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Cry1F resistance among lepidopteran pests: A model for improved resistance management?

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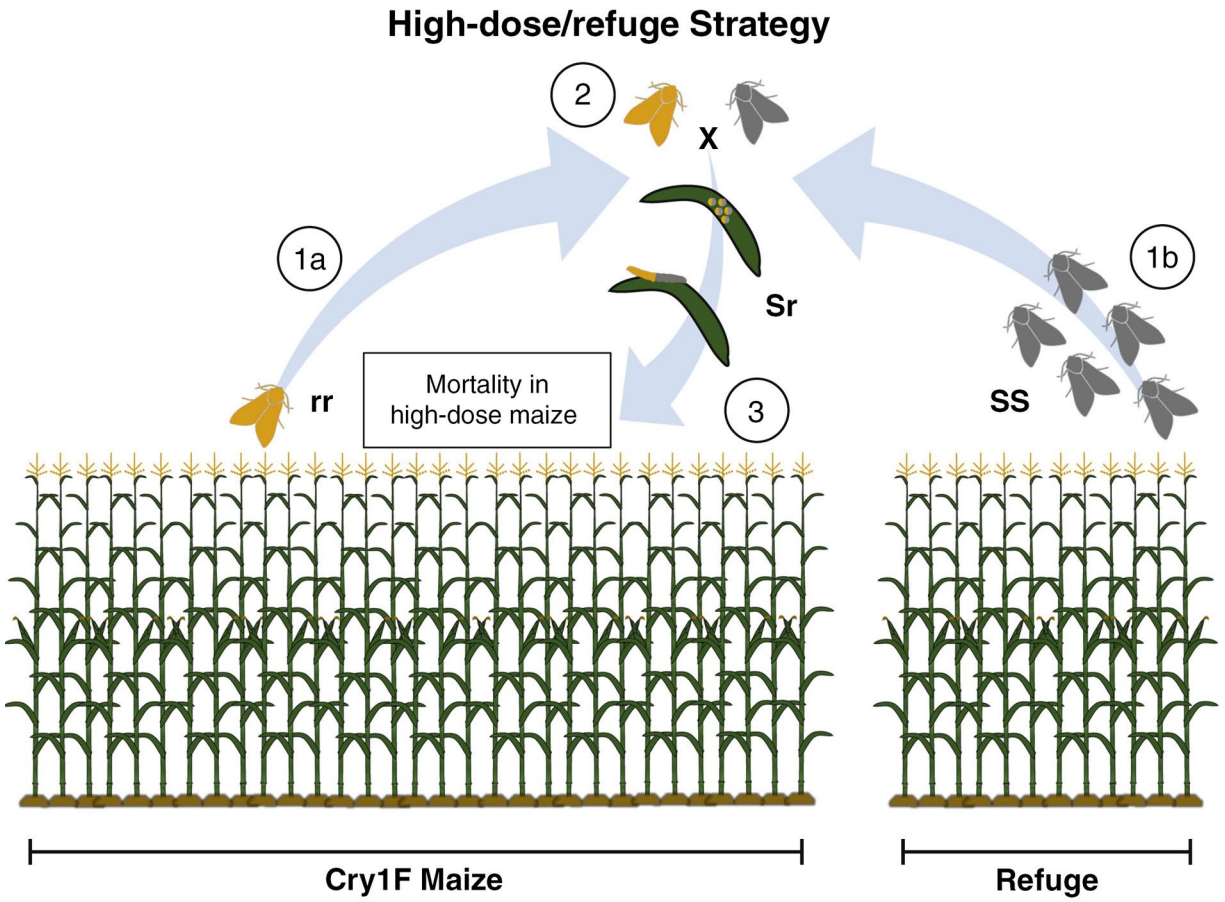
Abstract

