described in Wu et al. (2009) and Kaur et al. (2019).

Each diet over-lay bioassay for BZ-SS consists of series concentrations of Cry1A.105 or Cry2Ab2 protein, ranging from 0.001, 0.00316, 0.01, 0.0316, 0.1, 0.316, 1, 3.16, and10 μg/cm^2, while an additional concentration of 31.6 μg/cm^2 was added in each bioassay for all other insect populations. The Bt protein solutions were prepared with 0.1% Triton X-100 nonionic detergent to obtain uniform spreading over the diet surface. Bioassays were performed in 128-cell trays (CD International, Pitman, NJ) as described in Yu et al. (2021). In the bioassay, approximately 0.8 ml of a liquid diet (Southland Products, Lake Village, AR) was placed into each cell of the 128-cell trays using syringes (Becton, Dickinson and Company, Franklin Lakes, NJ). An amount of 50 μL (for Cry1A.105) or 100 μL (for Cry2Ab2) toxin solution mentioned above was applied to the diet surface in each cell. In addition, each bioassay also included a buffer-treated negative control and a 0.1% Triton-treated blank control. After the Bt solution on the diet surface dried, one neonate (< 24 h) of an insect population was released on the diet surface in each cell. After larval inoculation, cells were sealed with vented lids (C-D International, Pitman, NJ). In each bioassay, there are four replications with 16-32 larvae in each replicate. There were two bioassays for Cry1A.105-RR'; one was conducted along with BZ-SS, and the two F1 hybrid populations, while another was performed along with Cry1A.105-BC' and Cry1A.105-F2'. Bioassay trays were placed in growth chambers at 26 C, ~50% r.h., and a photoperiod of 8: 16h (D:L). Number of dead larvae, number of living larvae that are ≤ 2nd instars, and larval body weight were recorded after 7 days of the neonate release.

4.2.4 Data Analysis

A measurement of 'practical mortality' described in Chapter 2 was calculated based on the formula: practical mortality (%) = 100 x (number of dead larvae + number of living larvae that are ≤ 2nd instars)/total number of larvae assayed (Huang et al., 2006). The observed practical mortality for each replication in a bioassay was corrected to the negative

control mortality (Abbott, 1925). Probit model (PROC PROBIT, SAS Institute, 2010) was used to calculate the LC_{50} values and the associated 95% confidence limits (95% CLs) (Finney, 1971) for each bioassay, except for Cry1A.105-RR'. For Cry1A.105-RR', data collected from the two bioassays were combined, and the combined data were analyzed with the probit model to estimate the LC_{50} value and 95% CI. In addition, based on the observed dose-response data obtained from the diet over-lay bioassays with Cry1A.105 protein, four concentrations (1.0, 3.16, 10.0, and 31.6 µg/cm^2) appeared to be appropriate concentrations for discriminating the three insect genotypes (Cry1A.105-RR, -RS, and -SS). In the bioassays with Cry2Ab2, three concentrations (0.316, 1.0, and 3.16 µg/cm^2) seemed to be suitable to separate the three genotypes (Cry2Ab2-RR, -RS, and -SS). Thus, for each of the tests, corrected larval mortality data observed at these discriminating concentrations were also analyzed with a two-way analysis of variance (ANOVA) with insect populations and Bt concentrations as the two main factors (PROC GLM, SAS Institute, 2010). Treatment means were separated by LSMEANS tests at $\alpha = 0.05$ level. Similarly, as treated in the probit analysis, the mortality data of Cry1A.105-RR' observed at a Bt concentration in the two bioassays were combined for ANOVA.

Maternal effects of the Cry1A.105 and Cry2Ab2 resistance in *H. zea* were examined by comparing the calculated LC_{50}s and larval mortality at each discriminating concentration between the two F1 populations in a Test. Significant differences in the LC_{50} values and larval mortalities at the selected discriminating concentrations between the two F1 hybrid populations in a Test would suggest that resistance was not autosomal. Otherwise, if the LC_{50} values and the larval mortalities were similar between the F1 hybrids, the resistance was considered autosomal and not sex-linked or maternal effects (Huang et al., 1999)

Dominance levels of the Cry1A.105 and Cry2Ab2 resistance in *H. zea* were estimated in two ways: 1) Stone's dominance "D" value (Stone, 1968) referred to here as 'genetical'

dominance level and 2) (functional) effective dominance 'D_{ML}' (Bourguet et al., 2000). The Stone's dominance "D" value was estimated as

$$D = \frac{2X_2 - X_1 - X_3}{X_1 - X_3}$$

Here, X_1, X_2, and X_3 were the log LC_{50} values for the resistant homozygotes (Cry1A.105-RR, Cry1A.105-RR' and Cry2Ab2-RR'), and log LC_{50s} for the heterozygotes (Cry1A.105-F1, Cry1A.105-F1' and Cry2Ab2-F1'), and log LC_{50s} for the susceptible homozygotes (BZ-SS), respectively. The logarithm of doses, having a normal distribution that reflects animal tolerance to toxins, is used to measure the phenotypic value. The *D* value ranges from -1 to 1: a D = 1 indicates that the resistance is completely dominant; 0 < *D* < 1 indicates that the resistance is incompletely dominant, -1 < D < 0 indicates that the resistance is incompletely recessive; D = -1 indicates that the resistance is completely recessive; and D = 0 suggests that the resistance is neither dominant nor recessive (semi-dominant or codominant) (Stone, 1968).

