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Fitness costs and inheritance of Bt Cry2Ab2 resistance in fall armyworm, *Spodoptera frugiperda* (J.E. Smith)

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FITNESS COSTS AND INHERITANCE OF BT CRY2AB2 RESISTANCE IN FALL
ARMYWORM, *SPODOPTERA FRUGIPERDA* (J.E. SMITH)

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
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requirements for the degree of
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by
Binod Acharya
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ABSTRACT

Evolution of resistance in target pest populations is a major threat to the sustainability of transgenic crops expressing *Bacillus thuringiensis* (Bt) proteins. Fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is a cross-crop target pest of Bt corn, Bt cotton, and Bt soybean. This pest, thus far, is the only target pest species that has developed field resistance to Bt crops in multiple areas across countries. Cry2Ab2 is a common Bt protein expressed in transgenic corn and cotton targeting lepidopteran pests including *S. frugiperda*. The objective of this study was to characterize fitness costs and inheritance of Cry2Ab2 resistance in *S. frugiperda*. In the fitness cost test, performance of the Cry2Ab2-resistant, -susceptible, and two reciprocal F1 colonies of *S. frugiperda* was assayed on non-toxic diet and non-Bt corn leaf tissue. Biological parameters measured were 7-day larval weight, neonate-to-pupa development time, neonate-to-pupa survivorship, pupal weight, sex ratio, and egg production. In the inheritance study, larval mortalities of the resistant- and -susceptible parents, and eight other cross-strains were assayed using diet-incorporated and leaf tissue bioassays with Cry2Ab2. Maternal effects were examined by comparing the larval mortalities between the two F1 strains. Dominance levels of resistance were measured by comparing the larval mortalities of resistant, susceptible, and F1 heterozygous strains. Number of genes associated with the resistance was estimated by fitting the observed mortalities of F2 and backcross strains with the Mendelian monogenic inheritance model. There were no significant differences among the four insect strains for all the fitness parameters measured with few exceptions, suggesting that the resistance was not associated with fitness costs. The Cry2Ab2 resistance in *S. frugiperda* was likely inherited as a single, autosomal, recessive gene. Information generated from this study should be useful in assessing resistance risk and developing management strategies for the sustainable use of Bt crop technology.

CHAPTER 1: INTRODUCTION

1.1. Corn production in the United States of America

Field corn, also called as maize (*Zea mays* L.) is one of the economically important crops in the world. Although the origin and ancestry of corn is contested, it probably descended from an annual species of teosinte and was domesticated several thousand years ago, somewhere in present-day Mexico (Benz and Long, 2000; Piperno and Flannery, 2001). In the United States (U.S.), conventional corn varieties were cultivated as an open-pollinated crop for centuries until commercial hybrid seeds became available in 1930s. Introduction of hybrid corn seeds in the corn growing region of Midwest sharply increased the crop yield and the technology progressively diffused to the rest of the nation, rendering corn as one of the major agricultural crops of the country (Ryan and Gross, 1943; Griliches, 1960). For the past six years, corn was planted on an average of 37.6 million hectares (92.95 million acres) annually, with a total plantation area of 38.2 million hectares (94.5 million acres) in 2016 (NASS, 2016). In the U.S. corn production is mainly concentrated in the states of Illinois, Iowa, Indiana, eastern portions of South Dakota and Nebraska, western Kentucky and Ohio, and northern Missouri. Although it is a staple food of many countries for millions of people, it is mainly used for animal feed and biofuel production in North America (NASS, 2016). Other industrial uses include starch, sweeteners, oil, beverages, industrial alcohol etc. In addition, approximately 10-20% of corn grain produced in the U.S. is exported, making U.S. the world's largest producer and exporter of corn. Recently, higher corn prices are associated with strong demand of ethanol production, which has encouraged farmers to increase their corn acreage (USDA Economic Research Service, 2016).

1.2. Major corn insect pests and their control

Insects are a major group of pests that attack corn plants in the field and in storage, eventually hampering the quantity and quality of the product. Corn rootworms are probably the most serious insect pests of corn plants in the U.S. Rootworms damage corn both as larvae and adults (Levine and Oloumi-Sadeghi, 1991). The corn rootworm complex includes several species, namely the northern corn rootworm (*Diabrotica barberi* Smith and Lawrence), western corn rootworm (*Diabrotica virgifera virgifera* LeConte), Mexican corn rootworm (*Diabrotica virgifera zea* Krysan and Smith), and southern corn rootworm (*Diabrotica undecimpunctata howardi* Barber). The U.S. Corn Belt is threatened more by *D. virgifera virgifera* and *D. barberi* than closely related *D. undecimpunctata*, as larvae of the latter species cannot diapause in the colder climate of the North. *D. barberi* can exhibit a type of behavioral modification, “extended diapause”, where eggs are capable of remaining dormant in the soil through two winters and one growing season before hatching in the second season (Levine et al., 1992). *D. virgifera virgifera*, an exclusive corn feeder, is reported to have altered its ovipositional behavior, thereby reducing the effectiveness of crop rotation in controlling this pest, especially in the Eastern Corn Belt (Levine et al., 2002).

Several other soil-dwelling insect pests that feed on corn seeds or young seedlings represents another group of serious corn pests. For example, larvae of black cutworm (*Agrotis ipsilon* Hufnagel) may notch the stems of corn seedlings right below the soil, forcing the plant to wilt, or may cut through the stalks, resulting in reduced crop stand. Larvae of several species of click beetles, collectively called wireworms, may feed on the germ of corn kernels and cut off small roots of young plants. Seedcorn maggot (*Delia platura* Meigen) feeds on corn seeds and

the damaged seeds may not emerge. White grub attack on corn field results in stressed, stunted, wilted, discolored and dead seedlings, reducing crop stand (Carter et al., 1982).

Many lepidopteron insects feed on the above-ground parts of corn plants. The major corn borer species in the U.S. are the European corn borer (*Ostrinia nubilalis* Hübner) and the southwestern corn borer (*Diatraea grandiosella* Dyar). Both attack whorl, leaf midrib, can bore into the stalk, and feed on silks and ears. In Louisiana and several other states in the mid-south region of the U.S., the sugarcane borer (*Diatraea saccharalis* Fabricius) larvae tunnels into the pith of stalk, lessening the vitality of plant. In addition, the fall armyworm (*Spodoptera frugiperda* J. E. Smith) feed on foliage and in corn ears. Corn earworm larvae (*Heliothis zea* Boddie) normally feed near the tip of corn ear but may go progressively down towards the base, resulting in developing a track of damaged kernels. Western bean cutworm (*Striacosta albicosta* Smith) larvae penetrate ears and damage kernels (Carter et al., 1982).

Historically, a range of insect pest management strategies were practiced by corn growers. Up to the early twentieth century, an effort to find the chemicals to control insect pests was underway, but the effective chemical compounds were not yet synthesized, and the crop protection specialists had to rely on knowledge of pest biology and cultural practices (Gaines, 1957; Kogan, 1998). With the advent of organosynthetic insecticides in 1940s, pest management began to heavily depend on the chemical compounds. The Post-World War II period saw a surge of many chlorinated hydrocarbons and organophosphates used as pesticides. However, the criticism began to develop on public health and environmental risks of overreliance on chemical insecticides in late 1950s and 1960s (Carlson, 1962; Doult and Smith, 1971; Pimental, 2005). The need for more environmentally friendly and safer strategies was highlighted in successive years (Van den Bosch and Stern, 1962), which led to public policy changes in 1970s that put

more emphasis on integrated pest management (IPM) to control insect pests. One fallout of the excessive use of chemical pesticides was the development of resistance by pests to the pesticides designed to kill them. For instance, corn rootworms developed resistance to various groups of chemical insecticides (Meinke et al., 1998; Parimi et al., 2006) and so did the many lepidopteran pests (Brown, 1981; McCaffery, 1998).

1.3. Transgenic technology and Bt corn

As conventional approaches to insect-pest control were increasingly becoming less effective, the new idea of transgenic technology was about to come into realization. With the discovery of recombinant DNA technology in 1970s, coupled with advancement in tissue culture techniques and elucidation of genetics of bacterium *Agrobacterium*, it became possible for scientists to transfer genes of interest from one organism to another. As early as 1983, scientists sought to apply genetic engineering to insect, weed and external stress management in crops (Barton and Brill, 1983). Now, a wide array of genetically engineered crop products are on the market such as non-browning apple and potato, ring spot virus resistant papaya, high-oleic acid soybean, drought-tolerant corn, and glyphosate resistant cotton, soybean, and alfalfa. Application of genetic engineering to control specific phytophagous insect pests by incorporating one or more genes from a soil dwelling bacterium *Bacillus thuringiensis* (Bt) into the genome of plants is a huge success story of the past two decades with a lot of environmental and economic benefits (Vaeck et al., 1987; Grassler and Fraley, 1989; Hutchison et al., 2010; Tabashnik, 2010; National Academies of Sciences, Engineering, and Medicine, 2016).

Bt is an aerobic, gram positive, endospore forming bacterium first discovered by a Japanese scientist in 1901 (Gill et al., 1992; Madigan and Martinko, 2005). During the sporulation phase, Bt produces insecticidal proteins as parasporal crystals. Those crystals consist

of crystal (Cry) and cytolitic (Cyt) toxins, together called as δ -endotoxins (Bravo et al., 2007). Bt also synthesizes insecticidal proteins during the vegetative growth phase which are called vegetative insecticidal proteins (Vips) (Palma et al., 2014). The Cry proteins are toxic to the specific group of insects. Bt has been registered as a microbial insecticide in the U.S. since 1961 (U.S. EPA, 1998). Bt microbial insecticides or Bt crops pose no risk to the human health and to the environment (Mendelshon et al., 2003; Comas et al., 2014; National Academies of Sciences, Engineering, and Medicine, 2016). So far, insect resistant varieties of cotton, corn, poplar, and soybean are in commercial production, Bt corn and Bt cotton being the top two in terms of acreage and production. In 1996, when Bt corn was first commercialized in the U.S., it was planted in fewer than 300,000 hectares of land (James, 1997). Bt corn represented just 19 percentage of total corn acreage in 2001, but by 2015, it already occupied 53.7 million hectares which accounted to 81 percentage of total corn acreage in the U.S., which was approximately one-third of all land planted to corn worldwide that year (James, 2015). In the southern region of the U.S., transgenic field corn was first commercially planted in 1999 (Buntin et al., 2004).

Many Bt corn products are available on the market which express different Bt proteins and their efficiency towards various target pests differs. Broadly, the Bt proteins Cry3Bb1, mCry3A, eCry3.1Ab, and Cry34/35Ab1 are effective against below-ground coleopteran insect pests and Bt proteins Cry1F, Cry1Ab, Cry1Ac, Cry1A.105, Cry2Ab2, and Vip3A are effective against above-ground lepidopteran pest species. The Bt proteins may be stacked with traits that express crop tolerance to herbicides such as glyphosate or glufosinate. Before 2010, all Bt corn planted in the U.S. expressed only a single toxin for a target pest. In case they expressed more than one Bt toxin, the different toxins targeted different insects. These are referred as the first generation Bt crops (Huang, 2015). The recent Bt products express more than one Bt

proteins targeting the same pest species and are often referred to as second generation pyramided Bt products (Huang, 2015).