The Cry1Fa protein from the bacterium *Bacillus thuringiensis* (Bt) is known for its potential to control lepidopteran pests, especially through transgenic expression in maize and cotton. The maize event TC1507 expressing the cry1Fa toxin gene became commercially available in the United States in 2003 for the management of key lepidopteran pests including the European corn borer, *Ostrinia nubilalis,* and the fall armyworm, *Spodoptera frugiperda.* A high-dose/refuge strategy has been widely adopted to delay evolution of resistance to event TC1507 and other transgenic Bt crops. Efficacy of this strategy depends on the crops expressing a high dose of the Bt toxin to targeted pests and adjacent refuges of non-Bt host plants serving as a source of abundant susceptible insects. While this strategy has proved effective in delaying *O. nubilalis* resistance, field-evolved resistance to event TC1507 has been reported in *S. frugiperda* populations in Puerto Rico, Brazil, and the southeastern United States. This paper examines available information on resistance to Cry1Fa in *O. nubilalis* and *S. frugiperda* and discusses how this information identifies opportunities to refine resistance management recommendations for Bt maize.

Introduction

The Cry1Fa protein from *Bacillus thuringiensis* subsp. *aizawai* was first described by Chambers *et al.* [1] as active against a subset of lepidopteran insects, including larvae of the European corn borer, *Ostrinia nubilalis* (Hübner) and the beet armyworm, *Spodoptera exigua* (Hübner). As with other insecticidal Cry proteins, the Cry1Fa mode of action involves recognition of binding sites on the midgut brush border membrane and formation of toxin pores that lead to osmotic cell death, compromising the integrity of the midgut epithelial barrier and allowing bacteria to reach the hemocoel and cause septicemia, which ultimately kills the insect host [2]. Since its discovery, the Cry1Fa toxin has become widely recognized for its potential to control lepidopteran pests when produced in transgenic crop plants such as maize and cotton.

Insect-resistant transgenic maize event TC1507 expressing the *cry1Fa* toxin gene was commercialized as Herculex I. The technology was jointly developed by Pioneer Hi-Bred International, Inc. (DuPont Pioneer) and Dow AgroSciences LLC. As with other Bt events targeting lepidopteran pests of maize, TC1507 was developed to provide growers a simple and highly effective tool to control certain key lepidopteran larval pests [3]. The Cry1Fa concentration in TC1507 maize has been shown to vary among tissue types [4], but in general it is considered to express Cry1Fa at high enough concentrations to kill most susceptible target pest species. Based on laboratory studies of insect resistance to Bt toxins [5], resistance to TC1507 and other Bt maize events producing a high dose (25 times the LC_{99}) of a single Bt toxin was expected to be monogenic, recessive and autosomal. Based on these assumptions, insect resistance management (IRM) plans for TC1507 relied on the high-dose/ refuge strategy [6, 7]. According to this strategy it is assumed that TC1507 expresses a high dose of the toxin that will kill at least 99% of susceptible insects in the field [6]. The non-Bt refuge is intended to produce an abundance of susceptible homozygous insects that would mate with the few resistant homozygous insects emerging from Bt maize fields, thereby producing susceptible heterozygotes that would be controlled by the Bt maize plants (Box 1) [5, 8]. In the case of *O. nubilalis*, it appears that the initial assumptions of the high-dose/refuge strategy have been met, as there has not been a detectable change in susceptibility to Cry1Fa maize in over 10 years of commercial availability [9*] in the United States. This lack of field resistance is noteworthy because resistance in *O. nubilalis* can be readily selected under laboratory conditions [10] and the resistance alleles can be detected in field populations [9*]. In contrast, there is growing evidence from diverse geographies for resistance to Cry1Fa maize in *S. frugiperda*, which



Box 1. High-dose refuge strategy used to delay the evolution of resistance in Bt crops.
(1a) Few homozygous resistant insects will emerge from Cry1F maize
(1b) multiple homozygous susceptible insects will emerge from the non-Bt maize refuge
(2) homozygous susceptible insects will mate with homozygous resistant insects
(3) functionally recessive resistance will generate heterozygous offspring that will eventually die with the high-dose expressed in Cry1F maize.

is an important maize pest in much of Latin America [11*, 12]. Development of resistance to TC1507 may have been predicted considering evidence that TC1507 does not meet the assumption of high dose for *S. frugiperda* [13]. The differences between *O. nubilalis* and *S. frugiperda* in their propensity to evolve resistance, and our understanding of resistance in the two species suggest that there are opportunities to refine IRM recommendations for Bt maize. In the following sections we will explore the various factors that have been identified in both species that may influence future resistance management recommendations.