Effective dominance, D_{ML}, is the functional dominance level of survival at a given insecticide dose (Bt protein concentration) (Bourguet et al., 2000). In this study, D_{ML} was measured at each of the selected discriminating concentrations mentioned above using the formula:

$$D_{ML} = (ML_{RS} - ML_{SS}) / (ML_{RR} - ML_{SS})$$

Here, ML_{SS}, ML_{RR}, and ML_{RS} are the practical mortality of SS-BZ, homozygous resistant populations, and the F1 heterozygous populations at a discriminating Bt concentration, respectively. The D_{ML} value ranges from 0 to 1: D_{ML} = 1 suggests that resistance is completely dominant, D_{ML} = 0 means that resistance is completely recessive, 0 < D_{ML}<0.5 represents that resistance is incompletely recessive, and 0.5< D_{ML}<1 denotes that the resistance is incompletely dominant. Because larval mortalities of BZ-SS observed in the diet

over-lay bioassays at 1.0, 3.16, and 10.0 µg/cm^2 of Cry1A.105 were 100% (see results), the larval mortality of BZ-SS at Cry1A.105 concentration of 31.6 µg/cm^2 which was not included in the bioassays was also considered to be 100% in estimating D_{MLs}.

Finally, Chi-square (χ^2) tests were used to determine if the observed mortalities in the F2 and backcross populations at the selected discriminating concentrations fit the single gene Mendelian model (Lande, 1981; Tabashnik, 1991). For the same reason mentioned above, the larval mortality of BZ-SS at 31.6 µg/cm^2 of Cry1A.105 was considered to be 100% in calculating the expected mortality of Cry105-F2' and Cry1A.105-BC' populations in Chi-square tests. If the data fitted the single-gene model, the resistance was considered to be controlled by a single (or a few tightly linked) gene. Otherwise, if the data did not fit the single-gene model, the resistance was considered to be controlled by more than one gene.

4.3 Results

4.3.1 Inheritance of Cry1A.105 Resistance in *H. zea*

4.3.1.1 Resistance Ratio and Maternal Effects of Cry1A.105 Resistance

In Test-I, probit analysis showed that relative to the known Bt susceptible population (BZ-SS), Cry1A.105-RR in diet overlay dose-response bioassays showed a resistance ratio of 2,469-fold (Table 4.4). Cry1A.105-F1$_a$ had a LC_{50} value of 2.49 µg/cm^2 with a 95% CI of 1.87 to 3.35 µg/cm^2 and Cry1A.105-F1$_b$ exhibited a LC_{50} of 1.63 µg/cm^2 with a 95% CI of 0.88 to 2.93 µg/cm^2 (Table 4.4). The difference in LC_{50}s between the two F1 populations was not significant based on their overlapped 95% CLs. In Test-2 with the backcrossed-and-reselected populations, relative to BZ-SS, the resistant population (Cry1A.105-RR') exhibited a resistance ratio of 740-fold (Table 4.4). The LC_{50} values were 9.29 µg/cm^2 with a 95%CI of 6.16 to 16.39 µg/cm^2 for Cry1A.105-F1'$_a$ and 7.04 µg/cm^2 with a 95% CI of 5.39 to 9.68 µg/cm^2 for Cry1A.105-F1'$_b$. The difference in LC_{50}s between the two F1 populations was also not significant based on the overlapped 95% CIs (Table 4.4). In addition, ANOVA

showed that larval mortalities were similar between the two F1 hybrid populations at each of the four discriminating concentrations assayed and for both Test-I and Test-2 (Table 4.3). Thus, the results from both the probit analysis and ANOVA suggest that the Cry1A.105 resistance in *H. zea* was autosomal and not associated with sex-linkage or maternal effects.

