The European corn borer and Bt corn in the United States

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The European corn borer, Ostrinia nubilalis Hübn er (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 Hübn er (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. 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. 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.

Transgenic corn plants that express insecticidal proteins from Bacillus thuringiensis (Berlin) (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. 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, exceeding 78% 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.

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). 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.

Keywords: transgenic maize, GM crops, Bacillus thuringiensis, Cry1Ab, Cry1F, Ostrinia nubilalis
The IRM strategy that has received the most attention from both industry and regulatory agencies involves the “high dose/refuge” (HDR) concept.\(^1\)\(^,\)\(^2\) 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.\(^3\)

In spite of what has been perceived as intense selective pressures and a likelihood for resistance evolution, \textit{O. nubilalis} populations apparently remain susceptible to all the currently deployed Cry toxins that have been registered as PIPs by the USEPA.\(^4\)\(^,\)\(^5\) 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,\(^6\)\(^,\)\(^7\)\(^,\)\(^8\)\(^,\)\(^9\) 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\(^10\) or where there has been documented field control failures,\(^11\)\(^,\)\(^12\)\(^,\)\(^13\)\(^,\)\(^14\)\(^,\)\(^15\)\(^,\)\(^16\) certain aspects of the pest’s biology or attributes of the technology do not comply with the assumptions of the high dose/refuge concept.\(^1\)

In this review, we describe what has been learned with regard to \textit{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 \textit{O. nubilalis} biology and ecology. Additionally, recent developments in IRM specific to \textit{O. nubilalis} that may affect the continued sustainability of this technology are discussed.

**Bt Resistant \textit{O. nubilalis} Colonies**

Selection experiments among a variety of insect pest species have repeatedly shown the potential for development of resistance to Bt toxins\(^17\) including \textit{O. nubilalis}.\(^18\)\(^,\)\(^19\) Outside the laboratory, Bt resistance has also been documented in populations of \textit{Plodia interpunctella},\(^20\) \textit{Plutella xylostella}\(^21\) and \textit{Trichoplusia ni}\(^22\) 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 \textit{Brassica juncea} (Fuller) to Cry1Ab-expressing corn in South Africa,\(^23\)\(^,\)\(^24\) in \textit{Spodoptera frugiperda} to Cry1F-expressing corn in Puerto Rico\(^25\) and in the coleopteran pest, \textit{Diabrotica virgifera virgifera} to Cry3Bb1-expressing corn in the US.\(^26\) Field control failures of Cry1Ac-producing cotton have also recently been reported for the pink bollworm, \textit{Pectinophora gossypiella} in the Gujarat State of India.\(^27\) Additionally, Tabashnik et al.\(^28\) have reported resistance to Cry1Ac-producing cotton among US populations of \textit{Heliothis 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 \textit{O. nubilalis} and Bt corn where susceptibility and field performance of the technology have been sustained for more than 15 y.\(^29\)

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 \textit{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.

**Table 1. Summary of Bt resistant strains of \textit{O. nubilalis}**

<table>
<thead>
<tr>
<th>Name</th>
<th>Selection/Isolation</th>
<th>Resistance Ratio</th>
<th>On-Plant Survival</th>
<th>Inheritance</th>
<th>Number of generations</th>
<th>Relevant Citations</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-I</td>
<td>MVP Formulation of Cry1Ac</td>
<td>162 Not Tested Not Tested Not Tested</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KS-SC-R</td>
<td>Dipel-ES</td>
<td>65 - -</td>
<td>Incompletely dominant/autosomal</td>
<td>1 or few</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSTT</td>
<td>Cry1Ab</td>
<td>1,200 - -</td>
<td>Intermediate/autosomal</td>
<td>5-10</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>ELs-H</td>
<td>Cry1Ab</td>
<td>3,000 - -</td>
<td>Intermediate/autosomal</td>
<td>5-10</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>SKY*</td>
<td>Cry1Ab</td>
<td>815 - -</td>
<td>Incompletely recessive/autosomal</td>
<td>&gt; 2</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>Cry1F-Selected</td>
<td>Cry1F</td>
<td>&gt; 3,000 + ++</td>
<td>Recessive/autosomal</td>
<td>1</td>
<td>43, 44</td>
<td></td>
</tr>
<tr>
<td>HAM County*</td>
<td>Cry1F</td>
<td>&gt; 3,000 + ++</td>
<td>Recessive/autosomal</td>
<td>1</td>
<td>53</td>
<td></td>
</tr>
</tbody>
</table>

*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.

