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Bacillus thuringiensis* toxin resistance mechanisms among Lepidoptera: progress on genomic approaches to uncover causal mutations in the European corn borer, *Ostrinia nubilalis

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Transgenic plants that express *Bacillus thuringiensis* (Bt) crystal (Cry) protein toxins (Bt crops) effectively control feeding by the European corn borer, *Ostrinia nubilalis*, although documented resistance evolution among a number of species in both the laboratory and field has heightened concerns about the durability of this technology. Research has provided major insights into the mutations that alter Bt toxin binding receptor structure and function within the midgut of Lepidoptera that directly impacts the efficacy of Bt toxins, and potentially leads to the evolution of resistance to Bt crops in the field. In this manuscript we provide an overview of available data on the identification of genes involved in high levels of resistance to Cry toxins, with emphasis on resistance described for *O. nubilalis* as the main target of Bt corn.

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Introduction

Transgenic Bt crops were first commercialized in 1996 with the approval of Cry1Ab-expressing Bt maize hybrids for the control of the European corn borer, *Ostrinia nubilalis* [1], and were followed by additional transgenic maize hybrids that express both Cry1Ab and Cry1F toxins [2]. This introduction of Bt maize marked the start of a quintessential shift in the pest status of *O. nubilalis* in the United States, which prior to that time, was the most damaging maize pest in the United States [3]. Widespread adoption of Bt hybrids among producers [4,5] was credited for stark reductions in both the *O. nubilalis* population size and level of associated crop damage [6]. In addition, Bt maize also revolutionized how *O. nubilalis* and other pest insect

populations are managed, in that insect resistance management (IRM) strategies became an essential component for technology stewardship aimed to delay the onset of functional resistance [7]. Current IRM tactics are based on the high-dose/refuge (HDR) strategy [8–10] that has three basic assumptions; firstly, resistance alleles within insect populations are rare and recessive, secondly, transgenic toxins are expressed at a dose sufficient to render heterozygotes functionally susceptible, and thirdly, refuges of non-Bt maize grown near or blended within Bt maize fields are capable of producing an excess of homozygous susceptible individuals that will mate at random with any rare homozygous resistant individuals. Cry1Ab susceptibility has been retained among *O. nubilalis* populations for nearly 20 years after the introduction of the Bt maize varieties in the United States, and may be a testament to the successful implementation of the HDR strategy [6,11,12]. Field damage by *O. nubilalis* has not been reported despite estimates of resistance allele frequencies exceeding modeling thresholds, which would otherwise suggest compromised durability [13*]. These low observed Cry1F resistance allele frequencies may not be surprising considering effective population suppression provided by Cry1Ab [6] and the fitness costs associated with Cry1F resistance [14] (explanations of fitness costs in relation to Bt resistance found in [15–17]).

In contrast to *O. nubilalis*, functional resistance defined as the temporal reduction in susceptibility in an insect population after exposure to toxins under relevant field conditions [18,19], has been documented among species of Lepidoptera that feed on Bt maize. These include the African stem borer, *Busseola fusca*, resistant to Cry1Ab-expressing Bt maize in South Africa [19], and fall armyworm, *Spodoptera frugiperda*, with high levels of resistance toward Cry1F transgenic maize in Puerto Rico [20,21] and Brazil [22]. Additionally, among the cotton pest species, *Pectinophora gossypiella* show high tolerance to transgenic Cry1Ac cotton plants in the field [23] and *Helicoverpa armigera* and *Helicoverpa zea* show a decreased susceptibility to Cry1Ac over time [24–27]. Instances of field-evolved resistance might suggest that the biology of all lepidopteran pest insects may not fit within the basic tenants of the HDR strategy nor abide by parameters used to model product durability [28]. Recent reconfiguration of evolutionary models suggests that, even when exposed to high toxin doses, lack of random mating or

small increases in the dominance of resistance alleles can negatively affect success of the HDR strategy [29**]. Empirical evidence indeed demonstrates that field-evolved resistance traits are dominant or incompletely recessive in the case of *H. armigera* [26,30], *H. zea* [31], and *B. fusca* [32]. Furthermore, low-dose insecticidal exposures have been implicated in the rapid evolution of field resistance in a coleopteran maize pest, the western corn rootworm (*Diabrotica virgifera virgifera*) [33,34].