Table 4.3 Larval Mortalities (% mean ± sem) of Different *H. zea* Populations at Four Cry1A.105 Concentrations in Diet-Overlay Bioassays [*]

Insect Population	Cry1A.105 Concentration (μg/cm$^{2)}$			
	1.00	3.16	10.00	31.60
		Test-I		
BZ-SS	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a	Not available
Cry1A.105-RR	0.0 ± 0.0 g	17.2 ± 8.7 f	31.3 ± 6.8 ef	60.9 ± 3.0 bcd
Cry1A.105-F1$_a$	33.3 ± 9.2 def	50.8 ± 9.1 cde	79.4 ± 6.0 bc	89.8 ± 1.5 ab
Cry1A.105-F1$_b$	55.2 ± 8.3 cde	65.9 ± 4.0 bcd	68.0 ± 7.8 bc	79.2 ± 4.8 bc
Pooled F1	44.3 ± 7.1	58.3 ± 5.5	73.7 ± 5.0	84.5 ± 3.5
ANOVA	$F_{3,45}$ = 159.76, P < 0.0001 for population; $F_{3,45}$ = 35.91, P < 0.0001 for Bt concentration; and $F_{8,45}$ = 4.64, P = 0.0004 for interaction.			
		Test-II		
BZ-SS	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a	Not available
Cry1A.105-RR'	21.0 ± 2.5 h	28.6 ± 4.6 gh	48.1 ± 2.5 efg	52.8±6.1 efg
Cry1A.105-F1'$_a$	28.3 ± 4.5 gh	28.9 ± 2.7 gh	51.6 ± 4.1 efg	67.2 ± 6.9 b-e
Cry1A.105-F1'$_b$	20.4 ± 6.5 h	35.9 ± 3.0 fgh	60.2 ± 5.5 b-f	68.8 ± 6.8 b-e
Pooled F1'	24.3 ± 4.0	32.4 ± 2.3	55.9 ± 2.6	68.0 ± 4.5
Cry1A.105-F2'	35.2 ± 3.2 fgh	53.6 ± 6.5d-g	60.0 ± 3.5 c-f	84.4 ± 3.1bc
Cry1A.105-BC'	62.5 ± 7.1 b-f	64.1 ± 7.0 b-f	78.9 ± 5.5 bcd	85.9 ± 3.9 b
ANOVA	$F_{5,85}$ = 139.88, P < 0.0001 for insect population; $F_{3,85}$ = 54.04, P < 0.0001 for Bt concentration; and $F_{14,85}$ = 1.84, P = 0.0459 for interaction			

[*] Means followed by the same letter within each test are not significantly different (LSMEANS tests, α = 0.05). If a mean was followed by four or more letters, an abbreviation with only the first and last letters was presented; for example,'b-f' means 'bcdef.'

4.3.1.2 Dominance Levels of Cry1A.105 Resistance

In Test-I, LC_{50} values of the combined F1 hybrid population in Test-I were 1.63 μg/cm^2 with a 95% CI of 0.88 to 2.93. Based on the nonoverlapped 95% CIs, LC_{50}s of the combined F1 populations were significantly greater than the LC_{50}s of SS-BZ but significantly less than the LC_{50}s of Cry1A.105-RR (Table 4.4). The estimated Stone's D value of the Cry1A.105 resistance in Test-I was 0.376. The results-I suggest that the Cry1A.105 resistance in Test-I was (genetically) incompletely dominant (Table 4.5).

In Test-II, LC_{50} value of the combined F1 hybrid population was 8.04 µg/cm^2 with a 95% CI of 6.24 to 10.88, which was significantly greater than the LC_{50}s of SS-BZ, but not significantly different compared to the LC_{50}s of the resistant populations based on the overlapped 95% CIs (Table 4.4). The results suggest that the Cry1A.105 resistance in *H. zea* in Test-II was closer to dominant. Additionally, the estimated Stone's D value of the Cry1A.105 resistance in Test-II was 0.773, also suggesting that the resistance was (genetically) incompletely dominant (Table 4.5).

For both Test-I and Test-II, the effective dominance levels of the Cry1A.105 resistance in *H. zea* decreased as the Cry1A.105 concentrations increased (Table 4.5). In Test-I, D_{ML} at 1.0 and 3.16 µg/cm^2 was 0.557 and 0.507, respective, suggesting a semi-dominant resistance at these two concentrations, while at 10.0 and 31.6 µg/cm^2 D_{ML}s decreased to 0.383 and 0.396, respectively, indicating an incompletely recessive resistance. In Test-II, D_{ML} at 1.0 and 3.16 µg/cm^2 was 0.958 and 0.947, respectively, suggesting a near completely dominant resistance at these two concentrations. At 10.0 and 31.6 µg/cm^2, the dominance level decreased to 0.850 and 0.678, respectively, indicating an incompletely dominant resistance (Table 4.5).

