2012

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Understanding successful resistance management

The European corn borer and Bt corn in the United States

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The European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae) has been a major pest of corn and other crops in North America since its accidental introduction nearly a hundred years ago. Wide adoption of transgenic corn hybrids that express toxins from *Bacillus thuringiensis*, referred to as Bt corn, has suppressed corn borer populations and reduced the pest status of this insect in parts of the Corn Belt. Continued suppression of this pest, however, will depend on managing potential resistance to Bt corn, currently through the high-dose refuge (HDR) strategy. In this review, we describe what has been learned with regard to *O. nubilalis* resistance to Bt toxins either through laboratory selection experiments or isolation of resistance from field populations. We also describe the essential components of the HDR strategy as they relate to *O. nubilalis* biology and ecology. Additionally, recent developments in insect resistance management (IRM) specific to *O. nubilalis* that may affect the continued sustainability of this technology are considered.

Introduction

The European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae), is an economically important insect pest of corn (*Zea mays* L.) and other crops in North America and Europe with yield losses up to 20% caused by larval feeding. Damage from larval infestations of corn was estimated to exceed US $1 billion annually in yield losses and control expenditures.[1] It is a cosmopolitan species, originally distributed in Europe and from there introduced into America, where it has now spread to most of Southern Canada and the US east of the Rocky Mountains. Larval feeding on corn plants results in physiological disruption of plant growth and structural damage to the corn plant. Although capable of developing on > 200 herbaceous plant species, *O. nubilalis* has a strong preference for corn as a host plant.[2] Chemical insecticides often are not effective against *O. nubilalis* infestations because once the larvae tunnel into the corn stalk they are protected from exposure; thus there is a narrow application window for growers. European corn borers typically have

one or two generations per year, although three or four generations can occur in some areas of its distribution.[3]

Transgenic corn plants that express insecticidal proteins from *Bacillus thuringiensis* (Berliner) (Bt) have become an effective method of protecting corn plants from damage by *O. nubilalis* and have been widely adopted throughout the US corn belt.[4,5] Transgenic corn hybrids expressing either the Cry1Ab or Cry1F insecticidal proteins from *B. thuringiensis* for control of *O. nubilalis* have been used commercially in North America since 1996 and 2002 respectively. In the US, approximately 65% of the total 37.3 million ha of maize in 2011 was planted with Bt hybrids targeting European corn borer, corn rootworm (Diabrotica spp) or stacked events that target both pests,[5] exceeding 70% in Iowa and South Dakota with likely higher levels of adoption in some counties. Widespread adoption of Bt corn has resulted in an area-wide suppression of *O. nubilalis* populations, which has economically benefitted both growers of Bt and non-Bt corn.[6]

The first pyramided Bt corn hybrids express a chimeric gene consisting of both Cry1Ab and Cry1F domains and co-expressed with a second lepidopteran active protein, Cry2Ab2. These corn hybrids were commercially available in 2009. The chimeric protein, referred to as Cry1A.105, has four domains that are derived from corresponding domains from Cry1Ab, Cry1Ac and Cry1F proteins: Domains I and II (identical to Cry1Ab and Cry1Ac), Domain III (almost identical to the Cry1F protein) and the C-terminal Domain (identical to Cry1Ac protein).[7]

The season-long and high expression levels of Bt proteins in transgenic corn and its widespread adoption are thought to impose considerable selection pressure for resistance on target pest populations of *O. nubilalis*, and the risk for resistance evolution is perceived to be high. As a consequence, insect resistance management (IRM) strategies designed to minimize selection pressures and prevent or at least delay resistance evolution have been considered essential to maintaining the viability of transgenic Bt plants for control and management of the European corn borer. Because plant-incorporated protectants (PIPs) involving Bt proteins are recognized as a safe and valuable method of insect pest control, the US Environmental Protection Agency (USEPA) has imposed management requirements on registered PIPs to prevent insects from developing resistance.[8]

Table 1. Summary of Bt resistant strains of *O. nubilalis*

Name	Selection/ Isolation	Resistance Ratio	On-Plant Survival**		Inheritance	Number of genes	Relevant Citations
			Vegetative	Reproductive			
S-I	MVP Formulation of Cry1Ac	162	Not Tested	Not Tested	Not Tested	Not Tested	30
KS-SC-R	Dipel-ES	65	-	-	Incompletely dominant/autosomal	1 or few	29
RSTT	Cry1Ab	1,200	-	-	Intermediate/autosomal	5-10	40
ELS-H	Cry1Ab	3,000	-	-	Intermediate/autosomal	5-10	40
SKY*	Cry1Ab	815	-	+	Incompletely recessive/autosomal	> 2	48
Cry1F-Selected	Cry1F	> 3,000	+	++	Recessive/autosomal	1	43, 44
HAM County*	Cry1F	> 3,000	+	++	Recessive/autosomal	1	53

*Isolated from field populations and identified as exhibiting < 99% mortality at diagnostic concentrations used for annual susceptibility monitoring programs. See text for details. ** - indicates no survival; + some survival suggested but less than on non-expressing plants; ++ survival not different from non-expressing plants.

