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Risk Assessment of Fall Armyworm Resistance To Transgenic Corn Containing Single or Pyramided Bacillus Thuringiensis Genes

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RISK ASSESSMENT OF FALL ARMYWORM RESISTANCE TO TRANSGENIC CORN CONTAINING SINGLE OR PYRAMIDED *BACILLUS THURINGIENSIS* GENES

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is a target pest of *Bacillus thuringiensis* (Bt) crops in North and South America. However, after more than two decades of the intense use of Bt crop technology, *S. frugiperda* has developed resistance to Bt corn in several countries. The objectives of this study were: 1) to characterize the inheritance and fitness costs of Cry1A.105 resistance in *S. frugiperda*; 2) to evaluate larval survival and plant injury of Cry1A.105-susceptibile, -resistant, and -heterozygous *S. frugiperda* populations on transgenic corn plants containing single or pyramided Bt genes; 3) to estimate the frequency of Cry2Ab2 resistance alleles in field populations of *S. frugiperda* in the U.S. southern region; and 4) to evaluate the phenotypic performance of different genotypes of *S. frugiperda* possessing single- or dual-Cry1A.105/Cry2Ab2 resistant genes on MON 89034 corn. Cry1A.105 and Cry2Ab2 are the two Bt proteins expressed in the Bt corn event MON 89034, a common Bt corn trait. The results showed that Cry1A.105 resistance in *S. frugiperda* was inherited as a single autosomal and non-recessive gene with no fitness costs. Three commonly planted pyramided Bt corn traits (Genuity® VT Double Pro™, SmartStax™, and Agrisure® Viptera™ 3111) were highly effective against the Cry1A.105-resistant *S. frugiperda*. Frequency of major Cry2Ab2 resistance alleles in field *S. frugiperda* populations was estimated to be 0.0023 in the U.S. southern region. One F_2 family from Georgia was confirmed to possess a major resistance allele to Cry2Ab2. A Cry1A.105/Cry2Ab2 dual-gene resistant *S. frugiperda* strain was generated by crossing the Cry1A.105-resistant and Cry2Ab2-resistant strains. Insect survival, growth, development, pupation, and reproduction of nine genotypes of *S. frugiperda* possessing Cry1A.105/Cry2Ab2 resistant alleles were evaluated on leaf tissue and whole plants of MON 89034. The nine insect genotypes were a Cry1A.105/Cry2Ab2-susceptible (aabb), a Cry1A.105 resistant/Cry2Ab2-

susceptible (AAbb), a Cry1A.105-susceptible/Cry2Ab2-resistant (aaBB), a Cry1A.105/Cry2Ab2-resistant (AABB), and five heterozygous (AaBb, AABb, AaBB, Aabb, aaBb) genotypes that were produced from various crosses among aabb, AAbb, aaBB, and AABB. Laboratory bioassays and greenhouse tests exhibited that AABB was highly resistant to MON 89034. Genotypes containing one or two resistance alleles were overall susceptible to MON 89034, while those possessing three resistance alleles exhibited a significant level of resistant to the Bt plants. Information generated from this study should be valuable in assessing resistance risk, refining resistance management modeling, and developing resistance management strategies for the sustainable use of the Bt corn technology.

CHAPTER 1. INTRODUCTION

1.1 Corn Production in the United States

Field corn (*Zea mays* L.) is the most widely planted field crop in the United States. In 2017, corn acreage in the United States was 90.9 million acres and the total harvest was 83.5 million acres with $48.5 billion production value (NASS, 2018). Forty-eight states in the United States planted corn in 2017; among these the states in the Corn Belt dominated the major corn planted acres. Illinois, Iowa and Nebraska were the top three states in corn production in United States (NASS, 2018). Corn also contributes significant value to the agriculture in Louisiana, which is usually the second most widely planted field crops following soybean in the state. In 2017, a total of 470,000 acres of corn was planted in Louisiana with a production value of $333.6 million (NASS, 2018).

1.2 Major Corn Insect Pests

There are various arthropod pests that damage field corn. Lepidopteran species are the major above-ground pests of corn plants, while coleopteran species are the most important pests that attack below ground plant tissues. The major lepidopteran species which damage corn in the U.S. mid-southern region include the corn earworm (*Helicoverpa zea* (Boddie)), fall armyworm (*Spodoptera frugiperda* (J.E. Smith)) and a complex of corn stalk borers (Siebert et al., 2012). Across the north central and mid-western region, European corn borer (*Ostrinia nubilalis* (Hubner)) and southwestern corn borer (*Diatraea grandiosella* Dyar) are the two major corn borer species (Ostlie et al., 1997; Huang et al., 2011). Yield losses of traditional non-Bt corn by a corn borer complex of the sugarcane borer, *Diatraea saccharalis* (F.) and *D. grandiosella* are estimated at up to 28% in mid-southern states (Sankula and Blumenthal, 2004). Recently, it is also reported that *S. frugiperda* infestations occur frequently across the southern region of the

U.S. in conventional non-Bt and Bt corn varieties, especially when fields are planted after the optimum seeding dates (Hardke et al., 2011).

1.3 *Spodoptera frugiperda* (J. E. Smith)

S. frugiperda has historically been one of the most common pests of field corn in the southern U.S. (Pitre and Hogg, 1983; Buntin, 1986; 2004). Just like any other Lepidoptera, it has four stages in its life cycle including egg, larval, pupa and adult stages. *S. frugiperda* has a wide host range of more than 80 plant species, including many major field crops such as corn, cotton, soybeans, sorghum, rice, alfalfa, and many vegetable plants (Winter, 2010). *S. frugiperda* is susceptible to cold and in the mainland U.S. it just can overwinter in southern Texas and Florida (Sparks, 1979). Each year, populations of *S. frugiperda* migrate from areas including south Florida, Caribbean islands, south Texas, Mexico, or Central America (Sparks, 1979; Adamczyk et al., 1997; Buntin, 1986) with the adults ovipositing on seasonal hosts during a northerly migration. Traditional control strategies such as chemical and cultural control are not effective and often provide unsatisfactory suppression of *S. frugiperda* in field corn. For example, almost immediately after larval hatching, neonates move into the whorl region of corn plants where they are protected from foliar insecticide sprays (Harrison, 1986; Castro, 2002; Bokonon-Ganta et al., 2003; Siebert et al., 2008a). Those insecticides which are generally efficient against other pests, such as the corn earworm, typically provide poor control of *S. frugiperda* (Young, 1979; Guillebeau and All, 1990). Regional populations of *S. frugiperda* have developed resistance to several classes of insecticides including carbamates, organophosphates, and pyrethroids (Adamczyk et al., 1999). Currently, transgenic corn products containing *Bacillus thuringiensis* (Bt) proteins have become a more viable option for controlling *S. frugiperda*.

1.4 Transgenic Bt Corn Technology

Bacillus thuringiensis (Bt) is a rod shaped soil bacterium that produces specific crystalline (Cry) endotoxin during the reproductive stages and vegetative insecticidal proteins (VIP) during the vegetative growth stages that are toxic to specific insect species (Vaeck et al., 1989; Gasser and Fraley, 1989). Bt proteins were used as microbial pesticides under various trade names including Sporeine®, Thuricide®, Able™, Biobit®, and Dipel® to control many crop pests (Baum et al., 1999; Kaur et al., 2000; NPTN, 2000). Bt pesticides are considered as friendly to the environment, people, soil decomposers, pollinators, parasitoids, and wildlife. Bt toxins are highly diverse, highly effective, and relatively cheap. These merits have made it widely used all over the world for controlling lepidopteran, coleopteran larvae and several dipteran pests (Baum et al., 1999; Kaur, 2000).

The major target pests of Bt toxins are specific insect species. Bt bacteria produce proteinaceous parasporal crystalline inclusions. Upon ingestion by the insects, these Bt inclusions are solubilized in the midgut, releasing proteins called delta-endotoxins. The Cry toxin is then inserted into the insect gut cell membrane, paralyzing the digestive tract and forming a pore, which makes the insect stop eating and starve to death (Dean, 1984).

Bt crops are the plants that have been bioengineered to express the Bt proteins. Since first being commercialized in 1996, Bt crops have gained an international attention and acceptance worldwide, especially among the U.S. corn and cotton producers. The first generation transgenic Bt corn hybrids (e.g. YieldGard® Corn Borer) were commercially planted in the U.S. in 1996. Since then, adoption of Bt corn has greatly increased because of the high efficacy against target pests (e.g. stalk borers) and ease-of-use for producers. The acreage of Bt corn cultivars has increased rapidly in the U.S. and several other countries in the world (Huang et al., 2014). In

2016, Bt crops were planted over 98.84 Mha in 23 countries, including 53.95 Mha of Bt corn, 21.34 Mha of Bt cotton and 23.55 Mha of Bt soybean worldwide (James, 2017). For the first time in 2016, Brazil exceeded the United States to rank the first in acreages to plant Bt crops in the world. Bt corn hybrids initially aimed to reduce injury from corn stalk borers such as the European corn borer and Southwestern corn borer (Abel et al., 2000; Buntin et al., 2004; Castro et al., 2004b). Although the primary targets are corn stalk borers, Bt corn expressing Cry1Ab protein also suppresses foliar damage from corn earworm, but it is not very effective against *S. frugiperda*. These two species can be important in yield- and quality-limits in the southern U.S. corn fields (Buntin et al., 2004; Chilcutt et al., 2007). Since 2003 when Cry1F corn, event 1507, became commercially available, *S. frugiperda* has been listed as a target pest of Bt corn in both North and South America (Storer et al., 2010; Farias et al., 2014). Currently, *S. frugiperda* is also listed as a target pest in all commercial pyramided Bt corn and cotton varieties that target Lepidopteran species.

1.5 Bt Resistance

Resistance development in target pest populations has been a big challenge for the sustainable use of transgenic Bt crops (Alstad and Andow, 1995; Ostlie et al., 1997; Gould, 1998; Tabashinik et al., 2008). Resistance to Bt insecticides were earlier detected and reported in field populations of the diamondback moth, *Plutella xylostella* (L.) in the U.S. (Tabashnik, 1994), and cabbage looper, *Trichoplusia ni* (Hubner) in Canada (Kain et al., 2004). Major resistance genes to Bt crops have been found in laboratory selections in the tobacco budworm, *Heliothis virescens* (F.), (Gould et al.,1995; 1997), pink bollworm, *Pectinophora gossypiella* (Saunders) (Tabashnik et al., 2000), poplar leaf beetle, *Chrysomela populi* (L.) (Génissel et al., 2003), *D. saccharalis* (Huang et al., 2007a; 2007b; 2008; 2009), *O. nubilalis* (Pereira et al., 2008), *H. zea* in the U.S

(Tabashnik et al., 2008; Moar et al., 2008) and *Helicoverpa armigera* (Hübner) in Australia (Akhurst et al., 2003; Downes et al., 2007; Mahon et al., 2007) and China (Li et al., 2004; Xu et al., 2009).

A few early studies have evaluated the field efficacy of transgenic Bt corn against *S. frugiperda* (Buntin et al., 2000; 2004; Buntin, 2008; Siebert et al., 2008a). *S. frugiperda* can survive well on traditional non-Bt corn and even on the Cry1Ab corn varieties across the U.S. southern region. *S. frugiperda* is one of the notable target pests which have evolved several cases of field resistance that resulted in control failures with Bt crops in the world. The first report of field-derived resistance was the *S. frugiperda* population in Puerto Rico to Cry1F expressing corn in 2006 (Matten et al., 2008; Tabashnik et al., 2008; Storer et al., 2010). Then other three cases of field resistance of *S. frugiperda* to Cry1F Bt corn were also reported in Brazil (Farias et al., 2014), the U.S. mainland (Huang et al., 2014), and recently in Argentina (Chandrasena et al., 2018).

1.6 Bt Resistance Management

To delay resistance development, the U.S. and Canada as well as a few other countries have implemented an insect resistance management (IRM) plan named the 'high dose/refuge' strategy for planting Bt crops (Ostlie et al., 1997; Gould, 1998; Baute, 2004). This strategy firstly aims to use "high-dose" Bt plants to kill ≥ 95% resistant heterozygotes of the target pests (FIFRA Scientific Advisory Panel, 1998; U.S. EPA, 2001). The "high dose" property can prevent the resistance alleles of the heterozygous insects to be transmitted into the next generation. Secondly, a certain size of area is planted of non-Bt varieties that serve as refuge for the susceptible insects. The susceptible insects emerged from the non-Bt plants should mate with the rare resistant homozygous individuals that have survived from the Bt crop. If the frequency

of resistance is very low (e.g. <0.001), majority of offspring carrying resistance alleles will be heterozygous and the heterozygotes should be killed by the "high does" Bt crops (Huang et al., 2011). Through this strategy, the resistance allele frequency in the target pest populations can be maintained at low levels for a long-period of time.

One of the key assumptions of the 'high dose/refuge' IRM strategy is that the Bt corn plants must produce a "high dose" to be able to kill the resistant heterozygotes to prevent the resistance alleles passing to the next generations. In other word, the resistance should be functionally recessive (Huang et al., 2011). Thus, understanding the genetic basis of resistance in target pest is essential for IRM. In 2011, we isolated two Cry1A.105-resistant *S. frugiperda* strains using an F_2 screen with field populations collected from south Florida (Huang et al., 2016). These two populations have demonstrated significant level of resistance to purified Cry1A.105 protein and whole plants of Cry1A.105 corn. The objective 1 of this study is to characterize the inheritance and fitness costs of Cry1A.105 resistance in *S. frugiperda*.

In addition to the "high dose/refuge" strategy mentioned above, a gene-pyramiding strategy has also been utilized to develop transgenic plants that express multiple Bt toxins for targeting a same insect pest (Ghimire et al., 2011). Since 2010, these second generation Bt corn hybrids expressing pyramided Bt genes (e.g. Genuity®SmartStax™; Agrisure® Viptera™ 3111) have been available for controlling both above- and below-ground insect pests in the U.S. The use of pyramided Bt corn hybrids is expected more powerful to delay resistance development in target pests populations. Because pyramided Bt crops express two or more Bt proteins with dissimilar mode of action for a target pest, and thus once one of the proteins is out of control for the pest, the remaining proteins can still take effect. A recent study showed that several pyramided Bt corn products are effective against the Cry1F-resistant *S. frugiperda* and thus these

pyramided traits should provide a means for Cry1F resistance management in the insect (Niu et al., 2013; 2014).

Up to now, based on the mode of action or cross-resistance patterns, all Bt proteins targeting moth pests expressed in corn can be categorized into only three groups 1) Cry1 which including Cry1Ab, Cry1Ac, Cry1F, and Cry1A.105, 2) Cry2Ab2, and 3) Vip3A. As mentioned above, field resistance in *S. frugiperda* to Cry1F has occurred in several regions of the world (Storer et al., 2010; 2012; Huang et al., 2014). In addition, studies have shown that some levels of cross resistance existed in *S. frugiperda* between Cry1F and Cry1A (Huang et al., 2014; Niu et al., 2013). Cry1A.105 is one of the two Bt proteins expressed in the Bt corn event MON89034, which is one of the most used Bt traits in the current planted pyramided Bt corn products. Objective 2 of this study was to evaluate the larval survival and plant injury of Cry1A.105-susceptibile, -resistant, and -heterozygous populations of *S. frugiperda* on transgenic corn products containing single or pyramided Bt genes. Data generated from this study should be useful to determine the cross-resistance pattern of the Cry1A.105 resistance to other major Bt corn traits.

Another Bt gene in MON89034 is Cry2Ab2. Compared to Cry1 proteins (e.g. Cry1Ab, Cry1Ac, Cry1F), Cry2Ab2 has different binding sites in insect midguts and thus it has a dissimilar mode of action (Storer et al., 2012). Since 1999, pyramided Bt cotton containing Cry2Ab2, along with Cry1Ac, has been commercially planted in the U.S. and Australia. As mentioned above, another key assumption for the "high dose refuge" IRM strategy is the initial resistance alleles of target pest populations in the field should be rare (e.g. <0.001) (Andow and Alstad, 1998). Monitoring of resistance evolution o is of great importance for the long-term efficacy of Bt crop technologies. A few studies have investigated the allele frequency in *S.*

frugiperda populations to the commonly used Cry1F and Cry1A.105 protein in several regions of the world (Velez et al., 2013; Farias et al., 2014; Huang et al., 2014; 2016; Li et al., 2016). However, because Cry2Ab2 is a relatively new Bt protein used in Bt corn, the allele frequency to the commonly used Cry2Ab2 protein in pyramided Bt crops in *S. frugiperda* was unknown before the current study. In this study, an F_2 screening method was used to estimate the Cry2Ab2 allele frequency in *S. frugiperda* populations collected from multiple states in the U.S. southern region (Objective 3). The results generated from this study could be used to determine if the resistance allele frequency was low enough to meet the rare resistance allele assumption of the "high dose/refuge" IRM strategy.

Since 2010, pyramided Bt corn traits have gradually replaced the single-gene Bt crops in the United States and several other countries. However, most studies related to Bt resistance have just dealt with single-gene Bt resistance. In the objective 3 of the study, a single-gene Cry2Ab2-resistant strain of *S. frugiperda* was generated (Niu et al., 2016a). In addition, as mentioned above, a single-gene Cry1A.105-resistant strain of *S. frugiperda* was already available when the current study initiated (Huang et al., 2016). Studies have shown that both the Cry1A.105 and Cry2Ab2 resistances in the two resistant strains were controlled by a separate single autosomal gene (Acharya et al., 2017; Niu et al., 2017). By crossing the two well-documented single-gene Bt -resistant strains, a dual-Bt gene resistant strain of *S. frugiperda* that was resistant to both Cry1A.105 and Cry2Ab2 proteins in the plants was established in the laboratory. The availability of the dual-Bt gene resistant *S. frugiperda* strain provided an opportunity to determine the phenotypic performance of different insect genotypes containing single- and multiple-Bt resistance alleles and thus to generate essential parameters needed for refining resistance management modeling for the pyramided Bt crop technology.

1.7 Objectives

1. Characterize the inheritance and fitness costs of Cry1A.105 resistance in *S. frugiperda*;

2. Evaluate larval survival and plant injury of Cry1A.105-susceptibile, -resistant, and – heterozygous *S. frugiperda* populations on transgenic corn plants containing single or pyramided Bt genes;

3. Estimate the frequency of Cry2Ab2 resistance alleles in field populations of *S. frugiperda* in the U.S. southern region; and

4. Evaluate the phenotypic performance of different genotypes of *S. frugiperda* possessing single- or dual-Cry1A.105/Cry2Ab2 resistant genes on MON89034 corn.

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CHAPTER 2. INHERITANCE AND FITNESS COSTS OF CRY1A.105 RESISTANCE IN TWO STRAINS OF *SPODOPTERA FRUGIPERDA* (J.E. SMITH)[1]

2.1 Introduction

With the high selection pressure imposed by the broad adoption of *Bacillus thuringiensis* (Bt) crops (James, 2016), evolution of resistance has become a great challenge to the continued success of Bt crops. Recently, field-evolved resistance with control problems have reported in several major target pest species of Bt corn and Bt cotton (van Rensburg, 2007; Storer et al., 2010; Dhurua and Gujar, 2011; Gassmann et al., 2011; Farias et al., 2014a; 2014b; Huang et al., 2014; Kranthi, 2015; Dively et al., 2016). In fall armyworm, *Spodoptera frugiperda* (J.E. Smith), field-evolved resistance to Bt crops, particularly to the Cry1F protein, has developed in several geographical regions of the American Continents (Storer et al., 2010; Farias et al., 2014a; 2014b; Huang et al., 2014; Li et al., 2016). Therefore, implementing effective insect resistance management (IRM) strategies is urgently needed for this important cross-crop pest species (Yang et al., 2016).

Cry1A.105 (Biosafety Clearing-House, 2014) is one of the two Bt proteins produced in the event MON 89034. In the U.S., corn hybrids containing MON89034 was first commercialized in 2010 (US-EPA, 2010). Due to the similar gene structure among Cry1F and Cry1A proteins (e.g. Cry1A.105, Cry1Ab, Cry1Ac), studies have shown that there is a high level of cross-resistance among these proteins in *S. frugiperda* (Huang et al., 2014; Niu et al., 2016), hence the widespread resistance to Cry1F corn would be expected to impact the effectiveness of Cry1A.105. Because Cry1A.105 is a relatively new protein used in Bt crops, knowledge of

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Cry1A.105 resistance is very limited. Recently, two Cry1A.105-resistant *S. frugiperda* strains were successfully established using an F_2 screen from field populations collected from Florida, U.S. (Huang et al., 2016). The objective of this study is to analyze the genetic basis and fitness costs of these two strains. Information generated from this study should be valuable in understanding resistance mechanisms, assessing risk of resistance evolution, and developing resistance management strategies for Bt maize.

2.2 Material and Methods

2.2.1 Insect Sources

A Cry1A.105-susceptible (SS) and two Cry1A.105-resistant (RR32 and RR67) *S. frugiperda* strains were used as the original insect sources for this study (Table 2.1). SS was collected from corn fields near Weslaco, Texas in 2013 and has been shown to be susceptible to Cry1A.105, Cry2Ab2, Cry1F and Vip3A proteins in diet, as well as to corn leaf tissue and whole corn plants expressing these proteins (Huang et al., 2014; 2016; Niu et al., 2016; Yang et al., 2017). RR32 and RR67 were established by using an F_2 screen of two-parent families collected from corn fields in Collier County, Florida in 2011. Both RR32 and RR67 have demonstrated a significant level of resistance (>116-fold) to the Cry1A.105 protein. The two resistant strains also survived and developed well on whole plants of Cry1A.105 corn plants in the greenhouse. Larvae of all three insect strains were reared on corn leaf tissue or a meridic diet (Ward's Stonefly Heliothis diet, Rochester, NY) as described in Huang et al. (2016). Before RR32 and RR67 were used in the current study, they had been backcrossed with SS twice and reselected for resistance with Cry1A.105 corn plants as described in Dangal and Huang et al. (2015).