Table 4.4 Concentration-Responses of Different *H. zea* Populations to Cry1A.105 in Diet Overlay Bioassays

Insect Population	No. Larvae Assayed	Slope ± SE	LC_{50}(95%CL) ($\mu g/cm^2$)	χ^2	*P*-value	Resistance Ratio
			Test-I			
BZ-SS	620	1.82 ± 0.20	0.008 (0.006, 0.010)	14.75	0.6785	-
Cry1A.105-RR	640	1.50 ± 0.26	19.75 (13.12, 36.97)	22.29	0.0729	2469
Cry1A.105-F1a	635	1.20 ± 0.11	2.49 (1.87, 3.35)	26.75	0.2208	311
Cry1A.105-F1b	551	0.78 ± 0.12	1.63 (0.88, 2.93)	36.69	0.0118	204
Combined F1	1186	0.82 ± 0.10	1.73 (1.10, 2.55)	63.35	0.0061	217
			Test-II			
BZ-SS	1408	2.75 ± 0.28	0.023 (0.019, 0.028)	28.45	0.0124	-
Cry1A.105-RR’	2285	0.65 ± 0.10	17.01 (10.75, 35.96)	42.99	0.0586	740
Cry1A.105-F1’a	1214	0.94 ± 0.12	9.29 (6.16, 16.39)	28.85	0.0503	404
Cry1A.105-F1’b	1215	1.04 ± 0.10	7.04 (5.39, 9.68)	24.4	0.1423	306
Combined F1	2429	0.99 ± 0.08	8.04 (6.24, 10.88)	54.62	0.0394	350
Cry1A.105-F2’	1204	0.56 ± 0.05	2.13 (1.45, 3.32)	29.49	0.2892	93
Cry1A.105-BC’	1088	0.37 ± 0.07	0.10 (0.02, 0.23)	48.68	0.0045	4

Table 4.5 Effective and Stone's Dominance Levels of the Cry1A.105 Resistance in *H. zea*

Resistant Population	Bt Concentration (μg/cm^2)	Dominance Level	Functional Dominance
Cry1A.105-RR	Stone's D value	0.376	In completely dominant
	1.00	0.557	Codominant
	3.16	0.504	Codominant
	10.0	0.383	Incompletely recessive
	31.6	0.396	Incompletely recessive
Cry1A.105-RR'	Stone's D value	0.773	Incompletely dominant
	1.00	0.958	Near completely dominant
	3.16	0.947	Near completely dominant
	10.0	0.850	Incompletely dominant
	31.6	0.678	Incompletely dominant

4.3.1.3 Test for Fitting a Mendelian Monogenic Model

Chi-square tests showed that the observed larval mortalities of the Cry1A.105-F2 population in Test-I fitted well ($P > 0.05$) with the expected mortalities based on the Mendelian monogenic model at three of the four discriminating concentrations (Table 4.6). In fact, the only significant difference from single-gene model, which was for the F2 population at 31.6 μm/cm^2 0.0314, had a *P* value of 0.0314 in Chi-square tests, which is close to 0.05. In Test-II, observed larval mortalities of the Cry1A.105-BC' population also fitted well ($P > 0.05$) with the expected mortalities based on the monogenic model for all four discriminating concentrations (Table 4.6). Altogether, the results suggest that the Cry1A.105 resistance in *H. zea* was most likely controlled by a single locus.

4.3.2 Inheritance of Cry2Ab2 Resistance in *H. zea*

4.3.2.1 Maternal Effects of the Cry2Ab2 Resistance

In diet over-lay bioassays, BZ-SS exhibited a LC_{50} of 0.071 μg/cm^2. Compared to LC_{50} of BZ-SS, Cry2Ab2-RR' demonstrated a resistance ratio of 34-fold (Table 4.7). Probit analysis

showed that Cry2Ab2-F1'$_a$ exhibited a LC_{50} value of 0.52 µg/cm^2 with a LC_{50} of 0.39 to 0.66 µg/cm^2 and Cry2Ab2-F1'$_b$ had a LC_{50} of 0.77 µg/cm^2 with a 95% CI of 0.62 to 0.97 µg/cm^2 (Table 4.7). The difference in the LC_{50}s between the two F1 hybrid populations was not significant based on their overlapped 95% CLs (Table 4.7). ANOVA showed that larval mortalities between the two F1 hybrid populations were also not significantly different at each of the three discriminating concentrations. Thus, the results suggest that the Cry2Ab2 resistance in *H. zea* was also autosomal and not associated with sex-linkage or maternal effects (Table 4.8).

Table 4.6 Testing for Monogenic Inheritance of the Cry1A.105 Resistance in the Cry1A.105-RR' *H. zea* Population.

Insect Population	Bt Concentration	No. Larvae Assayed	No. Observed Dead Larvae	No. Expected Dead Larvae	χ^2	*P*-Value
Cry1A.105-F2'	1.00	128	45	54.3	2.77	0.0961
	3.16	125	67	60.4	1.40	0.2367
	10.0	119	71	77.3	1.47	0.2253
	31.6	64	54	46.2	4.63	0.0314
Cry1A.105-BC'	1.00	128	80	79.6	0.005	0.9436
	3.16	128	82	84.7	0.25	0.6171
	10.0	128	101	99.8	0.07	0.7913
	31.6	64	55	53.8	0.17	0.6801

Table 4.7 Concentration-Response of Different *H. zea* Populations to Cry2Ab2 in Diet Overlay Bioassays