The IRM strategy that has received the most attention from both industry and regulatory agencies involves the "high dose/refuge" (HDR) concept.[9-14] With this approach, insects that feed on the Bt corn are exposed to an extremely high dose of toxin. This is complemented with a refuge, usually non-Bt corn, that provides a population of susceptible insects that are not exposed to Bt toxin. Consequently, rare resistant moths that develop on Bt corn, instead of mating with each other, mate with individuals among the overwhelming number of susceptible moths from the refuge. The high dose is intended to reduce the fitness of heterozygotes such that inheritance of resistance is functionally recessive. This process essentially dilutes resistance genes and maintains a population of susceptible insects.[11]

In spite of what has been perceived as intense selective pressures and a likelihood for resistance evolution, *O. nubilalis* populations apparently remain susceptible to all the currently deployed Cry toxins that have been registered as PIPs by the USEPA.[15-17] While it is not possible to determine whether this lack of resistance is a consequence of regulatory IRM mandates, the predictions from initial theoretical models about Bt susceptibility and the HDR concept,[11,18-23] appear to have been realized. Moreover, in other insects when there has been an apparent increase in resistance allele frequencies among field populations of lepidopteran pests targeted by Bt corn[24] or where there has been documented field control failures,[25,26] certain aspects of the pest's biology or attributes of the technology do not comply with the assumptions of the high dose/refuges concept.[27]

In this review, we describe what has been learned with regard to *O. nubilalis* resistance to Bt toxins either through laboratory selection experiments or isolation of resistance from field populations. We also describe the essential components of the HDR strategy as they relate to *O. nubilalis* biology and ecology. Additionally, recent developments in IRM specific to *O. nubilalis* that may affect the continued sustainability of this technology are discussed.

Bt Resistant *O. nubilalis* Colonies

Selection experiments among a variety of insect pest species have repeatedly shown the potential for development of resistance to Bt toxins[28] including *O. nubilalis*.[29-33] Outside the laboratory, Bt resistance has also been documented in populations of *Plodia interpunctella*,[34] *Plutella xylostela*[35] and *Tricoplusia ni*[36] in response to repeated applications of Bt sprays. More recently, field evolved resistance to transgenic Bt crops resulting in control failures has been reported in *Brusseola fusca* (Fuller) to Cry1Ab-expressing corn in South Africa,[26,27] in *Spodoptera frugiperda* to Cry1F-expressing corn in Puerto Rico[25] and in the coleopteran pest, *Diabrotica virgifera virgifera* to Cry3Bb1-expressing corn in the US.[37] Field control failures of Cry1Ac-producing cotton have also recently been reported for the pink bollworm, *Pectinophora gossypiella* in the Gujarat State of India.[38] Additionally, Tabashnik et al.[24] have reported resistance to Cry1Ac-producing cotton among US populations of *Helicoverpa zea* based on analysis of more than a decade of resistance monitoring data. These recent reports of field evolved resistance contrast with what has been observed with *O. nubilalis* and Bt corn where susceptibility and field performance of the technology have been sustained for more than 15 y.[23]

A critical gap in our ability to assess the factors that contribute to the sustainability of IRM is the lack of resistant colonies that exhibit the ability to survive on transgenic plants and that represent resistance that is likely to evolve in the field. Since the initial introduction of Bt corn, a number of resistant strains of *O. nubilalis* have been isolated and characterized with varying levels of resistance and ability to survive on transgenic plant tissues (**Table 1**). These strains have provided valuable information regarding potential resistance mechanisms, resistance inheritance, fitness costs of resistance, cross resistance among different Bt toxins and most importantly, have provided a means to evaluate assumptions of the HDR strategy.

Laboratory selections. The first Bt resistant strains of *O. nubilalis* to be reported were identified through laboratory selection with commercial formulations of Bt incorporated into artificial rearing diet. Bolin et al. reported selection with a Bt formulation (MVP®) that contains only Cry1Ac resulting in resistance levels up to 162-fold after 17 generations, although not evaluated for survival on Cry1Ac expressing corn. Huang et al.[29] incorporated the Bt formulation Dipel®-ES into rearing diet for seven

generations resulting in a strain with 73-fold levels of resistance to the Bt formulation. The resistance was reported as being conferred by an incompletely dominant autosomal gene, and as a consequence, the usefulness of the HDR strategy for resistance management of Bt maize was called into question.[29] However, the Dipel-resistant and unselected control *O. nubilalis* larvae were similar in susceptibility to Cry1Ab expressing corn hybrids, and the resistant strain was unable to complete development on Bt transgenic plants expressing Cry1Ab toxins.[39] The resistance identified in this strain has been associated with reduced trypsin-like proteinase activity in the gut resulting in a slower rate of pro-toxin activation.[39-41] Moreover, Li et al.[41] demonstrated that plant enzymes hydrolyze Cry1Ab protoxin to one that is functionally activated. Although Dipel resistance and reduced proteinase activity may be common among field populations,[33] these populations are not resistant to Cry1Ab expressing hybrids and a role in resistance evolution for transgenic Bt corn appears unlikely.