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generations resulting in a strain with 73-fold levels of resistance to the Bt population. 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.30 The resistance identified in this strain has been associated with reduced Cry1Ab-like protease activity in the gut resulting in a slower rate of protoxin activation.31,32 Moreover, Li et al.31 demonstrated that plant enzymes hydrolyze Cry1Ab protoxin to one that is functionally activated. Although Dipel resistance and reduced protease 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 (<35-fold) after 10–15 generations of selection in a number of different populations.34 Continued selection by exposure to either Cry1Ab fermentation product or purified toxin throughout larval development in these same strains35 yielded >800-fold resistance after 60 generations of repeated exposures. Reciprocal crosses of resistant and susceptible parental populations and backcrosses to the F1 progeny to a susceptible parental population suggest that resistance in these populations is inherited as an intermediate trait and is conferred by multiple genes36 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.37,38 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 inabil-

ity to survive on Cry1Ab expressing plant tissue.39 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, labora-
tory selection with the Cry1F protein has allowed isolation of an O. nubilalis strain40 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.41 Pereira et al.42 described laboratory selection of O. nubilalis larvae to grow and develop on Cry1F expressing plants. In addi-
tion, 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 LC99 derived from baseline susceptibility studies have been conducted since the initial registration of Cry1Ab expressing events in 1996.43,44,45 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.46 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.47 Results of these tests indicated that significant Cry1Ab resistance had been isolated from the Kandiyohi popu-
lation.48 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 selection, and after 30 generations of selection, the highest concentration of Cry1F that could be tested (12.0 μg/cm2) did not cause significant mortality. Using this concentra-
tion as a lower limit for the LC99 of the resistant strain, the resistance ratio (LC99 for selected/LC99 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 F1 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.49 Genetic linkage maps with segregating markers show that the Cry1F resistance trait is controlled by a single quantitative trait locus (QTL) on linkage group 12 that supports the hypothesis that a single locus is responsible for resistance.50 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.51 Importantly, the Cry1F resistant strain described above is the first resistant O. nubilalis strain that is capable of sur-

viving on Cry1F expressing corn tissues. Greenhouse experiments with Cry1F-expressing corn hybrids indicated that some resis-
tant larvae survived the high dose of toxin delivered by Cry1F-
expressing plants, although F1 progeny of susceptible by resistant crosses had fitness close to zero.52 For vegetative stage plants, there appeared to be a reduction in survival of the resistant colony relative to survival on the non-expressing isolate. 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 isolate. 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 addi-
tion, since the resistance was isolated from a relatively small field collection, the frequency of this resistance may be higher than that observed for Cry1Ab.

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of Cry1Ab. The resulting resistant strain exhibited > 800-fold resistance to Cry1Ab that was primarily conferred by an autoso-
mal and incompletely recessive genetic factor. Analysis of the backcross progeny resulting from mass mating of the parental resistant strain with F1 generation indicated that the resistance was conferred by a small number of loci with major effects on Cry1Ab resistance. In greenhouse experiments with isoline and Cry1Ab expressing
plants, no survivors were found on vegetative stage Bt plants. However, the F1 resistant larvae and the F2 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. 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 fre-
quency 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 in the adult stage. Because the development of the F1 progeny was signifi-
cantly 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 lev-
els 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. 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.