It could be argued that despite *a priori* acknowledgement that resistance phenotypes were likely to develop following widespread adoption of Bt crops and implementation of IRM plans aimed to circumvent or delay the onset of resistance, that the HDR strategy has been a mixed bag of success. Although field-evolved Bt resistance has been rare in terms of number of species [4], the impact has been great due to the effects on large scale agricultural production. The occurrence of resistance might suggest that our biological, ecological, or genetic data are insufficient for the design of long-term sustainable IRM plans [28,35**], or potentially to maintain the durability of certain crop protection technologies in the short-term [36]. In many cases, Bt technologies may have been compromised due to insufficient knowledge of the complex interactions among genetic, biological and ecological factors that affect the rate at which resistant phenotypes develop [37]. The remainder of this review will focus on the molecular genetic basis of Bt toxin resistance among Lepidoptera, and provides a brief summary of current knowledge and a synthesis of genomic complexities governing the control of certain resistance traits that may constitute future interests of the research community. The field of genomics may indeed provide data that could be helpful for developing more sustainable of IRM strategies.

Receptor-mediated resistance

Inroads have been made with regards to elucidating the molecular genetic mechanisms that confer Bt resistance to lepidopteran insects, which has stemmed from results of biochemical, genetic, reverse genetic, and genomic experiments. Note that due to space limitations Bt mode of action will not be presented, but readers are directed to several reviews on the topic [38–40]. Additionally, descriptions of many methods described herein are presented by [41].

Aminopeptidase N (APN) proteins were initially identified as candidate resistance genes due to *in vitro* ability to bind Cry1A toxins [42,43], and later implicated in the Bt mode of action following transcript knockdown by RNA interference (RNAi) [44–46]. APN's function as a Cry1Ac receptor is dependent upon post-translational addition of N-acetylgalactosamine (GalNAc) [47,48], although GalNAc independent binding has also been demonstrated [49]. An ancestral tandem duplication generated up to

eight APN orthologs within the lepidopteran lineage [50–52], which was confirmed in *O. nubilalis* by genetic linkage and physical mapping of orthologs onto genomic inserts of the OnB1 BAC library [53,54**]. Furthermore, Crava *et al.* [55] demonstrated that Cry1Ab specifically bound the product of the *O. nubilalis apn1* gene (*Onapn1*), whereas Cry1Fa toxin bound to *Onapn3a* and *Onapn8* proteins. More importantly, resistance to Cry toxins was associated with reduced levels of the aminopeptidase N1 (*apn1*) transcript among *S. exigua* [56], *Diatrea saccharalis* [45], *Trichoplusia ni* [57**], and *O. nubilalis* [54**], and structural mutations in *H. armigera apn1* [58], which suggests involvement in Bt resistance evolution.

Alkaline phosphatases (ALPs) are hydrolytic enzymes that dephosphorylate a variety of molecules, and membrane-bound versions in the midgut of *Manduca sexta* and *Heliothis virescens* bind Cry1Ac toxins in a GalNAc-dependent fashion [59,60], which was similarly shown for the close relative to *O. nubilalis*, Asian corn borer, *O. furnacalis* [61]. Due to differences in temporal expression during larval development, *M. sexta* ALPs bind Cry1Ab prior to APNs [62], but ALP binding to Cry1Ab appears to be more crucial for toxicity in *M. sexta* compared to binding with Cry1Ac [63]. Furthermore, reduced expression of midgut ALP at the protein and mRNA levels are associated with increased resistance to Cry1 toxins among *H. armigera*, *H. virescens*, and *S. frugiperda* [64**,65**,66,67]. The importance of ALP binding in the Cry1Ac toxin mode of action was demonstrated by incubation with an ALP toxin-binding fragment prior to ingestion by Cry1Ac resistant *H. armigera*, which resulted in reversion to a susceptible phenotype [67].

Cadherin belongs to a group of cell adhesion proteins, and the *M. sexta* ortholog, BT-R1, was shown to bind Bt Cry1A toxins *in vitro* [68]. Cadherin from susceptible *O. nubilalis* similarly binds Cry1Ab, Cry1Ac and Cry1F toxins [69,70]. *In vivo* binding further demonstrated the functional role of cadherin in binding Cry1A toxins among *O. nubilalis* [71], *Bombyx mori* [72] and *H. virescens* [73]. Structural mutations described within the cadherin gene were initially associated with Cry1Ac resistance among a laboratory strain of *H. virescens* [74] and allelic variants associated with Cry1Ac resistance from field-derived *P. gossypiella* [75] and *H. armigera*. Despite these prior associations, correlations between the segregation of cadherin alleles derived from *O. nubilalis* Cry1Ab or Cry1F resistant parents and resistant progeny within F₂ and backcross pedigree designs have not been correlated [35**,54**,76].