Insect Population	No. Larvae Assayed	Slope ± SE	LC_{50}(95%CL) (μg/cm^2)	χ^2	*P*-Value	Resistance Ratio
BZ-SS	638	3.97 ± 0.49	0.071 (0.061,0.083)	2.48	0.9911	-
Cry2Ab2-RR'	561	4,11 ± 0.52	2.42 (2.07, 2.83)	6.40	0.7810	34
Cry2Ab2-F1'$_a$	416	1.98 ± 0.22	0.52 (0.39, 0.66)	14.65	0.4027	7
Cry2Ab2-F1'$_b$	1087	1.63 ± 0.13	0.77 (0.62, 0.97)	33.53	0.0548	11
Combined F1'	1503	1.76±0.12	0.66 (0.55, 0.78)	60.86	0.0107	9
Cry2Ab2-F2'$_a$	767	1.52 ± 0.12	1.50 (1.17, 1.92)	28.52	0.1592	21
Cry2Ab2-F2'$_b$	754	1.25 ± 0.11	1.29 (0.96, 1.71)	20.31	0.5635	18
Combined F2'	1521	1.38±0.08	1.40 (1.16, 1.68)	50.19	0.3108	20

4.3.2.2 Dominance Levels of Cry2Ab2 Resistance

The combined Cry2Ab2-F1' hybrid population had a LC_{50} of 0.66 μg/cm^2, which was significantly greater than the LC_{50} of BZ-SS, but significantly less than the LC_{50} of Cry2Ab2-RR'. The results of the dose-response bioassays indicate that the Cry2Ab2 resistance in *H. zea* was non-recessive or non-dominant. The estimated Stone's D value for Cry2Ab2-RR' was 0.264, suggesting that the resistance was 'genetically' incompletely dominant (Table 4.9). The effective dominance levels, D_{MLs}, of the Cry2Ab2-RR' at the three discriminating concentrations also decreased as the Cry2Ab2 concentrations increased. D_{ML} value was 0.733 at 0.316 μg/cm^2, suggesting that the resistance was incompletely dominant at this concentration, 0.489 at 1.0 μg/cm^{2,} indicating that the resistance was nearly semi-dominant; while it was down to 0.186 at 3.16 μg/cm^2, suggesting that the resistance was functional incompletely recessive at this higher concentration (Table 4.9).

Table 4.8 Larval Mortalities (% mean ± sem) of Different *H. zea* Populations at Three Cry1A.105 Concentrations in Diet-Overlay Bioassays*

Insect Population	Cry2Ab2 Concentration (µg/cm^2)		
	0.316	1.00	3.16
SS-BZ	100 ± 0 a	100 ± 0 a	100 ± 0 a
Cry2Ab2-RR'	8.1 ± 4.1hi	6.2 ± 3.7 i	65.5 ± 5.9 cde
Cry2Ab2-F1'$_a$	36.5 ± 9.3 efg	66.7 ± 3.0 cd	95.2 ± 3.0 a
Cry2Ab2-F1'$_b$	28.8 ± 3.5fgh	41.6 ± 4.4 defg	92.0 ± 2.1ab
Pooled Cry2Ab2-F1'	32.6 ± 4.8	54.1 ± 5.3	93.6 ± 1.8
Cry2Ab2-F2'$_a$	9.8 ± 4.9 hi	41.0 ± 4.6 defg	62.3 ± 4.9 cde
Cry2Ab2-F2'$_b$	15.8 ± 3.0 ghi	44.4 ± 4.8 def	74.1 ± 4.8 bc
Pooled Cry2Ab2-F2'	12.9 ± 2.7	42.8 ± 3.1	68.3 ± 3.8
ANOVA	$F_{5,54}$ = 121.18, P < 0.0001 for insect population; $F_{2,54}$ = 141.05, P < 0.0001 for Bt concentration; and $F_{10,54}$ = 8.46, P < 0.0001 for interaction		

* Means followed by the same letter in the table test are not significantly different (LSMEANS tests, α = 0.05).

4.3.2.3 Test for Fitting a Mendelian Monogenic Model

Chi-square tests showed that observed larval mortalities of Cry2Ab2-F2'$_b$ at Cry2Ab2 concentration of 1.0 µg/cm^2 fitted well (P > 0.05) with the single-gene Mendelian model. However, observed mortalities of Cry2Ab2b-F2'$_b$ at the other two concentrations (0.316 and 3.16 µg/cm^2) and for Cry2Ab2-F2'$_a$ at all three concentrations were all significantly different (P < 0.05) from the expected mortalities based on the monogenic model (Table 4.10). The available data indicate that the resistance in Cry2Ab2-RR' was likely controlled by more than one gene.