Laboratory selections conducted with North American and European populations and with fermentation products of Cry1Ab producing cells incorporated into larval rearing diet resulted in low levels of resistance (< 15-fold) after 10–15 generations of selection in a number of different populations.[31] Continued selection by exposure to either Cry1Ab fermentation product or purified toxin throughout larval development in these same strains[32] yielded > 800-fold resistance after 60 generations of repeated exposures. Reciprocal crosses of resistant and susceptible parental populations and backcross of the F_1 progeny to a susceptible parental population suggest that resistance in these populations is inherited as an intermediate trait and is conferred by multiple genes[42] as might be expected given the probable rarity of a resistance allele conferring high levels of resistance and the relatively small population sizes (< 500) used to initiate selections.[43,44]

While all of these studies provide evidence for genetic adaptation and confirm the potential for Bt resistance evolution in *O. nubilalis*, the utility of these strains for validating assumptions of the HDR strategy are limited by the apparent complexity of the resistance mechanisms and more importantly, by their inability to survive on Cry1Ab expressing plant tissue[40] (Siegfried B.D., unpublished). Importantly, the lack of a single major resistance allele that confers the ability to survive and develop on Bt corn plants in spite of repeated attempts to select for resistance supports the contention that such a gene is rare among field populations.

In contrast to the results of selection with Cry1Ab, laboratory selection with the Cry1F protein has allowed isolation of an *O. nubilalis* strain[45,46] that exhibits high levels of resistance and characteristics of what has been described as a "Mode I" pattern where high resistance is conferred by a single, recessive genetic factor that causes loss of toxin binding to midgut receptors.[28] Pereira et al.[45] described laboratory selection experiments with exposure to partially purified recombinant Cry1F protein throughout larval development. The conditions of the selection experiment were similar in many respects to the conditions reported by Siqueira et al.[32] with Cry1Ab selection. However, the response to selection with Cry1F was more rapid and the level of resistance achieved was much higher. There was a 1,000-fold increase in Cry1F concentrations used in the exposure experiments after only seven generations of selection, and after 30 generations of selection, the highest concentration of Cry1F that could be tested (12,000 ng/cm²) did not cause significant mortality. Using this concentration as a lower limit for the LC_{50} of the resistant strain, the resistance ratio (LC_{50} for selected/LC_{50} for control) was greater than 3,000. Concentration-response bioassays of reciprocal parental crosses indicated that the resistance to Cry1F was autosomal and recessive, and bioassays of the backcross of the F_1 generation with the selected strain were consistent with the hypothesis that a single locus, or a set of tightly linked loci, is responsible for the resistance.[47] Genetic linkage maps with segregating markers that show that the Cry1F resistance trait is controlled by a single quantitative trait locus (QTL) on linkage group 12 supports the hypothesis that a single locus is responsible for resistance.[47] These results are consistent with the "Mode I" pattern of Bt resistance, although the loss of toxin binding to midgut receptors has yet to be confirmed.[48]

Importantly, the Cry1F resistant strain described above is the first resistant *O. nubilalis* strain identified that is capable of surviving on Cry1F expressing corn tissues. Greenhouse experiments with Cry1F-expressing corn hybrids indicated that some resistant larvae survived the high dose of toxin delivered by Cry1F-expressing plants, although F_1 progeny of susceptible by resistant crosses had fitness close to zero.[46] For vegetative stage plants, there appeared to be a reduction in survival of the resistant colony relative to survival on the non-expressing isoline. However, based on the number of surviving larvae recovered and their advanced development, it is likely that at least some of these larvae would have pupated and emerged as adults. On reproductive stage plants, there was no significant difference between the survival rate of the resistant colony on Cry1F-expressing plants and the isoline. These data strongly suggest that a single major genetic factor confers high levels of resistance to Cry1F, which allows the larvae to grow and develop on Cry1F expressing plants. In addition, since the resistance was isolated from a relatively small field collection, the frequency of this resistance may be higher than that observed for Cry1Ab.

Isolation of resistant field populations. Annual assessments of Bt susceptibility involving diagnostic bioassays based on the upper end of the 95% confidence interval of the LC_{99} derived from baseline susceptibility studies have been conducted since the initial registration of Cry1Ab expressing events in 1996.[15,16,49] In the approximately 15 y that Cry1Ab susceptibility has been assessed and among almost 200 different populations that have been assayed, only one population (Kandiyohi County, MN 2001) was identified that did not exhibit mortality in excess of 99% in the diagnostic bioassay.[15,16] USEPA registrations of Bt corn events mandate that if resistance is suspected, a series of additional tests must be initiated to determine whether the resistance is heritable, to quantify the magnitude of resistance, and to measure the ability of the resistant strain to survive on Bt expressing corn plants.[9] Results of these tests indicated that significant Cry1Ab resistance had been isolated from the Kandiyohi population.[15,50] Survivors of initial diagnostic bioassays were further selected by exposure to corn leaf discs expressing Cry1Ab and subsequently to artificial diet treated with high concentrations

of Cry1Ab. The resulting resistant strain exhibited > 800-fold resistance to Cry1Ab that was primarily conferred by an autosomal and incompletely recessive genetic factor.[50] Analysis of the backcross progeny resulting from mass mating of the parental resistant strain with F_1 generation indicated that the resistance was conferred by a small number of loci with major effects on Cry1Ab resistance.[50]