Lepidopteran ATP-binding cassette (ABC) proteins are membrane-bound transporters [77], and a premature stop codon mutation in the ABC2 gene subfamily member C2 provided initial indication that it may function within the Cry1Ac resistance mechanism of *H. virescens* [78].

Analogous deletions and point mutations were subsequently linked to Cry1A toxin resistance among *Plutella xylostella* [79], Cry1Ab resistance in *B. mori* [80], and Cry1Ac and Cry1Ca tolerance among *S. exigua* [81]. A locus containing the *abcc2* gene was also implicated in an *O. nubilalis* Cry1F toxin resistance trait [35**] (see further discussion below). More recently, a truncation in the ABCA2 gene was genetically linked with Cry2A resistance in *H. armigera* and *H. punctigera* [82*], and expression of an ABCG subfamily member is reduced among Cry1Ac resistant *P. xylostella* larvae [83]. These studies established ABC transporters as an important player in both Bt toxin mode of action as well as a point at which mutations can confer resistance [84,85], even though the ABC transporter role in Bt toxin binding has not yet been established. Taken into context, identification of multiple mutations among independent genes leading to Bt resistance might suggest that species of Lepidoptera can attain some degree of tolerance in laboratory or field conditions by virtue of different evolutionary paths [15,86,87]. It remains to be seen if implication of ABC transporters in resistance of a large number of Lepidoptera will prove to be a universal underlying causal factor.

Genetic control of receptor dysregulation

Resistance has evolved by means of reduced expression of Bt toxin receptors in the *apn*, *alp* and *abcc* gene families (see above), and a number of instances demonstrate that transcriptional control assort independently of binding receptor genes such that one might deduce it to be under epistatic control (Box 1). Specifically, studies have highlighted the likely role of *trans*-regulatory mechanisms in the constitutive downregulation of transcription from genetic loci encoding APN1 [57**], APN1 and 3 [54**], and ABCC2 and 3 as well as ALP [65**]. Initial indications that gene regulation can be a factor in the evolution of Bt resistance among Lepidoptera came from the linkage between an endogenous transcriptional repression of *apn1* and upregulation of *apn6* with the Cry1Ac resistant *T. ni* strain GLEN-Cry1Ac-BCS [69]. Importantly, and in contrast to prior studies that only documented reduced transcript levels [45,56], Tiewisiri *et al.* [57**] also demonstrated that the genetic control of Cry1Ac resistance segregated independent of the *T. ni apn1* locus within backcross pedigrees. Analogously, a trait that results in the constitutive down-regulation of the *Onapn1* transcript and confers Cry1Ab tolerance in the *O. nubilalis* Cry1Ab^R colony was mapped to two quantitative trait loci (QTL) with high-throughput single nucleotide polymorphism (SNP) markers [88], and confirmed that both QTL for Cry1Ab resistance segregated independently of *apn* loci [66,88]. These data suggest that QTL for Cry1Ab and Cry1Ac resistance may directly modulate the transcription at certain unlinked *apn* genes, which is hypothesized to occur through the control of a *trans*-regulatory mechanism. Although simple from a genetic standpoint, these studies profoundly affected the conceptual framework by