One of the impulses to shift from the single-toxin Bt crop to pyramided Bt crops was the concern of reduced susceptibility (developing resistance) of pests to the single toxin Bt crops. Cry2Ab2 is a common Bt protein expressed in many pyramided Bt cotton and Bt corn hybrids, such as the popular Bt corn products Genuity[®] VT Double Pro[®], Genuity[®] VT Triple Pro[®] and Genuity[®] SmartStax[®] (DiFonzo et al., 2017). Cry2Ab2 protein is expressed in the Bt corn event MON 89034 along with Cry1A.105. Cry1A.105 is a chimeric protein composed of portions of Cry1Ab, Cry1Ac, and Cry1F proteins (Head, 2006). MON 89034 was developed by *Agrobacterium*-mediated transformation of corn using the 2TDNA plasmid vector PV-ZMIR245 (Head, 2006). Since 2002, Bt cotton containing Cry2Ab2 gene has been commercially planted in the U.S., but Cry2Ab2 is a relatively new protein used in Bt corn products, which were first commercialized in 2010 (U.S. EPA, 2010). Whereas the major initial lepidopteran targets of Bt corn were stalk borers (e.g. *O. nubilalis*, *D. grandiosella*) and *H. zea* (Huang et al., 2011), the Bt corn can also be used to reduce *S. frugiperda* damage, which is one of the major pests in the southern region of the U.S. (Buntin et al., 2004; Siebart et al., 2008). Thus, *S. frugiperda* has been listed as a major target species of Bt corn event MON 89034.

1.4. Mode of action of Bt toxin

Understanding the mechanism of action of Bt toxins and how insects develop resistance provides the basis for taking measures to counter resistance (Bravo and Soberón, 2008).

However, the exact mode of action of Bt toxin is still a subject of ongoing research. The widely believed hypothesis of Bt toxicity holds that the protoxin is first solubilized in the alkaline environment of insect alimentary canal and proteolytically activated by endopeptidases. The

activated monomeric cry toxin binds to its receptors in varying affinity in the midgut epithelium which involves the proteolytic removal of N-terminal end of the toxin, exposing hydrophobic region of the toxin followed by oligomerization of toxin to form pre-pore structure. The pre-pore structure inserts into the midgut epithelial cell membrane, forming pore and cell lysis that kills the insect (Pardo-López et al., 2006; Bravo et al., 2007; Vachon et al., 2012).

At least three different kinds of Bt receptors are reported which include cadherin-like protein (Gahan et al., 2001; Morin et al., 2003; Flannagan et al., 2005; Sayed et al., 2007; Park and Kim, 2013; Ren et al., 2013), aminopeptidase N (Gill et al., 1995; Luo et al., 1997; Rajagopal et al., 2002; Herrero et al., 2005; Zhang et al., 2009; Tiewisiri and Wang, 2011), and alkaline phosphatase (McNall and Adang, 2003; Perera et al., 2009; Ning et al., 2010; Jurat-Fuentes et al., 2011; Caccia et al., 2012). Recent studies show that both the protoxin and trypsin-activated toxin bind to cadherin and form two different pre-pores. These two pre-pores are assembled before inserting into membrane of midgut cells, suggesting a dual mode of action of Bt (Gómez et al., 2014, Tabashnik et al., 2015). Another hypothesis of Bt toxicity holds that monomeric Cry toxins binds to a cadherin receptor which activates intracellular transduction pathway. The G protein is activated in the process, which activates adenylate cyclase, giving rise to increased cyclic AMP level. Eventually, protein kinase A is activated causing cell necrosis (Zhang et al., 2005; Zhang et al., 2006). The role of ATP-binding cassette (ABC) transporter proteins in Bt toxicity is being investigated recently (Gahan et al., 2010; Baxter et al., 2011; Atsumi et al., 2012; Heckel, 2012; Tanaka et al., 2013; Xiao et al., 2014; Tay et al., 2015).

1.5. Fall armyworm, *Spodoptera frugiperda* (J.E. Smith)

S. frugiperda has a tropical-subtropical origin in the western hemisphere. It survives throughout the year in tropical areas of South and Central America, Mexico and in a few sub-

tropical areas of the southern U.S. (Sparks, 1979). In the U.S. mainland, the insect overwinters in southern Florida and Texas, because the optimum temperature ranges it requires are not available elsewhere in the U.S. mainland (Sparks, 1986). The life cycle is completed in about one month in summer, two months in spring and nearly three months in winter (Capinera, 1999). A single female moth is capable of laying about 1500 dome-shaped eggs in her lifetime (Capinera, 1999). The larvae undergo six instars before pupating in the soil. The pupae emerge into adult moths with wingspan of about 32-40 mm. Adults are nocturnal in behavior (Capinera, 1999).

S. frugiperda does not have an ability to undergo winter diapause, and thus it must travel northward each spring if it is to infest crops in the temperate regions (Mitchell, 1979; Johnson, 1987). It has a wide host range of more than 80 plant species. Along with corn, it also attacks other major crops such as cotton, sorghum, soybean, rice, forages, and turf grasses. It is one of the most serious crop pests in the southern U.S. (Buntin et al., 2004). Based on its host specificity, at least two genetically differentiated strains of *S. frugiperda*, namely corn strain and rice strain, exist (Pashley, 1986). However, substantial controversy remains about whether they should be referred as “host strains” or “host forms” or “sibling species” (Cañas-Hoyos et al., 2014; Juárez et al., 2014). Polymorphism in cytochrome oxidase I gene can be used to identify different host strains (Levy et al., 2002). The corn strain population can be subdivided into four haplotype subgroups as defined by the COI marker (Nagoshi et al., 2007).

Populations of *S. frugiperda* from Florida and Texas differ significantly in terms of the relative proportion of different haplotypes (Nagoshi et al., 2008). Migration from Texas is the primary source of *S. frugiperda* infestations west of the Appalachian Mountains, while the Florida population migrates to the states located on the Atlantic coast. The two populations

probably mix within the states of Alabama-Georgia in the south and Mid-Atlantic region in the east (Nagoshi et al., 2012).

Larvae damage corn plants by devouring foliage, burrowing into the bud and whorl, damaging the husk or clipping the leaves, as well as feeding on the ears (Capinera, 1999). Traditionally, chemical control had been the most common method for *S. frugiperda* control in the U.S., but it is increasingly considered an inefficient method. Immediately after the eggs hatch, neonates move into and begin feeding the whorl region of corn plants where they usually escape foliar sprays of chemical insecticides (Harrison, 1986; Bokonon-Ganta et al., 2003). Moreover, there are reports of regional populations of *S. frugiperda* having developed resistance to several chemical insecticides including carbamates (carbaryl), organophosphates (methyl parathion) and pyrethroids (Young et al., 1979; Guillebeau and All, 1990; Adamczyk et al., 1999; Yu et al., 2003).

1.6. Bt resistance

WHO (1957) defined insecticide resistance as “the inherited ability of a strain of some organisms to survive doses of a toxicant that would kill the majority of individuals in a normal population of the same species”. Owing to the difficulty of defining “normal population” and its inapplicability to individual insects, various modifications have been suggested to the definition. Tabashnik et al. (2009) have defined field-evolved resistance as “genetically based decrease in susceptibility of a population to a toxin that is caused by exposure of the population to the toxin in the field”. Although it is possible for pests to develop resistance with continuous exposure to toxins in laboratory and not necessarily in the field (Tabashnik et al., 2003), field-evolved resistance is an issue of practical concern. Field-evolved resistance has been analyzed as the continuum with five categories ranging from incipient resistance to practical resistance,

depending on the percentage of resistant individuals in the insect population (Tabashnik et al., 2014). The term field-evolved resistance does not necessarily imply control failure or reduced efficacy in the field. Field-evolved resistance that results in reduced control efficacy or control failure can be defined as “field resistance” (Huang et al., 2011) or “practical resistance” as suggested in Tabashnik et al (2014).

So far there are at least seven documented cases of practical resistance (or field resistance) by different target pests to Bt crops reported, with >50% resistant individuals and reduced efficacy of crops in field (Van Rensburg et al., 2007; Storer et al., 2010; Dhurua and Gujar, 2011; Gassmann et al., 2011; Farias et al. 2014, Huang et al. 2014; Dively et al., 2016). Three years after the commercial introduction of TC1507 corn seeds expressing Cry1F protein in Puerto Rico, the reports of unusual damage by *S. frugiperda* began to appear in the island (Storer et al., 2010). Laboratory bioassays of field populations confirmed that the field resistance had developed in *S. frugiperda* to the Cry1F toxin in the plants. Field resistance of *S. frugiperda* to Cry1F corn was also documented in Brazil (Farias et al., 2014; Monnerat et al., 2015), and the southeast region in the mainland U.S. (Huang et al., 2014). This insect, thus far, is the only target pest that has developed field resistance to Bt corn at multiple locations across different countries and continents (Dangal and Huang, 2015). Other notable cases of field resistance include the African stem borer (*Busseola fusca* Fuller) resistance to Cry1Ab corn in South Africa (Van Rensburg, 2007), pink bollworm (*Pectinophora gossypiella* Saunders) resistance to Cry1Ac cotton in India (Dhurua and Gujar, 2011), *D. virgifera virgifera* resistance to Cry3Bb1 and mCry3A corn (with the cross resistance between these two toxins) in North Central Corn Belt of the U.S. (Gassmann et al., 2011, Gassmann et al., 2014), and *H. zea* resistance to Cry1Ab sweet corn in the U.S. (Dively et al., 2016).

1.7. Bt resistance management

The evolution of resistance to Bt crops can be attributed to the widespread use of transgenic plants that places a high selection pressure on the target pests (Ostlie et al., 1997; Gould, 1998; Tabashnik et al., 2003). Anticipating the evolution of resistance, the 1998 Science Advisory Panel recognized that resistance management programs should be based on the use of high dose of Bt plants and planting of non-Bt plants (U.S. EPA, 2001). This strategy of resistance management is called as “high dose/refuge strategy” and is mandated by the United States Environmental Protection Agency (U.S. EPA). This strategy relies on some important assumptions: 1. Bt plants should produce toxins in a sufficiently high dose that can kill all or nearly all heterozygotes, in other words, the resistance should be functionally recessive; 2. Non-Bt refuges should provide abundant susceptible adult insects to mate; and 3. Low initial resistance allele frequency (Ostlie et al., 1997; U.S. EPA, 2001; Huang et al., 2011).

Not always withstanding the assumption of recessive inheritance as required by the “high dose/refuge strategy”, studies have shown that the inheritance of resistance can vary from completely recessive to incompletely dominant (see section 1.9). In addition, non-compliance on refuge planting is a serious issue in the U.S. and elsewhere resulting in inadequate susceptible individuals which can exacerbate the problem of resistance development (Goldberger et al., 2005). Huang et al. (2011) have shown that the documented cases of field resistance are likely due to the violations of one or more assumptions of the “high dose/refuge strategy”. For example, the Cry1F maize planted in Puerto Rico, where the *S. frugiperda* has been shown to have developed resistance, cannot be considered as high-dose and the refuges are believed to be inadequate. Similarly, the field resistance of *B. fusca* to Cry1Ab maize in S. Africa and *P. gossypiella* resistance to Cry1Ac cotton in India is likely due to noncompliance in refuges

planting and a use of non-high dose Bt varieties (Huang et al., 2011). In India, commercial cotton hybrids expressing Cry1Ac protein were released in 2002. Central and southern India witnessed an exceptionally high adoption of Bt cotton, compromising refuges requirement, and field failures due to *P. gossypiella* occurred as early as in 2009 (Kathage and Qaim, 2012; Mohan et al., 2015). Recently, there are reports of *P. gossypiella* developing resistance to dual toxin cotton (Cry1Ac + Cry2Ab) in different parts of Gujarat, Andhra Pradesh, Telangana, and Maharashtra (Kranthi, 2015; Kasabe, 2016).