2.2.2 Genetic Crosses

To assess inheritance of the Cry1A.105 resistance, eight additional *S. frugiperda* strains (Table 2.1) were developed from two types of genetic crosses among the three original strains using the method as described in Camargo et al. (2017). For genetic crosses, pupae of SS, RR32, and RR67 were first divided by gender before eclosion. In each cross, 50-70 females from one strain were then mass-mated with 50-70 males from the other strain (Camargo et al., 2017). Four F_1 heterozygous-resistant strains were produced from reciprocal crosses between SS and the two resistant strains, which were denoted as 1) F_1-$32_f SS_m$, F_1 progeny from crossing RR32 females with SS males; 2) F_1-$32_m SS_f$, F_1 progeny from crossing RR32 males with SS females; 3) F_1-$67_f SS_m$, F_1 progeny from crossing RR67 females with SS males; and 4) F_1-$67_m SS_f$, F_1 progeny from crossing RR67 males with SS females. Four F_2 strains were generated by sib-mating of the four F_1 strains, which were 1) F_2-$32_f SS_m$, F_2 progeny from the sib-mating of F_1-$32_f SS_m$; 2) F_2-$32_m SS_f$, F_2 progeny from the sib-mating of F_1-$32_m SS_f$; 3) F_2-$67_f SS_m$, F_2 progeny from the sib-mating of F_1-$67_f SS_m$; and 4) F_2-$67_m SS_f$, F_2 progeny from the sib-mating of F_1-$67_m SS_f$.

For fitness cost study, to further minimize differences in genetic background among insect strains, RR32 and RR67 were backcrossed with SS one more time (a total of three backcrosses) and reselected for resistance with Cry1A.105 corn plants as described in Dangal and Huang et al. (2015). Two F_1 heterozygous-resistant strains (F_1-RS32 or F_1-RS67) were produced from reciprocal crosses between SS and the two re-backcrossed and reselected resistant strains. F_1-RS32 was the F_1 offspring of the reciprocal crosses between SS and RR32, and F_1-RS67 was the F_1 offspring of the reciprocal crosses between SS and RR67. Thus, a total of five *S. frugiperda* strains (SS, F_1-RS32, F_1-RS67, and the two backcrossed-and- reselected strains RR32 and RR67) were used in the fitness cost study (Table 2.1).

Table 2.1. *Spodoptera frugiperda* strains used in assessing inheritance and fitness costs of Cry1A.105 resistance.

Insect strain	Description
Original three insect strains used in assessing inheritance and fitness costs of resistance	
SS	A Cry1A.105-susceptible strain from Weslaco, Texas in 2013
RR32	A Cry1A.105-resistant strain isolated using an F_2 screen of a single-pair collected from Florida in 2011
RR67	A Cry1A.105-resistant strain isolated using an F_2 screen of a single-pair collected from Florida in 2011
F_1 heterozygous-resistant strains used in inheritance study	
F_1-32_fSS_m	F_1 progeny generated by crossing females of RR32 and males of SS
F_1-32_mSS_f	F_1 progeny generated by crossing males of RR32 and females of SS
F_1-67_fSS_m	F_1 progeny generated by crossing females of RR67 and males of SS
F_1-67_mSS_f	F_1 progeny generated by crossing males of RR67 and females of SS
Four F_2 strains used in inheritance study	
F_2-32_fSS_m	F_2 progeny produced from the sib-mating of F_1-32_fSS_m
F_2-32_mSS_f	F_2 progeny produced from the sib-mating of F_1-32_mSS_f
F_2-32	A mixed strain of approximately 50% F_2-32_fSS_m and 50% F_2-32_mSS_f
F_2-67_fSS_m	F_2 progeny produced from the sib-mating of F_1-67_fSS_m
F_2-67_mSS_f	F_2 progeny produced from the sib-mating of F_1-67_mSS_f
F_2-67	A mixed strain of approximately 50% F_2-67_fSS_m and 50% F_2-67_mSS_f
Two F_1 heterozygous-resistant strains used in fitness cost study	
F_1-RS32	Mixed F_1 progeny produced by reciprocal crosses between SS and RR32
F_1-RS67	Mixed F_1 progeny produced by reciprocal crosses between SS and RR67

2.2.3 Assessing Inheritance of Cry1A.105 Resistance in *S. frugiperda*

To assess the inheritance of Cry1A.105 resistance in *S. frugiperda*, larval mortality was measured using two assay methods: diet-incorporated and corn leaf tissue bioassays as described in Camargo et al. (2017). Cry1A.105 protein and the related buffer, as well as, a Cry1A.105 corn experimental line and its non-Bt corn isoline were provided by Monsanto Company (St. Louis, MO). All 11 insect strains listed in Table 2.1 were included in the corn leaf tissue bioassay. In the diet-incorporated bioassay, because of the autosomal inheritance in both RR32 and RR67 (see results), the two F_2 strains associated with each of the two resistant strains were combined into one strain each, named as F_2-32 and F_2-67, respectively. Thus a total of nine *S. frugiperda* strains were evaluated in the diet-incorporated bioassay (Huang et al., 2016). Our previous studies (Huang et al., 2016; Niu et al., 2016) suggested that the genotypes of SS and RR *S. frugiperda* could be discriminated well using the die-incorporated bioassay with Cry1A.105 concentrations from 10 to 100 µg/g. Thus, larval mortalities of the nine *S. frugiperda* strains were determined at three Cry1A.105 concentrations: 10, 31.6, and 100 µg/g. In addition, a negative control (diet treated with buffer only) and a blank control (diet treated with water only) were used in each bioassay.

In the diet-incorporated bioassay, approximately 1g of treated diet or untreated control diet was placed into each cell of 128-cell trays (Niu et al., 2013). One neonate (< 24 h) was placed into each cell. For the leaf tissue bioassay, leaves of the Cry1A.105 and isoline non-Bt corn plants (Huang et al., 2016) were removed from greenhouse-grown plants. It has been reported that there is a great variability of Cry1A.105 protein expression at the V2-V4 corn plants of the event MON 89034 (Monsanto, 2006). Expression or non-expression of the Bt toxin in the plants was confirmed using the ELISA-based assays (EnviroLogix, Quantiplate ™ kits,

Portland, ME) (Wangila et al., 2012). To ensure a relatively consistent expression of Cry1A.105 protein in the plant materials used in the bioassay, only fully-expanded leaves of the V5-V8 stage plants of the greenhouse-grown plants were used in the current study. Two previous studies (Huang et al., 2016; Niu et al., 2016) have shown that the tissues of the fully-expanded leaves of Cry1A.105 corn plants at these growth stages consistently provided high mortality (e.g. 100% mortality) against the susceptible *S. frugiperda* strain, while RR strains survived well, suggesting leaf tissue of the Cry1A.105 corn plants at these stages expresses sufficient level of the Cry1A.105 protein to discriminate RR from SS (Huang et al., 2016). In the bioassay, leaves were cut into pieces approximately 3 cm in length. Two to three pieces of leaf tissue were placed in each well of a 32-well C-D International tray (Bio-Ba-32, C-D International, Pitman, NJ). Four neonates (<24 h old) of an insect strain were then placed on the surface of the leaf tissue in each well and leaf tissue was replaced every 2-3 days (Niu et al., 2013).

For both assay methods, bioassay trays were put in environmental chambers maintained at 28°C, 50% RH, and a 16:8 (L: D) h photoperiod and larval mortality was checked after 7 days of neonate release. Larvae were considered dead if they did not respond after being touched with a camel hair brush (Niu et al., 2013). In each bioassay, there were four or eight replications and each replication consisted of 16-32 larvae in the diet-incorporated bioassay and 24-32 larvae in the leaf tissue test.

2.2.4 Analyzing Fitness Costs of Cry1A.105 Resistance in *S. frugiperda*

To determine if fitness costs were associated with the Cry1A.105 resistance, survival, growth, development and reproduction of the five *S. frugiperda* strains (SS, RR32, RR67, F_1-RS32, and F_1-RS67) were examined on non-Bt corn leaf tissue using a similar method as described in Dangal and Huang (2015). A non-Bt corn hybrid, DKC62-95 (Monsanto, St. Louis,

MO), was used in the fitness study. DKC62-95 corn seeds were planted in open fields at the Louisiana State University AgCenter's Macon Ridge Research Station in Franklin Parish and at the Central Research Station in East Baton Rouge Parish, Louisiana, U.S. The non-expression of Bt proteins in the non-Bt corn plants was confirmed using the ELISA-based assays mentioned above. Larvae of the five *S. frugiperda* strains were individually assayed on the leaf tissue removed from the field-grown V5-V8 plants (Dangal and Huang, 2015). In the assay, one neonate (<24 h old) was released on the surface of leaf tissue in each well of 32-well C-D International trays (Bio-Ba-32, C-D International, Pitman, NJ). Bioassay trays were placed in growth chambers maintained at the same conditions as used in the inheritance study. After seven days, to ensure enough space for the late instars, live larvae were transferred from the 32-well trays into the 8-well C-D International trays (Bio-Ba-8) containing the same leaf tissue with 1 larva/well. Leaf tissue was replaced every 2-3 days until pupation. In each bioassay, there were four replications (growth chambers) for each insect strain and each replication included 32 neonates (n = 4 x 32=128). Bioassay trays within a replication were placed in the same growth chamber. Live pupae from each treatment replication were transferred into 3.78-liter paper containers for adult emergence in the same growth chamber as they were reared. Larval survival and body weight of *S. frugiperda* reared on the non-Bt corn leaf tissue were checked 10 days after neonate release. Pupation and adult emergence were checked daily once the first pupa was observed. Sex ratio and pupal weight were also recorded for each treatment replication.

To measure reproduction, a pair of newly emerged (<24 h old) virgin male and female adults were placed into 3.78-liter paper containers (Huhtamaki Foodservice, De Soto, Kansas) for mating and oviposition as described in Zhang et al. (2014). For each insect strain, there were

four replications and each replication consisted of five pairs. Total number of eggs produced per female was estimated by weighing the total egg masses laid.

2.2.5 Data Analysis

Larval mortality data measured in the inheritance study were corrected based on the mortality observed on the non-Bt control diet or non-Bt corn leaf tissue using the method of Abbott (1925). The corrected mortality data, along with the insect survivorship rate recorded in the fitness cost study, were transformed using arcsine ($x^{0.5}$). All other biological parameters recorded in the fitness cost study including 10-day larval body weight, sex ratio, pupal weight, neonate-to-pupa development time, neonate-to-adult emergence time, and egg production were transformed into log (x + 1) scale to meet the assumption for analysis of variance (ANOVA). All transformed data were analyzed using one-way ANOVA with *S. frugiperda* strain as the main factor (SAS Institute, 2010). Treatment means were separated using Tukey's honest significant difference test at $\alpha = 0.05$ level.

Sex linkage or maternal effect of the Cry1A.105 resistance was examined by comparing larval mortality of F_1 progeny from the reciprocal crosses between SS and the two resistant *S. frugiperda* strains (Roush and Tabashnik, 1990). Functional dominance level, D_{ML}, of the Cry1A.105 resistance in RR32 and RR67 at each Cry1A.105 concentration in the diet-incorporated bioassay and leaf tissue tests was estimated using the method as described in Bourguet et al. (2000). The number of genes related to the Cry1A.105 resistance was estimated with χ^2-tests by fitting observed larval mortality data in the F_2 generations to the Mendelian single-gene models as described in Tabashnik (1991). Because the Cry1A.105 concentrations of 10 and 31.6 μg/g in the diet-incorporated bioassay did not fully discriminate heterozygous from homozygous resistant individuals (see results), tests for fitting the Mendelian monogenic model

were conducted for only the concentration of 100 µg/g. Fitness costs of resistance were evaluated by comparing survival, growth, development and reproduction among SS, resistant and F_1 heterozygous *S. frugiperda* strains.

2.3 Results

2.3.1 Inheritance of Cry1A.105 Resistance in *S. frugiperda*

The effect of *S. frugiperda* strain on larval mortality was significant for each of the three Cry1A.105 concentrations in the diet-incorporated bioassay ($F_{8,35} \geq 14.44$, $P < 0.0001$ across the three Bt concentrations) and for the leaf tissue test ($F_{10,41} = 20.54$, $P < 0.0001$). Larval mortality of SS ranged from 98.1-100% on Cry1A.105-treated diet and was 96.4% on Cry1A.105 corn leaf tissue (Table 2.2). Performance of RR32 and RR67 was somewhat inconsistent between the diet-incorporated bioassay and leaf tissue test. Mortality of RR32 at 31.6 and 100 µg/g in diet-incorporated bioassay was lower ($P < 0.05$) than that of RR67, while, on Bt leaf tissue, mortality of RR32 was greater ($P < 0.05$) than that of RR67. However, the overall mortalities of the two resistant strains on both Bt-treated diet and Bt corn leaf tissue were relatively low (averaged 8.2% for RR32 and 17.7% for RR67) and both were significantly less ($P < 0.05$) than the SS mortality across Bt concentrations and assay methods (Table 2.2).

Larval mortality of the two F_1 strains derived from reciprocal crosses of SS and each of the two resistant strains were similar ($P > 0.05$) across the three Cry1A.105 concentrations in diet-incorporation and on Bt corn leaf tissue (Table 2.2). Thus, sex-linkage or maternal effect was not evident for the Cry1A.105 resistance in both RR32 and RR67 strains. However, in some bioassays, larval mortality of the two F_1 reciprocal strains associated with RR67 was somewhat greater than that of F_1 strains associated with RR32. For example, F_1-67_fSS_m and F_1-67_mSS_f showed an average mortality of 60.7% at 31.6 µg/g and 87.3% on Cry1A.105 leaf tissue, while

the corresponding mortality for the two F_1 strains associated with RR32 was 22.2% and 55.7%, respectively. In general, mortality at 10 and 100 µg/g were similar ($P > 0.05$) among the four F_1 strains.

Table 2.2. Larval mortality (% mean ± sem) of Cry1A.105-susceptible (SS), -resistant (RR32 and RR67), F_1, and F_2 strains of *Spodoptera frugiperda* on Cry1A.105-diet at three concentrations and Cry1A.105 corn leaf tissue.

Insect strain [a]	Cry1A.105-treated diet (µg/g)[b]			Insect strain[b]	Cry1A.105 corn leaf tissue[b]
	10	31.6	100		
SS	100 ± 0.0d	98.1 ± 1.9e	100 ± 0.0d	SS	96.4 ± 2.1e
RR32	0.4 ± 0.4a	0.0 ± 0.0a	6.5 ± 6.5a	RR32	26.0 ± 2.5b
F_1-32_fSS_m	12.0 ± 5.6b	31.6 ± 2.4bc	76.1 ± 4.4bcd	F_1-32_fSS_m	52.2 ± 7.3bc
F_1-32_mSS_f	20.5 ± 3.8bc	12.8 ± 3.6ab	94.9 ± 1.7cd	F_1-32_mSS_f	59.2 ± 4.1bc
F_2-32	38.2 ± 5.7c	53.9 ± 3.6cd	58.2 ± 2.8bc	F_2-32_fSS_m	40.9 ± 3.2bc
				F_2-32_mSS_f	59.8 ± 3.1bc
RR67	0.2 ± 0.2a	23.0 ± 2.7bc	45.1 ± 9.3b	RR67	2.3 ± 2.3a
F_1-67_fSS_m	34.7 ± 4.7c	70.0 ± 3.8d	92.0 ± 1.6cd	F_1-67_fSS_m	84.5 ± 6.2de
F_1-67_mSS_f	34.8 ± 2.5c	51.3 ± 9.1cd	70.4 ± 10.4bc	F_1-67_mSS_f	90.0 ± 3.3de
F_2-67	21.9 ± 1.0bc	45.8 ± 7.8cd	70.8 ± 8.7bc	F_2-67_fSS_m	64.3 ± 3.9bcd
				F_2-67_mSS_f	70.0 ± 8.8cde
ANOVA	$F_{8,35}$=85.92 P <0.0001	$F_{8,35}$ =30.85 P <0.0001	$F_{8,35}$=14.44 P <0.0001	ANOVA	$F_{10,41}$ = 20.54 P < 0.0001

[a] Insect strain identifications are the same as listed in Table 1. In the diet-incorporated bioassay, F_2 progeny were pooled from F_2-32_fSS_m and F_2-32_mSS_f or F_2-67_fSS_m and F_2-67_mSS_f. There were 8 replications for assaying F_1-67_fSS_m and F_1-67_mSS_f, respectively, in both diet-incorporated and corn leaf tissue bioassays, while four replications were used in all other assays.
[b] Mean values within a column followed by a same letter are not significantly different at $\alpha = 0.05$ (Tukey's honest significant difference test).

Functional dominance level of the resistance for RR32, calculated based on the mortality at each of the three concentrations in the diet bioassay, ranged from 0.155 at 100 μg/g to 0.841 at 10 μg/g (Table 2.3). The corresponding D_{ML} values for RR67, ranged from 0.342 at 100 μg/g to 0.654 at 10 μg/g. D_{ML} based on the mortality in leaf tissue bioassay was 0.579 for RR32, and 0.097 for RR67 (Table 2.3). These results suggest that Cry1A.105 resistance in both RR32 and RR67 was non-recessive, but it ranged from incompletely recessive to incompletely dominant, depending on strain, Bt concentration and assay method.

Table 2.3. Effective dominance levels (D_{ML}) of two Cry1A.105-resistant strains of *Spodoptera frugiperda* calculated based on larval mortalities on Cry1A.105-diet at three concentrations and Cry1A.105 corn leaf tissue.

Insect strain[a]	Assay method	Cry1A.105 concentration (μg/g) or corn stage	D_{ML}	Result
RR32	Diet-incorporated	10	0.841	Incomplete dominant
		31.6	0.774	Incomplete dominant
		100	0.155	Incomplete recessive
	Bt corn leaf tissue	Vegetative plant stages	0.579	Moderate
RR67	Diet-incorporated	10	0.654	Incomplete dominant
		31.6	0.499	Moderate
		100	0.342	Incomplete recessive
	Bt corn leaf tissue	Vegetative plant stages	0.097	Incomplete recessive

[a] Insect strain identifications are the same as listed in Table 2.1.

In general, mortality of F_2 strains was significantly ($P < 0.05$) less than that of SS, but significantly ($P < 0.05$) greater than RR32 or RR67. Mortalities at each of the three Cry1A.105 concentrations and on Bt leaf tissue were similar ($P > 0.05$) between F_2-32 and F_2-67. χ^2 tests showed that, the observed mortality of F_2-67 in both Cry1A.105-diet and leaf tissue bioassays fitted the Mendelian monogenic model ($\chi^2_{df-=1} = 1.329$, $P = 0.2489$ for diet-incorporated bioassay

and $\chi^2_{df=1}$= 0.116, P = 0.7334 for leaf tissue bioassay) (Table 2.4). χ^2 tests also showed that mortality of F_2-32 on Cry1A.105 diet fitted the monogenic model ($\chi^2_{df=1}$ = 3.76, P = 0.0525). However, the difference between the observed and expected mortality of F_2-32 on Bt leaf tissue was significant at the $\alpha = 0.05$ level, but not at the $\alpha = 0.01$ level (Table 4). Based on these results, the Cry1A.105 resistance in both RR32 and RR67 was likely controlled by a single or a few closely linked genes.

Table 2.4. χ^2 tests for Mendelian monogenic model for Cry1A.105 resistance in two strains of *Spodoptera frugiperda*.

Insect strain[a]	Assay method	Cry1A.105 concentration or corn stage	Pooled F_2				
			n	No. dead larvae		χ^2 test	
				Expected	Observed	χ^2	*P*-value
F_2-32	Diet-incorporated	100 μg/g	64	44.4	37.2	3.76	0.0525
	Bt corn leaf tissue	Vegetative plant stage	192	112.2	96.7	5.187	0.0228
F_2-67	Diet-incorporated	100 μg/g	64	49.2	45.3	1.329	0.2489
	Bt corn leaf tissue	Vegetative plant stage	192	131.1	128.9	0.116	0.7334

[a] Insect identifications are the same as listed in Table 2.1. Mortality data observed on Bt diet and Bt leaf tissue were corrected with the corresponding mortalities on non-Bt diet and non-Bt corn leaf tissue, respectively, before they were used for χ^2 -tests.

2.3.2 Fitness Costs of Cry1A.105 Resistance in *S. frugiperda*

The effect of *S. frugiperda* strain on 10-day larval survivorship was significant ($F_{4,12}$ = 9.51, P = 0.0011), while the effect on 10-day larval body weight was not significant ($F_{4,12}$ = 2.69, P = 0.0823) (Table 2.5). There were no significant ($P > 0.05$) differences in the survivorship among SS, RR32, F_1-RS67 and F_1-RS32, or among RR32, RR67 and F_1-RS67. However, the 10-day survivorship of RR67 (94.5 %) appeared to be greater ($P < 0.05$) than that of SS (67.2%) and

F_1-RS32 (64.8%) (Table 2.5). The 10-day larval body weight ranged from 253.5 mg/larva for F_1-RS67 to 320 mg/larva for RR67 (Table 2.5).