Resistance evolution in target pest species

The high-dose/refuge strategy has been widely adopted to manage resistance evolution for transgenic crops expressing Bt toxins [7]. The most direct way to test and validate the high-dose model is to characterize resistant insect strains [14]. Generally, laboratory-selected strains are used to describe the potential for evolution of resistance in the field [15]. However, in instances were field resistance has been reported, resistance characterization is fundamental to understand the factors influencing the evolution of resistance [16] and potential remediation strategies. Instances of field-evolved resistance suggest that certain assumptions of the high-dose refuge IRM strategy have not been met. The following descriptions of Cry1Fa resistance in *O. nubilalis* and *S. frugiperda* provide important examples of how resistance can be delayed when assumptions of the high-dose/refuge strategy are met and the consequences when they are not fulfilled as in the case of *S. frugiperda*.

Laboratory resistance in *O. nubilalis*

A laboratory colony of *O. nubilalis* obtained from field collections throughout the central United States Corn Belt in 1996 was selected in the laboratory for resistance to Cry1Fa by exposure to surface treated artificial diet. The selected strain developed more than 3000-fold resistance after 35 generations of selection and survived when feeding on

maize plants expressing Cry1Fa [10]. The inheritance of resistance in this strain was evaluated using concentration-response bioassays of reciprocal and parental crosses and was characterized as autosomal and recessive. Furthermore, bioassays of the backcross of the F1 generation with the Cry1F resistant strain suggested that resistance was conferred by a single locus or a set of tightly linked loci [15]. Additional experiments performed on Cry1Fa maize hybrids showed that resistant larvae readily survived on reproductive-stage but not on vegetative-stage TC1507 maize tissue, and that survival of heterozygotes on TC1507 was not significant. The results from this study provided the first evidence that if the assumptions of the high-dose/refuge strategy are fulfilled, this approach is appropriate to delay Cry1Fa resistance evolution in *O. nubilalis* [15]. This observation is supported by the fact that *O. nubilalis* populations have remained susceptible to Cry1Fa maize 13 years after the first commercial release [9*]. Additional experiments evaluating cross-resistance in the resistant *O. nubilalis* strain indicated susceptibility to Cry1Ab and Cry9C, and showed only low levels of cross-resistance (~7 fold) to Cry1Ac. This cross-resistance phenotype suggested that resistance involved alterations of binding sites shared by Cry1Ac and Cry1Fa [17], although difference in Cry1Fa binding could not be confirmed experimentally [18]. Weak recessive fitness costs were associated with resistance in this strain [19], which would further contribute to delaying resistance in the high-dose/refuge strategy [20]. These data suggested that maize hybrids expressing Cry1Ab and Cry1Fa were likely to be compatible for resistance management in *O. nubilalis* [10], which has become an important component of current pyramided events for *O. nubilalis* control.

Field resistance in *S. frugiperda*

Maize hybrids expressing the Cry1Fa protein were the first Bt event to demonstrate satisfactory *S. frugiperda* control [3, 21]. Although Cry1Fa maize hybrids were commercialized in the United States in 2003, in Puerto Rico they had been grown for hybrid development, parental seed production, and efficacy trials since 1998 [22]. In 2006, unexpected damage to TC1507 maize was reported in Puerto Rico and Cry1Fa resistance in *S. frugiperda* was subsequently documented [23, 24]. Storer *et al.* [23] and Blanco *et al.* [25] confirmed that field failures in Puerto Rico were associated with a high-level of resistance to the Cry1Fa protein, ranging from 1000 to 26,000-fold depending on whether mortality or growth inhibition were tested. Resistance to Cry1Fa in *S. frugiperda* represented the first case of resistance leading to withdrawal of a Bt product from the market [13, 16]. In Brazil, event TC1507 was introduced in the 2009/2010 season, and reports of economic damage and field evolved resistance emerged after a couple of years [11*]. The inheritance of Cry1Fa resistance in *S. frugiperda* populations originated from Puerto Rico and Brazil was characterized as autosomal, highly recessive [11*, 16, 23, 26*, 27], and monogenic [11*, 16, 27]. However, experiments with TC1507 maize plants and resistant *S. frugiperda* from Brazil suggested incomplete recessive resistance [28]. A more detailed report of the current *S. frugiperda* situation in Latin America is provided in the Blanco *et al.* manuscript in this special issue (*Current Opinion in Insect Science* 15).