To solve the problem of non-compliance in planting of structured refuges, a new seed mixture strategy called “refuge-in-the-bag (RIB)” has been approved by U.S. EPA for planting pyramided Bt corn in the Corn Belt since 2010. Although models show that RIB could be an effective strategy in some circumstances (Carroll et al., 2013), larval movement from non-Bt plants to Bt plants is a serious concern. For instance, larvae of *O. nubilalis* may move between Bt and non-Bt plants in the seed mixture and get exposed to some dose of the toxin, an event that contradicts the requirement of adequate susceptible insects should be harbored in the refuge plants (Mallet and Porter, 1992; Gould, 1998). In general, block (structured) refuges may be more effective than seed mixtures in pests with larvae that are more mobile and have inherently low susceptibility to Bt toxins (Brévault et al., 2015). Furthermore, the pollen-mediated gene flow from Bt crops to refuge plants, which is already a matter of concern in structured refuge (Chilcutt and Tabashnik, 2004) could be even more serious under RIB strategy. As a result of this kind of gene flow, a portion of kernels in refuge ears may express Bt toxin and the larvae feeding on those ears are selected to Bt toxin compromising the purpose of refuge plants to harbor susceptible insects (Yang et al., 2014).

In conjunction with the “high dose/refuge strategy”, another approach to delay resistance evolution is the use of pyramided Bt products which express more than one toxins that target the same pest species. The key assumption of this strategy is that insects resistant to one toxin will be killed by the other toxin in the pyramid (Roush, 1998). In fact, application of compounds with multiple modes of action is useful not only in insecticide resistance management but also in resistance management with weed, fungicide and drugs (Brent and Holloman, 1995; Bergstrom et al., 2004; Beckie, 2006). Pyramided Bt crops are progressively replacing the single toxin Bt crops. Single-gene Cry1Ac cotton was progressively and completely replaced in the U.S. from 2003 to 2011 by pyramided Bt cotton that produces two Bt toxins (Cry1Ac/Cry2Ab or Cry1Ac/Cry1F) (Brévault et al., 2013). Although the actual data is not available, pyramided Bt corn is believed to have surpassed the first generation Bt corn in total acreage. Mathematical models indicate resistance development will be delayed with pyramided Bt toxins (Roush, 1998; Zhao et al., 2003). Empirical studies seem to agree with this conclusion. Combinations of Cry1Ac and Cry2Ab2 (Bollgard II) cotton in general is superior over Cry1Ac only (Bollgard) cotton against the major cotton lepidopteran pests (Sivasupramaniam et al., 2008). Efficacy provided by pyramided Bt corn was reported to be either statistically equal to or better than single toxin Bt corn in several major pests including *D. saccharalis*, *H. zea* and *S. frugiperda* (Siebart et al., 2012; Niu et al., 2014).

The pyramid strategy, however, works better only if cross resistance among toxins is absent. Gene pyramiding should be done in such a way that the crop expresses protein that can overcome the insect resistance to other protein(s) in the pyramid (Moar and Anilkumar, 2007). If selection for resistance to one Bt toxin results in cross-resistance to another toxin in the pyramid, the pyramided Bt crops fare no better than single toxin Bt crops (Roush, 1998; Tabashnik et al.,

2009). Different cases of cross-resistance of Bt toxins in several lepidopteran and coleopteran insects have been documented (Tabashnik et al., 2000; Ferré and Van Rie, 2002; Brévault et al., 2013; Jakka et al., 2016). Cross-resistance normally occurs when pesticides share similar binding sites or similar detoxifying pathways (Wu, 2014). It is hypothesized that Cry1 and Cry2 proteins have different binding sites and thus insect show very low or no cross-resistance to these proteins (Xu et al., 2005; Hernández-Rodríguez et al., 2008; Brévault et al., 2009; Wu et al., 2009; Vélez et al., 2013; Huang et al., 2014; Wu, 2014). However, *H. zea* strain selected for Cry1Ac cotton had increased survival on two-toxin (Cry1Ac and Cry2Ab) cotton indicating that cross-resistance can still evolve between Cry1A and Cry2A (Brévault et al., 2013).

The sustainability of Bt crops perhaps rests on the extent growers integrate Bt crops as a component of IPM, and not as the silver bullet. There are several reported cases of regional populations of insects being suppressed to the level below the economic threshold due to the widespread use of Bt crops. For example, the population density of *O. nubilalis* across Iowa, Illinois, Minnesota, Nebraska, and Wisconsin (Hutchison et al., 2010; WI Department of Agriculture, 2014) and Pennsylvania (Bohnenblust et al., 2014), *P. gossypiella* in Arizona (Carrière et al., 2003; Liesner, 2015) and tobacco budworm (*Heliothis virescens*) in Mississippi Delta and Louisiana (Adamczyk and Hubbard, 2006; Micinski et al., 2008) has been drastically reduced over the years. Populations of *D. saccharalis* on corn, sorghum, and rice have been very low since 2010 in the mid-southern region of the U.S. (Fangneng Huang, personal communication). Switching to non-Bt crops in those areas for a certain period of time may not only be helpful in delaying resistance evolution but could also be economically advantageous (National Academies of Sciences, Engineering, and Medicine, 2016).

1.8. Fitness costs of resistance

Although not explicitly assumed in the “high dose/refuge strategy” of insecticide resistance management described in section 1.7, fitness costs can greatly influence the evolution of resistance (Gassmann et al., 2009). Fitness costs of Bt resistance happen if the fitness of insect individuals carrying at least one allele of resistance in the absence of selection is lower than the fitness of individuals lacking resistant alleles (Gassmann et al., 2009). Broadly, fitness refers to the ability of an organism to survive, reproduce and pass its genes to the next generation (Orr, 2009). Laboratory experiments assessing insect fitness usually involves measuring growth, development, and reproductive parameters. In some cases, fitness costs may be obvious such as developmental time (Minkoff and Wilson, 1992) and in some cases, they can be less noticeable such as in the case of reduced overwintering success (McKenzie, 1990). Fitness costs are often associated with resistance and can be used in insect resistance management. For non-recessive fitness costs, development of resistance in insect populations in field can be delayed or even reversed if there is absence of selection pressure for long period time (Tabashnik et al., 2005). In cases where resistance to Bt has been detected, there has been a decline in resistance level after the selection pressure is removed (Gassmann et al., 2009). Although most fitness costs are recessive, non-recessive costs help in delaying resistance as they can strongly select against resistant genotypes (Gassmann et al., 2009).

Fitness costs are commonly assessed by comparing one or more fitness components such as life-cycle traits (survival, neonate to emergence period), body weight and fertility parameters between insect strains with and without resistant allele, in the absence of selection pressure (e.g. insecticide). Since this method indicates fitness costs only when the components under study are significantly lower in resistant strains than in the susceptible strains, it is theoretically a less

comprehensive approach. Another method involves monitoring the stability of resistance in heterozygous populations in the absence of Bt toxin over several generations. This method detects the reduction in frequency or level of resistance over time (Gassmann et al., 2009). Researchers should be careful of following fallacies on fitness cost study: a. comparisons made between unrelated strains, b) experiments performed under optimal laboratory conditions to which some strains may be acclimatized, and c) experimental conditions with little or no relevance to field conditions (Bourguet, 2004; Gassmann et al., 2009).

There are numerous published studies on fitness costs regarding resistance to chemical insecticides as well as Bt toxins. For example, Oswald et al. (2012) found no fitness costs associated with Cry3Bb1 resistance in *D. virgifera virgifera*. However, in laboratory selected *O. nubilalis* strain, weak and recessive fitness costs were reported to be associated with the Cry1F resistance (Pereira et al., 2011). Studies on fitness costs of Bt resistance in *S. frugiperda* have shown contrasting results. In two separate studies, Vélez et al. (2014) & Jakka et al. (2014) showed a lack of strong fitness costs associated with a Cry1F-resistant population from Puerto Rico, but Dangal and Huang (2015) reported non-recessive fitness costs associated with resistant-populations from Florida and Puerto Rico.

1.9. Inheritance of resistance

Knowledge of the genetic basis of resistance is imperative to develop effective resistance management strategies (Bourguet, 2004; Tabashnik and Carrière, 2007). In the pesticide resistance literature, it is commonly hypothesized that laboratory selection tends to exhibit polygenic inheritance, whereas field selection favors monogenic response (Roush and McKenzie, 1987; Ffrench-Constant, 2013). It is probably because laboratory selections with usually small populations draw in common phenotypic variations where the alleles conferring high level of

resistance are rare. Moreover, adaptation to laboratory conditions and selection for resistance to toxins may cause inbreeding and genetic bottlenecks, reducing genetic variation (Fritz et al., 2016). In contrast, in field high selection intensities are presumed to be associated with insecticide application and the selection on novel variation are more likely which favor resistance alleles of major effect (Roush and McKenzie, 1987). For example, the finding of inheritance of Cry1F resistance in laboratory-selected *O. nubilalis* was consistent with monogenic model of resistance (Pereira et al., 2008). This hypothesis, however, is not always substantiated by published data both for conventional insecticides (Groeters and Tabashnik, 2000) and Bt insecticides (Gahan et al., 2001). Single major gene is responsible for 40 to 80% of resistance in laboratory selected strains of tobacco budworm, *Heliothis virescens* (Gahan et al., 2001). This means that laboratory selected strains are useful tool to study field-evolved resistance. However, compared with laboratory strains derived from single field populations, the screen of F1 and F2 progeny in laboratory derived from field populations across the range of geographic locations provide better prediction (Wu, 2014).

Studies on inheritance of resistance usually involves assessment of dominance level, detection of maternal effect and number of genes controlling resistance. Dominance relationships are usually measured in three different ways: the dominance of insecticide resistance or Stone's dominance, effective dominance or dominance level of survival at a given insecticide dose and the dominance of relative fitness. The three approaches may not be directly correlated with each other (Bourgeut et al., 2000). Presence of maternal effect is inferred if the F1 populations generated from reciprocal crosses of parental resistant and susceptible strains perform differently when presented with the toxin. Identification of number of genes conferring resistance involves sophisticated molecular techniques but they could be estimated by statistical tests.