The effect of *S. frugiperda* strain on sex ratio and pupal weight was not significant ($F_{4,12}$ = 0.77, P = 0.5630 for sex ratio and $F_{4,12}$ = 1.81, P = 0.1909 for pupal weight), while the effect on pupation time was significant ($F_{4,12}$ = 8.95, P = 0.0014) (Table 2.5). Across the five *S. frugiperda* strains, the average sex ratio was 1:1.01 (male: female) and the average pupal weight was 183.9 mg/pupa (Table 2.5). Average neonate-to-pupa development time for SS was 13.4 days, which was 0.5 to 1 day longer (P < 0.5) than the other four strains. No significant (P > 0.05) differences in development time was among the two resistant and two F_1 strains (Table 2.5).

The effect of *S. frugiperda* strain on emergence time was significant for both sexes ($F_{4,12}$ = 9.24, P = 0.0012 for male and $F_{4,12}$ = 6.32, P = 0.0056 for female) (Table 2.5) and the effect on neonate-to-adult emergence rate was also significant ($F_{4,12}$ = 5.7, P = 0.0083). The neonate-to-adult emergence time (20.8 days for male and 21.4 for female) of SS was approximately one day longer than the other four strains for both sexes. The difference was significant (P < 0.05) when it was compared to RR32, F_1-RS32 and F_1-RS67, but not significant (P > 0.05) for RR67. No significant (P > 0.05) differences were observed in the adult emergence time among RR32, RR67, F_1-RS32, and F_1-RS67 (Table 2.5). The emergence rate (64.8%) of RR67 was significantly (P < 0.05) greater than that of F_1-RS32 (34.4%) and SS (36.7%), while the difference was not significant (P > 0.05) among SS, RR32 and F_1-RS32, or among SS, RR32 and F_1-RS67 (Table 2.5). The effect of egg production was not significant ($F_{4,12}$ = 1.85, P = 0.1850) (Table 2.5). Across the five *S. frugiperda* strains, each successfully-mated female produced an average of 1,040 eggs (Table 2.5).

Table 2.5. Survival, growth, development, and reproduction of Cry1A.105-susceptible, -resistant, and F_1 strains of *Spodoptera frugiperda* feeding on non-Bt corn leaf tissue.

Insect strain[a]	10-d Survivorship %	10-Larval Weight mg/Larva	Sex Ratio (Male/female)	Pupal Weight (mg/pupa)	Neonate-to-Pupa Time (day)	Neonate-to-Adult Emergence Time (day)		Neonate-to-Adult Emergence Rate (%)	No. Eggs/Pair
						male	female		
SS	67.2±6.1a	256.4±16.6a	1.08±0.15a	185.0±3.1a	13.4±0.3b	20.8±0.3b	21.4±0.5b	36.7±4.8ab	899.3±167.4a
RR32	82.0±5.5ab	282.9±11.0a	0.76±0.10a	188.9±2.7a	12.3±0.1a	18.9±0.4a	20.4±0.2a	45.3±6.8abc	924.5±168.9a
RR67	94.5±2.3b	320.0±7.9a	0.98±0.19a	183.5±2.4a	12.9±0.3a	19.8±0.5ab	20.9±0.4ab	64.8±5.1c	949.0±209.0a
F_1-RS32	64.8±8.1a	279.7±18.7a	1.13±0.05a	181.8±1.4a	12.7±0.3a	19.1±0.4a	20.4±0.3a	34.4±3.4a	895.0±76.0a
F_1-RS67	85.7±3.9ab	253.5±16.5a	1.00±0.22a	180.2±2.4a	12.5±0.3a	19.2±0.4a	20.3±0.3a	60.2±8.4bc	1530.3±119.8a
ANOVA	$F_{4,12}$=9.51 P=0.0011	$F_{4,12}$=2.69 P=0.0823	$F_{4,12}$=0.77 P=0.5630	$F_{4,12}$=1.81 P=0.1909	$F_{4,12}$=8.95 P=0.0014	$F_{4,12}$=9.24 P=0.0012	$F_{4,12}$=6.32 P=0.0056	$F_{4,12}$=5.7 P=0.0083	$F_{4,12}$=1.85 P=0.1850

[a] Insect strain identifications are the same as listed in Table 2.1. Mean values followed by a same letter within a column were not significantly different at $\alpha = 0.05$ (Tukey's HSD test).

2.4 Discussion

Results of this study suggest that, the Cry1A.105 resistance in both RR32 and RR67 *S. frugiperda* strains was likely inherited as a single (or a few tightly linked) autosomal gene and the resistance was functionally non-recessive, but ranged from incomplete recessive to incomplete dominant, depending on strain, Bt concentration and assay method. Both RR32 and RR67 on non-Bt leaf tissue did not show less fitness than the susceptible insect strain for all life history parameters measured, suggesting that the Cry1A.105 resistance in *S. frugiperda* was not associated with fitness costs.

To analyze if the Cry1A.105 resistance in RR32 and RR67 is controlled by the same alleles, we conducted a reciprocal inter-strain cross between RR32 and RR67 and examined larval survival of the F_1 progeny on Cry1A.105 corn leaf tissue using the same method described above. The two reciprocal F_1 strains from the inter-strain crosses were highly resistant to the Bt corn leaf tissue as their resistant parental strains (data not shown). Because of the non-recessivity of the resistance in both RR32 and RR67, data generated from the inter-strain crosses could not conclusively demonstrate that the genetic basis is the same in both strains. In addition, previous studies (Huang et al., 2014; 2016) indicate that the Cry1A.105 resistance in RR32 and RR67 was likely due to cross resistance with Cry1F-resistant genes in the insect. However, Cry1F resistance in another *S. frugiperda* strain that was isolated from the same insect populations that was used in the current study was shown to be recessive on Cry1F corn leaf tissue (Camargo et al., 2017), and the resistance was associated with fitness costs (Dangal and Huang, 2015). Furthermore, the Cry1F resistance in *S. frugiperda* was found to be recessive to incompletely recessive in all other populations that have been examined (Storer et al., 2010; Vélez et al., 2013; Farias et al., 2016; Leite et al., 2016). Nevertheless, the lack of fitness costs and the non-

recessive resistance observed in the current study for both RR32 and RR67 suggest that Bt resistance, even for the same target pest, can be expressed quite differently on plants containing dissimilar Bt traits. The results indicate that strain and trait-specific knowledge of resistance may be needed to develop effective IRM strategies for Bt crops.

In the U.S. and several other countries, a "high dose/refuge" strategy (Ostlie et al., 1997; US-EPA, 2001) has been implemented for managing resistance to Bt crops. Two key assumptions for the success of this strategy are that the resistance should be functionally recessive and the initial resistance allele frequency in field pest populations should be rare (e.g. <0.001) (Huang et al., 2011). A previous study (Huang et al., 2016) showed that Cry1A.105 resistance alleles in field populations of *S. frugiperda* from the U.S. southeast region are no longer rare, estimated to be 0.0328 with a 95% credibility interval of 0.020-0.049. The non-recessive resistance character and the lack of fitness costs documented in the current study, plus the relatively high resistance allele frequency, indicate a relatively high potential risk of resistance development of *S. frugiperda* to single-gene Cry1A.105 corn. It should be noted that, the single-gene Cry1A.105 corn used in this study is for experimental use only and not available for commercial planting. As mentioned above, Cry1A.105 is one of the two Bt genes in event MON 89034; the other Bt protein in the event is Cry2Ab2. MON 89034 has been incorporated into several commonly used pyramided Bt corn hybrids (DiFonzo, 2017), and studies have documented that Cry1A.105-resistant *S. frugiperda* are not cross-resistant to Cry2Ab2 or Vip3A, and Bt corn plants expressing one or both of these proteins are effective against the Cry1A.105-resistant *S. frugiperda* (Niu et al., 2016). A significant concern is that, due to the cross-resistance between Cry1F and Cry1A (Huang et al., 2014; Bernardi et al., 2015; Yang et al., 2016), Cry1A.105 corn plants are not effective against the Cry1F-resistant *S. frugiperda*. Some of these

'pyramided' Bt corn products effectively a single-gene hybrids against the Cry1F-resistant *S. frugiperda* populations. Knowledge in resistance development under such situation is lacking and need to be investigated in the future.

Based on the knowledge generated from earlier studies of resistance to chemical insecticides and some Bt toxins, high levels of Bt resistance were thought to be typically controlled by a single, largely recessive gene with fitness costs (Tabashnik et al., 1994; Huang et al., 1999; Bourguet et al., 2000; US-EPA 2001; Ferré and van Rie, 2002; Gassmann et al., 2009; Huang et al., 2011). However, the results of more recent studies do not appear to agree with this general conclusion. The most notable example of dominant resistance are the field resistance of African stem borer, *Busseola fusca* (Fuller), to Cry1Ab corn, which could be a key factor for the rapid resistance development in the field for both target pests (Campagne et al., 2013). Other notable non-recessive resistance cases are in the resistance of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte to Cry3Bb1 corn (Petzold-Maxwell et al., 2012; Hoffmann et al., 2015), *Helicoverpa armigera* to Cry1Ac cotton (Kranthi et al., 2006; Nair et al., 2010; Jin, 2013), and *Pectinophora gossypiella* to Cry1Ac cotton (Nair et al., 2016). In addition, fitness costs of major resistance to single-gene Bt plants have also been evaluated in several cases associated with the same seven insect species and the same six Bt proteins. The results showed that the majority of these cases were not associated with significant fitness costs. More significantly, lack of fitness costs were found for the three most notable field resistance cases, called 'practical resistance' in Tabashnik et al. (2013), which include the resistance of African stem borer to Cry1Ab corn, western corn rootworm, *Diabrotica virgifera virgifera* LeConte to Cry3Bb1 corn (Gassmann et al., 2011), and *S. frugiperda* to Cry1F corn (Jakka et al., 2014; Vélez et al., 2014; Leite et al., 2016). We understand that the number of cases that have been

examined is still limited and major resistant genes for some primary target pests (e.g. European corn borer, *Ostrinia nubilalis* (Hübner), vs Cry1Ab corn) to Bt crops are yet to be identified. However, such a high rate of non-recessive resistance to Bt crops provides a cautionary evidence against the assumption that resistance is functionally recessive in an IRM plan for single-gene Bt crops. It should also be pointed out that, in the U.S. and several other countries, single-gene Bt cotton has already been phased out from the market, and it is expected that single-gene Bt corn will be completely replaced by pyramided Bt corn in the near future. Validation of IRM assumptions for pyramided Bt traits is still a great challenge. Two recent studies showed that the dual- or multiple-gene Bt resistance to Cry1A/Cry2A or Cry1A/Cry2A and Cry1F proteins in *S. frugiperda* is functionally recessive on the corresponding pyramided Bt corn plants (Santos-Amaya et al., 2015; Bernardi et al., 2017). More studies to characterize dual- or multiple-gene Bt resistance are warranted to generate the necessary information for developing robust IRM strategies for Bt crops.

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CHAPTER 3. PERFORMANCE OF CRY1A.105-SELECTED FALL ARMYWORM (LEPIDOPTERA: NOCTUIDAE) ON TRANSGENIC MAIZE PLANTS CONTAINING SINGLE OR PYRAMIDED BT GENES[2]

3.1 Introduction

Transgenic crops (e.g. maize, cotton, and soybean) containing *Bacillus thuringiensis* (Bt) genes have been widely planted for controlling some major insect pests (James, 2014). As with many other pest management tools, evolution of resistance in the pest populations is a threat to the sustainable use of Bt crop technology. Great efforts in implementation of resistance management plans have been made since the first commercialization of Bt crops in 1996 (Ostlie et al., 1997; Huang et al., 2011; Matten et al., 2012; Tabashnik et al., 2013). However, due to the intensive use of Bt crops over the last 20 years, field resistance resulting in insect control problems has occurred in at least four major target species and in several countries (van Rensburg, 2007; Storer et al., 2010; Dhurua and Gujar, 2011; Gassmann et al., 2011; Farias et al., 2014a; 2014b; Huang et al., 2014).

Fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is a target of both Bt maize and Bt cotton in North and South America, as well as a target of Bt soybean in Brazil (Farias et al., 2014a; Yang et al., 2016). Up to now, *S. frugiperda* is the first and only target insect that has developed field resistance to Bt crops at multiple locations across different countries and continents (Storer et al., 2010; Farias et al., 2014a; 2014b; Huang et al., 2014). In Puerto Rico, Cry1F maize (event TC1507) was commercially planted to control *S. frugiperda* in 2003, while field control problems occurred three years later (Storer et al., 2010). Similarly, in Brazil, Cry1F maize was first commercially available in the 2009/2010 season for controlling *S. frugiperda* and

[2]This chapter previously appeared as Niu et al., 2016b. Performance of Cry1A. 105-selected fall armyworm (Lepidoptera: Noctuidae) on transgenic maize plants containing single or pyramided Bt genes. Crop Protect. 88, 79-87.

other lepidopteran pests. Field resistance in *S. frugiperda* was documented in 2011, and currently the resistance has spread throughout the Western Bahia region of the country (Farias et al., 2014a; 2014b). In addition, field resistance of *S. frugiperda* to Cry1F maize has also been documented in some areas of the southern United States (Huang et al., 2014).

To slow the development of resistance, maize hybrids containing two or more pyramided Bt genes have been commercialized in the United States and several other countries (Ghimire et al., 2011; Matten et al., 2012; Buntin and Flanders, 2015). Relative to the single-gene Bt maize, these pyramided Bt maize products are usually more effective against some target pests, especially Noctuidae species such as the corn earworm (*Helicoverpa zea* [Boddie]) and *S. frugiperda* (Burkness et al., 2010; Niu et al., 2014; Yang et al., 2013; 2015). The widespread Cry1F resistance in *S. frugiperda* has sparked concerns about the durability of the pyramided Bt crops (Huang et al., 2014; Bernardi et al., 2015; Santos-Amaya et al., 2015; Yang et al., 2016). One of the common Bt proteins expressed in some pyramided Bt maize is Cry1A.105. This Bt toxin is a chimeric protein incorporating domains I and II from Cry1Ab or Cry1Ac, domain III from Cry1F, and the C-terminal domain from Cry1Ac (Biosafety Clearing-House, 2014). During 2011, two Cry1A.105-resistant strains of *S. frugiperda* were isolated from field populations collected in Florida (Huang et al., 2016). In this study, we evaluated the survival and plant injury of these two Cry1A.105-resistant populations, along with a susceptible population and two F_1 heterozygous genotypes, on commercial and experimental Bt maize hybrids/lines containing single or pyramided Bt genes (hereafter, 'maize products' refers to both commercial hybrids and non-commercially experimental lines). Information generated from this study should be useful in understanding the cross-resistance among the commonly used Bt maize traits and developing effective resistant management strategies for the sustainable use of Bt maize technology.

3.2 Materials and Methods

3.2.1 Insect Sources

Three populations of *S. frugiperda* including a Cry1A.105-susceptible strain (SS) and two Cry1A.105-resistant (FL32 and FL67) strains were used as the original insect sources in the study. SS was collected from maize fields near Weslaco, Texas, in 2013. SS was susceptible to purified proteins of Cry1A.105, Cry2Ab2, and Cry1F, as well as to maize leaf tissue and whole plants expressing Cry1A.105, Cry2Ab2, Vip3A, and Cry1F proteins (Huang et al., 2014; 2016). FL32 and FL67 were isolated from two single-pairing families collected from maize fields in Collier County, Florida, in 2011 (Huang et al., 2016). Both FL32 and FL67 have been shown to possess major resistance alleles to Cry1A.105 maize plants by using an F_2 screen and have demonstrated a significant level of resistance (>116-fold) to the Cry1A.105 protein. The two resistant populations also survived and developed well on whole plants of Cry1A.105 maize in the greenhouse (Huang et al., 2016). In the laboratory, larvae of the three populations were reared individually on maize leaf tissue or a meridic diet (Ward's Stonefly Heliothis diet, Rochester, NY) as described in Niu et al. (2013). Before FL32 and FL67 were used in the current study, they had been backcrossed with SS twice and reselected for resistance with Cry1A.105 maize leaf tissue, as described in Dangal and Huang (2015).

In addition, two F_1 heterozygous genotypes, FL32-RS and FL67-RS, were developed by reciprocal crosses of the two resistant strains with SS. FL32-RS was a mixture of the two F_1 heterozygous genotypes produced from the reciprocal crosses of FL32 with SS, while FL67-RS was a mixture of the two F_1 heterozygous genotypes produced from the reciprocal crosses of FL-67 with SS.

3.2.2 Maize Products

Performance of the five insect populations (SS, FL32, FL67, FL32-RS, and FL67-RS) described above was examined against 12 maize products, which consisted of four non-Bt and eight Bt maize products (Table 3.1). The eight Bt maize products included five single-Bt and three pyramided Bt maize hybrids/lines. The five single-Bt maize products were Cry1AP, Herculex® I (abbreviated product ID, HX1), YieldGard® (YG), Cry2AP, and Cry2APH; and the three pyramided products were Genuity®VT Double Pro™ (VT2P), Genuity® SmartStax™ (SMT), and Agrisure® Viptera™ 3111 (VIP3). Cry1AP, Cry2AP, and Cry2APH were three non-commercially experimental lines provided by Monsanto Company (St. Louis, MO). Cry1AP contains a single Bt gene encoding Cry1A.105, which targets aboveground lepidopteran pests including *S. frugiperda* (Huang et al., 2014). Both Cry2AP and Cry2APH contain a single Bt gene encoding Cry2Ab2, but Cry2APH expresses a higher level of the Cry2Ab2 protein than does Cry2AP (Huang et al., 2014; Niu et al., 2016). HX1 expresses the Cry1F protein (event TC1507) and YG contains the Cry1Ab gene for controlling lepidopteran pests. VT2P expresses Cry1A.105 and Cry2Ab2, SMT expresses these two proteins plus the Cry1F protein, and VIP3 expresses both Cry1Ab and VIP3A; all of which target aboveground maize lepidopteran pests (Buntin and Flanders, 2015). In addition, SMT also produces Cry3Bb1 and Cry34/35AB, and VIP3 also expresses mCry3A. These three Bt proteins target the belowground maize rootworms *Diabrotica* spp (Coleoptera: Chrysomelidae), with no activity for moth pests. Each of the four non-Bt maize products tested in this study was closely related to one or two of the Bt maize products (Table 3.1).

Two seeds of a maize product were planted in each 18.9-liter plastic pot containing ~5 kg of standard potting soil mixture (Perfect Mix™, Expert Gardener products, St. Louis, MO) in a

greenhouse located in Baton Rouge, LA, as described in Wangila et al. (2012). The non-expression for each non-Bt maize product or expression of the expected Bt protein(s) for each Bt maize product was confirmed using ELISA-based assays (EnviroLogix, Quantiplate ™ kits, Portland, ME). In this study, performance of the five populations of *S. frugiperda* on the non-Bt and Bt maize products was evaluated by two methods: leaf tissue bioassay in the laboratory and whole-plant test in the greenhouse.

3.2.3 Leaf Tissue Bioassay in the Laboratory

Two independent trials were performed with the leaf tissue bioassay in the laboratory. Trial-I evaluated SS on nine maize products, and FL32 and FL67 on all 12 maize products listed in Table 3.1. Owing to limited insect supply, SS was not evaluated on the three pyramided maize products in Trial-I. Trial-II examined all five insect populations on 11 of the 12 maize products (Cry2AP was not included due to limited seed supply). In the leaf tissue bioassay, fully-expanded leaves from maize plants at the V5-V8 stages were removed from greenhouse-grown plants and used in the leaf tissue bioassay described in Niu et al. (2013). The leaves were cut into pieces of approximately 3-4 cm in length. Two to three pieces of leaf tissue were then placed in each well of 32-well C-D International trays (Bio-Ba-32, C-D International, Pitman, NJ). Four neonates (<24 h old) of each population were placed on the surface of the leaf tissue in each well (Niu et al., 2013). Bioassay trays containing leaf tissues and neonates were placed in growth chambers maintained at 28°C, 50% RH, and a 16-h:8-h (L:D) photoperiod. Larval mortality was recorded on the 7th day after release of the neonates. In each trial, there were four replications for each combination of maize product and insect population, and each replication contained 32 neonates in eight wells ($n = 32 \times 4 = 128$).

3.2.4 Whole-Plant Test in the Greenhouse

As in the leaf tissue bioassay, two independent trials were conducted in the greenhouse tests: each trial evaluated the performance of all five insect populations on 11 of the 12 maize products listed in Table 3.1 (Cry2AP was not included in the greenhouse tests owing to limited seed supply.) In each trial, four neonates (<24 h old) of an insect population were manually placed into the whorl of a plant at the V5-V9 stage. Treatments in each trial were replicated four times in a randomized complete block design with one pot (2 plants) per replication. Maize leaf injury ratings were checked using the Davis scale of 1 (no damage or few pinholes) to 9 (most leaves with long lesions) (Davis et al., 1992) on the 14th day after larval inoculation. Plants containing live larvae were recorded immediately after rating the leaf injury as described in Niu et al. (2014).

3.2.5 Data Analysis

In both leaf tissue bioassay and whole-plant test, the performance of each insect population was similar among the four non-Bt maize products in each trial; thus, data on larval survival in both tests, as well as the leaf injury ratings in the whole-plant test, were pooled across the four non-Bt maize products. To normalize treatment variances for data analysis, the raw data of larval survivorship rate (recorded from the leaf tissue bioassays) and percentages of plants with live larvae (recorded in the whole-plant tests) (Niu et al., 2014) were transformed using the arcsine of ($x^{0.5}$), while data on the leaf injury rating were transformed to the log(x+1) scale (Zar, 1984). The transformed data were analyzed with a two-way analysis of variance for each of the two leaf tissue bioassays and greenhouse tests (SAS Institute, 2010), with maize product and insect population as the two main factors. In addition, because the overall results between the two greenhouse trials were generally consistent, data for each variable measured in the

Table 3.1. Non-Bt and Bt maize products evaluated in this study.