Cross-resistance tests indicated that Cry1Fa-resistant *S. frugiperda* larvae exhibited resistance to Cry1A toxins, although at much lower levels than resistance observed for Cry1Fa [16, 23, 29, 30]. However, while high levels of cross-resistance to Cry1Aa were observed in resistant populations from Brazil [30], populations from Puerto Rico showed no cross-resistance against Cry1Aa, Cry1Ba, or Cry2Aa, although inherent *S. frugiperda* susceptibility to these toxins was low [16]. This discrepancy between resistant strains from Puerto Rico and Brazil may represent the effect of genetic variability among *S. frugiperda* populations from diverse geographies [31]. Furthermore, no cross-resistance was detected in populations from Puerto Rico to the Vip3Aa protein [16]. Cross-resistance to commercial Bt pesticides XenTari WG and DiPel ES (Valent Biosciences, Libertyville, IL) was also evaluated in a strain from Puerto Rico; results indicated no differences in susceptibility compared to a susceptible strain [29]. Resistance in this strain has been found to involve reduced Cry1Fa toxin binding to a site shared with Cry1Ab and Cry1Ac toxins [26*].

Multiple factors, most of them shared between Puerto Rico and Brazil, are thought to have contributed to the rapid evolution of resistance to Cry1Fa in *S. frugiperda* populations in these geographies: (1) an isolated ecosystem, in the case of Puerto Rico, that restricts movement and enables local selection; (2) a tropical environment that allows for year-round cultivation of maize with multiple insect generations exposed to selection pressure in a single growing year; (4) long history of use of formulated Bt insecticides; (5) the affected Bt maize lines not being adapted to tropical conditions; and (6) a severe drought in 2006 in Puerto Rico that forced *S. frugiperda* populations to move to irrigated Cry1Fa maize causing intense selection pressure. In addition to these conditions, Cry1Fa maize has been shown not to represent a high dose crop for *S. frugiperda* [13, 28]. Recent detection of TC1507-resistant *S. frugiperda* in Florida and North Carolina [32*] may be a result of the known migratory behavior of *S. frugiperda* from Puerto Rico through the Caribbean [33]. Consequently, it would be predicted that the same resistance mechanism would be present in *S. frugiperda* from Puerto Rico and southeastern United States, although this hypothesis has not been tested experimentally.

Frequency of resistance alleles in *O. nubilalis* field populations

The frequency of Cry1Fa resistant alleles in *O. nubilalis* has been estimated using F_1 and F_2 screens and annual

susceptibility monitoring of Midwestern United States populations based on diagnostic and concentration response bioassays [9*]. The F_2 screen involves collecting a large number of individuals from the field and establishing single-female family lines [34]. The offspring of each collected female are inbred by sib-mating and their offspring (i.e., the F_2 of the collected generation) are screened with a discriminating concentration for tolerance to the toxin. The F_2 screen allows potentially heterozygous offspring from a field-collected homozygous resistant female to mate with each other, generating 1/4 of homozygous resistant offspring. Through back-calculation of the frequency of family lines containing a resistant allele, the frequency of the resistance alleles in the sampled population can be estimated. The F_1 screen involves mating individuals collected from the field with a previously described resistant laboratory strain [10] which is known to be homozygous for resistance. The offspring are tested using discriminating bioassays to distinguish resistant homozygotes from susceptible homozygotes and heterozygotes. Estimates of the frequency of resistance alleles among field populations of *O. nubilalis* using an F_1 screen, indicated that resistance alleles could be detected even during 2003, the first year of Cry1Fa maize commercialization. Resistant allele frequencies ranged between 0.029 in 2003–2005 and 0.025 in 2006–2008, indicating no net increase in frequency. Results from the F_2 screen estimated similar frequencies in 2008 and 2009 (≈ 0.009 and 0.014, respectively), confirming the presence of resistance alleles [9*]. Further, the susceptibility of *O. nubilalis* to Cry1Fa has been monitored annually using diagnostic and concentration response bioassays since 2003, and these results supported the observations obtained with the F_1 and F_2 screens [9*]. Taken together, the results from the monitoring and the F_1 and F_2 screens suggest that the frequency of Cry1Fa resistant alleles in *O. nubilalis* populations was higher than expected, even prior to the introduction of Cry1Fa maize [9*]. Lack of reports of unexpected damage in Bt fields by *O. nubilalis* is further evidence that the high-dose/refuge strategy is effective in delaying resistance evolution in this pest even when a high frequency of resistance alleles is detected in the field.