Although the “high dose/refuge strategy” of resistance management requires recessive, monogenic inheritance of resistance, requirements are not always met. The dominance of Bt resistance greatly varies and has been found ranging from completely recessive to dominant (Ferré et al., 2008). For example, highly recessive and monogenic inheritance of resistance in *S. frugiperda* to Cry1F protein in Puerto Rico (Storer et al., 2010) sharply contrasts with the dominant Cry1Ab inheritance in *B. fusca* (Campagne et al., 2013). Moreover, a strain of *D. saccharalis* that was highly resistant to both purified Cry1Ab protein and Cry1Ab corn plants was found to be incompletely dominant on several commercial Cry1Ab corn hybrids with a D_{ML} level of 0.32-0.78 (Ghimire et al., 2011; Wangila et al., 2012). The incompletely dominant inheritance character of the Cry1Ab resistance in *D. saccharalis* was also documented with Bt corn leaf tissue bioassays (Wu et al., 2007; Ghimire et al., 2011; Wangila et al., 2012). Recently, Jin et al. (2013) reported that two field-selected populations of the cotton bollworm, *Helicoverpa armigera*, that were highly resistant to Cry1Ac, was dominant at a diagnostic concentration in diet and on Cry1Ac cotton leaves.

The dominance level may vary across different species, type of toxin or even for different strains of a same species (Jin et al., 2013). The dominance of resistance, however, is not an entirely intrinsic property of alleles, and can be influenced by the dose of toxin. Expression of the Cry proteins in high enough levels can convert an incompletely dominant resistance to a functionally recessive resistance (Bourguet et al., 2000). In one study where inheritance of Cry1Ab resistance in *D. saccharalis* was examined, the effective dominance level was found to be dose dependent, resistance being incompletely recessive at low dose and completely recessive at high dose (Wu et al., 2009). In some cases, field-selected resistance may involve diverse genetic basis including both recessive and non-recessive alleles (Zhang et al., 2012). Such a

diversity and variation on findings suggest non-recessive resistance to Bt crops in target species is not uncommon and thus species-toxin-specific knowledge of inheritance of resistance is required (Janmaat et al., 2004).

1.10. Objectives

The objectives of the present study were to determine if fitness costs were associated with the Cry2Ab2 resistance and to characterize the inheritance of the resistance in *S. frugiperda*.

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CHAPTER 2: ANALYSIS OF FITNESS COSTS AND INHERITANCE OF BT CRY2AB2 RESISTANCE IN FALL ARMYWORM, *SPODOPTERA FRUGIPERDA* (J.E. SMITH)

2.1. Introduction

Bacillus thuringiensis (Bt) is a widespread, soil living, gram positive bacterium (Gill et al., 1992). The bacterium produces insecticidal crystalline (Cry) proteins during sporulation and vegetative insecticidal proteins (Vips) during vegetative phase (Bravo et al., 2007; Palma et al., 2014). Microbial pesticides containing Bt proteins have been deployed for the past several decades in the United States (U.S.) and elsewhere to control agricultural pests and medically important insect vectors (Sanahuja et al., 2011). Bt has been registered as a microbial insecticide in the U.S. since 1961 (U.S. EPA, 1998).

With the advancement of biotechnology, it is possible to genetically engineer crops that express Bt proteins which are toxic to the target insect-pests yet safe for human and non-target organisms (Mendelshon et al., 2003; Comas et al., 2014; National Academies of Sciences, Engineering, and Medicine, 2016). Transgenic plants that express the Bt insecticidal proteins have been commercialized since 1996 to control different agricultural pests. Bt crops have greatly changed the landscape of pest management by suppressing target pest populations, reducing application of conventional insecticides, and economically benefitting farmers (Carrière et al., 2003; Hutchison et al., 2010; Kathage and Qaim, 2012; National Academies of Sciences, Engineering, and Medicine, 2016). In 2015, Bt crops were planted on 84 million hectares globally (James, 2015). Although varieties of Bt cotton, eggplant, field corn, sweetcorn, poplar, and soybean are in commercial production, Bt corn and Bt cotton are the two most widely planted agricultural Bt crops. Currently, Bt corn and Bt cotton occupy 81% and 84% of the total corn and cotton acreage in the U.S., respectively (USDA Economic Research Service, 2016).

Transgenic Bt crops are deployed to control a range of lepidopteran and coleopteran insect pests. Broadly, the Bt proteins Cry3Bb1, mCry3A, eCry3.1Ab, and Cry34/35Ab1 are effective against corn coleopteran insect pests and the Bt proteins Cry1F, Cry1Ab, Cry1Ac, Cry1A.105, Cry2Ab2, and Vip3A target the lepidopteran insect-pests of corn and cotton.

Due to the strong selection pressure imposed by the widespread adoption of Bt crops, evolution of resistance in target pest populations has been a major threat to the sustainability of transgenic Bt crop technology (Huang et al., 2011; Tabashnik et al., 2013). Thus far, at least seven cases of field resistance and control failure are documented which include the resistance developed by fall armyworm, *Spodoptera frugiperda* (J.E. Smith), to Cry1F maize in Puerto Rico (Storer et al., 2010), Brazil (Farias et al., 2014; Monnerat et al., 2015), and the U.S. mainland (Huang et al., 2014); African stem borer, *Busseola fusca* (Fuller), to Cry1Ab maize in South Africa (Van Rensburg, 2007); pink bollworm, *Pectinophora gossypiella* (Saunders), to Cry1Ac cotton in India (Dhuria and Gujar, 2011); western corn rootworm, *Diabrotica virgifera virgifera* (LeConte), to Cry3Bb1 and mCry3A maize in Northern U.S. (Gassmann et al., 2011, Gassmann et al., 2014; Jakka et al., 2016); and recently by corn earworm, *Helicoverpa zea* (Boddie), to Cry1Ab and Cry1A.105 + Cry2Ab2 sweet corn in Maryland, U.S. (Dively et al., 2016).

S. frugiperda is a major economic pest of corn in many regions of North and South America (Cruz and Turpin, 1983; Buntin et al., 2004). It does not undergo winter diapause and hence is only a sporadic pest in temperate regions but is a serious pest of southern U.S. where it overwinters in southern Texas and Florida (Sparks, 1979; Johnson, 1987; Nagoshi et al., 2012). Although chemical-based control measures are deployed for its control, they are largely unsuccessful due to resistance development (Young, 1979; Guillebeau and All, 1990; Yu, 1991).

S. frugiperda is a cross-crop target pest of Bt corn, Bt cotton, and Bt soybean. The first transgenic corn expressing the Cry1F protein was registered in the U.S. in 2001 for controlling various lepidopteran pests including *S. frugiperda* (Siebert et al., 2008). This pest is, thus far, the only target pest species that has developed field-resistance to Bt crops in multiple areas across different countries and continents (Dangal and Huang, 2015).

To delay resistance evolution, the “high dose/refuge strategy” and “gene-pyramiding” are implemented in the U.S. and several other countries (U.S. EPA, 2001; Zhao et al., 2003; Baker et al., 2008; Tabashnik et al., 2009; Huang et al., 2011). In the “high dose/refuge strategy”, non-Bt plants (refuges) are planted along with high dose Bt plants so that the refuges can harbor adequate susceptible insects to mate with the rare resistant individuals that survive on Bt plants. Heterozygous progenies are killed by the high dose of toxin expressed in Bt plants. The success of this strategy depends on some important assumptions: 1) the resistance should be functionally recessive and the plant should express Bt proteins in a high enough dose so that >95% of heterozygous individuals are killed; 2) random mating between susceptible and resistant homozygote individuals, and 3) low initial resistance allele frequency in the field pest populations (Gould, 1998; U.S. EPA, 2001; Tabashnik et al., 2009; Huang et al., 2011). Although the strategy requires recessive inheritance of resistance, the inheritance may vary across the spectrum from completely recessive to dominant. Thus, it is important to study the inheritance of resistance in each target pest species in all relevant Bt toxins to develop effective insect resistance management (IRM) strategies for the sustainable use of Bt crop technology (Wu, 2014).

Resistance evolution is also influenced by fitness costs (Tabashnik et al., 2005; Gassman et al., 2009). If the insect genotypes conferring at least one resistance allele have lower fitness

than insect genotypes lacking resistance allele in the absence of Bt proteins, fitness costs occur (Gassmann et al., 2009). Presence of fitness costs is commonly assessed by comparing fitness parameters such as survival, growth, development, and reproduction among Bt-resistant, -susceptible, and heterozygous strains or by analyzing the stability of Bt resistance in heterozygous populations over extended period of time. Laboratory studies and mathematical models show that fitness cost could play a major role in delaying evolution of resistance by selecting against resistant genotypes (Gassmann et al., 2009).

Cry2Ab2 is a common Bt protein expressed in transgenic corn and cotton targeting lepidopteran pests including *S. frugiperda*. In Bollgard II cotton, Cry2Ab2 is expressed along with Cry1Ac. In Bt corn, Cry2Ab2 is expressed in the transformation event MON 89034 which expresses Bt proteins Cry2Ab2 and Cry1A.105. In a narrow pool of Bt proteins targeting *S. frugiperda*, the pest has already developed field resistance to Cry1F protein in multiple regions. Because the Cry1F shares similar resistance mechanisms, and/or cross-resistance to other Cry1 proteins such as Cry1Ab, Cry1Ac, and Cry1A.105 (Hernández-Rodríguez et al., 2013; Vélez et al., 2013; Huang et al., 2014; Yang et al., 2016a;), Cry2Ab2 would essentially be the only fully-active Bt protein targeting this pest in many currently used Bt corn and Bt cotton varieties.

A Cry2Ab2-resistant strain of *S. frugiperda* was recently established using an F2 screen of a two-parent family collected from Georgia, U.S. (Niu et al., 2016). The resistant family is the first-ever strain of *S. frugiperda* that is highly resistant to Cry2Ab2 protein in diet bioassays as well as to Cry2Ab2 corn plants. The availability of this resistant strain provided a unique opportunity to analyze the fitness costs and inheritance of Cry2Ab2 resistance in *S. frugiperda*. Information generated from this study should be valuable in monitoring evolution of the

resistance to Cry2Ab2 in *S. frugiperda* and developing effective IRM strategies for the sustainable use of Bt corn technology.

2.2. Materials and methods

2.2.1. Sources of Bt protein and corn materials

Cry2Ab2 protein and the associated buffer were provided by Monsanto Company (St. Louis, MO). Once received from the company, Cry2Ab2 protein was stored at -80 °C, while the related buffer was maintained around 4-8 °C in a refrigerator. Before the Cry2Ab2 protein was used in bioassays, it was dissolved in the buffer under room conditions to make desired concentrations.

Seeds of a Cry2Ab2 corn line and a non-Bt corn iso-line were provided by Monsanto Company (St. Louis, MO). Corn seeds were planted in plastic pots filled with standard potting mixture (Perfect Mix, Expert Gardener products, St. Louis, MO) in a greenhouse of the Louisiana State University Agricultural Center in Baton Rouge, LA as described in Wu et al (2007). Two plants per pot were maintained and the plants were provided with irrigation water and fertilizer when necessary. Corn leaves at the vegetative stage (V4-V7) were used in the bioassays. Presence and absence of Bt proteins in the Bt and non-Bt corn plants were confirmed using an ELISA-based assay (EnviroLogix, Quantiplate™ kits, Portland, ME).