Table 4.9 Effective and Stone's Dominance Levels of the Cry2Ab2 Resistance in *H. zea* Based on the Larval Mortality in Diet-Overlay Bioassays

Bt Concentration(µg/cm^2)	Dominance Level	Functional Dominance
Stone's D value	0.264	In completely dominant
0.316	0.733	In completely dominant
1.00	0.489	Co-dominant
3.16	0.186	Incompletely recessive

Table 4.10 Test for Monogenic Inheritance of the Cry2Ab2 Resistance in *H. zea*

Insect Population	Bt Concentration (μg/cm^2)	No. Larvae Assayed	No. Observed Dead Larvae	No. Expected Dead Larvae	χ^2	*P*-Value
Cry2Ab2-F2'$_a$	0.316	64	11	29.0	20.4	0.0000
	1	64	29	38.3	5.6	0.0178
	3.16	63	47	56.1	13.5	0.0002
Cry2Ab2-F2'$_b$	0.316	64	9	26,5	19.7	0.0000
	1	64	28	30.3	0.33	0.5657
	3.16	64	41	55.9	31.38	0.0000

4.4 Discussion

Genetic analysis of this study showed that the Cry1A.105 resistance in the Louisiana population of *H. zea* (Cry1A.105-RR) was most likely inherited as a single autosomal gene and the resistance was (genetically) incompletely dominant based on Stone's D value. As mentioned above, prior to the current study, inheritance of Cry1A.105 resistance in *H. zea* has not been investigated yet. However, genetic bases of the Cry1Ac resistance in the old-world bollworm, *Helicoverpa armigera*, has been investigated in at least seven studies including three in China (Xu et al. 2005; Liang et al., 2008; Jin et al., 2013), three in India (Kranthi et al., 2006; Nair et al., 2010; Kaur et al., 2011), and one in Pakistan (Alvi et al., 2012). *H. armigera* in the Old World is a close species to *H. zea* in the New World, and it is the primary insect pest of cotton and targeted by Bt cotton in China, India, and Pakistan. The Cry1Ac resistance in *H. armigera* was found to be inherited as a single autosomal gene for at least six of the seven studies, but the dominance levels estimated at the tested discriminating doses or Bt cotton varied greatly, ranging from completely recessive (in one study) to completely dominant. In addition, another study by Akhurst et al. (2003) also reported that the Cry1Ac resistance in an Australia *H. armigera*

population was incompletely recessive. *H. armigera* is also the primary pest targeted by Bt cotton in Australia.

Cry1A.105 is not a natural protein produced by the *Bt* bacteria. It is a bioengineered chimeric protein consisting of domains I and II from the original Cry1Ab/Cry1Ac, domain III from Cry1F, and the C-terminal domain from Cry1Ac (BCH 2021). Binding sites of Cry1A.105 protein in larval midguts have not been investigated for *H. zea*. Competition binding assays for two other lepidopteran pests, *Ostrinia nubilalis*, and *Spodoptera frugiperda*, showed that the Cry1A.105 shares the same binding sites with Cry1Ab, Cry1Ac, and Cry1Fa proteins in the two species (Hernández-Rodríguez et al. 2013). In addition, cross-resistance between Cry1A.105 and other Cry1A proteins (e.g. Cry1Ab and Cry1Ac) has been well-documented in several pest species targeted by Bt crops, including *H. zea* (Niu et al., 2021; Yu et al., 2021; Lin et al., 2022). Thus, it is reasonable to assume that Cry1A.105 may also share the same midgut binding sites with other Cry1A proteins in *H. zea*, and thus it has a high possibility that the mode of action of Cry1A.105 to *H. zea* is the same or very similar as it observed in *O. nubilalis* and *S. frugiperda*. The similarity in the inheritance between the Cry1A.105 resistance and Cry1Ac resistance observed in the two closely related species may also suggest that the mechanisms of the Cry1A.105 resistance in *H. zea* and Cry1Ac resistance in *H. armigera* are similar. Additional studies are warranted to document this assumption.

As observed in the Cry1A.105 resistance, inheritance of the Cry2Ab2 resistance in the Louisiana population of *H. zea* was also autosomal and (genetically) incompletely dominant based on Stone's D value. However, unlike the Cry1A.105 resistance, the Cry2Ab2 resistance was likely controlled by more than one gene. The results of the current study were similar as the inheritance reported for the Cry2Ab2 resistance in a Texas *H. zea* population (Yang et al., 2020),

suggesting a possible similarity in the resistance mechanisms between Texas and Louisiana populations. In addition, the inheritance of Cry2Ab resistance was also investigated in a laboratory-selected strain of *H. armigera* in Australia, in which the resistance was found to be controlled by a single autosomal and recessive gene (Mahon et al. 2007). Thus, compared to the Cry1A resistance, the inheritance of Cry2Ab2 resistance appeared to be more complicated in the insects. Additional cross studies have been arranged to generate more data so that more concrete conclusions about the inheritance of the Cry2Ab2 resistance in *H. zea* can be made.