Frequency of resistance alleles in field populations of *S. frugiperda*

After the first report of *S. frugiperda* Cry1Fa resistance in Puerto Rico in 2006, populations from both Puerto Rico and the southeastern United States were monitored using different methodologies to detect potential changes in susceptibility. Storer *et al.* [13] monitored *S. frugiperda* populations in 2010 and 2011 from Puerto Rico, Texas, Florida, Alabama, and Mississippi using concentration range bioassays. In this study, the majority of the collections from Puerto Rico showed high levels of Cry1Fa resistance, whereas populations from southeastern United States exhibited susceptibility similar to the reference laboratory colony [13]. Additional studies evaluated the frequency of Cry1Fa resistance alleles in populations from Puerto Rico (2010–2013), Florida and Texas (2010–2011) [16]. The nature of Cry1Fa resistance inheritance in *S. frugiperda* (i.e., autosomal, recessive and conferred by a single locus) allowed F_1 screens using the resistant Puerto Rican population and evaluating the offspring with discriminating bioassays to estimate the frequency of resistance alleles [16]. Using this approach, resistance alleles were detected in both Florida and Texas. In Florida, the frequency of resistance alleles was as high as 0.13 in some populations, with localized differences; while in Texas the frequency was much lower (0.02) but still detectable [16]. These results are consistent with *S. frugiperda* gene flow studies that indicate significant gene exchange between Florida and Puerto Rico and limited gene exchange between Florida and Texas [33, 35]. As stated above, the higher frequency of resistance alleles detected in Florida might be in part the result of migration of resistant individuals from Puerto Rico to Florida. However, local variance between Florida counties suggests localized differences in selection pressures [16]. In Puerto Rico, the frequency of Cry1Fa resistance remained high between 2010 and 2013, even after withdrawal of TC1507 maize from the local market. This observation may be explained by the resistance allele being fixed in the local populations and/or by the absence of significant fitness costs associated with resistance [36, 37], as further discussed below.

Additional studies using F_2 screens with populations from Florida collected in 2011 showed relatively high frequencies of resistant alleles (0.29) [32*]. In addition, the susceptibility of populations collected between 2012 and 2013 on non-Bt maize from Florida, Louisiana and Georgia, and populations from Bt maize fields with unexpected damage from Florida and North Carolina was assessed with concentration response bioassays. Populations from non-Bt maize exhibited 18.8 to >85.4-fold resistance to purified Cry1Fa protein, while populations from fields with unexpected damage from Florida and North Carolina showed >85.4-fold resistance [32*]. The presence of field resistance in south Florida was also evaluated with field trials using Cry1Fa and pyramided Bt maize products. Field trials in Florida showed reduced efficacy and control failure of natural *S. frugiperda* populations [32*]. The results from F_1 and F_2 screens suggest that these methods are more sensitive for early detection of resistant alleles [16], while concentration range bioassays are generally insensitive to small changes in allele frequencies [34, 38]. Overall, these results suggest that resistance allele frequencies in *S. frugiperda* were high prior to the introduction of Cry1Fa maize [16], similar to results with *O. nubilalis*. However, in contrast to *O. nubilalis*, the lack of a high dose hindered the ability of the high-dose/refuge strategy to delay resistance in *S. frugiperda*.