2.2.2. Sources of insects

A Bt-susceptible (SS) and a Cry2Ab2-resistant (RR) strains of *S. frugiperda* were used as the original insect sources (Table 2.1). SS strain was collected from non-Bt corn fields near Weslaco, Texas in 2013. This strain has been documented to be susceptible to Bt proteins Cry2Ab2, Cry1F, and Cry1A.105 as well as corn plants expressing one or more of these Bt proteins (Huang et al., 2014; Niu et al., 2014; Dangal and Huang, 2015). SS has been maintained

in the Corn and Small Grain Insect Research Laboratory at the Louisiana State University Agricultural Center in Baton Rouge, LA. It has not been exposed to any Bt proteins or insecticides. RR strain was derived from one of the 211 two-parent families established using single pair mating of feral individuals collected from seven locations in four states (Texas, Louisiana, Georgia, and Florida) in 2012-2013 (Niu et al., 2016). RR has been documented to be highly resistant to both purified Cry2Ab2 protein in the diet and Cry2Ab2 corn plants in the greenhouse (Niu et al., 2016). To ensure a similar genetic background between the two strains, before RR was used in the current study, it was backcrossed to SS two times and reselected using Cry2Ab2 corn leaf tissue for two generations. The reselections were done in 8- well trays (C-D International, Pitman, NJ), where each well contained 4 to 5 pieces of leaf tissues of Cry2Ab2 corn line. Newly hatched neonates were released at a rate of 20-30 per well for a total of 5-7 trays. Fresh leaf tissues were added every three days. A week after the release, the survivors were transferred into meridic (WARD's Stonefly *Heliothis*) diet.

2.2.3. Genetic crosses

Using the SS and the backcrossed-and-reselected RR mentioned above, three types of crosses were performed to generate eight additional genetic strains: 1) two F1 strains, F1A_{RmSf} and F1B_{RfSm}, produced by the reciprocal crosses between SS and RR; 2) two F2 strains, F2A_{RmSf} and F2B_{RfSm}, obtained by sib-mating of the two F1 strains, respectively; and 3) four backcross strains, BC_{F1AmRf}, BC_{F1AfRm}, BC_{F1BmRf}, and BC_{F1BfRm}, generated by reciprocal crosses between RR and each of the two F1 strains (Table 2.1). Because the resistance was recessive (see results), backcrosses were conducted by crossing RS with RR only (Roush et al., 1986; Tabashnik, 1991).

Table 2.1. Strains of *Spodoptera frugiperda* used in the study and their description

Insect strain	Description
SS	Bt-susceptible strain originally collected from Texas in 2013. It was susceptible to Cry2Ab2, Cry1F, and Cry1A.105.
RR	Cry2Ab2-resistant strain originally developed from F2 screen and re-selected on Cry2Ab2-corn leaf tissue.
F1A _{RmSf}	F1 progeny generated by crossing RR males with SS females
F1B _{RfSm}	F1 progeny generated by crossing RR females with SS males
F2A _{RmSf}	F2 progeny generated by sib-mating of F1A _{RmSf}
F2B _{RfSm}	F2 progeny generated by sib-mating of F1B _{RfSm}
BC _{F1AmRf}	Backcrossed progeny generated by crossing F1A _{RmSf} males with RR females
BC _{F1AfRm}	Backcrossed progeny generated by crossing F1A _{RmSf} females with RR males
BC _{F1BmRf}	Backcrossed progeny generated by crossing F1B _{RfSm} males with RR females
BC _{F1BfRm}	Backcrossed progeny generated by crossing F1B _{RfSm} females with RR males

2.2.4. Assessing fitness costs of the Cry2Ab2 resistance in *Spodoptera frugiperda*.

To determine if the Cry2Ab2 resistance in *S. frugiperda* is associated with any fitness costs, biological parameters including 7-day larval body weight, pupation rate, pupation time, pupal weight, sex ratio, and egg production of SS, RR, and the two F1 hybrid strains of *S. frugiperda* were evaluated on non-Bt diet and non-Bt corn leaf tissue using similar methods to Dangal and Huang (2015).

2.2.4.1. Relative fitness of SS, RR, F1A_{RmSf}, and F1B_{RfSm} on non-Bt diet

In the non-Bt diet assay, approximately 1 g of a meridic (WARD's Stonefly Heliothis) diet was placed in each cell of the 128-cell trays (C-D International, Pitman, NJ), upon which one neonate (<24 h old) of *S. frugiperda* was placed. The bioassay trays were then placed in growth chambers maintained at 26⁰C, ~50% r.h., and a photo period of 16h:8h (L: D). The

bioassays were run using a randomized block design where growth chambers acted as blocks. There were four blocks (growth chambers) with 30 larvae per block for each insect strain ($n = 4 \times 30 = 120$). After 7 days, live larvae were transferred from the 128-cell trays to 30-ml plastic cups (Fill-Rite, Newark, NJ) each containing approximately 8 g of the same diet, and continuously reared in the same growth chambers until the pupal stage. Once first pupa was observed, pupation was checked daily. Biological parameters measured in the rearing were 7-day larval body weight, neonate-to-pupa development time, neonate-to-pupa survivorship, pupal weight, and sex ratio. A larva was considered dead if it did not move when prodded with a fine brush.

Pupae collected from the above rearing were separated by sex for each insect strain. A pair of newly emerged (<24 h old) virgin male and female adult of an insect strain were placed into each 3.78-L paper container, which was placed in a growth chamber at 26°C, >90% r.h., and a photo period of 14h:10h (L: D) for mating and reproduction as described in Niu et al. (2016). The egg masses produced per pair were weighed. Sample size of single-pairs (n) for RR, SS, F1A_{RmSf}, and F1B_{RfSm} were 11, 16, 19, and 15, respectively. The egg mass produced was analyzed with completely randomized design.

2.2.4.2. Relative fitness of SS, RR, F1A_{RmSf}, and F1B_{RfSm} on non-Bt corn leaf tissue

In the leaf tissue assay, leaves from the greenhouse-grown non-Bt corn (iso-line of Cry2Ab2 corn-line) plants at the vegetative stage V4-V7 were used. Two to three pieces (≈ 3 cm long) of the non-Bt corn leaves were placed in each well of 32-well trays (Bio-Ba-32, C-D International, Pitman, NJ), and a neonate (<24-hr old) was then placed on the leaf tissue in each well (1 larva/well) as described in Dangal and Huang (2015). One tray (or 32 larvae) for each strain was placed in each of four growth chambers ($n = 4 \times 32 = 128$). As mentioned above, the

assays were run with a randomized block design with growth chambers as the block factor. The growth chambers were maintained at 26⁰C, ~50% r.h., and a photo period of 16h:8h (L: D). Leaf tissue was replaced every 2-3 days. After 7 days, live larvae were transferred to the 8-well trays (C-D International, Pitman, NJ) containing the same non-Bt corn leaf tissue (1 larva/well) and reared in the same growth chambers until the pupal stage. As in the non-Bt diet assay, once the first pupa was observed, pupation was checked daily. Virgin adults were single-paired in the 3.78-L paper containers and eggs produced from each pair were collected as described in the non-Bt diet assays. Sample size of single-pairs (n) for RR, SS, F1A_{RmSf}, and, F1B_{RfSm} were 13, 16, 7, and, 12 respectively. The egg masses produced were analyzed with completely randomized design. Life history parameters measured in the leaf tissue assays were the same as recorded in the non-Bt diet assays.

2.2.5. Analysis of inheritance of Cry2Ab2 resistance in *Spodoptera frugiperda*

To assess inheritance of Cry2Ab2 resistance in *S. frugiperda*, larval survival of RR, SS, and the eight genetic-crossed strains of *S. frugiperda* listed in Table 2.1 were assayed using two assay methods: 1) diet-incorporated Cry2Ab2 protein bioassays and 2) bioassays using corn leaf tissue expressing Cry2Ab2 protein. In the diet-incorporated bioassay, three Cry2Ab2 concentrations, 10, 31.6, and 100 µg/g, plus a buffer only control were used in each bioassay. Three Cry2Ab2 concentrations were prepared by dissolving the appropriate amount of Cry2Ab2 protein in the buffer first and then the Bt solutions were mixed thoroughly in the meridic diet mentioned above. Approximately 1 g of the Bt-treated or non-treated diet was placed on each cell of the 128-cell trays (C-D International, Pitman, NJ) and in each cell one neonate was released on the surface of the diet. Bioassay trays with diet and larvae were placed in growth chambers maintained at 26⁰C, ~50% r.h., and a photoperiod of 16h:8h (L: D). The bioassays

were run with a randomized block design where four growth chambers acted as blocks. For each combination of insect strain and Bt concentration, there were 25 larvae per block ($n = 25 \times 4 = 100$). Larval mortality for each combination of insect strain and Bt concentration was recorded at 7th day of larval infestation.

Leaf tissue bioassay was carried out in the 32-well trays (C-D International, Pitman, NJ). The corn leaves were collected from the greenhouse-grown corn plants at V4-V7 stages. Collected leaves were cut into 2-3 cm pieces and 3-4 pieces of the leaf tissue were placed in each well of the 32-well trays. One neonate was then released on the surface of the leaf tissue in each well. Bioassay trays with leaf tissue and larvae were placed in the growth chambers at the same conditions as used in the diet-incorporated bioassays. Similar to the diet-incorporated bioassay, the bioassay was run with a randomized block design where four growth chambers acted as blocks. For each insect strain, there were 32 larvae (in a tray) per block ($n = 32 \times 4 = 128$). Larval mortality of each insect strain was checked at 7th day of larval infestation.

2.2.6. Data analysis

The 7-day larval mortalities of the ten strains used in the inheritance study on Cry2Ab2 diet or Cry2Ab2 leaf tissue were corrected for the control mortalities using the formula in Abbott (1925). The various life history data recorded in the fitness cost tests and the corrected 7-day mortalities obtained in the inheritance study were found to be normally distributed (SAS Institute, 2010). Data from diet and leaf bioassays for fitness cost study and from leaf assays for inheritance study were analyzed using one-way analysis of variance (ANOVA) where insect strain was the main factor (SAS Institute, 2010). Data from the diet-incorporated bioassays for inheritance study were analyzed with a two-way ANOVA (SAS Institute 2010) with Cry2Ab2

concentration and insect strains as the two major factors including the interaction of these factors. Treatment means were separated using Tukey's HSD at the $\alpha = 0.05$ level.

Sex-linkage of resistance was assessed by comparing the 7-day larval mortality of the two F1 hybrid progenies derived from the reciprocal crosses between SS and RR. Student's *t* test was also employed to compare if the mortality was significantly different between the two F1 strains in each toxin concentration. An absence of significant differences in mortality response between the two strains was considered as an indicator of absence of sex-linkage.

Effective dominance level (D_{ML}) of resistance at a given Cry2Ab2 concentration or on Cry2Ab2 leaf tissue was quantified as the formula in Bourguet et al. (2000).

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

where ML_{RS} , ML_{SS} and ML_{RR} are the respective mortalities for F1 heterozygous, susceptible and resistant insects. The value of D_{ML} ranges from 0 to 1; where 0 refers that the resistance is completely recessive, while 1 means that the resistance is completely dominant. In this study, because the resistance was autosomal (see results), the mortality data of the two F1 strains were pooled in calculating the D_{ML} values. In addition, because larval mortalities at the Cry2Ab2 concentration of 10 $\mu\text{g/g}$ in the diet-incorporated bioassay were low, ranging from 3.1 to 13.8% (see results) across the four insect strains, D_{ML} was not calculated for this concentration.