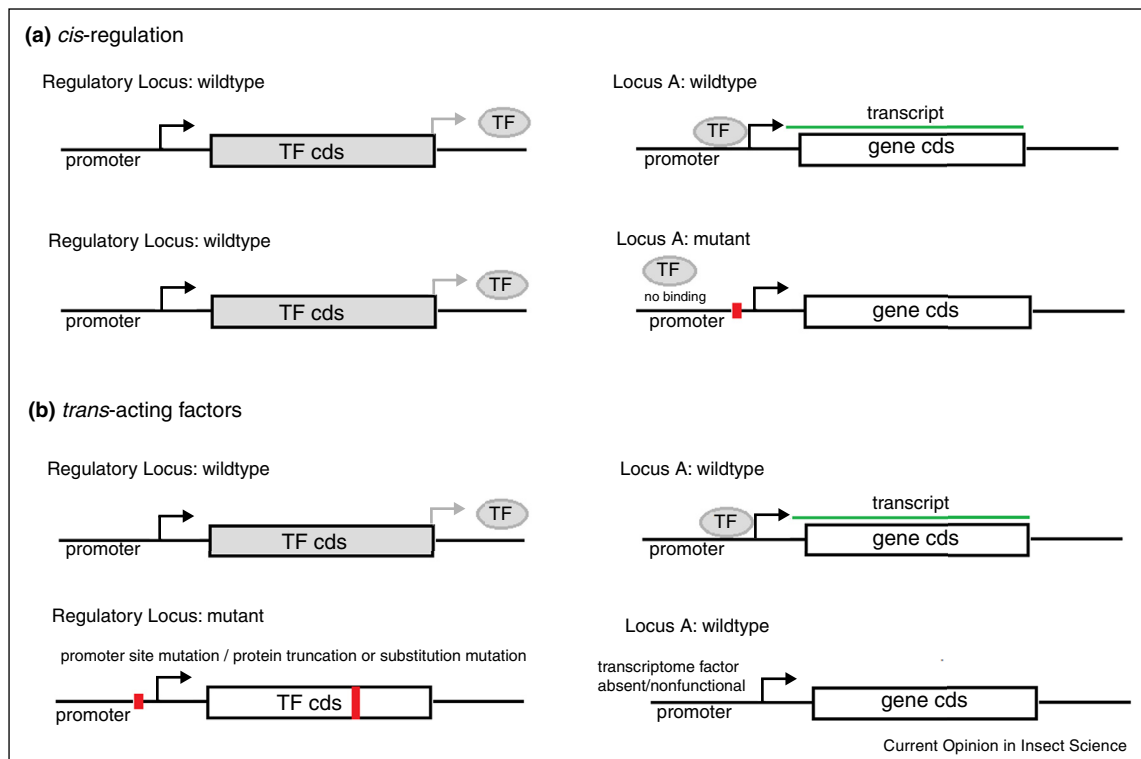
Box 1 Contributions of gene regulation in the evolution of Bt resistance

Structural mutations that cause gross alterations in the proteins encoded by Bt toxin receptors in the insect midgut, such as truncations and frameshifts, are relatively straightforward to discern in the context of potential changes in toxin-receptor interactions; especially in cases when known Bt toxin-binding domains are altered or eliminated from the mature proteins. Similarly, the downregulation of transcript levels are surmised to result in concomitant reductions in protein levels, which for example would decrease Bt receptor titers in the midgut and disrupt the normal toxin mode of action. In this latter case, the causal basis of transcriptional dysregulation can be manifested by way of either *cis*-acting or *trans*-acting mutations, which respectively involve changes in regulatory elements on the same DNA strand in proximity to the gene coding sequence and those that can act by means of a diffusible factor encoded at a different genetic locus (Figure 1).

Interestingly, in three instances wherein mutations in genes encoding *trans*-regulatory factors have been implicated in the Bt toxin resistance mechanisms that result from the downregulation of aminopeptidase N, alkaline phosphatase, and ABCC transporter genes [54**,57**,65**], these mutations have affected the quantitative expression of transcripts from two or more genes. In the case of *T. ni* [57**] and *O. nubilalis* [54**], *trans*-regulatory mutations affect transcription from related members of midgut-expressed aminopeptidase N gene family, which may have retained a degree of coordinated control due to probable duplication from an ancestral copy. For *O. nubilalis* a *trans*-regulatory mutation appears to result from the combined effect of two independent QTL which may coordinate the down-regulation of *apn1* and *apn3* transcripts, although it remains unknown if these two factors act independently on the two separate genes or contribute additively. It remains unclear if this *O. nubilalis* dysregulation of *apn1* and *apn3* results from an analogous *trans*-acting factor controlling *apn1* expression in *T. ni* that was linked to the *abcc2* locus [79], but considering the dissimilarities between *apn3* expression it is conceivable that the *trans*-acting factors in *O. nubilalis* could result from mutations at another points in the aminopeptidase N gene regulatory pathway.