In greenhouse experiments with isoline and Cry1Ab expressing plants, no survivors were found on vegetative stage Bt plants. However, both resistant larvae and the F_1 progeny of resistant *x* susceptible parents were able to survive on reproductive corn 15 d after infestation, although the larvae that survived were found feeding on silk, ear shanks, ear tips, kernels and pollen accumulated in leaf axils, which are known to express lower concentrations of Cry1Ab.[51-53] Therefore, in bivoltine strains of *O. nubilalis*, progeny from the second generation may be capable of developing on transgenic plants but are unlikely to survive on high-Cry1Ab-expressing tissues of vegetative-stage plants in the subsequent generation. This does not preclude an increased frequency of Cry1Ab resistance alleles for the second generation, where the resistance does not appear to be functionally recessive. However, it should be noted that survival was measured at 15 d after infestation and does not reflect survivorship to the adult stage. Because the development of the F_1 progeny was significantly delayed on the reproductive-stage plants, it is likely that a much higher proportion of larvae would be unable to complete development than reflected by the 15 d survival. Although the resistance identified in this population colony exhibited high levels of resistance in diet bioassays and was able to feed on Cry1Ab-expressing leaf tissue, there was no evidence of feeding or survival on whole plants expressing Cry1Ab.[50] Moreover, in each year since the strain was initially collected, additional collections obtained from the same area have not shown unusual survival at the diagnostic concentration.[16]

As previously described for Cry1Ab, baseline susceptibility of *O. nubilalis* populations was established[54] and used to estimate the upper end of the 95% confidence interval for the LC_{99} as a diagnostic Cry1F concentration for annual assessments of Cry1F susceptibility among field populations of *O. nubilalis* that is coordinated through the Agricultural Biotechnology Stewardship Technical Committee (ABSTC). This concentration was validated with field populations in 2001 and 2002 prior to the commercial release of Cry1F-expressing hybrids. In both years of validation experiments, however, at least one population was observed that exhibited < 99% mortality suggesting that either the calculated diagnostic concentration was inaccurate or that there was a higher frequency of resistant individuals than expected among field populations.

In 2004, in only the second year of commercial availability for corn hybrids expressing Cry1F, a field collection obtained from Hamilton County, IA exhibited significantly reduced mortality at the diagnostic concentration and an elevated LC_{50} value for Cry1F.[55] Because the initial collection consisted of only 11 egg masses, multiple generations of rearing were required to obtain sufficient numbers of neonates for bioassays. In contrast to the other populations examined in 2004, the colony established from Hamilton County collections exhibited much lower mortality rates (50.7%) at the diagnostic Cry1F concentration, which was consistent with the elevated LC_{50} and EC_{50} observed for Cry1F in this collection. As with the Kandiyohi County, MN population that exhibited Cry1Ab resistance, a series of experiments was initiated to document inheritance of resistance, to quantify the magnitude of resistance and to assess survival on Cry1F-expressing plants.

The results of these additional tests[55] indicated that F_3-F_4 progeny of individuals collected from Hamilton County in 2004 that survived exposure to the Cry1F diagnostic concentration possessed high levels of resistance to the Cry1F protein. After pooling the survivors of the initial diagnostic bioassay and rearing for four additional generations, mortality had declined to < 3% at the diagnostic Cry1F concentration. Additionally, neonate larvae from the strain selected from survivors of the initial diagnostic bioassay exhibited rates of survival and growth on 1 cm-diam leaf discs cut from whorl stage plants expressing Cry1F that were not statistically different from survival and growth on leaf discs cut from non-expressing plants.

Greenhouse experiments involving exposure of the resistant Hamilton County populations to both vegetative and reproductive stage plants were also conducted. While increased feeding and some larval survival was observed on vegetative stage plants, it appears likely that even with the high level of resistance, the ability of these insects to complete development was compromised. However, on reproductive stage plants, the resistant insects survived equally well and grew to similar sizes on isoline and Cry1F expressing plants. Although emerging adults could not be recovered from the plants, late instars recovered from the transgenic plants were able to complete development and emerge as adults under laboratory rearing conditions.

The primary trait expressed by the Hamilton County colony was highly recessive, which is the identical pattern observed in the Cry1F resistant laboratory colony[46] and described previously. The resistance that was identified in the Hamilton County population resembles that which developed in the laboratory selected strain described previously in that the resistance in both strains was highly recessive, apparently conferred by a single genetic factor, and both exhibited significant survival on Cry1F expressing plant tissues. To determine whether the resistance identified in the field population was the same as that selected in the laboratory, a simple crossing experiment was conducted between individuals from the lab selected colony and the strain derived from the Hamilton County collection. Since the resistance was highly recessive for both strains, the F^1 progeny should be susceptible to Cry1F if the resistance is conferred by two separate loci. However, if the resistance is conferred by the same locus, then the F^1 progeny will be resistant. In 13 families, the F_1 progeny derived from each strain exhibited nearly 100% survival at a Cry1F concentration that was 10-fold higher than the original diagnostic concentration confirming that the resistance is conferred by the same locus.