To test whether the inheritance of resistance fitted the Mendelian monogenic model of inheritance, a Chi-Square test was employed to compare if the observed mortalities of the F2 and backcrossed strains fitted the expected mortalities. The null hypothesis tested is that resistance is controlled by one locus with two alleles. If the null hypothesis is true, the F2 strains are expected to consist of 25% RR, 50% heterozygous (RS) and 25% SS individuals and the backcross strains

should contain 50% RS and 50% RR individuals. If the observed mortality of RR, SS and F1 are P_{RR} , P_{SS} and P_{RS} , respectively, under the null hypothesis, the expected mortality (p_1) of F2 strains can be estimated as:

$$p_1 = (P_{RR} + 2 P_{RS} + P_{SS})/4,$$

and that for the backcross strains (p_2) should be

$$p_2 = 0.5 (P_{RR} + P_{RS}).$$

Chi-square value is calculated as:

$$\chi^2 = \frac{(O - np_i)^2}{np_i(1 - p_i)},$$

where O is the observed number of dead insects in the F2 or backcross strains. The null hypothesis is rejected if the calculated χ^2 value is greater than the book value of χ^2 at $\alpha = 0.05$ and $df=1$. As mentioned above, because the 7-day larval mortality of SS, RR, and F1 hybrid strains at the Cry2Ab2 concentration of 10 $\mu\text{g/g}$ was not a good discriminating concentration, tests for fitting the monogenic model were performed for 31.6 $\mu\text{g/g}$ and 100 $\mu\text{g/g}$ in the diet-incorporated assay and for the leaf tissue assay.

2.3. Results

2.3.1. Fitness costs of the Cry2Ab2 resistance in *Spodoptera frugiperda*

2.3.1.1. Relative fitness of SS, RR, F1A_{RmSf}, and F1B_{RfSm} on non-Bt diet

The effect of insect strain on the 7-day larval weight reared on non-Bt diet was significant ($F_{3,9} = 6.64$, $P = 0.0117$), while it was not significant for all other life history parameters measured ($F_{3,26.4} = 0.52$, $P = 0.52$ for egg production and $F_{3,9} \leq 2.82$, $P \geq 0.0992$ for other parameters) (Table 2.2). The average weight of an SS larvae after feeding on non-Bt diet for 7 days was 49.8 mg. The 7-day average larval weight of RR (35.0 mg/larva) was not

significantly different ($P > 0.05$) from SS. The average weight of each of the two F1 hybrid strains was significantly greater than the weight of RR ($P < 0.05$) but not SS ($P > 0.05$) (Table 2.2).

Neonate-to-pupa survival rate of the four insect strains feeding on non-Bt diet ranged from 41.7-73.3% with an overall average of 55% (Table 2.2). Males and females had a similar larval developmental rate. On average, a male neonate needed 16.0 days to develop to the pupal stage, and a female took 15.7 days. The average weight of a male pupa of the four insect strains was 180.3 mg, while a female pupa weighed 173.4 mg. Sex ratios (male/female) of the four strains ranged from 0.8 to 1.5 with a grand average of 1.05. Egg productions of the four strains ranged from 57.8-72.8 mg/pair with a grand average of 64.5 mg/pair (Table 2.2).

2.3.1.2. Relative fitness of SS, RR, F1A_{RmSf}, and F1B_{RfSm} on non-Bt corn leaf tissue

The effect of insect strain on egg production was significant ($F_{3,18.6} = 6.21$, $P = 0.0042$), while it was not significant for all other life history parameters measured ($F_{3,9} \leq 2.52$, $P \geq 0.1237$) (Table 2.3). SS female feeding on the non-Bt corn leaf tissue laid an average of 34.5 mg eggs, which was not significantly different ($P > 0.05$) compared to the egg production of RR (61.4 mg/pair) or F1A_{RmSf} (58.8 mg/pair), but it was significantly less ($P < 0.05$) than the eggs (91.6 mg/pair) produced by F1B_{RfSm}.

Larval weight of the four insect strains after 7 days feeding on non-Bt corn leaf tissue ranged from 58.0 to 70.9 mg/larva with a grand average of 64.1 mg/larva (Table 2.3). Similar to the non-Bt diet assay, a grand average of 55.1% larvae survived after 7 days feeding on non-Bt corn leaf tissue. Males and females also had a similar larval developmental rate on non-Bt corn leaf tissue, but larvae feeding on non-Bt leaf tissue apparently developed somewhat faster than on non-Bt diet. On average, a male neonate on non-Bt corn leaf tissue needed 13.9 days to

Table 2.2. Survival, growth, development, and egg production (mean \pm sem) of Bt-susceptible (SS), Cry2Ab2-resistant (RR), and two reciprocal F1 (F1A_{RmSf} and F1B_{RfSm}) strains of *Spodoptera frugiperda* on non-Bt diet*

Insect Strain	7-day larval weight (mg/larva)	Neonate-to-pupa survival %	Neonate-to-pupa development time (day)		Pupal mass (mg/pupa)		Sex ratio (m:f)	Egg mass per pair (mg)
			Male	Female	Male	Female		
SS	49.8 \pm 10.5 ab	56.7 \pm 11.0 a	16.0 \pm 0.7 a	16.2 \pm 1.0 a	182.2 \pm 3.6 a	168.9 \pm 10.5 a	0.8 \pm 0.1 a	64.6 \pm 8.8 a
RR	35.0 \pm 8.8 b	41.7 \pm 10.4 a	16.5 \pm 0.9 a	16.4 \pm 1.1 a	182.9 \pm 5.8 a	166.9 \pm 14.0 a	1.5 \pm 0.8 a	57.8 \pm 8.2 a
F1A _{RmSf}	59.5 \pm 12.5 a	48.3 \pm 12.0 a	15.5 \pm 1.1 a	15.1 \pm 1.0 a	180.3 \pm 8.2 a	171.4 \pm 3.3 a	0.8 \pm 0.1 a	62.9 \pm 7.2 a
F1B _{RfSm}	58.3 \pm 14.0 a	73.3 \pm 9.9 a	15.8 \pm 1.1 a	15.2 \pm 0.9 a	175.6 \pm 12.6 a	186.3 \pm 8.2 a	1.1 \pm 0.25 a	72.8 \pm 8.9 a
ANOVA	$F_{3,9} = 6.64$	$F_{3,9} = 1.41$	$F_{3,9} = 0.64$	$F_{3,9} = 2.82$	$F_{3,9} = 0.15$	$F_{3,9} = 0.85$	$F_{3,9} = 0.43$	$F_{3,26.4} = 0.52$
P-value	0.0117	0.3021	0.6106	0.0992	0.9297	0.5005	0.7350	0.6744

*Mean values within a column followed by a same letter are not significantly different at $\alpha = 0.05$ (Tukey's HSD tests).

Table 2.3. Survival, growth, development, and egg production (mean \pm sem) of Bt-susceptible (SS), Cry2Ab2-resistant (RR), and two reciprocal F1 (F1A_{RmSf} and F1B_{RfSm}) strains of *Spodoptera frugiperda* on non-Bt corn leaf tissue*

Insect Strain	7-d larval weight (mg/larva)	Neonate-to-pupa survival %	Neonate to pupa development time (day)		Pupal mass (mg/pupa)		Sex ratio (m:f)	Egg mass per pair (mg)
			Male	Female	Male	Female		
SS	58.0 \pm 9.2 a	54.3 \pm 7.4 a	14.1 \pm 0.7 a	13.9 \pm 0.7 a	203.9 \pm 5.0 a	198.4 \pm 4.9 a	1.7 \pm 0.7 a	34.5 \pm 8.0 b
RR	60.2 \pm 8.8 a	57.8 \pm 4.9 a	14.2 \pm 0.6 a	13.9 \pm 0.5 a	194.9 \pm 2.9 a	199.3 \pm 4.3 a	1.2 \pm 0.3 a	61.4 \pm 8.1 ab
F1A _{RmSf}	67.3 \pm 13.3 a	53.5 \pm 5.8 a	13.8 \pm 0.5 a	13.7 \pm 1.0 a	208.0 \pm 3.9 a	196.5 \pm 4.5 a	1.1 \pm 0.3 a	58.8 \pm 15.1 ab
F1B _{RfSm}	70.9 \pm 13.2 a	54.7 \pm 7.9 a	13.4 \pm 0.6 a	13.5 \pm 0.5 a	201.3 \pm 6.5 a	195.3 \pm 6.2 a	1.5 \pm 0.07 a	91.6 \pm 10.7 a
ANOVA	$F_{3,9} = 2.45$	$F_{3,9} = 0.11$	$F_{3,9} = 2.20$	$F_{3,9} = 0.52$	$F_{3,9} = 2.52$	$F_{3,9} = 0.14$	$F_{3,9} = 0.46$	$F_{3,18.6} = 6.21$
	$P = 0.1305$	0.9505	0.1577	0.6783	0.1237	0.9360	0.7189	0.0042

*Mean values within a column followed by a same letter are not significantly different at $\alpha = 0.05$ (Tukey's HSD tests).

develop to the pupa stage, and a female took 13.8 days. Pupae developing from larvae feeding on non-Bt corn leaf tissue were also somewhat heavier than those from non-Bt diet. On average, male and female pupae of the four insect strains weighed 202.0 and 194.4 mg, respectively. On the leaf tissue, relatively more males than females survived to the pupal stage; sex ratios (male/female) of the four strains ranged from 1.1 to 1.7 with an average of 1.38 (Table 2.3).

2.3.2. Inheritance of Cry2Ab2 resistance in *S. frugiperda*.

2.3.2.1. Overall results of the analysis of variance for the 10 insect strains and performance of SS and RR on Cry2Ab2-treated diet and Cry2Ab2 corn leaf tissue

In the two-way ANOVA of the data observed from the diet-incorporated bioassay, a significant effect of insect strain ($F_{9,87} = 42.28$, $P < 0.0001$), Cry2Ab2 concentration ($F_{2, 87} = 398.90$, $P < 0.0001$), and the interaction between Cry2Ab2 concentration and insect strain ($F_{18, 87} = 12.30$, $P < 0.0001$) was detected. The 7-day mortality of SS at the Cry2Ab2 concentration of 10 $\mu\text{g/g}$ was as low as 11.8%, but reached 43% at 31.6 $\mu\text{g/g}$ and 96.8% at 100 $\mu\text{g/g}$, with significantly higher mortalities at higher concentrations (Table 2.4). Similarly, the mortalities of two F1 strains were significantly higher as toxin concentration increased. In contrast, mortality of RR was low across all three Cry2Ab2 concentrations and ranged from 3.1 to 6.1% that did not differ across the toxin concentrations (Table 2.4).

In the one-way ANOVA of the data collected from the leaf tissue test, the effect of insect strain on larval mortality was significant ($F_{9,27} = 56.18$; $P < 0.0001$) (Table 2.5). After 7 days of feeding on Cry2Ab2 corn leaf tissue, 97.6% SS larvae were killed, which was considerably ($P < 0.05$) greater than the corresponding mortality (5.7%) of RR (Table 2.5).