Maize product ID	Bt gene	Maize product	Traits [a]	Event
NBt	No	Pioneer 31P40	Non-Bt	Closely related to Pioneer 31D59
	No	DKC 61-22	Non-Bt	Closely related to DKC 61-49 and DKC 61-21
	No	N78N-GT	Non-Bt	Closely related to N78N-3111
	No	ExpL	Non-Bt experimental line	Closely related to Cry1A.105Ln and Cry2Ab2Ln
Cry1AP	Cry1A.105	Cry1A.105Ln	Experimental line	Not available
HX1	Cry1F	Pioneer 31D59	Herculex®I	TC1507
YG	Cry1Ab	DKC 69-70	YieldGard®	MON810
Cry2AP	Cry2Ab2	Cry2Ab2Ln	Experimental line with low expression of Cry2Ab2 protein	Not available
Cry2APH	Cry2Ab2	Cry2Ab2Hn	Experimental line with high expression of Cry2Ab2 protein	Not avialable
VT2P	Cry1A.105, Cry2Ab2	DKC 64-04	Genuity® VT Double Pro™	MON89034
SMT	Cry1A.105, Cry2Ab2, Cry1F, Cry3Bb1, Cry34/35Ab	DKC 62-08	Genuity® SmartStax®	MON89034+ TC1507 + MON88017+DAS-59112-7
VIP3	Vip3A, Cry1Ab, mCry3A	N78N-3111	Agrisure® Viptera™ 3111	Bt11+MIR162+MIR604

[a] Maize products that are not labeled as an experimental line are commercial hybrids.

greenhouse trials were pooled across the two trials. The pooled data were then analyzed using mixed models with trial as a random factor (SAS Institute, 2010). Analysis with the mixed models was not performed for leaf tissue bioassays due to the differences in the insect populations and maize products evaluated between the two trials. For each trial and the combined data, treatment means were separated using LSMEANS tests at $\alpha = 0.05$ level.

Both test methods showed cross-resistance of the Cry1A.105-resistant *S. frugiperda* to the Cry1F maize product (see Results). Thus, the effective dominance levels (D_{ML}) of the two Cry1A.105-resistant populations on the leaf tissue and whole plants of Cry1AP and HX1 were estimated by using the method described in Roush and McKenzie (1987). To calculate the dominance levels, the observed larval survival data of an insect population on a Bt maize product were first corrected to the survival on the non-Bt maize products using the method described in Abbott (1925). The corrected-survivorship rates were then used to calculate the dominance levels for each of the two test methods. D_{ML} for the leaf tissue bioassay was estimated based only on Trial-II because F_1 heterozygous insect populations were not included in Trial-I, whereas for the whole-plant tests, D_{ML} was based on pooled data from the two trials.

3.3 Results

3.3.1 Larval Survival of *S. frugiperda* on Leaf Tissue of Non-Bt and Bt Maize Products Containing Single or Pyramided Genes

The effects of insect population, maize product, and their interaction on larval survivorship were all significant for both trials of the leaf tissue bioassay (Table 3.2). The overall performance of each of the three insect populations (SS, FL32, and FL67) that were evaluated in both trials was consistent between the two trials across all the maize products, with few exceptions. In general, larvae of the three populations survived well on leaf tissue of the non-Bt maize products with a 7-day survivorship of 57.6-73.0% in Trial-I and 44.1-72.3% in Trial-II

Table 3.2. Analysis of variance on the data from the leaf tissue bioassay in the laboratory and whole-plant test in the greenhouse.

Parameter & trial	Source of variation	df_1, df_2 [a]	*F*-value	*P*-value
	Leaf tissue bioassay			
Larval survival in Trial-I [b]	Insect	2, 100	67.31	<0.0001
	Maize	8, 100	281.26	<0.0001
	Insect*maize	13, 100	38.63	<0.0001
Larval survival in Trial-II	Insect	4, 120	41.33	<0.0001
	Maize	7, 120	195.71	<0.0001
	Insect*maize	28, 120	9.59	<0.0001
	Whole-plant test			
Leaf injury rating in Trial-I	Insect	4, 161	5.70	0.0003
	Maize	7, 161	140.04	<0.0001
	Insect*maize	28, 161	3.61	<0.0001
Leaf injury rating in Trial-II	Insect	4, 171	7.95	<0.0001
	Maize	7, 171	148.91	<0.0001
	Insect*maize	28, 171	2.67	<0.0001
Leaf injury rating-pooled	Insect	4, 374	13.03	<0.0001
	Maize	7, 374	284.8	<0.0001
	Insect*maize	28, 374	5.33	<0.0001
Larval survival in Trial-I	Insect	4, 164	0.71	0.5852
	Maize	7, 164	22.12	<0.0001
	Insect*maize	28, 164	0.95	0.5450
Larval survival in Trial-II	Insect	4, 171	1.37	0.2478
	Maize	7, 171	28.78	<0.0001
	Insect*maize	28, 171	0.91	0.6027
Larval survival-pooled	Insect	4, 374	2.00	0.0933
	Maize	7, 374	54.00	<0.0001
	Insect*maize	28, 374	1.53	0.0438

[a] df_1 is the degree of freedom of the nominator and df_2 is the degree of freedom for the denominator (error).

[b] In Trial-I of the leaf tissue bioassay, survival of the SS population on the three pyramided Bt maize products (VT2P, SMT, and VIP3) was not evaluated.

(Fig. 3.1). SS on leaf tissue of Cry1AP (Cry1A.105) maize showed a mortality of >99% in both trials. FL32 on Cry1AP maize leaf tissue showed a survivorship of 70.3% in Trial-I and 53.9% in Trial-II, while the corresponding survivorship of FL67 was somewhat lower, i.e., 26.6-32.0% in the two trials (Fig. 3.1). In Trial-II, which included the two F_1 heterozygous populations, leaf tissue of Cry1AP was effective against both RS populations, with a 7-day mortality of 100% for FL32-RS and 93% for FL67-RS (Fig. 3.1). Thus, the effective dominance level, D_{ML}, based on the leaf tissue bioassay was 0 for FL32 and 0.21 for FL67, indicating recessive or incompletely recessive resistance on the Cry1AP leaf tissue (Table 3.3).

FL32 and FL67 also exhibited significant cross-resistance to HX1 (Cry1F) maize. HX1 maize leaf tissue killed 92.2-96.1% SS larvae in the 7-day assays in both trials, while FL32 and FL67 showed a survivorship rate of 70.3-73.4% and 20.3-31.3%, respectively (Fig. 3.1). In Trial-II, the larval survivorship of FL32-RS (14.8%) was not significantly ($P > 0.05$) different from that of SS (7.8%), but significantly ($P \leq 0.05$) lower than that of FL67-RS (37.1%). The survivorship of FL67-RS was similar ($P > 0.05$) to that of FL67 (Fig. 3.1). The calculated D_{ML} based on the leaf tissue bioassay with the HX1 maize was 0.27 for FL32 and 1 for FL67, suggesting that the resistance of the two populations was more dominant on HX1 (Cry1F) leaf tissue than on Cry1AP (Cry1A.105) leaf tissue (Table 3.3).

Leaf tissue of Cry1Ab maize was not effective against any of the five populations of *S. frugiperda*. SS on Cry1Ab leaf tissue exhibited a survivorship rate of 31.2-49.2% in the two trials, and the other four populations showed a survivorship of 28.1-62.5% (Fig. 3.1). However, neither of the Cry1A.105-resistant populations showed any cross-resistance to the maize products containing the Cry2Ab2 protein (Cry2AP and Cry2APH). Larval survivorship of SS on leaf tissue of the low-expressing Cry2Ab2 line (Cry2AP) was 16.4% in Trial-I, and the

corresponding survivorship of FL32 and FL67 was even lower, ≤5.5%. On the high-expressing Cry2Ab2 maize line (Cry2APH), mortality of the five insect populations was 99.2-100% in the two trials (Fig. 3.1). The three pyramided Bt maize products, VT2P, SMT, and VIP3 were effective against both Cry1A.105-susceptible and -resistant *S. frugiperda*. Leaf tissue of these three Bt maize products killed 100% of SS in Trial-II (the only trial where SS was evaluated on pyramided products), and 75.0–100% of the other four populations in the two trials (Fig. 3.1).

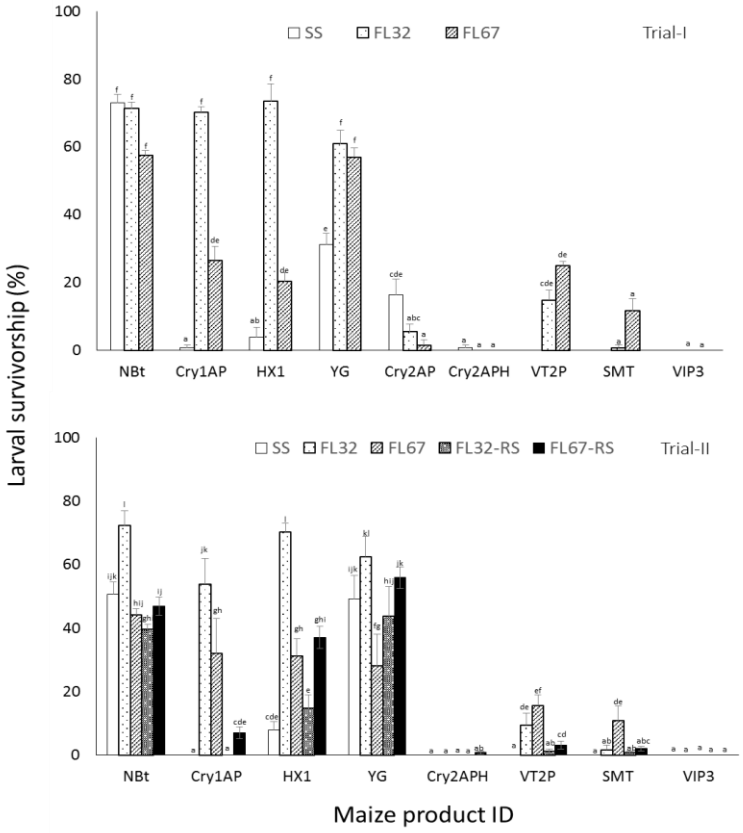

Fig. 3.1. Larval survivorship (mean ± sem %) of Cry1A.105-susceptible (SS) and -resistant populations (FL32 and FL67) of *Spodoptera frugiperda* after 7 days of feeding on leaf tissue of non-Bt and Bt maize products expressing single or multiple Bt proteins. Mean values followed by a same letter are not significantly different (α =0.05; LSMEANS test).

Table 3.3. Effective dominance level (D_{ML}) of two Cry1A.105-resistant populations (FL32 and FL67) of *Spodoptera frugiperda* on leaf tissue and whole plants of Cry1A.105 (Cry1AP) and Cry1F (HX1) maize products.

Test method	Maize product ID	Insect	Effective dominance D_{ML}[a]
Leaf tissue bioassay	Cry1AP	FL32	0.00
		FL67	0.21
	HX1	FL32	0.27
		FL67	1.00[b]
Whole-plant test	Cry1AP	FL32	0.75
		FL67	0.50
	HX1	FL32	0.40
		FL67	0.86

[a] D_{ML} ranges between 0 and 1; $D_{ML} = 0$ means that the resistance is completely recessive, while $D_{ML} = 1$ indicates completely dominant resistance.
[b] The calculated value based on the survival data observed in the leaf tissue bioassay was 1.15.

3.3.2 Leaf Injury Ratings of *S. frugiperda* to Non-Bt and Bt Maize Containing Single or Pyramided Genes in the Whole-Plant Tests

Leaf injury ratings caused by the five populations of *S. frugiperda* were generally consistent between the two trials in the greenhouse. The effects of maize product, insect population, and their interaction on leaf injury ratings were all significant for each of the two trials and for the pooled data analysis (Table 3.2). There were no significant ($P > 0.05$) differences in the leaf injury ratings of non-Bt maize plants among the five insect populations for each trial and for the pooled data. After 14 days, when the trials were terminated, all five populations had caused heavy leaf injuries to the non-Bt maize plants, with an overall average leaf injury rating of 7.4 for the pooled data (Fig. 3.2).

SS caused little damage to Cry1A.105 (Cry1AP) maize plants, with a leaf injury rating of 1.6 for the pooled data (Fig. 3.2). Compared to SS, both resistant populations caused significantly ($P \leq 0.05$) greater injury to Cry1AP. The leaf injury ratings of Cry1AP caused by

FL67 (4.9 in Trial-I and 5.8 in Trial-II) were somewhat greater than those caused by FL32 (3.0 in Trial-I and 3.7 in Trial-II). The difference between the two resistant populations was not significant ($P > 0.05$) for the analysis based on each of the individual trials, but significant ($P \leq 0.05$) for the pooled data analysis (Fig. 3.2). The leaf injury rating to Cry1AP caused by the two heterozygous populations (FL32-RS and FL67-RS) was significantly ($P \leq 0.05$) greater than that caused by SS. In general, FL32-RS and FL32 caused similar ($P > 0.05$) injury to Cry1AP, while the leaf injury caused by FL67-RS was somewhat lower than that caused by FL67. The difference between FL67-RS and FL67 was not significant ($P > 0.05$) for Trial-II, but significant ($P \leq 0.05$) for Trial-I and the pooled data analysis (Fig. 3.2).

As observed in the leaf tissue bioassay, both FL32 and FL67 showed cross-resistance to Cry1F (HX1) maize in the whole-plant test. HX1 was effective against SS and had little leaf injury. Compared to SS, both FL32 and FL67 caused significantly greater ($P \leq 0.05$) leaf injury to HX1. The leaf injury to HX1 caused by the two resistant populations was similar ($P > 0.05$) for both trials, with an average leaf injury rating of 5.4 for the pooled data (Fig. 3.2). The leaf injury levels of HX1 in each trial were similar ($P > 0.05$) between the two RS populations, but overall the injury in Trial-II was greater than in Trial-I. Based on the pooled data analysis, the leaf injury rating (an average of 2.9) on HX1 plants infested with FL32-RS or FL67-RS was significantly greater ($P \leq 0.05$) than that caused by SS, but significantly less ($P \leq 0.05$) than on plants infested with the resistant populations (Fig. 3.2).

Again, Cry1Ab maize (YG) was generally not effective against any of the five populations, with an average leaf injury rating of 6.0 for the pooled data (Fig. 3.2). In contrast, little or no leaf injury was observed on plants of Cry2APH and the three pyramided Bt maize hybrids infested with any of the five populations. In the pooled analysis of the two trials, the two

resistant populations caused an average leaf injury rating of 1.2 to Cry2APH, 1.3 to VT2P, 1.3 to SMT, and 1.1 to VIP3; with few exceptions, none of these values were significantly different from those infested with SS (1.0-1.5) or RS (1.2-2.5) across the four maize products (Fig. 3.2).

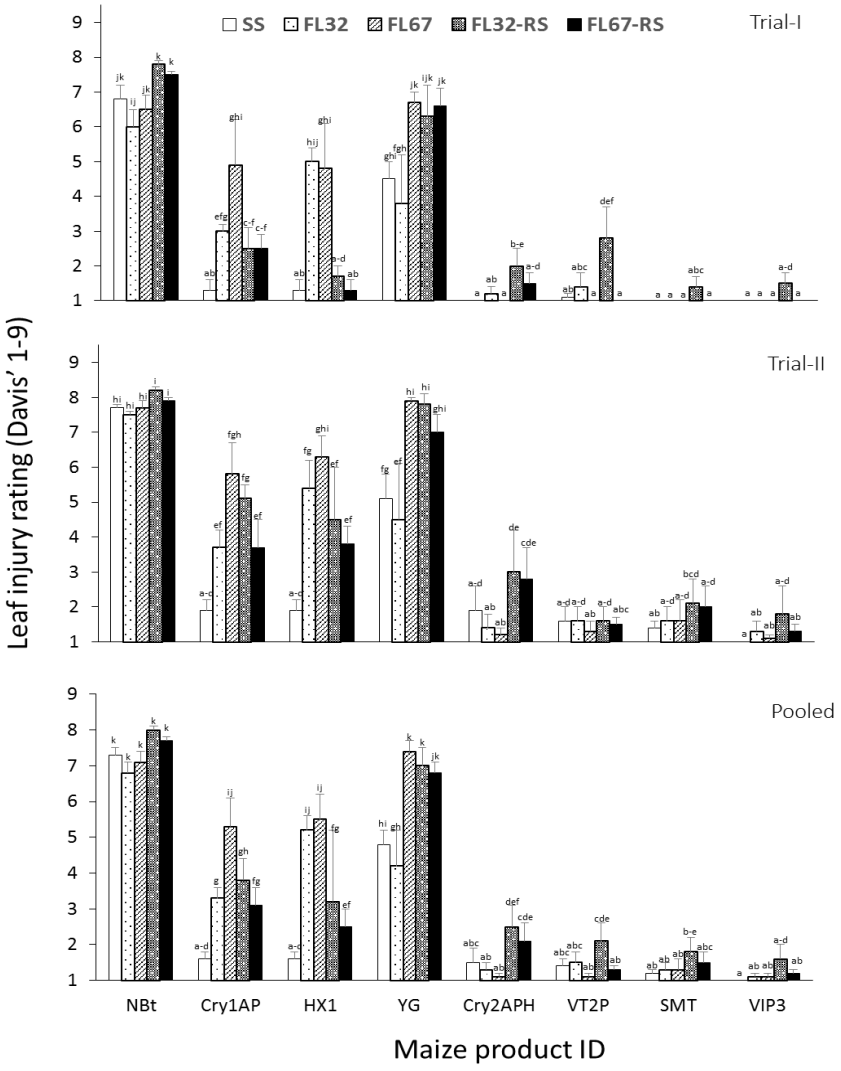

Fig. 3.2. Leaf injury ratings (mean ± sem) of Bt-susceptible (SS), -heterozygous (FL32-RS, FL67-RS), and -resistant (FL32, FL67) populations of *Spodoptera frugiperda* on non-Bt and Bt maize plants expressing single or multiple Bt proteins. Higher values indicate greater injury. Mean values followed by a same letter are not significantly different (α =0.05; LSMEANS test). Only the first and the last letters are presented over the bar if four or more letters were needed for the LSMEANS tests. For example, the label "c-f" over the bar representing the survivorship of FL32-RS on Cry1AP in Trial-I means "cdef".

3.3.3 Larval Survival of *S. frugiperda* on Non-Bt and Bt Maize Containing Single or Pyramided Genes in the Whole-Plant Tests

In the whole-plant test, the effect of insect population and the interaction of insect population and maize product on larval survival of *S. frugiperda* was not significant in the analysis for either of the two single trials, but the effect of maize product was significant for each trial and the pooled data analysis (Table 3.2). In addition, the interaction effect was also significant in the pooled data analysis. Larvae of the five populations survived well on non-Bt maize plants in both trials, and there were no significant ($P > 0.05$) differences in the larval survival rates among the five populations for either of the trials or for the pooled data analysis. Across the five populations and two trials, live larvae were observed on 52.4–72.9% of the non-Bt plants 14 days after larval release (Fig. 3.3). In contrast, no live larvae were observed from Cry1AP plants infested with SS in either trial, while 41.7–62.5% of the Cry1AP plants infested with FL32 or FL67 contained live larvae. The larval survival rate on Cry1AP plants was not significantly ($P > 0.05$) different between the two resistant populations, suggesting that both FL32 and FL67 were highly resistant to these plants (Fig. 3.3). The survival rate of RS on Cry1AP was similar ($P > 0.05$) between FL32-RS and FL67-RS in each of the two trials and in the pooled data analysis. In general, the survival rate of the two RS populations on Cry1AP was numerically greater than that of SS, but lower than that of the two resistant populations. In the pooled data analysis, the difference relative to SS was significant ($P \leq 0.05$) for both RS populations, while, relative to resistant populations, it was significant for FL67-RS, but not for FL32-RS. The calculated D_{ML} based on the pooled data was 0.75 for FL32 and 0.50 for FL67, suggesting that the resistance in the two populations was intermediate to incompletely dominant when it was measured on whole Cry1AP (Cry1A.105) maize plants in the greenhouse (Table 3.3).

Data on larval survival in the whole-plant tests also showed that both FL32 and FL67 were cross-resistant to Cry1F (HX1) maize plants. At 14 days after larval release, live larvae were found in 12.5% and 0% of HX1 plants infested with SS in Trial-I and Trial-II, respectively, while these values were 50.0–54.2% for FL32 and 37.5–66.7% for FL67. The difference between SS and the two resistant populations was, in most cases, significant ($P \leq 0.05$) for each trial and for the pooled data analysis (Fig. 3.3). The survivorship of the two RS populations on HX1 was somewhat greater than that of SS, ranging from 16.7% to 54.2% in the two trials. Relative to SS, the difference was significant ($P \leq 0.05$) for FL67-RS in Trial-I and the pooled analysis, while the difference relative to resistant populations was not significant ($P > 0.05$) in the pooled analysis for either FL32-RS or FL67-RS (Fig. 3.3). The calculated D_{ML} based on the pooled data from the two trials was 0.40 for FL32 and 0.86 for FL67, suggesting that the resistance to Cry1F maize was intermediate to incompletely dominant when measured on whole plants of HX1 maize (Table 3.3).

The larval survival data from the greenhouse whole-plant tests also suggested that the single-gene Cry1Ab maize (YG) was ineffective against any of the five insect populations. For the two trials, an average of 37.5-75% of the YG plants contained live larvae after 14 days, which was not much lower than the survivorship rates observed on the non-Bt maize plants (Fig. 3.3). In contrast, whole plants of Cry2APH and the three pyramided Bt maize products were effective against all five populations. Across these four maize products and both whole-plant trials, live larvae were observed from an average of 3.6% of the plants infested with the two resistant and two F_1 heterozygous populations (Fig. 3.3).