The implication of these results is that Cry1F resistance among *O. nubilalis* populations in the Midwestern US may be higher than anticipated. Moreover, the resistance may have already been

present at relatively high frequencies prior to the introduction of Cry1F-expressing corn plants based on the initial validation experiments of the diagnostic Cry1F concentration. It should be noted that in each year since 2004, additional collections obtained from the same area have not shown unusual survival at the diagnostic concentration and there have been no reports of unexpected damage to Cry1F-expressing corn plants. Therefore, the HDR strategy that is in place for Cry1F expressing hybrids appears to be functioning even when the frequency of resistance may be higher than 10^{-3}, which is the theoretical value often cited in support of the HDR strategy.[10,14,43]

The success of the HDR strategy for *O. nubilalis* and Bt corn may also be associated with incomplete resistance and fitness costs, factors that are not usually considered in IRM planning.[23] Fitness costs are evident when homozygous resistant insects on a non-Bt plant have lower fitness than susceptible larvae on non-Bt plants.[23] Pereira et al.[57] and Crespo et al.[58] compared life-history traits and population growth rates of genotypes homozygous and heterozygous for resistance with susceptible genotypes to Cry1F and Cry1Ab, respectively. In both studies, the existence of weak and, recessive to incompletely recessive, fitness costs were indicated in both strains. However, the incomplete nature of the resistance where resistant homozygotes have reduced fitness on Bt plants than susceptible insects on non-Bt plants is clearly indicated in both strains (see above), and may be a more important factor in delaying resistance evolution.

O. nubilalis Resistance and HDR

The success of IRM for Bt corn targeted against *O. nubilalis* is apparently dependent on characteristics of the insect's biology and attributes of the technology that comply with assumptions of the HDR. Since the initial registrations of Bt corn in 1996, we have learned a great deal regarding biology and ecology of *O. nubilalis* and the genetic architecture of Bt resistance that has provided a means to validate the various components of the HDR strategy and their inherent assumptions.

Evidence of high dose expression. One of the assumptions underlying the HDR strategy is that resistance is functionally recessive, which means that the protein concentration in tissues fed on by homozygous susceptible *O. nubilalis* is sufficiently high that nearly all (> 99.9%) of larvae feeding as neonates fail to complete development, and insects heterozygous for resistance alleles are expected to suffer at least 95% mortality.[9] The definition of "high dose" as it relates to the IRM strategies that have been developed for *O. nubilalis* and Bt maize has generally been described as levels of expression in plant tissue that are 25-fold in excess of the concentration of toxin needed to kill 99.9% of susceptible larvae.[9] The "25-fold" definition was initially based on a conservative estimate derived from empirical data on the inheritance of resistance in species where resistance has been characterized by crossing resistant and susceptible parents.[9,10]

While it is difficult to experimentally compare the concentration of toxin in artificial diet that consistently causes high mortality of susceptible homozygotes (i.e., LC_{99}) with expression levels in plants, there seems to be a general consensus that Bt corn for *O. nubilalis* complies with the definitions that have been proposed. Field surveys of *O. nubilalis* infestations of Bt corn established that the Bt hybrids produce at least 99.99% control relative to non-Bt hybrids.[10,56] In addition, for all the laboratory lines selected either with Bt formulations or with Bt fermentation products, even very high levels of resistance were insufficient to allow the insects to develop on expressing plants[39,40] (Siegfried B.D., unpublished) and support the high-dose designation. For the Cry1Ab-resistant Kandiyohi population that was identified through annual monitoring of susceptibility, neither the resistant parental strain nor F$_1$ hybrids of resistant x susceptible parents were able to survive on vegetative stage plants. However, for reproductive stage plants, there was some indication that both the parental resistant line and the heterozygotes are able to feed and develop on lower expressing tissues such as silk and pollen[52,53] and that functional recessiveness may be somewhat dependent on the toxin concentration of the specific plant tissue.[50] For Cry1F expressing hybrids, the Cry1F resistance that is conferred by a single, highly recessive genetic factor, where only the resistant homozygotes are capable of developing on Cry1F expressing plants, confirms the high-dose nature of these plants.

Low resistance allele frequencies. One of the key assumptions of the high-dose/refuge strategy is that alleles conferring resistance to Bt toxins are rare, i.e., < 10^{-3},[59] which has been taken as a default when modeling the evolution of resistance to Bt toxins.[60] However, estimates of allele frequencies prior to selection pressure are difficult because recessive alleles in heterozygotes will be missed by most traditional bioassay methods[16,44] and reliable detection of allele frequencies less than about 10^{-2} is impractical. Andow and Alstad[44] described a method referred to as the F$_2$ screen that offers the advantage of potentially detecting recessive alleles for resistance in a heterozygous state. This methodology involves collecting a large number of individuals from the field and establishing single-female family lines. The offspring of each collected female are inbred within family lines. The offspring of these matings (i.e., the F$_2$ of the collected generation) are then screened at a discriminating concentration for tolerance to the toxin. The purpose of the inbreeding process is to allow potentially heterozygous offspring of the collected females to mate with each other, generating a significant and easily detectible fraction of homozygous resistant offspring. Through back-calculation of the frequency of family lines containing a resistant allele, the frequency of the resistance allele in the sampled population can be estimated. Although potential changes in frequency over time have not been examined, no major Cry1Ab resistant alleles have ever been recovered using this technique confirming that the frequency of alleles conferring resistance to Cry1Ab expressing plants is below 10^{-3} in all the populations examined to date.[61-64]