Fitness costs

Resistance alleles are often assumed to be associated with fitness costs [20], and the relative fitness of heterozygote individuals influences the response to selection and the rate of resistance evolution [39]. The study of fitness costs associated with resistance to Bt insecticidal proteins is fundamental to understanding resistance evolution and the evaluation of resistance management practices implemented to mitigate resistance to transgenic maize in the field [39].

Fitness costs of Cry1Fa resistance in *O. nubilalis*

The existence of fitness costs in laboratory-selected Cry1Fa resistant *O. nubilalis* was tested by comparing life-history traits and population growth parameters in the absence of Cry1F between the resistant strain, a susceptible strain with similar genetic background, and their reciprocal crosses. Comparison of life history traits (i.e. pupal weight, developmental time, growth rate, and number of eggs per female) and population growth parameters indicated weak and recessive fitness costs associated with Cry1Fa resistance [19]. The estimates of relative fitness in that study were obtained under controlled environmental conditions with artificial diet, and suggested that fitness costs associated with resistance are likely to be more apparent when larvae feed on maize plants and under field conditions [40]. Additional experiments were performed to determine if fitness cost of Cry1Fa resistance in *O. nubilalis* would be affected by the presence of host plant resistance by comparing fitness on three maize lines [41]. Two of the maize lines were susceptible to *O. nubilalis* (F1-hybrid B73xMO17 and WF9) [42] and the third line (B94) expressed moderate levels of the maize benzoxazinone DIMBOA, which confers resistance against *O. nubilalis* [43]. Larval survival and development were measured in the greenhouse using plants in the vegetative and reproductive stage. Both experiments demonstrated that B94 maize significantly affected survival and developmental rate in both the susceptible and resistant strains indicating no fitness cost of resistance [41] and supporting the results obtained in laboratory experiments [19]. The lack of fitness cost was further confirmed with choice and no-choice experiments on Cry1Fa maize tissue and the respective isoline, where no strong differences were observed between susceptible and Cry1Fa resistant *O. nubilalis* neonates [44].

Fitness costs of Cry1Fa resistance in *S. frugiperda*

The fitness costs of Cry1Fa resistance in *S. frugiperda* from Puerto Rico have also been evaluated using susceptible and resistant strains with similar genetic background and their reciprocal crosses [36, 37]. One study compared life-history traits (i.e. pupal weight, developmental time, growth rate, number of spermatophores per male, and number of eggs/larvae per female) and population growth rate parameters using artificial diet. Results from this study reported no major fitness costs in either heterozygotes or homozygous resistant insects [36]. Additional research compared biological performance of susceptible and Cry1Fa resistant larvae in artificial diet, in maize or soybean leaf tissue, or in the reproductive tissue of cotton. In this study, researchers measured larval survival, larval and pupal weights, developmental time, adult longevity, fecundity, fertility, and sex ratio [37]. In general, all of the measured parameters were influenced by the host plant but not by the strain. The only parameter that significantly differed between the susceptible and resistant strains was the larval developmental time, with resistant larvae exhibiting longer developmental times that resulted in a short asynchrony (<2 days) in peaks of adult emergence between susceptible and resistant strains [37]. Further research compared fitness parameters of two Cry1Fa resistant *S. frugiperda* populations collected from Florida and Puerto Rico with a susceptible strain and the respective reciprocal crosses. Assessed biological parameters included survival, growth and developmental time, and were measured in untreated artificial diet and non-Bt maize leaf tissue [45]. Results from this study showed that the Cry1Fa resistance from Puerto Rico and Florida was associated with a significant fitness cost in all the parameters measured, especially for Florida populations [45]. The discrepancy between this and previous studies may be attributed to different alleles being responsible for resistance among the tested populations or to differences in rearing techniques. To determine the relevance of the negligible fitness costs reported in a resistant strain from Puerto Rico, researchers followed the proportion of homozygous resistant individuals in a heterogeneous strain through 12 generations of rearing on meridic diet, using diagnostic Cry1Fa bioassays to determine the percentage of resistant neonate larvae [37]. In a different study, two strains with a fixed resistance allele frequency of 50% were tested for seven generations of rearing in meridic diet and diagnostic Cry1Fa bioassays were used to estimate the frequency of resistant alleles in each generation [36]. While the first study found no changes in the proportion of homozygous resistant individuals in the population after 12 generations, the second study found that the frequency of resistance alleles slightly decreased after seven generations in the two lines tested. However, is important to consider that both studies measured different parameters. Jakka *et al.* [37] only reported mortality, underestimating the frequency of resistance alleles since heterozygotes would not be detected. Velez *et al.* [36] estimated the frequency of resistance alleles, yet it is uncertain if the slight decrease of resistance alleles represented a true fitness cost or was the result of random drift. The consistent levels of resistance in Puerto Rico in the absence of apparent selection [13, 16] and the results from measurements of fitness parameters are more consistent with a lack of fitness costs. Additional evidence for the lack of fitness costs of Cry1Fa resistance in *S. frugiperda* from Puerto Rico was provided by choice and no-choice experiments on Cry1Fa maize and the respective isoline. Similar to *O. nubilalis*, no strong behavioral differences were observed