As described above, the calculated Stone's D values that were based on the LC_{50} values of the three insect genotypes showed that both the Cry1A.105 and Cry2Ab2 resistances in the Louisiana populations of *H. zea* were (genetically) incompletely dominant. In addition, the estimated effective dominance levels, D_{ML}s, based on the selected discriminating concentrations (1.0, 3.16, 10.0, and 31.6 μg/cm^2 for Cry1A.105 and 0.316, 1.0, and 3.16 μg/cm^2 for Cry2Ab2), showed that both Cry1A.105 resistance and Cry2Ab2 resistance in *H. zea* were functionally non-recessive, ranging from incompletely recessive to incompletely dominant. The reported expression levels of Cry1A.105 and Cry2Ab2 proteins in MON 89034 maize grains (5.9 μg/g for Cry1A.105 and 1.3 μg/g dry weight for Cry2Ab2) (US-EPA, 2010) appeared to be well within the ranges of the selected discriminating concentrations used in the current diet over-lay bioassays. In addition, as mentioned above, Yang et al. (2020) reported the D_{ML}s of the Texas Cry2Ab2 resistant population also varied from incompletely recessive and incompletely dominant at the selected concentrations from 1.0 to 31.6 μg/cm^2, while the resistance was functionally dominant on cotton leaf tissue expressing the Cry1Ab and Cry2Ae proteins. A recent analysis of globally published data showed that all six cases of practical resistance (=field resistance) with field control problems of Bt crops, whose dominance levels had been evaluated,

were associated with functional non-recessive resistance (Huang, 2021). The functional no-recessive property of the Cry1A.105 and Cry2Ab2 resistance in *H. zea* was observed in the current study, and Yang et al. (2020) provided empirical data to demonstrate that the Bt maize plants expressing Cry1A.105 and Cry2Ab2 appeared do not produce the necessary 'high dose' for either of the two proteins to kill >95% heterozygotes (RS) of the pest populations. The non-recessive property of the Cry1A.105 and Cry2Ab2 resistance documented in the current study could be an important factor that has contributed to the widespread occurrence of the Cry protein resistance in *H. zea* in the southern region of the United States. The results suggest that resistance management strategies that are more effective for non-recessive resistance (non-high dose) systems are needed for the sustainable use of the Bt crop technology.

CHAPTER 5. SUMMARY AND CONCLUSIONS

Since 1996, transgenic maize hybrids expressing *Bacillus thuringiensis* (Bt) proteins have been used to protect the crop from damage by the herbivory. The use of Bt crops (e.g., maize and cotton) has dramatically reduced the applications of chemical insecticides in the field. Along with the great economic and environmental benefits gained from the wide adoption of Bt crops, the rapid development of Bt resistance has challenged the sustainable use of transgenic crop technology. To date, resistance to Bt maize and Bt cotton that have led to field control problems has occurred in more than 20 cases, involving almost all major target pest species, all Cry proteins used in Bt crops, and across at least six countries. Cry1A.105 and Cry2Ab2 are two pyramided Bt insecticidal proteins expressed in the maize event MON 89034. This event is among the first commercialized pyramided Bt maize traits targeting above-ground lepidopteran pests, including the corn earworm *Helicoverpa zea* (Boddie). Compared to the earlier transgenic plants that contained only a single Bt gene, the use of pyramided traits expressing more than one Bt protein targeting the same insect was expected to enhance pest control efficacy, broaden the target pest spectrum, and delay resistance evolution.

H. zea is an economically important cross-crop pest species targeted by both Bt maize and Bt cotton in the United States, including Louisiana. Studies reported that MON 89034 maize had been effective in controlling *H. zea* during its initial years of commercial use. However, in recent years, problems in controlling *H. zea* with MON 89034 have frequently been observed, and the field control problems have been documented to be caused by resistance development to the Cry1A and Cry2A proteins in the plants. Further investigations showed that the Cry1A/Cry2A protein resistance has widely occurred in *H. zea* populations. The Cry protein resistance has placed a great challenge to the sustainability of the Bt crop technology in the southern region of

the United States. Thus, effective mitigation programs to manage the Cry protein resistance are urgently needed. The objectives of this study were to 1) determine the current status and distribution of the resistance to Cry1A.105 and Cry2Ab2 in *H. zea* populations in the southeastern United States; 2) estimate the allele frequencies of the two Bt proteins in *H. zea* field populations with F2 isolines generated from a mass-mating method, and 3) assess the inheritance of the Cry1A.105 and Cry2Ab2 resistance in the insect.

To determine the current status and distribution of Cry1A.105 and Cry2Ab2 resistance in *H. zea*, 31 insect populations were collected from major maize planting areas across seven states of the region during 2018 and 2019 and assayed against the two Bt proteins. Diet over-lay bioassays showed that most of the populations collected during the two years were significantly resistant to the Cry1A.105 protein. Most of the populations collected during 2019 were also resistant to Cry2Ab2, while significant variances were observed in the susceptibility of the populations collected during 2018 to Cry2Ab2. The results showed that Cry1A.105 and Cry2Ab2 resistance in *H. zea* is widely distributed in the regions. The resistance to Cry1A.105 appeared to have plateaued, while selection for Cry2Ab2 resistance is likely still occurring. Effective measures to mitigate the Cry1A/Cry2A resistance in *H. zea* need to be developed and implemented to ensure the sustainable use of Bt crop biotechnology.