Similar estimates of Cry1F resistance frequencies from field populations of *O. nubilalis* have yet to be reported. However, based on the ease with which resistance can be selected for in the laboratory and the identification of the same resistance allele among field populations, it appears likely that the frequency of Cry1F resistance is higher than 10^{-3} in field populations. Given that annual assessment of *O. nubilalis* susceptibility have provided strong evidence that field populations remain susceptible

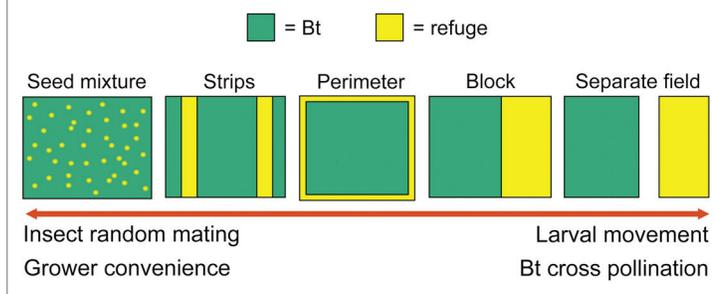**Biological and practical considerations for refuge placement**

■ = Bt   □ = refuge

Seed mixture | Strips | Perimeter | Block | Separate field

Insect random mating
Grower convenience

Larval movement
Bt cross pollination

Figure 1. Refuge configurations for lepidopteran Bt corn with a continuum of biological and practical considerations for refuge placement; e.g., seed mixtures are best for maximizing insect random mating but separate fields are best for minimizing larval movement issues.

to Cry1F, it appears likely that the HDR strategy that has been mandated for Bt corn is robust enough to maintain susceptibility even with an allele frequency that is possibly greater than 10^{-3}.

Refuge size and placement. Another key component of the HDR strategy involves a refuge of non-Bt corn to provide large numbers of homozygous susceptible insects to mate with the rare RR individuals capable of developing on Bt corn. The size and placement of the refuge is critically important to ensure that resistant and susceptible insects will mate more or less randomly.[14] For the refuge to be effective, susceptible homozygotes should out number SR and RR insects by a ratio of 500:1 or more[9,10] assuming a low resistance allele frequency ($> 5 \times 10^{-2}$).[65]

Currently structured refuges with non-Bt corn are the most common type of refuge used for managing *O. nubilalis* resistance to Bt corn. Growers that plant Bt corn are required to plant a specific proportion of their crop into a non-Bt variety, either within (strips or blocks), adjacent (edges or headlands) or within a designated distance (separate fields) from the Bt cornfield[66] (**Fig. 1**). There is general agreement that non-Bt corn provides the best refuge to increase the probability that susceptible corn insects will mate with resistant corn insects from Bt corn. Production of susceptible insects from weeds or other host plants as an unstructured refuge has been considered; however, unstructured refuges are inadequate replacements for non-Bt corn for managing *O. nubilalis* resistance.[67,68] Other types of corn, such as popcorn and sweet corn, could be substituted for field corn and may be categorized as "super" refuges because *O. nubilalis* production, at least in the case of popcorn, can be several-fold higher than that of field corn.[69]

Non-Bt hybrids used for refuge should be selected based on equivalent maturity to Bt hybrids, planted in similar fields within the same planting window, and managed with similar fertilization, weed and pest management and irrigation practices.[70] Otherwise moths could emerge from Bt and refuge hybrids at different times, leading to assortative (nonrandom) mating between resistance and susceptible individuals, and thus, weaken the refuge strategy. In the US corn belt, this is a particular concern for

first generation *O. nubilalis* moths that have a tendency to oviposit on taller, usually early planted corn.[71]

Refuge size and placement are important considerations for resistance management in order to maximize random mating between potentially resistant moths from Bt corn fields and refuge moths. In the corn belt a 20% non-Bt refuge has been the standard recommendation, which in most cases should provide plenty of susceptible insects that exceed the recommended ratio. Proximity of the refuge depends on the distance moths move before mating. In the case of *O. nubilalis*, males and females often fly a half mile or more before mating,[72,73] thus placement of structured refuges for lepidopteran Bt corn is recommended a half mile or less.[74] Management of *O. nubilalis* resistance to Bt corn since 1996 is probably due to the successful implementation of the HDR strategy.[23,75] Short-term laboratory and greenhouse studies support the refuge strategy for other lepidopteran pests[76,77] along with a recent evaluation of field studies for the control of sweetpotato whitefly, *Bemisia tabaci*.[78]

Another type of refuge option is seed mixtures, which is becoming a viable option for corn growers, but previously had been discouraged. A seed mixture simply means the Bt and non-Bt seeds are mixed in the same bag, which is convenient for growers to plant and avoids size and placement concerns. Besides mating behavior, another important biological consideration is plant-to-plant movement of larvae. Such movement is primarily a concern with seed-mixture and narrow-strip refuges as larval movement among Bt and non-Bt plants could violate the high-dose component of HDR.[79,80] This could occur if a young larva (neonate) tastes a Bt plant, becomes sick, and moves to a non-Bt plant. In this scenario if a larva with one copy of a resistance gene (heterozygote) has greater fitness than a susceptible insect then the high-dose component of the HDR strategy could be compromised.[11,81]