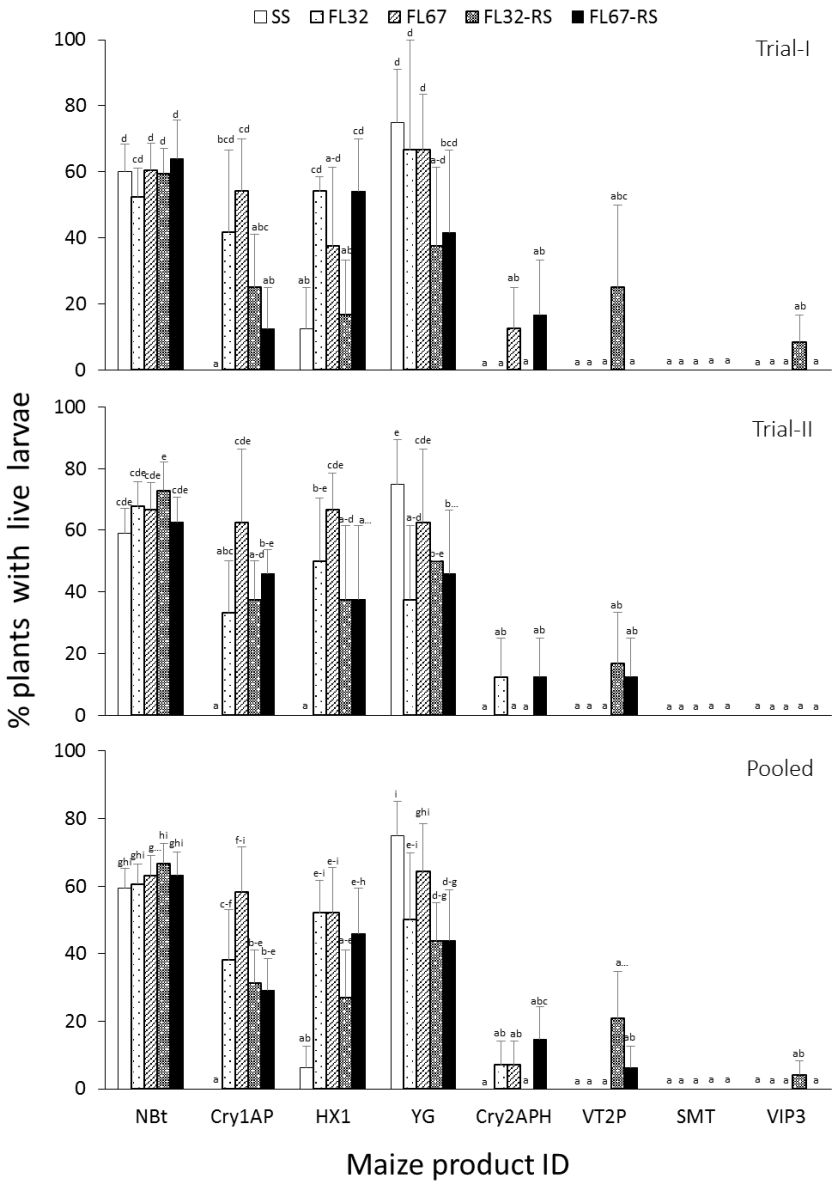

Fig. 3.3. Percent plants (mean ± sem) containing live larvae of Bt-susceptible (SS), -heterozygous (FL32-RS, FL67-RS), and -resistant (FL32, FL67) populations of *Spodoptera frugiperda* on non-Bt and Bt maize plants expressing single or multiple Bt proteins. Mean values followed by a same letter are not significantly different ($\alpha = 0.05$; LSMEANS test). Only the first and the last letters are presented over the bar if four or more letters were needed for the LSMEANS tests. For example, the label "a-d" over the bar representing the survivorship of FL67 on HX1 in Trial-I means "abcd".

3.4 Discussion

A previous study demonstrated that FL32 and FL67 were resistant to the Cry1A.105 protein, allowing the larvae to survive and develop on whole Cry1A.105 maize plants (Huang et al., 2016). In the present study, these two populations also survived well on the Cry1A.105 maize product in the leaf tissue bioassay and whole-plant test. The results further validate that both FL32 and FL67 are highly resistant to Cry1A.105 maize.

Understanding the functional dominance level of resistance is important in resistance management. This study showed that the effective dominance level, D_{ML}, of the Cry1A.105 resistance in *S. frugiperda* appeared to vary depending on the insect population, Bt maize product, and test method. Resistance in FL32 and FL67 on leaf tissue of Cry1A.105 maize was recessive to incompletely recessive, while on whole Cry1A.105 plants it was moderate to incompletely dominant. Several possible reasons might explain the observed variation. First, the genetic basis of the Cry1A.105 resistance in the two populations might not be the same, resulting in different dominance levels in the two populations. Second, the level of Cry1A.105 protein can vary in different plant tissues and plant growth stages (Monsanto, 2009; US-EPA, 2010), which could cause differences in survival of the RS larvae between feeding on whole plants in the greenhouse and on leaf tissue in containers in the laboratory. The variation in the dominance levels observed on different test plant materials suggests that careful experimental designs are needed for evaluating the 'high-dose' qualification of Bt maize against *S. frugiperda*.

The current study also showed that both Cry1A.105-resistant populations of *S. frugiperda* were highly cross-resistant to Cry1F maize. The cross-resistance was incompletely recessive for FL32 but dominant for FL67 in leaf tissue bioassay, while it was incompletely dominant in the whole-plant tests for both populations. Cross-resistance of Cry1F-resistant *S. frugiperda* to

Cry1A.105 protein or Cry1A.105 maize has been reported in two previous studies (Huang et al., 2014; Bernardi et al., 2015); Cry1F and Cry1A.105 have similar structures and thus selection for resistance to one is expected to confer resistance to the other. A previous study also showed that Cry1A.105 resistance alleles in the field populations of *S. frugiperda* collected in 2011 from Florida, U.S. was relatively abundant, reached 0.056 with a 95% credibility interval of 0.032-0.087 (Huang et al., 2016). It was suspected that the relatively high level of Cry1A.105-resistance allele frequency detected in *S. frugiperda* populations in Florida might be a result of the selection of Cry1F resistance, together with the cross-resistance between Cry1F and Cry1A.105 (Huang et al., 2016). Both FL32 and FL67 used in this study were isolated from field populations in which Cry1F-resistance alleles were already abundant (Huang et al., 2014; 2016). The high level of 'cross-resistance' of FL32 and FL67 to Cry1F maize documented in the current study supports this interpretation. Both Cry1F and Cry1A.105 proteins are expressed in some current pyramided Bt maize products (Buntin and Flanders, 2015). The significant cross-resistance between Cry1F and Cry1A.105 in *S. frugiperda* plus the non-recessive nature of the resistance could diminish the durability of some pyramided Bt maize technology if effective resistance management plans are not implemented, especially in areas where Cry1F resistance has already widely occurred (Bernardi et al., 2015).

As reported for Cry1F resistance (Niu et al., 2013; Huang et al., 2014), the Cry1A.105-resistant populations of *S. frugiperda* are not cross-resistant to Cry2Ab2 or Vip3A; thus, both FL32 and FL67 are still susceptible to the pyramided Bt maize products containing one of these two Bt genes. In the current Bt maize market, five Bt proteins (Cry1Ab, Cry1A.105, Cry1F, Cry2Ab2, and Vip3A) are available for controlling aboveground lepidopteran targets, including *S. frugiperda* (Huang et al., 2016). Up to now, all commercial pyramided Bt maize products

targeting lepidopteran pests contain one or two of the three Cry1 proteins plus either Cry2Ab2 or Vip3A (Buntin and Flanders, 2015). As shown in the current study, Cry1Ab maize is ineffective against *S. frugiperda*. Thus, the cross-resistance between Cry1A.105 and Cry1F essentially makes these pyramided maize products function as a single-gene Bt maize product against Cry1F/Cry1A.105-resistant *S. frugiperda*. Currently, university and industry scientists are evaluating new Bt maize products with triple modes of action, i.e., containing one of the three Cry1 proteins plus both Cry2Ab2 and Vip3A. It is expected that these new Bt maize products will soon become commercially available. Results of the current study support the use of these new triple-Bt maize products to manage the Cry1F/Cry1A.105-resistant populations of *S. frugiperda*. More importantly, we believe that integrated pest management tactics with diversified mortality factors must be implemented along with Bt crops to ensure the long-term success of Bt crop technology (Hutchison, 2015; Yang et al., 2016).

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CHAPTER 4. F_2 SCREEN FOR RESISTANCE TO *BACILLUS THURINGIENSIS* CRY2AB2-MAIZE IN FIELD POPULATIONS OF *SPODOPTERA FRUGIPERDA* (LEPIDOPTERA: NOCTUIDAE) FROM THE SOUTHERN UNITED STATES[3]

4.1 Introduction

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

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is a target pest of Bt maize and Bt cotton in North and South America (Farias et al., 2014; Huang et al., 2014). In recent years, field resistance to Cry1F maize in *S. frugiperda* has been documented in Puerto Rico (Storer et al., 2010), Brazil (Farias et al., 2014), and the U. S. mainland (Huang et al., 2014). Up to date, *S. frugiperda* is the only target pest that has developed field resistance to Bt crops in multiple locations across different countries and continents (Dangal and Huang, 2015). Cry2Ab2 is one of the two pyramided Bt genes in the event MON 89034, which has been incorporated into some pyramided Bt maize hybrids. In 2010, maize hybrids containing MON 89034 became commercially available for controlling above-ground lepidopteran pests including *S. frugiperda* (Flanders, 2014). The wide occurrence of the Cry1F resistance in *S. frugiperda* makes it even

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more important to preserve the Cry2Ab2 susceptibility in the target pest populations to ensure the sustainable use of the Bt maize technology. However, because Cry2Ab2 is a relatively new Bt gene used in transgenic maize, information about Cry2Ab2 resistance in target pests of Bt maize is still very limited.

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

4.2 Materials and Methods

4.2.1 Insect Collection, Rearing, and Development of F_2 Two-Parent Families

During 2013-14, 3rd to 5th instars of *S. frugiperda* were collected from non-Bt maize fields at seven geographical locations across four states of the southern U.S.: Texas (TX), Louisiana (LA), Georgia (GA) and Florida (FL). The seven locations included one site in Hidalgo County, TX; two sites in LA, one each in Franklin and Rapides parishes; one site in Tift

County, GA; and three sites in FL, one each in Miami-Dade, Hendry, and Collier counties. Field collected larvae were reared on meridic diet until the pupal stage as described in Niu et al. (2013). F_2 two-parent families were developed by single-pair mating of the individuals derived from the field collections as described in Yang et al. (2013). For each two-parent family, ≈55 viable F_1 pupae were used in sib-mating to generate the F_2 generation of the family.

4.2.2 Screening of F_2 Neonates to Identify Potential Positive Families

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

used for defining the Cry1F and Cry1A.105 resistance as described in Huang et al. (2014; 2016); a PPF means that the family had ≥1 large live larvae (≥3rd instar) after 7 days in the F_2 screen.

4.2.3 Confirmation of Resistance on Whole Plants of Cry2Ab2 Maize

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

Thus, a total of five laboratory strains (GA-15, FL-CL-14, Mixed-A, Mixed-B, and FL-CL-21) were tested for larval survival on whole Cry2Ab2 plants in the greenhouse. For the greenhouse tests, 25 neonates of the 2nd or 3rd generations of each family/group were inoculated

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

4.2.4 Estimation of Cry2Ab2 Resistance Allele Frequency

Expected allele frequencies to Cry2Ab2 maize plants and the corresponding 95% credibility intervals (CI) were calculated using the Bayesian analysis method described in Andow and Alstad (1998; 1999). The probability (detection power) that a resistance allele was detected in a family if one had actually existed in the family was computed using the methods described in Stodola and Andow (2004). Based on our observations, a 1:1 sex ratio and 100% F_1 pupal emergence were used in computing the detection power (Dangla and Huang, 2015).

4.2.5 Susceptibility of Cry2Ab2-Maize-Selected Families to Cry2Ab2 Protein Incorporated in Meridic Diet

The F_2 screen and greenhouse confirmation tests showed that one family from Georgia (GA-15) possessed a major resistance allele to Cry2Ab2 maize plants (see results). To further verify if the survival of GA-15 in the F_2 screen and whole plant tests was due to resistance to the Cry2Ab2 protein in the plants, susceptibility of GA-15, along with TX-SS, to Cry2Ab2 protein

was determined using the diet-incorporated bioassay method described in Niu et al. (2013). Cry2Ab2 protein and its buffer solution were provided by Monsanto Company (St. Louis, MO). Six Cry2Ab2 protein concentrations (0.1, 0.316, 1, 3.16, 10, and 31.6 µg/g) plus two controls (blank and negative) were used in assaying TX-SS and six higher concentrations (1, 3.16, 10, 31.6, 100, and 316 µg/g) plus the two controls were used in assaying GA-15. Distilled water was used for the blank control, while the buffer only was used for the negative control. Larval mortality was recorded on the 7th day after neonate inoculation (Niu et al., 2013). Each Cry2Ab2 concentration and control were replicated four times with 16-32 larvae in each replicate.

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

4.3 Results

4.3.1 Development of Two-Parent Families

In 2013 and 2014, a total of 253 single pairs of *S. frugiperda* were established from the moths derived from the field-collected larvae in the four states (Table 4.1). From these single-pair matings, a total of 215 F_2 two-parent families were successfully established and produced sufficient F_2 (≥120 neonates) progeny for F_2 screening. Among the 215 F_2 families, 32, 101, 17, and 65 were derived from collections in TX, LA, GA and FL, respectively. Of the 101 Louisiana families, 57 were established from Franklin Parish and 44 were from Rapides Parish. Of the 65 Florida families, 14 were developed from Miami-Dade Co., 16 were from Hendry Co., and 35 were from Collier Co.

Table 4.1. Performance of F_2 two-parent families of seven populations of *Spodoptera frugiperda* collected from Texas (TX), Louisiana (LA), Georgia (GA), and Florida (FL) in F_2 screen for resistance to Cry2Ab2 maize. [a]

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

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

4.3.2 Baseline Performance of TX-SS on Leaf Tissue of Non-Bt and Cry2Ab2 Maize Lines

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

4.3.3 Survival of F_2 Families in F_2 Screen

All of the 215 F_2 families mentioned above were screened on Cry2Ab2 maize leaf tissue (Table 4.1). Among the 32 Texas-Hidalgo families, a total of 47 larvae from 9 families survived on Cry2Ab2 leaf tissue after 4 days (Table 4.1). After 7 days, 16 larvae from 6 families survived, which included 14 small larvae (≤2nd instar) and 2 large larvae (≥3rd instars). The two large larvae were from the same family, TX-29 (Table 4.2).

In the 57 families from Louisiana-Franklin, 21 families had some larvae surviving with a total of 79 individuals after 4 days (Table 4.1). After 7 days, 10 larvae from two families were still alive. All of the 10 survivors were small, ≤2nd instar. From 44 Louisiana-Rapides families, 20 had some larvae surviving to 4 days with a total of 50 survivors. After 7 days, 6 larvae from 3 families were surviving and all were 2nd instar or smaller (Table 4.1).

From 17 Georgia-Tift families, 63 larvae from 12 families survived through 4 days on the Cry2Ab2 leaf tissue (Table 4.1). At 7 days, 12 larvae from 4 families were still alive. The

survivors included six large larvae (≥3rd instars) from a single family, GA-15 (Table 4.2). Other survivors were 2nd instars or smaller.

Table 4.2. Potentially positive families that might possess Cry2Ab2 resistance alleles in *Spodoptera frugiperda* collected from Texas, Louisiana, Georgia and Florida.[a]

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

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

In the 14 Florida-Miami/Dade families, a total of 20 larvae from 5 families survived through 4 days, while four families still had 12 live larvae after 7 days, which included 11 small larvae and one large larva (Table 4.1). The large larva belonged to the family FL-MD-8 (Table 4.2). Of the 16 Florida-Hendry families, eight families had live larvae after 4 days with a total of 16 survivors, which was reduced to one family and one small larva on the 7th day. For the 35 Florida-Collier families, 21 families had 231 live larvae at 4 days, which reduced to 11 families with 64 larvae at 7 days. These survivors consisted of 42 small larvae and 22 large larvae (Table 4.1). The large larvae were recovered from families of FL-CL-7 (1 larva), FL-CL-10 (1 larva), FL-CL-13 (1 larva), and FL-CL-14 (19 larvae) (Table 4.2).

Thus, based on the criteria mentioned above, the F_2 screen showed that seven families qualified as PPFs, which included one from the Texas-Hidalgo population (TX-29); one from the Georgia-Tift population (GA-15), one from the Florida-Miami/Dade population (FL-MD-8) and four from the Florida-Collier population (FL-CL-7, FL-CL-10, FL-CL-13, and FL-CL-14) (Table 4.2).

4.3.4 Performance of PPFs on Whole Plants of Cry2Ab2 Maize in the Greenhouse Confirmation Tests

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

On whole plants of Cry2Ab2 maize in the greenhouse, GA-15 caused severe plant injury after 11 days with a leaf injury rating of 9 on the 1-9 scale (Table 4.3) and two large live larvae (4^{th} instars) were recovered from two of the four plants infested with GA-15 neonates. The other two plants were also severely damaged, indicating that larvae had moved away from these two Cry2Ab2 plants. In contrast, all Cry2Ab2 plants infested with *S. frugiperda* larvae from other PPFs/groups (FL-CL-14, FL-CL-21, Mixed-A, and Mixed-B) were not injured and no live larvae

were found (Table 4.3). Thus, only GA-15 family was confirmed to carry a major resistance allele against Cry2Ab2 maize plants.

Table 4.3. Performance of potentially positive families/groups and a negative family of *Spodoptera frugiperda* on whole plants pf Cry2Ab2 maize in the greenhouse.

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

[a] Larval survival and plant injury of TX-SS on the Cry2Ab2 maize and non-Bt maize plants were evaluated after 14 days of neonate release. TX-SS didn't cause any leaf injury (leaf injury rating of 1 on a scale of 1-9 with 1 being the least) and no live larvae were recovered from the Cry2Ab2 plants, while on non-Bt maize plants, TX-SS caused significant leaf damage rated 7.4 and 50% plants contained large live larvae ($\geq 5^{th}$ instars).
[b] FL-CL-21 was a negative family which was treated as a control to confirm the criteria for major resistance alleles.

4.3.5 Major Resistance Allele Frequency

The F_2 screen and whole plant confirmation tests showed that of the 17 families collected from Georgia-Tift Co., one F_2 family, GA-15, possessed a major resistance allele to Cry2Ab2 maize plants (Table 4.4), while major resistance alleles, based on the criteria mentioned above, were not evident in the other 214 families. Based on the Bayesian analysis, the expected Cry2Ab2 resistance allele frequency associated with a major allele for the Georgia population was 0.0274 with a 95% CI of 0.0035 to 0.0766. The corresponding frequency with 95% possibility was <0.0224 for the Texas population, <0.0073 for the Louisiana populations, and

<0.0113 for the Florida populations (Table 4.4). The expected frequencies in the four states were not significantly different based on their overlapping 95% CIs. Thus, the Cry2Ab2 major resistance allele frequency for the combined populations collected from the four states was estimated to be 0.0023 with a 95% CI of 0.0003 to 0.0064 (Table 4.4). The F_2 screen had a detection power of 97.0% for identifying a resistance allele if one existed in a family.

Table 4.4. Expected frequency and/or corresponding 95% credibility interval (CI) of resistance alleles to Cry2Ab2 in field populations of *Spodoptera frugiperda* from Texas, Louisiana, Georgia and Florida.

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

4.3.6 Minor Resistance Allele Frequency

Besides GA-15, results of the F_2 screen also showed that six other F_2 families had some survival on Cry2Ab2 maize leaf tissue and had at least one large survivor ($\geq 3^{rd}$ instars). These six families were not confirmed to possess major resistance alleles because they could not develop to the adult stage in the laboratory or the progeny of the families could not survive on whole plants of the Cry2Ab2 maize in the greenhouse. Based on the criteria described above, these six families were considered to possess minor resistance alleles to the Cry2Ab2 maize. The

six families were TX-29 from the Texas population, FL-MD-8 from the Florida-Miami/Dade population, and FL-CL-7, FL-CL-10, FL-CL-13, and FL-CL-14 from the Florida-Collier population. The estimated minor resistance allele frequency with 95% possibility was <0.0073 for the Louisiana populations and <0.0408 for the Georgia population (Table 4.4). It was 0.015 with a 95% CI of 0.0019 to 0.042 for the Texas population, 0.0232 with a 95% CI of 0.0086 to 0.0449 for the Florida populations, and 0.0082 with a 95% CI of 0.0033 to 0.0152 for the combined populations across the four states.

4.3.7 Susceptibility of TX-SS and GA-15 to Cry2Ab2 Protein

The estimated LC_{50} value for TX-SS was 20.8 µg/g with a 95% CI of 12.9 to 51.6 µg/g (Table 4.5). The LC_{50} for GA-15 could not be calculated with the probit analysis because of the low mortality (42.2%) at the highest concentration of 316 µg/g in the bioassay. Therefore, the LC_{50} value for GA-15 was considered >316 µg/g. Using the LC_{50} value of TX-SS as a standard, the resistance ratio to Cry2Ab2 protein in GA-15 was >15-fold. The results of the bioassays further confirmed that the survival of GA-15 on leaf tissue and whole plants of the Cry2Ab2 maize was due to a resistance to the Cry2Ab2 protein in the plants.