Resistance monitoring is a foundation of insect resistance management (IRM) programs. Several methods have been used in Bt resistance monitoring. Among these, F2 screen is commonly used to detect heterozygous resistant individuals, especially when the resistance is recessive. During the last two decades, the F2 screening method has been used in monitoring Bt resistance allele frequencies in field populations of several major pest species targeted by Bt crops. However, due to the difficulty in the establishment of *H. zea* F2 isoline via single-pair

mating, Bt resistance allele frequencies in the insect have so far been investigated in only three studies. In this study, 103 F2 isolines were established by mass-mating of *H. zea* populations collected during 2018 and 2019 in four southeastern U.S. states, Louisiana, Mississippi, Georgia, and South Carolina. Dissection of mated females showed that at least 92.7% of the isolines originated from single matings, suggesting that the applied mass-mating method is a suitable approach to establish *H. zea* F2 isolines. F2 screen showed that resistance allele frequencies in these isolines were high to the two Bt proteins expressed in MON 89034 maize hybrids, averaging 0.405 for Cry1A.105 and 0.330 for Cry2Ab2. The observed high resistance allele frequencies confirm that resistance to Cry1A/Cry2A in the insect is common in the southeastern region. In addition, the F2 screen showed that resistance allele frequencies (0.490 for Cry1A.105 and 0.541 for Cry2Ab2) in the populations collected in 2019 were significantly greater than the frequencies (0.312 for Cry1A.105 and 0.081 for Cry2Ab2) in the populations collected in 2018, also indicating that selection for the Cry protein resistance was likely still ongoing in the field.

Knowledge of the genetic bases of resistance is critically important in resistance monitoring, risk assessment, and IRM. Learning about the inheritance of resistance includes the information about the dominance level, distinguishing between monogenic or polygenic, and sex linkage aspects (maternal effects) of a certain resistant insect population. These inheritance traits of resistance are highly associated with the theories of appropriating preventative management programs for planting Bt crops, such as the use of the ‘high dose/refuge’ IRM. For these reasons, numerous studies have been performed to investigate the inheritance of Bt resistance in many insect pest species targeted by Bt crops. However, due to the well-known difficulty in selection and maintenance of the Bt-resistant *H. zea* colonies in the laboratory, to date, inheritance of Bt

resistance in *H. zea* has been evaluated in only two cases, one with a Vip3A resistance and another with Cry2Ab resistance in two Texas populations.

By using an F2 screening method described in Chapter 3 of this dissertation, a Cry1A.105-single-protein resistant population and a Cry2Ab2-single-protein resistant population of *H. zea* were established from the field-collected individuals in Louisiana in 2018 and 2019. Both resistant strains have been documented to possess major resistance alleles that allowed the homozygous resistant individuals to survive and complete larval life cycle (neonate-to-pupa) on the corresponding Bt maize ears. The availability of these well-documented Bt-resistant populations provided an opportunity to characterize the Cry1A.105 and Cry2Ab2 resistance in *H. zea*. In this study, various genetic crosses and backcrosses were performed between a known-susceptible population and the single-protein Cry1A.105 population or single-protein Cry2Ab2 resistant populations of *H. zea*. Susceptibilities of these susceptible, resistant F1, F2, and backcrossed *H. zea* populations to Cry1A.105 and Cry2Ab2 proteins were assayed using diet over-lay bioassays. The results showed that the Cry1A.105 resistance in *H. zea* was inherited as a single, autosomal, and non-recessive gene, while the Cry2Ab2 resistance in the insect was more likely to be autosomal, non-recessive, and polygenic. The non-recessive property of the Cry1A.105 and Cry2Ab2 resistance documented in the study could be an important factor that has contributed to the widespread occurrence of the resistance of *H. zea* to the Cry protein maize/cotton in the southern region of the United States. The results suggest that resistance management strategies that are more effective for non-recessive resistance (non-high dose crop) systems are needed for the sustainable use of the Bt crop technology. The information generated from this study is useful in resistance risk assessment, refining resistance modeling, and

improving IRM programs to mitigate the great challenge of the Cry protein resistance in *H. zea* for the sustainable use of Bt crop technology in the southern United States.

APPENDIX. COPYRIGHT INFORMATION FOR CHAPTER 1

Extended investigation of field-evolved resistance of the corn earworm Helicoverpa zea (Lepidoptera: Noctuidae) to Bacillus thuringiensis Cry1A.105 and Cry2Ab2 proteins in thesoutheastern United States

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