In a strip or block refuge, most larvae that move will encounter the same type of plant (Bt or refuge) because *O. nubilalis* larvae tend to disperse within rows rather than between rows.[80,82] In mixed-seed fields, however, larval movement among Bt and refuge plants is more likely to occur. Gould[11] and Davis and Onstad[80] consider survival of larvae moving off of Bt corn to be a key parameter for the development of insect resistance. Recent research on larval behavior, however, suggests that some aspects of larval dispersal could reduce concerns. Many lepidopteran neonates, including *O. nubilalis*, disperse off their host plant before feeding.[83-85] By means of gut dissections, Razze et al.[85] determined that only a small percentage (~15%) of *O. nubilalis* neonates (within 2 d of egg eclosion) had fed on Bt tissues before dispersing off the plant. Other studies have reported *O. nubilalis* neonate deterrence of Bt-corn leaf tissue or Bt-incorporated diet.[86-88] On the other hand, other research suggests that larval movement will remain an important consideration for resistance management. With on-plant tests Prasifka et al.[88] estimated the relative survival of susceptible larvae moving off of Bt corn was about 60% (susceptible/resistant = 43%/71%), supporting the conclusion of Davis and Onstad[80] that 50% mortality (relative to resistant larvae) is a realistic consequence for susceptible larvae

feeding on Bt maize before dispersal. Furthermore, threats to the high-dose strategy could occur if older larvae from a non-Bt plant move to Bt plants and survive. Movement of older *O. nubilalis* larvae occurs frequently among vegetative corn plants when high densities of larvae are present (R.L.H., unpublished). There are similar high-dose violation concerns with ear tissues when non-Bt corn plants are fertilized with pollen from Bt corn.[89] Theoretically, larvae could be exposed to low-levels of Bt or even high-dose tissue in close proximity to low or non-expressing tissues. Again, if such conditions results in the survival of heterozygous larvae then IRM could be compromised. Corn earworm, *Helicoverpa zea*, mortality is influenced by Bt pollinated sweet corn[90] and similar Bt and non-Bt cross pollination of corn could be an important factor for European corn borer.[91]

When corn borer Bt corn was introduced, the size recommendations for refuge ranged from 5% to 40% depending on the type of Bt corn, which was confusing to growers. After several meetings between academic, government and regulatory scientists, organized by the NC-205 Regional Research Committee, with considerable discussion and use of insect resistant management models, a minimum 20% refuge recommendation was established with refuge placed within half a mile of the Bt cornfield.[65] In the cotton growing areas in the Southern US the refuge recommendation for Bt corn is 50% or more because corn is an important refuge source for managing cotton bollworm, *H. zea* (a.k.a., corn earworm), resistance to Bt cotton.[92]

Corn has multiple pests so IRM recommendations often are influenced by the biology of all these pests. In particular, refuge recommendations were altered slightly after the introduction of Bt corn for rootworm, Diabrotica spp, in 2003. The 20% or greater refuge recommendation remained the same in the Corn Belt; however, the placement recommendation for rootworm Bt corn was changed to adjacent to the Bt cornfield. Rootworm beetles are more likely to mate within the field compared with corn borer moths, thus the refuge for rootworms had to be closer to the Bt cornfield to increase the chances that resistance beetles would mate with susceptible beetles. The best proximity strategy will vary depending on the biology of each targeted pest species (**Fig. 1**). Seed mixtures are the best strategy for maximizing random mating of adults but the riskiest strategy when larval movement or Bt pollen contamination are important factors. Refuge placement for lepidopteran Bt corn is probably best optimized with separate blocks or fields, but in the case of coleopteran Bt corn within a field or even seed-mixture strategy may be optimal. From a grower perspective, though, refuge placement that is most convenient may be the most important factor, especially if compliance is an issue (**Fig. 1**).

Recent Developments

The introduction of pyramided corn producing two or more Bt proteins with different modes of action targeting the same pest has dramatically changed options for managing corn pest resistance to Bt corn. Two or more toxins results in "redundant killing" and reduces chances that insects will evolve resistance,[55] especially when each of the toxins satisfy high-dose criteria,

which is the case for Bt corn varieties developed for *O. nubilalis*. Pyramided corn also provides a wider spectrum of control for other lepidopteran pests. Pyramided corn opens the door for consideration of smaller non-Bt refuges in both corn and cotton areas and the possibility of mixing or blending Bt and non-Bt seeds in the corn belt. Providing non-Bt refuge through seed mixtures is an especially attractive and practical option for growers.[88] As discussed previously, larval movement was the biggest obstacle for using seed mixtures for *O. nubilalis* control. This issue requires further study, but initial IRM models suggest that even with some movement, seed mixtures with pyramided corn will endure longer than single-toxin hybrids.[93]