As previously described for Cry1Ab, baseline susceptibil-
ity of O. nubilalis populations was established and used to estimate the upper end of the 95% confidence interval for the LC50 as a diagnostic Cry1F concentration for annual assessments of Cry1F susceptibility among field populations of O. nubila-
lis that is coordinated through the Agricultural Biotechnology Stewardship Technical Committee (ABSCTC). This concentra-
tion 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, 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 LC50 value for Cry1F; however 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 LC50 and EC50 observed for Cry1F in this collection. As with the Kandiyohi County, MN popu-
lation 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 indicated that F1, F2 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, neo-
imate larvae from the strain selected from survivors of the initial diagnostic bioassay exhibited rates of survival and growth on high-Cry1Ab-expressing 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 reproduc-
tive 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 com-
promised. 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 and described previously. The resistance that was identified in the Hamilton County popu-
lation 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 identi-
fied 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 F2 progeny should be suscep-
tible to Cry1F if the resistance is conferred by two separate loci. However, if the resistance is conferred by the same locus, then the F2 progeny will be resistant. In 13 families, the F2 progeny derived from each strain exhibited nearly 100% survival at a Cry1F con-
centration 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.4,9,14,43

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.49 Fitness costs are evident when homoyzgous resistant insects on a non-Bt plant have lower fitness than susceptible larvae on non-Bt plants.9,10 Pereira et al.9 and Crespo et al.4 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.6 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.6 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.28

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.6,25 In addition, all for 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.6,64 (Singfried 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_{2} 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,6,65 and that functional recessiveness may be somewhat dependent on the toxin concentration of the specific plant tissue.60 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}, 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 never be recovered using this technique confirming that 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^{-5} 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^{-5} in field populations. Given that annual assessment of O. nubilalis susceptibility have provided strong evidence that field populations remain susceptible
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. For the refuge to be effective, susceptible homozygotes should outnumber SR and RR insects by a ratio of 500:1 or more, assuring a low resistance allele frequency (> 5 × 10⁻³). For the refuge to be effective, susceptible homozygotes should outnumber SR and RR insects by a ratio of 500:1 or more, assuring a low resistance allele frequency (> 5 × 10⁻³).

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 non-Bt crop to provide homoygous susceptible insects to mate with the rare RR individuals capable of developing on Bt corn. In mixed-seed fields, however, larval movement among Bt and non-Bt flowering plants provides a means for larvae to disperse between Bt genotypes. In a strip or block refuge, most larvae that move will encounter the same type of plant (Bt or refuge) because they will mate with resistant corn insects from Bt corn. In the absence of refuges, susceptible insects may be exposed 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.

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Figure 1. Refuge configurations for lepidopteran Bt corn with a continuum of biological and practical considerations for refuge placement. (a) seed mixtures are best for maximizing insect random mating but separate fields are best for minimizing larval movement issues.
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 (H.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. Theoretically, larvae could be exposed to low-levels of Bt or even high-dose tissue in dense proximity to low or non-expressing tissues if field conditions result in the survival of heterozygous larvae then IRM could be compromised. Corn earworm, Helicoverpa zea, mortality is influenced by Bt pollinated sweet corn and similar Bt and non-Bt cross pollination of corn could be an important factor for European corn borer. 

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. In the cotton growing areas in the Southern US the refuge recommended 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.

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 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, 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. 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.

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. 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, and Bt corn hybrids allowing seed mixtures for both Diabrotica and O. nubilalis were registered in 2011.

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.

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.

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. 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 “bulge-proof” to O. nubilalis injury, more effective than any previous technology for pest 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 not 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 resistance 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 recessive nature 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 insects is a major advance because of Bt 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 have evolved resistance to Bt corn under field conditions is growing especially in instances where 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 Com 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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References

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68. Liu Y, Takahashi DR. Experimental evidence that refuge delay insect adaptation to Bacillus thuringiensis. Proc Natl Sci USA 1997; 94:4645-10; http://dx.doi.org/10.1073/pnas.94.9.4645.


73. King J, Ostrand DW, Holdcraft RL, Mason SE, Holdcraft RL, King J. Impacting the model of cross-pollination and low toxin expression in corn kernels on adaptation of European corn borer (Lepidoptera: Crambidae) to transgenic insect-resistant crops. Environ Entomol 2011; 40:667-9. PMID:22084774; http://dx.doi.org/10.1603/EC11081.


75. King J, Ostrand DW, Holdcraft RL, Mason SE, King J, Holdcraft RL. How to model the impact of cross-pollination and low toxin expression in corn kernels on adaptation of European corn borer (Lepidoptera: Crambidae) to transgenic insect-resistant crops. Environ Entomol 2011; 40:667-9. PMID:22084774; http://dx.doi.org/10.1603/EC11081.