Table 2.4. 7-day larval mortality (% mean \pm sem) of ten strains of <i>Spodoptera frugiperda</i> in diet-incorporated bioassays		
Cry2Ab2 Concentration ($\mu\text{g/g}$)	Insect Strain ^a	Mean \pm sem ^b
10	SS	11.8 \pm 2.8 ghi
10	RR	3.1 \pm 1.0 i
10	F1A _{RmSf}	13.8 \pm 5.1 fghi
10	F1B _{RfSm}	7.5 \pm 2.8 hi
10	F2A _{RmSf}	32.6 \pm 4.5 cdef
10	F2B _{RfSm}	7.0 \pm 1.9 i
10	BC _{F1AmRf}	20.4 \pm 6.1 efghi
10	BC _{F1AfRm}	9.5 \pm 2.7 ghi
10	BC _{F1BmRf}	5.2 \pm 2.9 i
10	BC _{F1BfRm}	4.4 \pm 1.8 i
31.6	SS	43.0 \pm 2.1 c
31.6	RR	5.1 \pm 2.0 i
31.6	F1A _{RmSf}	41.4 \pm 3.9 cd
31.6	F1B _{RfSm}	28.0 \pm 6.2 cdefg
31.6	F2A _{RmSf}	33.7 \pm 5.6 cdef
31.6	F2B _{RfSm}	22.1 \pm 7.2 defghi
31.6	BC _{F1AmRf}	27.6 \pm 3.5 cdefgh
31.6	BC _{F1AfRm}	10.5 \pm 3.2 ghi
31.6	BC _{F1BmRf}	11.3 \pm 3.6 ghi
31.6	BC _{F1BfRm}	35.9 \pm 3.3 cde
100	SS	96.8 \pm 2.1 a
100	RR	6.1 \pm 1.7 i
100	F1A _{RmSf}	80.5 \pm 2.2 ab
100	F1B _{RfSm}	72.0 \pm 2.8 b
100	F2A _{RmSf}	70.8 \pm 1.3 b
100	F2B _{RfSm}	67.4 \pm 3.3 b
100	BC _{F1AmRf}	46.9 \pm 2.9 c
100	BC _{F1AfRm}	46.3 \pm 4.0 c
100	BC _{F1BmRf}	42.3 \pm 5.1 cd
100	BC _{F1BfRm}	42.4 \pm 2.7 c

^a Sample size (N, number of larvae) in diet-incorporated bioassay for each strain was 100.

^b Means within the same column followed by different letters were significantly different based on Tukey's HSD tests at $\alpha= 0.05$.

Table 2.5. 7-day larval mortality (% mean \pm sem) of ten strains of *Spodoptera frugiperda* in corn leaf tissue bioassays

Insect Strain ^a	Mean \pm sem ^b
SS	97.6 \pm 0.8 ab
RR	5.7 \pm 2.5 d
F1A _{RmSf}	99.1 \pm 0.9 a
F1B _{RfSm}	99.1 \pm 0.9 a
F2A _{RmSf}	78.3 \pm 5.8 b
F2B _{RfSm}	81.0 \pm 2.5 ab
BC _{F1AmRf}	54.7 \pm 2.2 c
BC _{F1AfRm}	52.6 \pm 6.2 c
BC _{F1BmRf}	54.3 \pm 5.4 c
BC _{F1BfRm}	42.2 \pm 5.2 c
ANOVA	$F_{9, 27} = 56.18, P < 0.0001$

^a Sample size (N, number of larvae) in each bioassay was 128.

^b Means within the same column followed by different letters were significantly different based on Tukey's HSD tests at $\alpha = 0.05$.

2.3.2.2. Sex linkage and effective dominance level (D_{ML}) of Cry2Ab2 resistance in *Spodoptera frugiperda*

The multiple comparison of Tukey's HSD tests showed no significant differences in larval mortality between the F1 strains generated from reciprocal crosses of SS and RR for all three Cry2Ab2 concentrations in the diet incorporated bioassay (Table 2.4) and for the corn leaf tissue tests (Table 2.5). Mortality of the two F1 strains was low (7.5-13.8%) at 10 $\mu\text{g/g}$ but reached 72.0% (F1B_{RfSm}) and 80.5% (F1A_{RmSf}) at 100 $\mu\text{g/g}$, which were significantly ($P < 0.05$) greater than the mortality of RR, but less than the mortality of SS (Table 2.4). On Cry2Ab2 leaf tissue, mortality of the two F2 strains was 99.1%, which was also considerably greater ($P < 0.05$) than the mortality of RR but not significantly different ($P > 0.05$) from SS (Table 2.5). Similarly, mean comparison between the two F1 strains based on the Student's *t*-test also showed that there

were no significant differences in the mortalities between the two F1 strains for each of the three Cry2Ab2 concentration and leaf tissue bioassays (Table 2.6). Thus, no sex-linkage or maternal effect was evident in the Cry2Ab2 resistance in *S. frugiperda* in this study.

Table 2.6. Larval mortality (% mean) of the two F1 reciprocal strains of *Spodoptera frugiperda* in diet-incorporated and leaf tissue bioassays

Assay Method	Cry2Ab2 concentration or plant Stage	Mean Mortality % (F1A _{RmSf})	Mean Mortality % (F1B _{RfSm})	t value	P-value
Diet-incorporated	10 µg/g	13.8	7.5	1.24	0.3025
	31.6 µg/g	41.4	28.0	2.09	0.1277
	100 µg/g	80.5	72.0	1.85	0.1621
Leaf tissue	V4 – V7	99.1	99.1	0.00	0.9881

Effective dominance levels were calculated based on the larval mortality at each of the toxin concentration in diet-incorporated bioassay and Cry2Ab2 leaf tissue test. Based on the diet-incorporated bioassays, the calculated D_{ML} was 0.22 at 31.6 µg/g and 0.23 at 100 µg/g, while it was -0.02 based on the Cry2Ab2 leaf tissue tests (Table 2.7). These results indicate that the Cry2Ab2 resistance in *S. frugiperda* was functionally recessive in the leaf tissue and incompletely recessive in the diet-incorporated assays.

Table 2.7. Effective dominance level (D_{ML}) of Cry2Ab2 resistance in *Spodoptera frugiperda* in diet-incorporated and leaf tissue bioassays

Assay method	Cry2Ab2 concentration or plant stage	Dominance level (D_{ML})	Conclusion
Diet-incorporated	31.6 µg/g	0.22	Incompletely recessive
	100 µg/g	0.23	Incompletely recessive
Leaf tissue	V4 – V7	-0.02	Completely recessive

2.3.2.3. Test for fitting the Mendelian monogenic model of inheritance

In general, 7-day larval mortality was similar between the two F2 strains for the leaf tissue tests and at each Cry2Ab2 concentration in the diet-incorporated bioassays except for 10 µg/g. At a Cry2Ab2 concentration of 31.6 µg/g in diet-incorporated bioassays, mortality of the two F2 strains was low (33.7% for F2A_{RmSf} and 22.1% for F2B_{RfSm}), but they had higher mortality at 100 µg/g concentration (70.8% and 67.4%, respectively), which was significantly greater than the mortality of RR, but significantly less than SS (Table 2.4). The mortality of the two F2 strains on Cry2Ab2 corn leaf tissue (78.3% for F2A_{RmSf} and 81.0% for F2B_{RfSm}) was also considerably greater ($P < 0.05$) than that observed for RR but the differences between the F2 strains and SS were not significant ($P > 0.05$) (Table 2.5).

Some variation in the larval mortality at Cry2Ab2 concentration of 10 µg/g and 31.6 µg/g in the diet-incorporated bioassays were observed among the four backcross insect strains, but the overall mortality at these two concentrations was low across the four insect strains, ranging from 4.4 to 35.9% (Table 2.4). There were no significant differences in the larval mortality among the four backcross strains at 100 µg/g in the diet-incorporated bioassay (ranged from 42.3-46.9%) (Table 2.4) as well as in the leaf tissue test (ranged from 42.2-54.7%) (Table 2.5).

χ^2 tests showed that the larval mortality data observed fitted well the expected data ($\chi^2 = 0.145$ to 2.55, $df = 1$, $P = 0.11$ to 0.703) based on the Mendelian monogenic model of inheritance for both the F2 and backcrossed strains, and in both the diet-incorporated and leaf tissue bioassays (Table 2.8). Thus, it is very likely that the Cry2Ab2 resistance is controlled by a single or a few tightly-linked genes.

Table 2.8. Test for fitting the Mendelian monogenic model for Cry2Ab2 resistance in *Spodoptera frugiperda*

Assay Method	Cry2Ab2 concentration	Insect strains	N ^a	Observed dead (O)	Expected dead (E)	χ^2	P-value
Diet-incorporated	31.6 $\mu\text{g/g}$	Pooled Backcross	400	84.8	79.0	0.530	0.470
		Pooled F2	200	56.0	58.5	0.145	0.703
	100 $\mu\text{g/g}$	Pooled Backcross	400	178.0	164.4	1.910	0.167
		Pooled F2	200	138.2	127.5	2.470	0.116
Cry2Ab2 leaf tissue	V4-V7 stage	Pooled Backcross	512	260.9	268.3	0.420	0.517
		Pooled F2	256	204.3	193.0	2.550	0.110

^a N = total number of insect individuals assayed.

2.4. Discussion

Although a few minor differences were observed among SS, RR, and the two F1 strains in the fitness cost study, the performance of SS and RR was similar on non-Bt diet or on non-Bt corn leaf tissue across all the life history parameters measured. In addition, the two F1 hybrid strains had comparable fitness to SS in both assay methods. Together with the results from the inheritance study, Cry2Ab2 resistance in *S. frugiperda* appears to be inherited as a single (or a few tightly-linked), autosomal, recessive gene and the resistance is not associated with significant fitness costs.

The presence of fitness costs, especially non-recessive fitness costs, could be important in delaying the evolution of resistance by selecting against resistant genotypes after selection pressure is removed (Gassmann et al., 2009). Past studies have shown that Bt resistance are associated with fitness costs in some cases (Carrière et al., 2001; Bird and Akhurst, 2004; Anilkumar et al., 2008; Gassmann et al., 2009; Pereira et al., 2011; Dangal and Huang, 2015). However, the lack of fitness costs in Bt resistance is not uncommon. In particular, a lack of

fitness costs has been documented in the three most notable cases of field resistance to Bt crops, which are the resistance of the *B. fusca* to Cry1Ab corn (Kruger et al., 2014); *D. virgifera virgifera*, to Cry3Bb1 corn (Petzold-Maxwell et al., 2012); and *S. frugiperda* to Cry1F corn (Jakka et al., 2014; Vélez et al., 2014; Leite et al., 2016). The similar results for these three cases indicate that the absence of fitness costs may play an important role in resistance development in the field. In addition, a lack of fitness costs was reported for the Cry1A.105 resistance in *S. frugiperda* (Niu et al., 2017), Cry2Ab2 and Cry1Ab resistance in the sugarcane borer, *Diatraea saccharalis* (Fabricius) (Wu et al., 2009a; Zhang et al., 2014b; Huang et al., 2015), and Cry1Ac resistance in *Plutella xylostella* Linnaeus (Sayyed and Wright, 2001). If the current finding of a lack of fitness costs for Cry2Ab2 resistance translates into field scenarios, it would suggest that having effective proactive resistance management strategies in place before field-evolved resistance occurs will be critical to preserve susceptibility to this protein.