4.4 Discussion

The F_2 screen in the current study identified that at least one (GA-15) out of the 215 two-parent families of *S. frugiperda* from populations collected in the southern U.S. possessed a major resistance allele to the Cry2Ab2 protein. It should be noted that the Cry2Ab2 resistance allele frequency calculated in this study for these populations is a conservative estimation. Due to the difficulty in establishing colonies from the very limited number of survivors after F_2 screen, resistance confirmation was not performed for all PPFs. Although we used the well-established procedures (Huang et al., 2014; 2016), the result of the current study still could not

Table 4.5. Susceptibility of a Cry2Ab2-susceptible strain (TX-SS) and family GA-15 of *Spodoptera frugiperda* on meridic diet containing Cry2Ab2 protein.

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

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

completely exclude the possibility that some PPFs (e.g. FL-MD-8; FL-CL-7, and FL-CL-13) might actually possess major alleles. The current study, as other published studies, did not provide any additional information to confirm the 'minor' resistance alleles. In addition, in the greenhouse confirmation tests, survival on non-Bt plants was evaluated only for the susceptible strain, but not for PPFs. However, based on our previous studies (Yang et al., 2013; Huang et al., 2014; 2016), the possibility that the observed mortality of the tested PPFs on Cry2Ab2 maize was not caused by the insecticidal Bt plants was extremely low. Our on-going studies also showed that a Cry2Ab2-resistant strain that was developed from GA-15 feeding on non-Bt maize leaves survived well, and developed to pupae and produced offspring normally (B.A. and F.H., unpublished data).

Several studies have shown that most Bt maize products do not produce a high dose against *S. frugiperda*, and even susceptible larvae could survival on some Bt maize plants (Niu et al., 2014; Yang et al., 2013). For this reason, in this study, the survivors of each *S. frugiperda*

family after the F_2 screen were separated into two groups based on their developmental stages and the families that had only small survivors ($\leq 2^{nd}$ instars) were not considered to carry resistance alleles. As mentioned above, the same method has been used in several previous studies (Niu et al., 2013; Yang et al., 2013; Huang et al., 2014; 2016). The greenhouse whole plant test for the family FL-CL-21 in the current study showed little plant injury and a 100% mortality on the Bt plants, also suggesting the chance that these small survivors possessed resistance alleles to the Bt plants was very low.

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

availability of the Cry2Ab2-resistant strain of *S. frugiperda* established in the current study warrants further studies to characterize the mechanisms of the resistance in the pest. The results of the current F_2 screen also suggest that the major Cry2Ab2 resistance allele frequency in *S. frugiperda* is apparently still relatively low in the southern U.S.

Besides the family that possessed a major resistance allele, six other families were considered to possess 'minor' resistance alleles to the Cry2Ab2 protein. The detection of the major resistance allele coupled with the relatively more common 'minor' resistance alleles in the field populations of *S. frugiperda* may have important implications for resistance management. Single gene Cry2Ab2 maize is not commercially available for controlling insect pests, but Cry2Ab2 is one of the two Bt proteins in MON 89034 that has been incorporated into many pyramided Bt maize products (Ghimire et al., 2011). The other Bt protein in MON 89034 is Cry1A.105 that is also active against *S. frugiperda* (Huang et al., 2016). Cry2Ab2 is dissimilar in protein structure from Cry1 proteins (e.g. Cry1A and Cry1F) and has different binding sites in the midguts of target insects, indicating that Cry2Ab2 represents a distinct mode of action from Cry1A and Cry1F proteins (Storer et al., 2012). Several studies have shown that a Cry1 resistant insect is usually not cross-resistant to Cry2Ab2 (Sivasupramaniam et al., 2008; Brévault et al., 2009; Wu et al., 2009; Vélez et al., 2013; Huang et al., 2014), suggesting that pyramiding Cry2Ab2 and Cry1A.105 (e.g. MON 89034) should be a good strategy for resistance management (Wu et al., 2009; Ghimire et al., 2011; Wangila et al., 2012).

However, as mentioned above, field resistance of *S. frugiperda* to Cry1F maize has occurred in some regions of the world (Storer et al., 2010; Farias et al., 2014; Huang et al., 2014). Studies have demonstrated that Cry1A.105 and Cry1F are cross-resistant to each other in *S. frugiperda* (Huang et al., 2014; 2016; Bernardi et al., 2015b). The cross-resistance between

Cry1F and Cry1A.105 could significantly reduce the activity of the Cry1A.105 protein in MON 89034, leaving the Cry2Ab2 protein only partially protected against Cry1F-resistant *S. frugiperda*. Thus, Cry1F-resistant individuals of *S. frugiperda* that possess resistance alleles (major or minor) to other Bt proteins could have an advantage in survival on pyramided Bt plants. Recently, a resistant strain of *S. frugiperda* to MON 89034 was selected from a Cry1F-resistant population in 10 generations in the laboratory (Santos-Amaya et al., 2015). More knowledge is needed to develop effective strategies to manage resistance evolution to pyramided Bt crops, especially when resistance/cross-resistance to one Bt protein has already occurred.

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CHAPTER 5. PHENOTYPIC PERFORMANCE OF DIFFERENT GENOTYPES OF FALL ARMYWORM POSSESSING SINGLE- OR DUAL-CRY1A.105/CRY2AB2 RESISTANT GENES ON MON 89034 CORN

5.1 Introduction

Due to the intensive use of Bt crops since it was commercialized in 1996, at least 24 cases of field resistance to Bt crops have been reported in different target pests in the world (Tabashnik and Carriere, 2017). Fall armyworm, *S. frugiperda* (J.E. Smith), was one of the notable target pests that has developed several cases of field resistance to Cry1F corn at multiple locations in different countries, including Puerto Rico in 2006, Brazil and USA 2011, and Argentina in 2013 (Storer et al., 2010; Farias et al., 2014; Huang et al., 2014; Chandrasena et al., 2018). All field resistance cases in *S. frugiperda* are related to the control failures of the single-gene Cry1F corn. In order to delay the development of resistance, pyramided Bt corn containing two or more pyramided Bt genes has gradually replaced the single-gene Bt crops (Ghimire et al., 2011; Matten et al., 2012; Buntin and Flanders, 2015). Compared to the single-gene Bt corn, pyramided Bt corn products are usually more effective against some target pests, especially for Noctuidae species such as the corn earworm (*Helicoverpa zea* [Boddie]) and *S. frugiperda* (Burkness et al., 2010; Niu et al., 2014; Yang et al., 2013; 2015). However, the elevated resistance allele frequency to single Bt proteins mentioned above should have increased the frequency of pest individuals possessing dual or multiple resistance alleles to different Bt proteins. Up to date, there have been limited studies for dual- or multiple-gene Bt resistance (Santos-Amaya et al., 2015; Bernardi et al., 2017; Horikoshi et al., 2016).

Recently, a single-gene Cry1A.105-resistant strain and a single-gene Cry2Ab2-resistant strain of *S. frugiperda* were developed by using F_2 screen (Huang et al., 2016; Niu et al., 2016a). Studies have shown that both the Cry1A.105 and Cry2Ab2 resistances in the two strains were

controlled by a separate single autosomal gene (Acharya et al., 2017; Niu et al., 2017). No cross resistance was detected between the Cry1A.105 and Cry2Ab2 resistant strains (Yang et al., 2017). By crossing the two well-documented single-gene Bt resistant strains, a dual-Bt gene resistant strain of *S. frugiperda* that was resistant to MON 89034 corn plants containing both Cry1A.105 and Cry2Ab2 proteins was established in the laboratory. The availability of the single-gene and dual-Bt gene resistant strains of *S. frugiperda* provided an opportunity to determine the phenotypic performance of different insect genotypes containing single- and multiple-Bt resistance alleles and thus to generate essential parameters needed for refining IRM modeling for the pyramided Bt crop technology.

5.2 Materials and Methods

5.2.1 Insect Sources

Three genotypes of *S. frugiperda* were used as the original insect sources for this study (Table 5.1). They were a susceptible genotype (aabb), a Cry1A.105-resistant (AAbb) genotype and a Cry2Ab2-resistant (aaBB) genotype. The genotype of aabb was collected from non-Bt corn fields in Franklin Parish, LA, in 2016. Field collected larvae were reared on meridic diet until the pupal stage as described in Niu et al. (2013). The baseline susceptibility of aabb was evaluated through a leaf tissue bioassay using different non-Bt and Bt lines/hybrids (Table 5.2). Genotype of AAbb was isolated from two single-pairing families collected from maize fields in Collier County, Florida, in 2011 (Huang et al., 2016). It was documented to possess a major resistance allele to Cry1A.105 corn plants, and could survive and develop well on whole plants of Cry1A.105 corn in the greenhouse (Huang et al., 2016; Niu et al., 2016b; 2017). Genotype of aaBB was developed from an F_2 screen of field populations collected from Georgia, U.S. in 2013 (Niu et al., 2016a). It possessed a major Cry2Ab2 resistance allele which allowed homozygous

Cry2Ab2-resistant individuals (aaBB) to survive and complete larval development on Cry2Ab2 corn plants (Niu et al., 2016a). No cross resistance was found between AAbb and aaBB insect strains (Yang et al., 2017). Recently, we analyzed the genetic basis and evaluated the fitness costs of the resistance in both AAbb and aaBB. The resistance in AAbb or aaBB was likely controlled by a separate single autosomal gene with no fitness costs (Niu et al., 2017; Acharya et al., 2017). Cry1A.105 resistance in AAbb was non-recessive (Niu et al., 2017), while Cry2Ab2 resistance in aaBB was completely recessive on Cry2Ab2 leaf tissue (Acharya et al., 2017), but non-recessive on whole Cry2Ab2 plants (Yang et al., 2017).

5.2.2. Establishment of Cry1A.105/Cry2Ab2-Dual Gene Resistant Genotype

A genotype of AABB was established from the F_2 generation developed from sib-mating of F_1 progeny, which was produced by reciprocal crossing between AAbb and aaBB. The F_2 generation of the crosses was selected three times with MON 89034 corn leaf tissue. Before AABB was used in the current study, it had been backcrossed with aabb twice and reselected for resistance with MON 89034 corn leaf tissue, as described in Dangal and Huang (2015). The baseline survival of AABB has also been examined using the leaf tissue of different non-Bt and Bt corn leaf issues.

5.2.3. Reciprocal Crosses of aabb, AAbb, aaBB, and AABB

Reciprocal crosses among the four *S. frugiperda* sources (aabb, AAbb, aaBB, and AABB) mentioned above were conducted in the laboratory by using the method as described in Camargo et al. (2017) to generate five different F_1 heterozygous genotypes containing single or multiple resistance alleles (Table 5.1). They were denoted as 1) Aabb, a mixture of the two F_1 heterozygous genotypes produced from the reciprocal crosses of aabb and AAbb; 2) aaBb, a mixture of the two F_1 heterozygous genotypes produced from the reciprocal crosses of aabb and aaBB; 3) AaBb, a mixture of the two F_1 heterozygous genotypes produced from the reciprocal

crosses of aabb and AABB; 4) AABb, a mixture of the two F_1 heterozygous genotypes produced from the reciprocal crosses of AAbb and AABB; and 5) AaBB, a mixture of the two F_1 heterozygous genotypes produced from the reciprocal crosses of aaBB and AABB.

Table 5.1. Nine genotypes of *Spodoptera frugiperda* used in the study.

Insect genotype notation	Description of insect genotypes
aabb	A Bt-susceptible genotype originally collected from Franklin Parish, Louisiana in 2016.
AAbb	A Cry1A.105-resistant and Cry2Ab2-susceptible *S. frugiperda* genotype isolated using an F_2 screen of a single-pair collected from Florida in 2011.
aaBB	A Cry1A.105-susceptible and Cry2Ab2-resistant *S. frugiperda* genotype isolated using an F_2 screen of a single-pair collected from Georgia in 2013.
AABB	A dual-Bt gene resistant genotype generated by crossing AAbb and aaBB. It was resistant to single gene Cry1A.105, Cry2Ab2 corn, as well as, to MON89034 expressing both Cry1A.105 and Cry2Ab2.
Aabb	A F1 genotype generated by crossing AAbb and aabb
aaBb	A F1 genotype generated by crossing aabb and aaBB
AaBb	A F1 genotype generated by crossing aabb and AABB
AABb	A F1 genotype generated by crossing AABB and AAbb
AaBB	A F1 genotype generated by crossing AABB and aaBB

5.2.2 Corn Products

To evaluate the baseline susceptibility of aabb and AABB genotypes, one non-Bt (DKC 62-95) and nine different Bt lines/hybrids described in Table 5.2 were used. The nine Bt lines/hybrids expressed five different Bt proteins against lepidopteran, including Cry1A.105, Cry1Ab, Cry1F, Cry2Ab2 and VIP3A proteins (Table 5.2). Performance of the nine genotypes of *S. frugiperda* was evaluated on one non-Bt corn (DKC 65-17) and one Bt corn (DKC 66-87) hybrids. Both corn hybrids were provided by Monsanto Company (St. Louis, MO). The Bt corn hybrid contained the Bt event MON 89034 which expressed two pyramided Bt proteins Cry1A.105 and Cry2Ab2 for controlling lepidopteran pests including *S. frugiperda* (Difonzo and Porter, 2018). The non-Bt product was genetically closely related to the MON 89034 corn hybrid.

Two seeds of each corn product were planted in each 18.9-liter pot containing ~5 kg of standard potting soil mixture (Perfect MixTM, Expert Gardener products, St. Louis, MO) in a greenhouse located in Baton Rouge, LA, as described in Niu et al. (2013; 2016b). The non-expression of the non-Bt corn plants or expression of the expected Bt protein(s) for each Bt corn plant was confirmed using ELISA-based assays (EnviroLogix, Quantiplate TM kits, Portland, ME). In this study, leaf tissue bioassay was conducted to determine the baseline susceptibility of aabb and AABB in the laboratory (Nin et al., 2013), while performance of the nine genotypes of *S. frugiperda* on the two corn hybrids was conducted with two methods: 1) leaf tissue bioassay in the laboratory and 2) whole-plant test in the greenhouse.

5.2.3 Leaf Tissue Bioassay to Determine The Baseline Susceptibility of aabb and AABB Genotypes

Baseline susceptibility of aabb and AABB genotypes was determined using non-Bt and Bt corn leaf tissue (Niu et al., 2013; 2016b). In the bioassay, fully-expanded leaves from corn plants at the V5-V8 stages were removed from greenhouse-grown plants and used in the bioassay. Leaves were cut into pieces approximately 3 cm in length. Two to three pieces of leaf tissue were placed in each well of a 32-well C-D International tray (Bio-Ba-32, C-D International, Pitman, NJ). Four neonates (<24 h old) of an insect strain were then placed on the surface of the leaf tissue in each well and leaf tissue was replaced every 2-3 days (Niu et al., 2013; 2016b). Bioassay trays were put in environmental chambers maintained at 26°C, 50% RH, and a 16:8 (L: D) h photoperiod and larval mortality was checked after 7 days of neonate release. Larvae were considered dead if they did not respond after being touched with a camel hair brush (Niu et al., 2013; 2016b). There were four replications and each replication consisted of 32 larvae.

Table 5.2. Larval mortality (mean ± sem) of aabb and AABB *Spodoptera frugiperda* genotypes on leaf tissue of non-Bt and Bt corn plants.

Corn line/hybrid	Corn trait	Event	Bt genes	Larval Mortality %[a]	
				aabb	AABB
DKC 62-95	Non-Bt corn, closely related to DKC 62-98	---	---	15.6±1.8ab	10.9±4.1a
Cry1A.105Ln	Experimental line	---	Cry1A.105	100.0±0.0g	23.4±4.5b
Cry2Ab2Ln	Experimental line	---	Cry2Ab2	92.2±2.7f	13.3±3.5a
DKC 62-98	Genuity® VT Double Pro™	MON89034	Cry1A.105, Cry2Ab2	100.0±0.0g	14.8±3.5ab
P1319HR	Herculex®I	TC1507	Cry1F	82.8±3.3e	14.8±4.7ab
P1197YHR	Optimum®Intrasect™	TC1507+Bt11	Cry1F, Cry1Ab	95.3±0.9f	70.3±5.2d
DKC 66-40	Genuity® SmartStax™	MON89034+ TC1507+MON88017+ DAS-59112-7	Cry1A.105, Cry2Ab2, Cry1F, Cry3Bb1, Cry34/35Ab	99.2±0.8g	52.3±4.1c
P1319VYHR	Optimum®Leptra®	TC1507+Bt11+MIR162	Cry1F, Cry1Ab, Vip3A	100.0±0.0g	100.0±0.0g
n/a	Trecepta™	MON89034+MIR162	Cry1A.105, Cry2Ab2, Vip3A	100.0±0.0g	100.0±0.0g
N60F-3111	Agrisure® Viptera™ 3111	Bt11+MIR162+MIR604	Vip3A, Cry1Ab, mCry3A	100.0±0.0g	100.0±0.0g
ANOVA		Insect		$F_{1,60} = 654.86$, $P < 0.0001$	
		Corn		$F_{9,60} = 156.39$, $P < 0.0001$	
		Insect x Corn		$F_{9,60} = 55.54$, $P < 0.0001$	

[a] Mean values followed by a same letter in the table are not significantly different ($P > 0.05$; Fisher's LSD significant difference tests).

5.2.4 Performance of Nine *S. frugiperda* Genotypes in Leaf Tissue Bioassay

To evaluate the performance of the nine *S. frugiperda* genotypes on leaf tissue, fully-expanded leaves at the V5-V8 stages were removed from the corn plants in the greenhouse and were cut into small pieces with ~1 cm length. One-two pieces of leaf tissue were then placed into each well of the 128-well C-D International trays (Bio-Ba-32, C-D International, Pitman, NJ). One neonate (<24 h old) of each *S. frugiperda* genotype was infested with brush on the surface of the leaf tissue in each well. The bioassay trays were placed on the shelves in a walk-in insect rearing room maintained at ~26°C, ~50%RH, and a 14h: 10h (L:D) photoperiod. After 3 days, the survivors of each treatment combination were then transferred from the 128-well bioassay wells into 30 ml rearing plastic cups containing the same leaf tissue with 1 survivor/cup. To ensure enough air circulation in the cups, the lids of cups were punched with a few holes using dissection noodles. All cups were held in plastic trays. Leaf tissue in the cups was replaced every 2-3 days until pupation. In each bioassay, there were four replications (trays) for each insect genotype and each replication consisted of 32 larvae (n = 4 x 32=128). The trays within a replication were placed on the shelves in the same walk-in insect rearing room mentioned above. Pupation for each treatment replication was checked daily once the first pupa occurred. Pupae from the four replications of each treatment combination were transferred into 3.78-liter paper containers for adult emergence in the walk-in insect rearing room. Development time to pupa stage was also recorded for each treatment replication.

To evaluate the reproduction, a pair of newly emerged (<24 h old) virgin male and female adults were placed into 3.78-liter paper containers (Huhtamaki Foodservice, De Soto, Kansas) for mating and oviposition as described in Zhang et al. (2014). Up to 11 pairs of adults were established for each treatment combination. Total number of eggs produced per pair was

estimated by weighing the total egg masses laid using the method as described in Zhou et al. (in submission).

5.2.4 Performance of Nine *S. frugiperda* Genotypes in Whole-Plant Tests

Two independent trials were conducted in the greenhouse to evaluate the performance of the nine genotypes of *S. frugiperda* on whole plants of the two corn hybrids mentioned in the leaf tissue bioassay. In each trial, five neonates (<24h old) of an insect genotype were released into the whorl of each plant at V7-V9 plant stages (Niu et al., 2014; 2016b). Each treatment combination of corn product and insect genotype was replicated four times in a randomized complete block design with one pot (2 plants) per replication. Corn leaf injury ratings were determined using the Davis scale of 1 (no damage or few pinholes) to 9 (most leaves with long lesions) (Davis et al., 1992) on the 9th day after larval inoculation. To secure that there were no escapes of *S. frugiperda* adults from the greenhouse, larval survival data was recorded when majority of the larvae on non-Bt corn plants were at the 3rd and 4th instars. Data recording at these stages could also avoid errors caused by the larval movement and minimize the effect of the cannibalism. In the greenhouse study, number of larvae and their developmental stage were recorded immediately after rating the leaf injury as described in Niu et al. (2014; 2016b).

To measure the treatment effect after the greenhouse tests, larvae collected from the greenhouse-grown plants were reared on the same corn leaf tissue from which the larvae were collected in the greenhouse. The laboratory rearing condition was the same as described in the leaf tissue bioassay, except that the rearing cups of each treatment combination were put into a plastic zip bag instead of into the rearing trays. To ensure air circulation, the zip bags containing insect rearing cups were also punched with many holes using dissection noodles. The zip bags for each block were then placed into a big plastic boxes treated as the block factor. A total of

eight plastic boxes (eight blocks from two trials) were put on the table in the walk-in insect rearing room. After the first pupa was observed, observation was done daily until all larvae pupated or died.

5.2.5 Data Analysis

As described in Niu et al. (2014; 2016b), the larval survival of *S. frugiperda* on whole plants in the greenhouse tests was measured as % plants containing live larvae after 9 days of neonate release. The larval mortality, pupation rate recorded in the corn leaf tissue bioassay and % plants with live larvae in the whole plant test were transformed using arcsine ($x^{0.5}$). Other biological parameters recorded in both leaf tissue bioassay and greenhouse tests including neonate-to-pupa development time, leaf injury ratings, larval growth index, pupa/plant and egg production/pair were transformed into log(x+1) scale to meet the assumption for analysis of variance (ANOVA). The larval growth index measured as the average larval stage at the data recording in the greenhouse: 1 = 1st instar, 2= 2nd instar, …., 5 = 5th instar. All transformed data were analyzed with a two-way analysis of variance (ANOVA) (SAS Institute, 2010), with corn product and insect genotype as the two main factors. In addition, to increase the power of the data analysis, data for each biological parameter measured in the greenhouse tests were pooled across the two trials. The pooled data were then analyzed using mixed models with trial as a random factor (SAS Institute, 2010). For each trial and the pooled data, treatment means were separated using LSMEANS tests at $\alpha = 0.05$ level.