between susceptible and Cry1Fa-resistant *S. frugiperda* neonates, although a small percentage of susceptible larvae abandoned Cry1Fa maize leaf tissue [44]. The lack of strong fitness costs associated with Cry1Fa resistance in *S. frugiperda* is also in agreement with the higher than expected initial resistant allele frequencies in field populations, and suggests that their presence will remain stable in the absence of selection pressure (e.g. Puerto Rico) [36].

The fitness costs of Cry1Fa resistance in *S. frugiperda* from Brazil have also been evaluated with near isogenic susceptible and resistant colonies and their reciprocal crosses [46]. Fitness parameters measured included developmental time, survival rates, sex ratio, adult longevity, timing of oviposition, fecundity, and fertility. The resistant colony showed 7% lower survival to adulthood and the mean generation time was two days shorter compared to the susceptible colony, yet reproductive parameters were similar between the colonies. Overall, the authors concluded no relevant fitness costs in the Cry1Fa resistant colony, indicating, as in the case of populations from Puerto Rico, stability of field resistance to Cry1Fa in *S. frugiperda* populations from Brazil [46].

Mechanisms of Cry1Fa resistance

A complicating factor in our understanding of Cry1Fa resistance in both *O. nubilalis* and *S. frugiperda* is that the molecular basis of resistance is still emerging. Such information is critical for understanding the underlying impacts to fitness if any, initial allele frequencies, and ultimately to IRM decisions that are dependent on lack of cross-resistance. Resistance in both species shows a number of similarities, including linkage to a single autosomal resistance allele, and absence of relevant fitness costs. However, the lack of Cry1Fa binding to resistant *S. frugiperda* [26*] but not to *O. nubilalis* [18], and the distinct cross-resistance pattern to Cry1A toxins [10, 29] support that Cry1Fa resistance genes may differ for the two species. High levels (>200-fold) of resistance to Cry toxins has been most often linked to alterations in the recognition of midgut receptors [47]. Analysis of Cry1Fa binding to brush border membrane vesicles (BBMV) from midgut epithelia of susceptible and Cry1Fa resistant strains of *O. nubilalis* described above suggested that reduced binding of Cry1Fa was not associated with resistance [18]. In addition, no differences in activity of luminal gut proteases or altered proteolytic processing of the toxin were observed when comparing susceptible and resistant strains [48]. Genetic mapping in that Cry1Fa-resistant *O. nubilalis* strain identified a single quantitative trait locus (QTL) associated with Cry1Fa resistance, which mapped to a single linkage group [49]. Fine mapping positively identified a 46.5 cM QTL region containing the Cry1Fa resistance gene. Within this region, an *abcc2*-like gene was detected [50*]. This detection is relevant because mutations in *abcc2* genes have been previously shown to be linked with resistance to Cry1Ac [51–55] and Cry2Ab [56] in diverse lepidopteran pests. Moreover, there is experimental evidence for ABCC2 proteins as Cry1Fa functional receptors in *Bombyx mori* (L.) [57]. Comparative transcriptome analyses between the Cry1Fa resistant and a near-isogenic susceptible *O. nubilalis* strain [10] revealed a different scenario involving differential constitutive expression of a number of genes previously associated with the mode of action of Cry toxins, suggesting the involvement of multiple path-ways [58*]. The resistant strain had higher expression of possible cadherin mutants and lower expression of aminopeptidase N, amylase and alkaline phosphatase genes compared to the susceptible strain. Most of these genes have been identified as Cry toxin receptors associated with resistance to Cry toxins in other species [59, 60, 61, 62, 63]. Lower expression of v-ATPase and protease activity were also observed in the Cry1Fa resistant strain of *O. nubilalis*, suggesting that altered midgut pH and reduced protease activity may contribute to Cry1Fa resistance in this insect [58*]. Similar observations of the association of gut pH and altered proteolytic activity have been described in *Heliothis virescens* (Fabricius) resistant to Cry1Ac and Cry2A [64] and *Aedes aegypti* resistance to *B. thuringiensis israelensis* (Bti) toxins [60].