The finding of lack of maternal effect/sex linkage for the Cry2Ab2 resistance in *S. frugiperda* in the current study is consistent with a wide body of published studies on Bt resistance. For example, Cry1F resistance in several other populations of *S. frugiperda* that have been evaluated was also autosomally inherited (Storer et al., 2010; Vélez et al., 2013; Leite et al., 2016; Santos-Amaya et al., 2016; Camargo et al., 2017). Similar results were also found for the Cry1Ab and Cry1Ac resistance in Asian corn borer (*Ostrinia furnacalis* Guenée) (Zhang et al., 2014a), Cry1Ac and Cry2Ab resistance in *Helicoverpa armigera* Hübner (Kranthi et al., 2006; Mahon et al., 2007), Cry1Ac resistance in *P. gossypiella* (Tabashnik et al., 2002), Cry1F resistance in *Ostrinia nubilalis* Hübner (Pereira et al., 2008), and Cry1Ab and Cry2Ab2 resistance in *D. saccharalis* (Wu et al., 2009c; Huang et al., 2015).

Past studies also have reported that high level resistance to Bt toxins is largely recessive (Bourguet et al., 2000; Ferré and van Rie, 2002; Mahon et al., 2007; Huang et al., 2011). The findings of recessive (or incompletely recessive) inheritance of the Cry2Ab2 resistance in *S. frugiperda* in the current study is also consistent with many studies, which include the Cry2Ab resistance in *H. armigera* (Mahon et al., 2007) and Cry1F resistance in *S. frugiperda* (Storer et al. 2010; Vélez et al., 2013; Camargo et al., 2017). However, some recent studies have shown that functionally non-recessive resistance to Bt crops can occur frequently, particularly against single toxin, non-high dose products. For example, the resistance of *B. fusca* to Cry1Ab corn was reported to be dominant (Campagne et al., 2013) and resistance to Cry3Bb1 corn in *D. virgifera virgifera* was found to be incompletely recessive to partially dominant (Petzold-Maxwell et al., 2012; Ingber and Gassmann, 2015). Non-recessive resistance to Bt crops also has been observed with Cry1Ac resistance in *H. armigera* (Nair et al., 2010; Jin et al., 2013), Cry1F resistance in *S. frugiperda* (Farias et al., 2016; Leite et al., 2016), and Cry1Ab and Cry2Ab2 resistance in *D. saccharalis* (Wu et al., 2009c; Ghimire et al., 2011; Wangila et al., 2012; Huang et al., 2015). Diverse genetic basis of resistance has been documented in *H. armigera* where Cry1Ac resistance is governed by recessive cadherin mutations as well as non-recessive resistance alleles (Zhang et al., 2012). All these findings imply that species-and toxin-specific knowledge on Bt resistance is needed to develop scientifically sound resistance management strategies.

The method employed here to determine the number of loci involved in the resistance is based on the expected mortality of F2 and backcross strains. The null hypothesis assumes that the resistance is controlled by one locus with two alleles (Tabashnik, 1991). Due to the complexity, two or more gene hypotheses were not tested, but the multiple tests with the diet-incorporated bioassays at two Cry2Ab2 concentrations as well as with corn leaf tissue bioassays

all pointed to a monogenic inheritance of the Cry2Ab2 resistance in *S. frugiperda*. Studies have shown that the number of genes conferring Bt resistance can vary depending on insect species, toxin types, and even selection regimes (Liang et al., 2008; Wang et al., 2016). However, in most cases, Bt resistance was found to be controlled by a single gene (or a few tightly-linked genes), such as the Cry1F resistance in *S. frugiperda* (Vélez et al., 2013; Santos-Amaya et al., 2016; Camargo et al., 2017) as well as the Cry2Ab2 resistance in *H. armigera* (Mahon et al., 2007) and *D. saccharalis* (Huang et al., 2015).

Documentation of a single, autosomal, recessive gene associated with the Cry2Ab2 resistance in *S. frugiperda* (particularly when confronted with Bt corn tissue) in the current study should have significant implications for resistance management. If the findings of the current study hold true under field conditions, it suggests that the recessive resistance assumption of the currently implemented “high-dose/refuge” IRM strategy is likely satisfied for managing the Cry2Ab2 resistance in *S. frugiperda*. Recessive resistance is a key requirement for the “high-dose/refuge” IRM strategy so that the heterozygous individuals carrying a single Bt resistance allele can be killed by the “high dose” Bt plants (Huang et al., 1999; Huang et al., 2011).

Besides the high dose/refuge strategy, a gene-pyramiding strategy has been implemented for Bt crop resistance management in the U.S. and globally. This strategy relies on the use of transgenic crops that produce two or more dissimilar Bt proteins targeting the same insect pests. To be effective, the pyramided proteins should lack cross-resistance so that resistance to one Bt protein does not confer resistance to others (Zhao et al., 2003; Moar and Anilkumar, 2007). In the current Bt crop market, none of the commercial Bt corn or Bt cotton express the Cry2Ab2 protein alone. In corn, Cry2Ab2 is one of the two genes in the event MON89034 which has been incorporated into some common commercial Bt corn hybrids such as Genuity® VT Double

Pro™, Genuity® VT Triple Pro™, Genuity®SmartStax™ and PowerCorn™ (Buntin and Flanders, 2015; DiFonzo et al., 2017). Other Bt genes in these corn products targeting lepidopteran pests are Cry1A.105 and Cry1F. In cotton, Cry2Ab2 is combined with the Cry1Ac protein in a common Bt cotton product, Bollgard II (Monsanto, 2012). These Bt corn and cotton plants are shown to control *S. frugiperda* along with other insect pests such as *H. zea* (Adamczyk et al., 2008; Siebert et al., 2012; Rule et al., 2014). Recently, another Bt protein, Vip3A, which has a novel mode of action, has been incorporated into some Bt corn and cotton varieties (Estruch et al., 1996). Many studies have shown that there is no cross-resistance among Cry1A, Cry2A and Vip3A proteins in *S. frugiperda* (Sivasupramaniam et al., 2008; Niu et al., 2013; Vélez et al., 2013; Huang et al., 2014; Niu et al., 2014; Santos-Amaya et al., 2015; Niu et al., 2016; Yang et al., 2016a; Yang et al., 2017) and in other target pests (Brévault et al., 2009; Wu et al., 2009b; Yang et al., 2015; Sivasupramaniam et al., 2008). However, cross-resistance among Cry1 (e.g. Cry1F to Cry1Ab, Cry1Aa, and Cry1A.105) proteins is very common (Hernández-Rodríguez et al., 2013; Huang et al., 2014; Bernardi et al., 2015). Because *S. frugiperda* has developed field resistance to Cry1F corn in multiple locations (Storer et al., 2010; Farias et al., 2014; Huang et al., 2014), and Cry1F cross-resistance with other Cry1 proteins, Cry2Ab2 would essentially be the only fully active Bt protein targeting this pest. Moreover, *H. zea* has shown decreased Cry1Ac susceptibility in some field populations of *H. zea* collected from the U.S. Cotton Belt (Yang et al., 2016b). Recently, Dively et al. (2016) reported that field resistance of *H. zea* to MON89034 sweet corn has occurred in Maryland. This highlights the importance of preserving susceptibility of Cry2Ab2 to its target pest populations such as *S. frugiperda* and *H. zea* for the sustainability of Bt crops. The information from the current study

should be useful in developing effective IRM strategies to prevent further spread of the resistance and preserve the Cry2Ab2 susceptibility of the field pest populations.

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CHAPTER 3: SUMMARY AND CONCLUSIONS

Genetically engineered crops expressing proteins from *Bacillus thuringiensis* (Bt proteins) have been commercialized since 1996, which has greatly changed the landscape of pest management in corn and cotton by suppressing target pest populations, reducing application of conventional insecticides, and economically benefitting farmers. Due to the strong selection pressure imposed by the widespread adoption of Bt crops, evolution of resistance in target pest populations has been a major threat to the sustainability of transgenic Bt crop technology.

The polyphagous insect, fall armyworm, *Spodoptera frugiperda* (J.E. Smith) is one of target pests of Bt corn, Bt cotton, and Bt soybean. It is a major economic pest of corn in many regions of North and South America. This pest, thus far, has developed field-resistance to Bt corn in Puerto Rico, Brazil, and U.S. mainland. Cry2Ab2 is a relatively new Bt protein that was deployed in transgenic corn to control lepidopteran pests including *S. frugiperda*. This particular Bt protein remains the chief Bt protein in Bt products to control *S. frugiperda* because the pest has already developed field resistance to Cry1F protein, and the resistance development to other Cry1-proteins is likely to hasten because of their cross-resistance to Cry1F. The proper knowledge on fitness costs and genetic basis of resistance is crucial because they are important factors that dictate the rate of evolution of resistance. Using an F2 screen, a Cry2Ab2-resistant strain *S. frugiperda* was established in the Corn and Small Grain Insect Research Laboratory at the Louisiana State University Agricultural Center, which paved the way to analyze the fitness costs and inheritance of Cry2Ab2 resistance in *S. frugiperda*.

In the fitness tests, larval survival, development, and reproduction of the Cry2Ab2-resistant, -susceptible, and two reciprocal F1 heterozygous strains were assayed on non-toxic diet and non-Bt corn leaf tissue. Biological parameters measured in the rearing were 7-day larval

body weight, neonate-to-pupa development time, neonate-to-pupa survivorship, pupal weight, sex ratio, and egg production. In inheritance study, larval mortalities of the resistant- and susceptible-parents, and 8 other cross-strains were assayed using diet-incorporated and leaf tissue bioassays. Maternal effects were examined by comparing the larval mortalities between the two reciprocally crossed F1 strains. Dominance levels of resistance were measured by comparing the larval mortalities of resistant, susceptible, and F1 heterozygous strains. Number of genes associated with the resistance was estimated by fitting the observed mortalities of F2 and backcross strains with the Mendelian monogenic inheritance model.

In the absence of selection pressure, there were no significant differences among the four insect strains for all the fitness parameters measured with few exceptions, suggesting that the resistance was not associated with fitness costs. Student's *t*-tests showed that there were no significant differences in the mortalities between the two F1 strains for each of the three Cry2Ab2 concentration and leaf tissue bioassays. Based on the diet-incorporated bioassays, the calculated D_{ML} was 0.22 at 31.6 $\mu\text{g/g}$ and 0.23 at 100 $\mu\text{g/g}$, while it was -0.02 based on the Cry2Ab2 leaf tissue tests which suggests that the Cry2Ab2 resistance in *S. frugiperda* was functionally recessive in the leaf tissue and incompletely recessive in the diet-incorporated assays. χ^2 tests showed that the larval mortality data observed fitted well the expected data based on the Mendelian monogenic model of inheritance for both the F2 and backcrossed strains, and in both the diet-incorporated and leaf tissue bioassays. In summary, the Cry2Ab2 resistance in *S. frugiperda* was likely inherited as a single, autosomal, and recessive gene. Information generated from this study should be useful in assessing resistance risk and developing management strategies for the sustainable use of Bt crop technology.

VITA

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