5.3 Results

5.3.1 Baseline Susceptibility of aabb and AABB Genotypes on Leaf Tissue of Non-Bt and Nine Bt Corn Lines/Hybrids

The effect of insect strain, corn line/hybrid, and their interaction on larval mortality was all significant (F = 654.86; df = 1, 60; $P < 0.0001$ for insect strains, F = 156.39; df = 9, 60; $P <$

0.0001 for corn lines/hybrids, and $F = 55.54$; df = 9, 60; $P < 0.0001$ for interaction) (Table 5.2). Larval mortalities on non-Bt corn leaf tissue were not significantly different ($P > 0.05$) between aabb (15.6%) and AABB (10.9%) (Table 5.2). Genotype aabb was susceptible to corn leaf tissue expressing Cry1A.105, Cry2Ab2, Cry1F, Cry1Ab and VIP3A proteins. Larval mortalities of aabb on leaf tissue of the nine Bt corn lines/hybrids ranged from 82.8 to100% (Table 5.2). In contrast, AABB was highly resistant to corn leaf tissue containing Cry1A.105 and/or Cry2Ab2, somewhat cross-resistant to leaf tissue containing Cry1F and Cry1Ab genes, but susceptible to corn leaf tissue expressing Vip3A protein. AABB on leaf tissue of Cry1A.105, Cry2Ab2, Herculex®I, and Genuity®VT Double Pro™ corn plants had a mortality of only 13.3 to 23.4%, which were generally similar ($P > 0.05$) to the mortality on the non-Bt corn leaf tissue (Table 5.2). The genotype of AABB showed 52.3% larval mortality on Genuity®SmartStax™ corn and 70.3% on Optimum®Intrasect™ corn hybrid, which were significantly greater ($P < 0.05$) than the mortality on the non-Bt corn hybrid, but lower ($P < 0.05$) than the mortality of aabb on Bt corn hybrids (Table 5.2). No survivors of AABB were observed on the three Bt corn hybrids containing Vip3A gene (Table 5.2).

5.3.2 Pupation, Neonate-to-Pupa Development Time and Egg Production of Nine Genotypes of *S. frugiperda* on Leaf Tissue of Non-Bt and MON 89034 Corn Plants

The effect of insect genotype, corn hybrids, and their interaction on neonate-to-pupation rate was all significant ($F = 3.80$; df = 8, 54; $P = 0.0013$ for insect genotype, $F = 838.57$; df = 1, 54; $P < 0.0001$ for corn hybrid, and $F = 7.89$; df = 8, 54; $P < 0.0001$ for interaction (Table 5.3). All nine genotypes of *S. frugiperda* pupated well with 57.8-83.6% pupation rate on the leaf tissue of the non-Bt corn; and these values were generally not significantly ($P > 0.05$) different among insect genotypes (Table 5.3). Only one pupa was produced from aabb that were reared on MON89034 corn leaf tissue, which represented a neonate-to-pupation rate of 0.8%. In contrast,

AABB reared on MON89034 leaf tissue presented a pupation rate of 32.0%, which was significantly lower ($P < 0.05$) than that on non-Bt corn products, but significantly greater ($P < 0.05$) than the pupation rates of any other eight genotypes on Bt leaf tissue (Table 5.3). An average of 0.0-10.9% pupation rates were observed for the five heterozygous genotypes on MON 89034 corn leaf tissue, which were generally not significantly different ($P > 0.05$) compared to the rate of aabb on the Bt leaf tissue (Table 5.3). The genotypes of AABb and AaBB had an average pupation rate of 8.1% on MON 89034 leaf tissue, which was, in generally, greater ($P < 0.05$) than the pupation rates observed for the other five genotypes containing one or two resistance alleles.

The effect of insect genotype, corn hybrid, and their interaction on neonate-to-pupa development time was also all significant ($F = 3.47$; df = 8,40; $P = 0.0040$ for insect genotype, $F = 393.36$; df = 1,40; $P < 0.0001$ for corn hybrid, and $F = 7.91$; df = 7,40; $P < 0.0001$ for interaction) (Table 5.3). Neonates of the nine genotypes on non-Bt leaf tissue spent 18.0-19.8 days to become pupae, which were significantly shorter ($P < 0.05$) than that observed on MON 89034 leaf tissue for all the nine genotypes. AABB spent 21.9 days on Bt leaf tissue to develop to the pupal stage, which was longer ($P < 0.05$) than that on non-Bt leaf tissue, but was generally significantly shorter ($P < 0.05$) than the time (23.0-29.0 days) spent for any other genotypes on the same leaf tissue. Except AABB, few significant differences ($P > 0.05$) in the development time were observed among the other eight genotypes on MON 89034 corn leaf tissue (Table 5.3).

Egg production varied very much, even within pairs of a same genotype. ANOVA with the limited data available showed that the main effect of insect genotype on egg reproduction was significant (F = 2.25; df = 8,84; P < 0.0336), while the effects of corn hybrid and their interaction were not significant (F = 2.03; df = 1,84; P =0.1079 for corn hybrid; F = 1.82;

Table 5.3. Pupation, neonate-to-pupa development time, and reproduction (mean ± sem) of nine genotypes of *Spodoptera frugiperda* feeding on leaf tissue of non-Bt and MON 89034 corn plants.

Corn trait	Insect Genotype	Pupation %[a]	Neonate-to-pupa time (day)[a]	No. Pairs[a]	No. Pairs with eggs[a]	No. eggs/pair[a]
Non-Bt	aabb	74.2 ± 8.6 fg	19.3 ± 0.3 bc	11	9	936.9 ± 183.6 cd
	Aabb	75.0 ± 8.0 fg	18.6 ± 0.2 abc	11	11	735.0 ± 123.2 bcd
	aaBb	77.3 ± 8.7 fg	18.5 ± 0.1 abc	11	11	1278.8 ± 117.6 d
	AaBb	77.3 ± 2.7 fg	19.5 ± 0.8 bc	10	8	964.6 ± 160.3 cd
	AAbb	78.9 ± 3.2 fg	18.0 ± 0.5 a	10	7	970.5 ± 154.3 cd
	aaBB	63.3 ± 3.5 ef	19.8 ± 0.6 c	10	7	509.2 ± 164.3 ab
	AABb	79.7 ± 2.0 g	18.4 ± 0.2 ab	10	10	909.2 ± 87.2 cd
	AaBB	83.6 ± 3.5 g	18.5 ± 0.1 abc	10	9	1141.5 ± 152.8 cd
	AABB	57.8 ± 2.7 e	19.8 ± 0.4c	10	8	596.8 ± 127.3 a-d
MON 89034	aabb	0.8 ± 0.8 a	29.0 h	0	0	--
	Aabb	10.9 ± 4.1 bc	25.2 ± 0.3 efg	5	4	915.3 ± 161.9 cd
	aaBb	0.0 ± 0.0 a	--	0	0	--
	AaBb	3.9 ± 2.0 ab	26.0 ± 1.0 fg	1	1	363 a-d
	AAbb	1.6 ± 0.9 a	26.5 ±0.5 gh	0	0	--
	aaBB	0.8 ± 0.8 a	23.0 de	0	0	--
	AABb	13.3 ± 2.0 c	24.4 ± 0.8 ef	5	3	373.7 ± 92.9 abc
	AaBB	3.1 ± 1.3 ab	27.2 ± 1.0 gh	0	0	--
	AABB	32.0 ± 7.7 d	21.9 ± 0.4 d	11	9	387.7 ± 88.8 a
ANOVA	Insect	$F_{8,54} = 3.80$ $P = 0.0013$	$F_{8,40} = 3.47$ $P = 0.0040$	--	--	$F_{8,84} = 2.25$ $P = 0.0336$
	Corn	$F_{1,54} = 838.57$ $P < 0.0001$	$F_{1,40} = 393.36$ $P < 0.0001$	--	--	$F_{1,84} = 2.03$ $P = 0.1079$
	Insect x Corn	$F_{8,54} = 7.89$ $P < 0.0001$	$F_{7,40} = 7.91$ $P < 0.0001$	--	--	$F_{3,84} = 1.82$ $P = 0.2885$

[a] Mean values followed by a same letter in a column are not significantly different ($P > 0.05$; Fisher's LSD significant difference tests).

df = 3,84; P = 0.2885 for interaction) (Table 5.3). For each insect genotype that was reared on non-Bt corn leaf tissue, 10-11 single-pairings were established for measuring egg production. Among these pairs, 7-11 pairs of each genotype produced an average of 596.8-1278.8 eggs/pair (Table 5.3). Compared to aabb adults derived from the larvae reared on non-Bt leaf tissue, the other eight genotypes produced similar ($P > 0.05$) eggs except for aaBB. On the MON 89034 leaf tissue, because of the high level of larval mortality for some genotypes, uneven pupation, adult emergence time and sex ratios, only four genotypes produced adults to make 1-11 single parings for each of the four genotypes (Aabb, AaBb, AABb, and AABB); and thus egg production was not measured for other five genotypes. Among the four genotypes, AABB had the most pairs (9 pairs) that produced eggs successfully with an average of 387.7 eggs/pair. In addition, four Aabb, one AaBb, and three AABb adult pairs also successfully laid eggs (Table 5.3). Based on the egg laying of the limited number of adult pairs available, the egg production (915.0 eggs/pair) of Aabb on MON89034 was generally not significantly different ($P > 0.05$) compared to the egg production of the insects reared on non-Bt corn leaf tissue. The other three genotypes including AaBb, AABb and AABB on MON 89034 produced an average of 363-387.7 eggs/pair, which were not significantly different ($P > 0.05$) among the three genotypes (Table 5.3).

5.3.3 Leaf Injury Ratings of Nine Genotypes of *S. frugiperda* to Non-Bt and MON 89034 Corn Plants in Whole-Plant Tests

Leaf injury ratings caused by the nine genotypes of *S. frugiperda* were generally consistent between the two trials in the greenhouse. The effects of insect genotype, corn hybrid, and their interaction on leaf injury ratings were all significant for each of the two trials (Trial-1: $F = 6.85$; df = 8,51; $P < 0.0001$ for insect genotype, $F = 456.12$; df = 1,51; $P < 0.0001$ for corn hybrid, and $F = 5.95$; df = 8,51; $P < 0.0001$ for interaction; Trial-2: $F = 8.84$; df = 8,50; $P <$

0.0001 for insect genotype, $F = 619.28$; df = 1,50; $P < 0.0001$ for corn hybrid, and $F = 14.96$; df = 8,50; $P < 0.0001$ for interaction) and for the pooled data analysis ($F = 12.80$; df = 8,118; $P < 0.0001$ for insect genotype, $F = 943.99$; df = 1,118; $P < 0.0001$ for corn hybrid, and $F = 16.39$; df = 8,118; $P < 0.0001$ for interaction) (Table 5.4). After 9 days, non-Bt plants were seriously injured with an average leaf injury rating of 5.0-6.2 for trial-1, 4.9-7.1 for trial-2, and 5.0-6.6 for the pooled data (Table 5.4). There were generally no significant differences ($P > 0.05$) in the leaf injury ratings among the nine insect genotypes for each of the two trials and for the pooled data analysis. In the same period on MON 89034 plants, aabb caused little or no injury with a rating of 1.5 in trial-1, 1 in trial-2 and 1.3 for the pooled data (Table 5.4). In contrast, AABB caused a leaf injury rating of 4.9 in both trials, which was significantly greater ($P < 0.05$) than the leaf injury observed on the plants infested with aabb, and, in many cases, not significantly different ($P > 0.05$) from the injury on non-Bt plants for each of the two trials and for the pooled data analysis (Table 5.4). MON 89034 was effective in protecting leaf injury from the genotypes possessing single or two resistance alleles with an average leaf injury ratings of 1.7-1.9 in trial-1, 1.0-1.4 in trial-2, and 1.4-1.6 in the pooled data, which were not significantly different ($P > 0.05$) than the values of aabb on MON89034 (Table 5.4). Compared to the heterozygotes possessing one or two resistance alleles, the leaf injury ratings caused by AaBB on MON89034 were not significant different in trial-1 (with a rating of 1.7), slightly but statistically significantly greater ($P < 0.05$) in trial-2 (a rating of 2.3) and for the pooled data analysis (a rating of 2.0). However, relative to the genotypes possessing one or two resistance alleles, AABb on MON 89034 caused significant greater ($P < 0.05$) leaf injury in each of the two trials (a rating of 3.0 in trial-1 and 2.8 in trial-2) and for the pooled data analysis (a rating of 2.9). The leaf injury rating caused by

AABb on MON 89034 was also significantly greater ($P < 0.05$) than that caused by AaBB in trial-1 and for the pooled data analysis (Table 5.4).

Table 5.4. Leaf injury ratings (mean ± sem) of non-Bt and MON 89034 corn plants recorded after 9 d infested with neonates of nine genotypes of *Spodoptera frugiperda*.

Corn trait	Insect Genotype	Trial-1[a]	Trial-2[a]	Pooled[a]
Non-Bt[a]	aabb	6.0 ± 0.4 c	7.0 ± 0.4 de	6.5 ± 0.3 e
	Aabb	5.8 ± 0.3 c	5.8 ± 0.2 cde	5.8 ± 0.2 de
	aaBb	6.2 ± 0.3 c	7.1 ± 0.4 de	6.6 ± 0.3 e
	AaBb	6.0 ± 0.1 c	5.7 ± 0.4 cde	5.8 ± 0.2 de
	AAbb	5.0 ± 0.5 c	5.1 ± 0.2 c	5.0 ± 0.2 d
	aaBB	6.0 ± 0.3 c	5.3 ± 0.6 c	5.6 ± 0.3 de
	AABb	5.9 ± 0.3 c	5.9 ± 0.3 cde	5.9 ± 0.2 de
	AaBB	5.9 ± 0.4 c	5.5 ± 0.6 cd	5.7 ± 0.4 de
	AABB	6.2 ± 0.2 c	4.9 ± 0.5 c	5.5 ± 0.3 de
MON 89034	aabb	1.5 ± 0.3 a	1.0 ± 0.0 a	1.3 ± 0.2 a
	Aabb	1.8 ± 0.2 a	1.4 ± 0.3 a	1.6 ± 0.2 ab
	aaBb	1.7 ± 0.1 a	1.2 ± 0.2 a	1.4 ± 0.1 a
	AaBb	1.8 ± 0.3 a	1.1 ± 0.1 a	1.5 ± 0.2 a
	AAbb	1.9 ± 0.1 a	1.2 ± 0.2 a	1.5 ± 0.2 ab
	aaBB	1.8 ± 0.3 a	1.0 ± 0.0 a	1.4 ± 0.2 a
	AABb	3.0 ± 0.2 b	2.8 ± 0.8 b	2.9 ± 0.3 c
	AaBB	1.7 ± 0.6 a	2.3 ± 0.4 b	2.0 ± 0.3 b
	AABB	4.9 ± 0.4 c	4.9 ± 0.3 c	4.9 ± 0.2 d
ANOVA	Insect	$F_{8,51} = 6.85$	$F_{8,50} = 8.84$	$F_{8,118} = 12.80$
		$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
	Corn	$F_{1,51} = 456.12$	$F_{1,50} = 619.28$	$F_{1,118} = 943.99$
		$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
	Insect x Corn	$F_{8,51} = 5.95$	$F_{8,50} = 14.96$	$F_{8,118} = 16.39$
		$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

[a] Mean values followed by a same letter in a column are not significantly different ($P > 0.05$; Fisher's LSD significant difference tests).

5.3.4 Larval Survival, Growth index, and Number of Pupae Produced Per Plant of Nine Genotypes of *S. frugiperda* on Non-Bt and MON 89034 Corn Plants in the Whole-Plant Tests

Similarly as observed in the leaf injury, the effects of insect genotype, corn product, and their interaction on larval survival were all significant for each of the two trials (Trial-1: $F = 5.33$; df = 8,51; $P < 0.0001$ for insect genotype, $F = 297.31$; df = 1,51; $P < 0.0001$ for corn hybrid,

and $F = 4.72$; df = 8,51; $P = 0.0002$ for interaction; Trial-2: $F = 3.46$; df = 8,50; $P = 0.0030$ for insect genotype, $F = 58.95$; df = 1,50; $P < 0.0001$ for corn hybrid, and $F = 6.54$; df = 8,50; $P < 0.0001$ for interaction) and for the pooled data analysis ($F = 6.36$; df = 8,118; $P < 0.0001$ for insect genotype, $F = 243.85$; df = 1,118; $P < 0.0001$ for corn hybrid, and $F = 8.96$; df = 8,118; $P < 0.0001$ for interaction) (Table 5.5). Larvae of the nine genotypes of *S. frugiperda* survived well on non-Bt corn plants in both trials. After 9 days, live larvae of the nine genotypes were observed on 83.3-100% non-Bt plants in trial-1, 66.7-100% in trial-2, and 79.2-91.7% for the pooled data (Table 5.5). With few exceptions, there were generally no significant differences ($P > 0.05$) in the larval survival on non-Bt corn plants among the nine genotypes. MON 89034 in the whole plant greenhouse tests was extremely effective against the Bt-susceptible genotype aabb; after 9 days of neonate release, no live larvae were found on MON 89034 plants infested with aabb. In contrast, AABB was highly resistant to MON 89034 plants in the whole plant tests. On MON 89034 plants infested with AABB, live larvae were recovered from the average of 83.3% plants in trial-1, 100% in trial-2, and 91.7% in the pooled data, which were not significantly different ($P > 0.05$) from the survivorship on non-Bt corn plants for each of the two trials and for the pooled data analysis (Table 5.5). MON 89034 in whole plant tests was also very effective against the five genotypes possessing one or two resistance alleles with an average of 0-16.7%, 8.3-25%, and 4.2-12.5% plants containing live larvae in trial-1, trial-2, and the pooled data, respectively (Table 5.5). The differences in the larval survival on MON 89034 were not significant ($P > 0.05$) among these five genotypes and aabb for each of the two trials and the pooled data analysis. On MON 89034 plants infested with AaBB, live larvae were observed in 8.3% plants in trial-1, 58.3% plants in trial-2, and 33.3 % plants in the pooled data. These values, compared to the larval survival of the five genotypes and aabb, were generally not significant ($P >$

0.05) for trial-1, but significant for trial-2 and the pooled data analysis. For MON 89034 plants infested with AABb, live larvae were recovered from 41.7% plants in trial-1, 77.8% in trial-2, and 57.1% in the pooled data which were significantly greater ($P < 0.05$) than that of aabb and the five genotypes containing one or two resistance alleles (Table 5.5). Compared to AaBB on MON 89034, the survival rates of AABb on MON 89034 were significantly greater ($P < 0.05$) in trial-1 and for the pooled data analysis, while not significantly different ($P > 0.05$) in trial-2 (Table 5.5).

The effects of insect genotype, corn product, and their interaction on larval growth index were significant for trail-2 ($F = 2.96$; df = 8,29; $P = 0.0150$ for insect genotype, $F = 12.90$; df = 1,29; $P = 0.0012$ for corn hybrid, and $F = 2.45$; df = 7,29; $P = 0.0420$ for interaction) and for the pooled data analysis ($F = 3.73$; df = 8,71; $P = 0.0011$ for insect genotype, $F = 26.32$; df = 1,71; $P < 0.0001$ for corn hybrid, and $F = 3.76$; df = 7,71; $P = 0.0016$ for interaction), while the main effects of insect genotype and the interaction in trial-1 were not significant ($F = 1.15$; df = 8,30; $P = 0.3621$ for insect genotype, $F = 16.49$; df = 1,30; $P = 0.0003$ for corn hybrid, and $F = 2.66$; df = 3,30; $P = 0.0661$ for interaction) (Table 5.5). On non-Bt corn plants, larval growth index among the nine insect genotypes ranged from 2.9-3.4 in trial-1, 2.9-3.4 for trial-2, and 3.0-3.4 for the pooled data. The differences in the larval growth on non-Bt plants were not significant ($P > 0.05$) among the nine insect genotypes. Larval growth index for the larvae recovered from MON 89034 plants infested with AABB was 3.0 in trial-1, 2.9 in trial-2, and 2.9 in the pooled data, which were not significantly different ($P > 0.05$) from that of the larvae recovered from non-Bt plants for each of the two trails and for the data analysis. The growth of the limited number of larvae recovered from MON 89034 infested with the five genotypes possessing one or two resistance alleles was somewhat inhibited, with an average growth index of 2-3 (2^{nd} and 3^{rd}

Table 5.5. Percent plants (mean ± sem) containing live larvae and larval growth index (mean ± sem) of non-Bt and MON 89034 corn plants recorded after 9 d infested with neonates of the nine genotypes of *Spodoptera frugiperda*.