In contrast, *P. xylostella* appears to have a single genome region (QTL) that has pleiotropic effects on the transcription of ABC transporter and alkaline phosphatase [65**]; that is, changes in *mapk4* were shown to affect the expression of two seemingly unrelated genes. The connection between *mapk4* and the regulatory pathways of Bt toxin resistance genes remains unknown but could involve indirect modulation of individual control switches for the two gene families or connection within a larger yet undescribed signal transduction cascade. Transcriptome-wide analyses of gene expression, such as the application of RNA-seq, hold the promise to help elucidate systemic changes in gene expression that are associated with these gene regulatory changes. Regardless, a small number of changes in gene regulatory cascades can result in a dramatic downstream effects. For instance, despite the introgression of an *O. nubilalis* Cry1F toxin resistance trait into a genetic background common to susceptible controls, several thousand transcripts showed significant quantitative changes between resistant and control larvae [89]. This highlights the complications still presented for such genomic-wide studies, which may be inherent to the global changes invoked by mutations a few genes that regulate entire pathways. A greater understanding of the interconnectedness of gene regulatory pathways may be needed in order to fully appreciate the complex system-wide changes observed between resistant and susceptible phenotypes, and moreover to fully grasp the role gene-gene interactions may play in the evolution of Bt resistance among species of Lepidoptera.

Figure 1



Differences between *cis*-regulatory and *trans*-regulatory controls involved in the alteration of gene transcription. **(a)** In the wildtype situation, promoter sequences facilitate binding of one or more transcription factors (TF) and support synthesis of nascent transcripts, whereas mutant situations have incurred mutations in upstream promoter sequence which prevent TF binding even when the TF is present. **(b)** Normal transcription can be interrupted when mutations occur at positions upstream within the gene regulatory network which either cause the diffusible TF to be non-functional or fail to be produced.

which Bt resistance is thought to develop; mainly that resistance can develop by transcriptional dysregulation involving gene-regulatory networks (Box 1).

Interestingly, the *T. ni* QTL for Cry1Ac resistance that controls the down-regulation of *apn1* [57**] was mapped to the *abcc2* locus [79]. Although any connection between *abcc2* and the transcriptional control of *apn1* remains unknown, these results might suggest a convergence between involvement of the *abcc2* locus and *trans*-regulatory control of the *apn1*. Recent evidence from *P. xylostella* may shed light into this control mechanism. Specifically, Guo *et al.* [65**] reported linkage between control of *alp* transcription and inheritance of a QTL positioned within the same genomic region as *abcc1*, 2, and 3. Conservation of *abcc1*, 2, and 3 mutations among resistant and susceptible strains led these researchers to suspect other genes within this genome interval may be the causal factor for Cry1Ac resistance. Successful use of RNAi to knockdown transcripts for the proximal mitogen-activate protein kinase 4 gene (*mapk4*) resulted in both upregulation of *alp* as well as *abcc2* and 3, and conversely endogenous down-regulation of these same genes were associated with elevated *mapk4* expression

among Cry1Ac resistant compared to susceptible *P. xylostella* larvae [65**]. These findings are significant in that a genetic factor at the *abcc2* locus (e.g. *mapk4*) was shown to control transcription of yet a different Bt toxin receptor, in this case *alp*. Even more importantly, these results implicate MAPK4 as a factor in *trans*-regulatory control of genes encoding known Bt toxin resistance genes. Supporting evidence may have come from results of ultra-high density genotyping-by-sequencing (GBS) marker-based QTL mapping that implicated a locus containing *abcc2* with segregation of an incompletely recessive *O. nubilalis* Cry1F_a resistance trait. Specifically, GBS markers adjacent to a marker for the *O. nubilalis* *abcc2* gene were more tightly associated with the QTL, which was interpreted to suggest that either another *abcc* gene or other linked genetic factor may be controlling Cry1F resistance in *O. nubilalis* [35**]. Recent whole-transcriptome and RNA-seq-based gene expression analyses indicated significant reductions in *alp* and *apn* transcripts in the midgut of Cry1F resistant compared to susceptible *O. nubilalis*, but ABC transporter-encoding transcripts were not identified within this pool of differentially-expressed genes [89*]. It will be interesting to see if *mapk4* has any involvement in controlling *apn*

expression and concomitant Cry1Ac or Cry1Ab toxin resistance traits in *T. ni* or *O. nubilalis*. Such information will provide a means to unravel the genetic architecture that leads to *O. nubilalis* Cry1F resistance.

Conclusions

Field-evolved resistance to Bt toxins expressed by transgenic crop plants represents a threat to current insect pest management practices, as well as a challenge to sustainable production practices. Laboratory models have proven valuable for the investigation of traits conferring Bt toxin resistance among species of Lepidoptera, and progress of this research has been facilitated by advances in molecular genetic-level and genomic-level analyses [36]. Devising effective IRM plans will undoubtedly require additional investigations into the genomic architecture of resistance, as well as the myriad of complex biological and ecological interactions of the insect that modulate the function of this genetic architecture.

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