Grower compliance in planting refuges has gradually decreased since early commercialization, especially following the commercialization of stacks of lepidopteran and coleopteran Bt corn varieties in 2004.[94,95] This trend toward increasing non-compliance among growers has motivated both industry and regulators to pursue the mixed-seed option because this strategy enables growers to be 100% compliant for refuge size and placement. Bt corn hybrids allowing seed mixtures for Diabrotica were registered by the US Environmental Protection Agency (USEPA) in 2010,[90] and Bt corn hybrids allowing seed mixtures for both Diabrotica and *O. nubilalis* were registered in 2011.[96]

Seed mixtures may be a viable option for managing resistance to European corn borer and possibly corn rootworm in the Corn Belt, but Bt corn is not high dose for many common maize pests. For example, lepidopteran Bt maize is not high dose for fall armyworm, *Spodoptera frugiperda*, corn earworm, *H. zea*, and cutworm species (family Noctuidae). Also, since coleopteran Bt maize is not high dose for corn rootworm even pyramided corn for rootworm may be susceptible to insect resistance, especially in light of recent evidence that rootworm resistance in the field may have evolved to single-toxin Bt corn.[37]

Transition to pyramided corn for lepidopteran and coleopteran Bt corn poses a challenge because the landscape, at least for a few years, will include a mosaic of single-toxin and multiple toxin corn. Such mosaics theoretically could foster the development of resistance to corn pyramids if insects develop resistance to single-toxin hybrids and if the same toxin is used in the pyramided hybrids.[59,77]

The fate of specific types of corn stacks and pyramids may be determined by the weakest link in the hybrid, that is, the pest most likely to evolve resistance. In the corn belt this weak link is unlikely to be *O. nubilalis* because Bt corn has satisfied high-dose criteria, but rather one or more of the insect pest species that does not satisfy these criteria, especially the rootworm. Bt corn for *O. nubilalis* established a high standard for growers, industry and regulators because it has been remarkably effective.[23] In doing so, it established the HDR strategy as the IRM strategy of choice, which is not necessarily the most robust IRM strategy for all insect pests of corn.

Conclusions

Nearly a hundred years after the European corn borer was accidently introduced into North America, effective management

and areawide suppression of pest populations has been made possible through the introduction of transgenic Bt corn. Prior to the introduction of this technology, *O. nubilalis* was difficult to control because larvae often escaped insecticide applications by boring into cornstalks. Bt corn circumvents this behavior by expressing a high dose of Bt toxin throughout the plant. When corn entomologists were introduced to this technology in the early 1990s, they were amazed that Bt corn was nearly "bulletproof" to *O. nubilalis* injury, more effective than any previous types of corn host plant resistance. Because of this effective control and the potential for intense selective pressures, insect resistance to Bt toxins was identified as the major threat to this technology.

Fortunately, a proactive insect resistance management program based on the high-dose refuge strategy was implemented with three major assumptions: (1) Bt plants must produce a high dose of toxin sufficient to kill most heterozygous insects (i.e., insects with one copy of resistance gene); (2) the initial frequency of resistance alleles is rare; (3) susceptible moths from non-Bt refuges intermingle and mate with rare resistant moths. Apparently these assumptions are met for *O. nubilalis* with current types of lepidopteran Bt corn because in spite of intense selective pressures for resistance evolution, *O. nubilalis* populations appear to remain susceptible. Whether this lack of resistance is a consequence of regulatory mandates for IRM practices is unknown, but the predictions from initial theoretical models about the sustainability of the technology when deployed in a manner consistent with the HDR strategy appear to have been realized.

Early surveys of Bt corn fields as well as the inheritance of resistance among selected populations supports the high-dose nature of Bt corn events. That heterozygotes from even the most resistant strains do not survive exposure to either Cry1Ab or Cry1F expressing corn plants supports the functional recessiveness of resistance to these plants. Annual surveys of *O. nubilalis* susceptibility and repeated attempts to select for resistance to Cry1Ab protein suggest a major allele that confers resistance is rare among field populations. In contrast, laboratory selection with the Cry1F protein has isolated an *O. nubilalis* strain that exhibits high resistance conferred by a single, recessive genetic factor. Since Cry1F resistance was isolated from a relatively small field collection suggests that the frequency of this resistance may be higher than observed for Cry1Ab. However, there is no indication that the frequency of this resistance has increased suggesting that the HDR strategy may be robust enough to delay resistance evolution even when the frequency of resistance is higher than anticipated.

Introduction of pyramided corn that produces two or more Bt proteins with different modes of action targeted for the same insect is a major advancement for IRM. For *O. nubilalis* resistance management, pyramids open the door to smaller refuges and the possibility of in-field refuges through the use of seed mixtures. Growers in particular will benefit from pyramids and seed mixtures because IRM compliance for refuge size and placement will no longer be an issue since refuge is literally in the bag.

This review has focused on the successful IRM of European corn borer. However, the list of pest species that that have evolved resistance to Bt crops under field conditions is growing especially in instances when the HDR assumptions are not satisfied. The fate of next generation transgenic products that include stacked and pyramided Bt events targeting multiple pest species may be determined by the weakest link in the hybrid; that is, the pest most likely to evolve resistance. In the Corn Belt this weak link is unlikely to be *O. nubilalis* because the assumptions of the HDR strategy appear to be satisfied. Bt corn for *O. nubilalis* has established a high standard for growers, industry and regulators because it has been both remarkably effective and durable.

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