Corn trait	Insect Genotype	Trial-1[a]		Trial-2[a]		Pooled[a]	
		% plants	Growth index	% plants	Growth index	% plants	Growth index
Non-Bt	aabb	83.3 ± 16.7 c	3.2 ± 0.1 c	100.0 ± 0.0 d	3.4 ± 0.2 c	91.7 ± 8.3 d	3.3 ± 0.1 e
	Aabb	83.3 ± 9.6 c	3.4 ± 0.3 c	75.0 ± 8.3 cd	3.2 ± 0.3 c	79.2 ± 6.1 cd	3.3 ± 0.2 e
	aaBb	91.7 ± 8.3 c	3.3 ± 0.2 c	75.0 ± 8.3 cd	3.3 ± 0.2 c	83.3 ± 6.3 d	3.3 ± 0.1 e
	AaBb	91.7 ± 8.3 c	3.3 ± 0.1 c	83.3 ± 9.6 cd	3.1 ± 0.2 bc	87.5 ± 6.1 d	3.2 ± 0.1 de
	AAbb	100.0 ± 0.0 c	2.9 ± 0.1 bc	83.3 ± 9.6 cd	3.1 ± 0.2 bc	91.7 ± 5.5 d	3.0 ± 0.1 cde
	aaBB	91.7 ± 8.3 c	3.3 ± 0.1 c	75.0 ± 16.0 cd	3.1 ± 0.4 bc	83.3 ± 8.9 d	3.2 ± 0.2 de
	AABb	100.0 ± 0.0 c	3.4 ± 0.2 c	66.7 ± 23.6 c	3.3 ± 0.4 bc	83.3 ± 12.6 d	3.4 ± 0.2 e
	AaBB	83.3 ± 9.6 c	3.2 ± 0.1 c	83.3 ± 9.6 cd	2.9 ± 0.2 bc	83.3 ± 6.3 d	3.0 ± 0.1 e
	AABB	91.7 ± 8.3 c	3.1 ± 0.1 c	66.7 ± 13.6 c	3.1 ± 0.2 bc	79.2 ± 8.8 cd	3.1 ± 0.1 e
MON89034	aabb	0.0 ± 0.0 a	--	0.0 ± 0.0 a	--	0.0 ± 0.0 a	--
	Aabb	16.7 ± 9.6 ab	3.0 bc	8.3 ± 8.3 a	3.0 bc	12.5 ± 6.1 ab	3.0 cde
	aaBb	0.0 ± 0.0 a	--	8.3 ± 8.3 a	2.5 bc	4.2 ± 4.2 a	2.5 b-e
	AaBb	0.0 ± 0.0 a	--	25.0 ± 16.0 ab	2.5 ± 0.5 bc	12.5 ± 8.8 ab	2.5 bcd
	AAbb	0.0 ± 0.0 a	--	8.3 ± 8.3 a	3.0 bc	4.2 ± 4.2 a	3.0 de
	aaBB	0.0 ± 0.0 a	--	8.3 ± 8.3 a	2.0 b	4.2 ± 4.2 a	2.0 ab
	AABb	41.7 ± 16.0 b	2.5 ± 0.5 ab	77.8 ± 22.2 cd	2.6 ± 0.3 bc	57.1 ± 14.0 c	2.5 ± 0.3 bc
	AaBB	8.3 ± 8.3 a	2.0 a	58.3 ± 8.3 bc	1.0 a	33.3 ± 10.9 b	1.5 ± 0.5 a
	AABB	83.3 ± 9.6 c	3.0 ± 0.2 bc	100.0 ± 0.0 d	2.9 ± 0.2 bc	91.7 ± 5.5 d	2.9 ± 0.1 cde
ANOVA	Insect	$F_{8,51}$ = 5.33 P < 0.0001	$F_{8,30}$ = 1.15 P = 0.3621	$F_{8,50}$ = 3.46 P = 0.0030	$F_{8,29}$ = 2.96 P = 0.0150	$F_{8,118}$=6.36 P<0.0001	$F_{8,71}$ = 3.73 P = 0.0011
	Corn	$F_{1,51}$ = 297.31 P < 0.0001	$F_{1,30}$ = 16.49 P = 0.0003	$F_{1,50}$ = 58.95 P < 0.0001	$F_{1,29}$ = 12.90 P = 0.0012	$F_{1,118}$=243.85 P<0.0001	$F_{1,71}$ = 26.32 P < 0.0001
	Insect x Corn	$F_{8,51}$ = 4.72 P = 0.0002	$F_{3,30}$ = 2.66 P = 0.0661	$F_{8,50}$ = 6.54 P < 0.0001	$F_{7,29}$ = 2.45 P = 0.0420	$F_{8,118}$=8.96 P<0.0001	$F_{7,71}$ = 3.76 P = 0.0016

[a] Mean values followed by a same letter in a column are not significantly different (P >0.05; Fisher's LSD significant difference tests).

instars) (Table 5.5). Live larvae recovered from MON 89034 plants infested with AaBB were also inhibited with an average of growth index of 1-2 for both trials and the pooled data. Larvae from MON 89034 plants infested with AABb had an average growth index of 2.5, 2.6, and 2.5 in trials 1 and 2, and the pooled data, respectively, which were not significantly different ($P > 0.05$) comparted to the value of the planted infested with AABB, but significantly less ($P < 0.05$) that the insect on non-Bt plants for each trial and the pooled data analysis (Table 5.5).

The effect of insect genotype was not significant on number of pupae produced from each plant for each trial and for the pooled data, but the effects of corn hybrid and their interaction were significant for the two trials and for the pooled data (Trial-1: $F = 1.69$; df = 8,51; $P = 0.1245$ for insect genotype, $F = 90.62$; df = 1,51; $P < 0.0001$ for corn hybrid, and $F = 2.71$; df = 8,51; $P = 0.0144$ for interaction; Trial-2: $F = 0.37$; df = 8,50; $P = 0.9321$ for insect genotype, $F = 82.58$; df = 1,50; $P < 0.0001$ for corn hybrid, and $F = 2.78$; df = 8,50; $P = 0.0127$ for interaction; Pooled: $F = 1.48$; df = 8,118; $P = 0.1723$ for insect genotype, $F = 178.56$; df = 1,118; $P < 0.0001$ for corn hybrid, and $F = 4.91$; df = 8,118; $P < 0.0001$ for interaction) (Table 5.6). An average of 0.4-1.3 pupae/plant in trial-1, 0.3-1.1 pupae/plant in trial-2, and 0.5-1.2 pupae/plant for the pooled data were produced from non-Bt plants across the nine insect genotypes. The differences in the number of pupae produced from non-Bt plants were generally not significantly different ($P > 0.05$) among the nine insect genotypes, with a few exceptions for AABB (Table 5.6). In the trial-2 and the pooled trial, AABB had significantly less ($P < 0.05$) pupa (0.3 in

Table 5.6. Number of pupa/plant (mean ± sem) produced from non-Bt and MON 89034 corn plants infested with neonates of nine genotypes of *Spodoptera frugiperda*.

Corn trait	Insect Genotype	Trial-1[a]	Trial-2[a]	Pooled[a]
Non-Bt	aabb	1.3 ± 0.4 de	1.1 ± 0.2 e	1.2 ± 0.2 d
	Aabb	1.1 ± 0.3 de	0.8 ± 0.2 de	1.0 ± 0.2 cd
	aaBb	1.2 ± 0.3 de	0.8 ± 0.3 de	1.0 ± 0.2 cd
	AaBb	1.1 ± 0.2 de	0.7 ± 0.3 cde	0.9 ± 0.2 cd
	AAbb	0.4 ± 0.3 abc	0.6 ± 0.2 cde	0.5 ± 0.2 b
	aaBB	1.0 ± 0.4 de	0.8 ± 0.3 de	0.9 ± 0.2 cd
	AABb	1.3 ± 0.1 e	0.5 ± 0.2 bcd	0.9 ± 0.2 cd
	AaBB	0.7 ± 0.2 cd	0.6 ± 0.2 cde	0.6 ± 0.1 bc
	AABB	0.7 ± 0.3 bcd	0.3 ± 0.1 abc	0.5 ± 0.2 b
MON89034	aabb	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
	Aabb	0.2 ± 0.1 ab	0.0 ± 0.0 a	0.1 ± 0.1 a
	aaBb	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
	AaBb	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
	AAbb	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
	aaBB	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
	AABb	0.1 ± 0.1 a	0.1 ± 0.1 ab	0.1 ± 0.1 a
	AaBB	0.0 ± 0.0 a	0.2 ± 0.2 ab	0.1 ± 0.1 a
	AABB	0.8 ± 0.2 cde	0.4 ± 0.1 bcd	0.6 ± 0.1 bc
ANOVA	Insect	$F_{8,51} = 1.69$	$F_{8,50} = 0.37$	$F_{8,118} = 1.48$
		$P = 0.1245$	$P = 0.9321$	$P = 0.1723$
	Corn	$F_{1,51} = 90.62$	$F_{1,50} = 82.58$	$F_{1,118} = 178.56$
		$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
	Insect x Corn	$F_{8,51} = 2.71$	$F_{8,50} = 2.78$	$F_{8,118} = 4.91$
		$P = 0.0144$	$P = 0.0127$	$P < 0.0001$

[a] Mean values followed by a same letter in a column are not significantly different ($P > 0.05$; Fisher's LSD significant difference tests).

trial-2 and 0.5 in the pooled data) than that observed for aabb (1.1 in trial-2 and 1.2 in the pooled data) on the non-Bt plants (Table 5.6). Because no live larvae were found from MON 89034 plants with aabb, no pupae were produced from those plants infested with aabb. MON 89034 was also very effective against the five genotypes possessing one or two resistance alleles with few pupae produced across all the five genotypes and the two trials; and the differences were not significantly different ($P > 0.05$) among the five insect genotypes for each of the two trials and for the pooled data analysis (Table 5.6). For MON 89034 plants infested with AABB, an average of 0.8 pupae/plant in trial-1, 0.4 pupae in trial-2, and 0.6 pupae/plant for the pooled data were produced, which were generally not significantly different ($P > 0.05$) from that of the non-Bt plants infested with AABB in both trials and the pooled data. In addition, these data for AABB were significantly greater ($P < 0.05$) than the values observed from MON 89034 plants infested with aabb and other seven genotypes (Table 5.6). A few pupa were produced from MON 89034 plants infested with AaBB or AABb in the two trials, which were not significant different compared to the pupa number of other heterozygous genotypes containing only one or two resistance alleles for both trials and for the pooled data analysis (Table 5.6).

5.4 Discussion

The performance of AABB on MON 89034 corn plants in the whole plant test was nearly the same as that on non-Bt corn plants. A few exceptions occurred in the corn leaf tissue bioassay that AABB on the MON 89034 leaf tissue showed a less pupation rate and a longer neonate-to-pupa development time than its performance observed on non-Bt leaf tissue. The difference between the performance of AABB on corn leaf tissue bioassay and on whole plant test might be due to the different level of Bt toxin expressions between the corn leaf tissue and whole corn plant. Nevertheless, the overall results validated that the Cry1A.105/Cry2Ab2-dual

Bt resistant genotype (AABB) of *S. frugiperda* was highly resistant to MON 89034 corn expressing both Cry1A.105 and Cry2Ab2 proteins. The genotype of AABB could complete the larval development (from neonates to pupae) on MON89034 plant tissue. Then, the adults of AABB emerged from the pupa could mate and produce offspring successfully.

On both leaf tissue bioassay and whole plant test, the overall performance of the genotypes containing two resistance alleles (AaBb, AAbb and aaBB) were not significantly different from that of aabb on leaf tissue and whole plants of MON 89034 corn. Two recent studies have showed that the dual- or multiple-gene Bt resistance to Cry1A/Cry2A or Cry1A/Cry2A/Cry1F plants in *S. frugiperda* is functionally recessive on the corresponding pyramided Bt corn plants (Santos-Amaya et al., 2015; Bernardi et al., 2017). The results in this study also indicate that MON 89034 is effective against the genotypes of *S. frugiperda* possessing one or two resistance alleles.

On MON 89034 corn leaf tissue, there was significant difference ($P \leq 0.05$) observed between the performance of Aabb and aaBb possessing one resistance allele. The genotype of Aabb had higher pupation rate and shorter neonate-to-pupa development time than the performance of aabb on the MON 89034 leaf tissue, while no pupa was observed for aaBb. Aabb and aaBb were the F_1 heterozygotes of the crossing between AAbb and aabb, and between aaBB and aabb, respectively. Previous studies have shown that the Cry1A.105 resistance in AAbb was functionally non-recessive with an effective dominance level of 0.097-0.579 on Cry1A.105 leaf tissue (Niu et al., 2017) and 0.5-0.7 on Cry1A.105 whole plants (Niu et al., 2016b). In contrast, Cry2Ab2 resistance in aaBB was inherited as a recessive gene to Cry2Ab2 corn leaf tissue with a dominance level of -0.02 (Acharya et al., 2017) and incompletely recessive in Cry2Ab2 whole plants with an effective dominance level of 0.34 (Yang et al., 2017). The non-recessive character

of the Cry1A.105 resistance in *S. frugiperda* to Cry1A.105 corn plants could result in the better performance of Aabb than aaBb on MON 89034 corn leaf tissue. Such different performance did not occur in the whole plant tests, in which both of the two genotypes performed nearly the same as the genotype of aabb on MON 89034 plants for either of the two trials or for the pooled data. However, the outperformance of the Cry1A.105 resistance in *S. frugiperda* on MON 89034 over the Cry2Ab2 resistance was more evident between the genotypes of AABb and AaBB. For most biological parameters measured in the current study, AABb performed better than AaBB on MON 89034. These parameters included pupation, development time, egg production in the leaf tissue bioassay, as well as leaf injury ratings, larval survival and larval growth index in the greenhouse trials. The results suggest that the effective dominance level of single-gene resistance could have significant effects on the dominance level of the dual- or -multiple gene resistance.

In summary, aabb genotypes of *S. frugiperda* was susceptible to Bt plants expressing single or any combination of the Cry1A.105, Cry2Ab2, Cry1F, and Vip3A proteins. The dual-gene resistant genotype, AABB, was highly resistant to MON 89034 corn plants expressing both Cry1A.105 and Cry2Ab2 proteins, somewhat cross-resistant to Bt corn also expressing Cry1F and Cry1Ab, but susceptible to the plants expressing the Vip3A protein. The single-gene Cry1A.105- or Cry2Ab2-resistant genotypes and their correspondent single-gene heterozygous genotypes could not survive on MON 89034 plants. However, the combinations of the dual-gene resistant genotypes possessing three resistance alleles exhibited a significant level of resistant to the pyramided Bt plants, especially for the genotype containing the two Cry1A.105 resistance alleles plus a Cry2Ab2 resistance allele (AABb). This study is the first time to characterize the phenotypic performance of all nine different genotypes related to a dual-gene Bt resistance in *S. frugiperda* to pyramided Bt corn. Data generated from this study should be useful in refining

resistance modeling, improving resistance risk assessment, and developing management strategies for the sustainable use of the pyramided Bt corn technology.

5.5 References

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CHAPTER 6. SUMMARY AND CONCLUSIONS

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is a target pest of *Bacillus thuringiensis* (Bt) crops in North and South America. However, after intense use of Bt crop technology, *S. frugiperda* has developed field resistance to Cry1F corn at multiple locations in three different countries (Storer et al., 2010; Farias et al., 2014; Huang et al., 2014; Chandrasena et al., 2018). To delay resistance development, the U.S. and a few other countries have implemented an insect resistance management (IRM) plan named the 'high dose/refuge' strategy for planting Bt crops (Ostlie et al., 1997; Gould, 1998; Baute, 2004). One of the key assumptions for this strategy is that the Bt corn plants must produce a 'high dose' to be able to kill the resistant heterozygotes to prevent the resistance alleles passing to the next generations. In other word, the resistance should be functionally recessive (Huang et al., 2011). Thus, the genetic basis of Bt resistance in target pest to Bt crops is essential in the resistance management. Cry1A.105 and Cry2Ab2 are the two Bt proteins expressed in a common pyramided Bt corn trait-MON 89034 event. During 2011, two Cry1A.105-resistant *S. frugiperda* strains were isolated using an F_2 screen with field populations collected from south Florida (Huang et al., 2016). These two populations have been documented to be high resistant to purified Cry1A.105 protein and whole plants of Cry1A.105 corn. The objective 1 of this study was to characterize the genetic basis and fitness costs of the two Cry1A.105-resistant *S. frugiperda* strains. The results indicated that the Cry1A.105 resistance in *S. frugiperda* was inherited as a single autosomal and non-recessive gene with no fitness costs. Both resistant populations might share the same genetic basis. The non-recessive resistance character and the lack of fitness costs documented in objective 1 could provide cautionary evidence against the assumption that resistance is functionally recessive in an IRM plan for single-gene Bt crops.

It was also reported that pyramided Bt corn could provide a 'high dose' effect against the single-gene resistant populations. In the objective 2, larval survival and plant injury of Cry1A.105-susceptibile, -resistant, and -heterozygous *S. frugiperda* populations on transgenic corn plants containing single or pyramided Bt genes were evaluated in leaf tissue bioassay in the laboratory and whole plants in the greenhouse. Three commonly planted pyramided Bt corn traits were used in this objective, including Genuity® VT Double Pro™, SmartStax™, and Agrisure® Viptera™ 3111. Results showed that low injury ratings and little or no insect survivorship of Cry1A.105-susceptibile, -resistant, and -heterozygous populations of *S. frugiperda* were observed on the three pyramided Bt corn traits. Cry2Ab2 and VIP3A genes were highly effective against the Cry1A.105-resistant *S. frugiperda* with no injury and no larval survivors. The results showed that these three pyramided Bt corn products could be used to manage the Cry1A.105-resistant *S. frugiperda*.

Another key assumption for the 'high dose/refuge' strategy is that the initial resistance alleles in the field populations of target pests should be very rare (e.g. < 0.001) (Andow and Alstad, 1998). Monitoring of resistance evolution in target insect species as part of the current IRM plan is of great importance for the long-term efficacy of Bt crop technology. Cry2Ab2 is a relatively new Bt protein used in the pyramided Bt corn. Studies related to the Cry2Ab2 resistance in *S. frugiperda* were limited. The objective 3 estimated the frequency of Cry2Ab2 resistance alleles in field populations of *S. frugiperda* and the results suggested that the Cry2Ab2 resistance allele frequency was relatively low (0.0023) in the U.S. southern region. One F_2 family from Georgia was confirmed to possess a major resistance allele to Cry2Ab2 and a laboratory strain of *S. frugiperda* that was highly resistant to the Cry2Ab2 protein in the plants was isolated from this F_2 family (Niu et al., 2016).

Since 2010, pyramided Bt crops have gradually replaced the single-gene Bt crops in the market. However, most Bt resistance studies had dealt with single-gene Bt resistance. There have been very limited studies for dual- or multiple-gene Bt resistance (Santos-Amaya et al., 2015; Bernardi et al., 2017; Horikoshi et al., 2016). In the objective 4, a Cry1A.105/Cry2Ab2 dual-gene resistant *S. frugiperda* strain was generated by crossing the Cry1A.105-resistant and Cry2Ab2-resistant strains as mentioned in the previous objectives. Insect survival, pupation, growth, development and reproduction of nine genotypes of *S. frugiperda* possessing Cry1A.105/Cry2Ab2 resistant alleles were evaluated on leaf tissue and whole plants of MON 89034. The nine insect genotypes were a Cry1A.105/Cry2Ab2-susceptible (aabb), a Cry1A.105 resistant/Cry2Ab2-susceptible (AAbb), a Cry1A.105-susceptible/Cry2Ab2-resistant (aaBB), a Cry1A.105/Cry2Ab2-resistant (AABB), and five heterozygous (AaBb, AABb, AaBB, Aabb, aaBb) genotypes that were produced from various crosses among aabb, AAbb, aaBB, and AABB. One leaf tissue bioassay in the laboratory and two whole plant tests in the greenhouse exhibited that AABB was highly resistant to MON 89034 expressing both Cry1A.105/Cry2Ab2 proteins, somewhat cross-resistant to the Bt plants also expressing Cry1F and/or Cry1Ab, but susceptible to plants also expressing Vip3A. Genotypes containing one or two resistance alleles were overall susceptible to MON 89034, while those possessing three resistance alleles exhibited a significant level of resistant to the MON 89034 plants, especially for the genotype AABb. The study performed in objective 4 is the first research that characterized the phenotypic performance of all nine possible genotypes related to a dual-gene Bt resistance in *S. frugiperda* to pyramided Bt corn. Information generated from this objective should be valuable in assessing resistance risk, refining resistance management modeling, and developing resistance management strategies for the sustainable use of the Bt corn technology.

APPENDIX: LETTER OF PERMISSION

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Author: Ying Niu,Graham P. Head,Paula A. Price,Fangneng Huang

Publication: Crop Protection

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Author: Ying Niu,Jawwad A. Qureshi,Xinzhi Ni,Graham P. Head,Paula A. Price,Robert L. Meagher,David Kerns,Ronnie Levy,Xiangbing Yang,Fangneng Huang

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VITA

Ying Niu is the only child of Mr. Ken Niu and Mrs. Yinglan Zhu. She was born and raised in the city of Chongqing in China. She received bachelor degree majoring in plant protection from Southwest University, Chongqing, China in 2010. Then she joined Louisiana State University in the fall of 2011 for pursuing the master degree in the Department of Entomology at Louisiana State University in Baton Rouge, LA. Her thesis research with Dr. Fangneng Huang has focused on the evaluation of fall armyworm resistance to *Bacillus thuringiensis*. She finished her master study in the summer of 2014 and then continued her doctoral research in the same lab of Dr. Fangneng Huang. Currently, she is a doctoral candidate in the Department of Entomology. Her dissertation research is about the risk assessment of fall armyworm resistance to transgenic Bt corn products, which is an extended study from her master research. She has already completed the requirements for the degree of Doctor of Philosophy and plans to graduate in the summer, 2018. Ms. Ying Niu plans to go back to China to start her faculty career in an agricultural research institute in her hometown, Chongqing, China.