Resistance to Cry1Fa in a *S. frugiperda* strain from Puerto Rico [26 *] was associated with reduced expression of selected alkaline phosphatase (ALP) genes that serve as high affinity Cry1Fa toxin-binding sites [65]. Cross-resistance and reduced binding of Cry1Ab and Cry1Ac but not Cry1Ca in this resistant strain identifies the phenotype as Mode 1 resistance [66]. In *Plutella xylostella* (L.), Mode 1 resistance involved reduced levels of a toxin binding ALP, as observed for *S. frugiperda*, as well as altered expression of ABCC genes. Both of these processes involved altered expression of a mitogen-activated protein kinase (MAPK) gene [51]. Further work is needed to determine if similar MAPK kinase genes are involved in resistance to Cry1Fa in *S. frugiperda*. Despite slight differences in genetic transmission and cross-resistance phenotype between *S. frugiperda* populations from Puerto Rico and Brazil (as described above), resistance in Brazilian strains was also associated with reduced Cry1Fa toxin binding [30].

Continued research to elucidate the causative mechanism and genes of Cry1Fa resistance in both *O. nubilalis* and *S. frugiperda* will provide crucial information to improve approaches to managing resistance in these two species.

Conclusions

This paper describes current knowledge on Cry1Fa resistance in two lepidopteran species, *O. nubilalis* and *S. frugiperda*, targeted by Bt maize. Resistance to Cry1Fa in laboratory selected *O. nubilalis* and field resistance in *S. frugiperda* from Puerto Rico and Brazil has been characterized as recessive, autosomal, monogenic [11*, 15, 16], and not linked to fitness costs [19, 36, 37, 46]. It remains to be determined if *S. frugiperda* Cry1Fa resistance from Puerto Rico, southern United States, and Brazil are independent resistant events or the results of insect migration [26*]. In the case of *O. nubilalis*,

resistance has been selected in the laboratory, but field populations remain susceptible even with reports of relatively high frequency of resistant alleles [9 *]. Taken together, these data support success of the high-dose/refuge strategy in cases when the assumptions of the model are met, as is the case of *O. nubilalis*. However, in cases when at least one of the major assumptions is not met, the likelihood of resistance evolution is higher. This is true in *S. frugiperda* and other species where the high-dose assumption has not been met [67]. In these cases, the use of pyramided crops expressing toxins with different modes of action and integrated pest management will be fundamental in delaying the evolution of resistance. Cross-resistance [16, 23] and binding studies in *S. frugiperda* [26*], suggest that pyramided maize events expressing Cry1Ca and Vip3A should be effective in controlling Cry1F-resistant *S. frugiperda* [16, 26*]. In contrast, Cry1Ab and Cry1Ac are not suitable for pyramiding with Cry1Fa because of cross-resistance [16, 23, 29] and evidence for shared binding sites [26*]. In conclusion, the lessons learned from Cry1Fa resistance in *O. nubilalis* and *S. frugiperda* highlight the importance of meeting the assumptions of the high-dose/refuge strategy for the successful delay of resistance. Additional pest management strategies will be necessary if the high-dose assumption is not met.

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