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ScienceDirect
Journal of Invertebrate Pathology 95 (2007) 192–197
Journal of INVERTEBRATE PATHOLOGY
www.elsevier.com/locate/yjipa

The diversity of Bt resistance genes in species of Lepidoptera

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Received 1 March 2007; accepted 19 March 2007
Available online 25 March 2007

Abstract

Although the mode of action of Cry1A toxins produced by *Bacillus thuringiensis* is fairly well understood, knowledge of the molecular mechanisms by which lepidopteran species have evolved resistance to them is still in its infancy. The most common type of resistance has been called "Mode 1" and is characterized by recessive inheritance, >500-fold resistance to and reduced binding by at least one Cry1A toxin, and negligible cross-resistance to Cry1C. In three lepidopteran species, *Heliothis virescens*, *Pectinophora gossypiella*, and *Helicoverpa armigera*, Mode 1 resistance is caused by mutations in a toxin-binding 12-cadherin-domain protein expressed in the larval midgut. These mutations all interrupt the primary sequence of the protein and prevent its normal localization in the membrane, presumably removing a major toxic binding target of the Cry1A toxins. In *Plutella xylostella*, however, Mode 1 resistance appears to be caused by a different genetic mechanism, as Cry1A resistance is unlinked to the cadherin gene. Mapping studies in *H. virescens* have detected an additional major Cry1A resistance gene, which on the basis of comparative linkage mapping is distinct from the one in *P. xylostella*. An additional resistance mechanism supported by genetic data involves a protoxin-processing protease in *Plodia interpunctella*, and this is likely to be different from the genes mapped in *Plutella* and *Heliothis*. Thus, resistance to Cry1A toxins in species of Lepidoptera has a complex genetic basis, with at least four distinct, major resistance genes of which three are mapped in one or more species. The connection between resistance genes and the mechanisms they encode remains a challenging task to elucidate.

Keywords: *Heliothis virescens*; *Plutella xylostella*; *Helicoverpa armigera*; *Pectinophora gossypiella*; *Bacillus thuringiensis*; Cry1A toxin; Genetics; Resistance

1. Introduction

Insecticide resistance is an evolutionary phenomenon, involving changes in allele frequencies of specific genes over time. Insecticidal toxins of the Cry1A family produced by *Bacillus thuringiensis* (Bt) used in agricultural pest control have exerted a strong selective effect on populations of insects. For at least three species of Lepidoptera, prolonged applications for pest control in the granary, open field, or greenhouse have led to the appearance of resistance [Indianmeal moth (McGaughey, 1985), diamondback moth (Tabashnik et al., 1990; Shelton et al., 1993), and cabbage looper (Janmaat and Myers, 2003)]. Bt-resistant strains have been developed by laboratory selection in many other species of insects. The widespread adoption of transgenic maize and cotton expressing Bt toxins has greatly increased the opportunity for resistance selection in the field. Anticipating, and preventing or delaying this process in other target pest species that have not yet evolved Bt resistance in the field requires an understanding of which genes are subject to Bt selection and how they confer resistance. A genetic approach to analyzing Bt resistance is thus a necessary complement to the biochemical and physiological

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0022-2011/$ - see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.jip.2007.03.008

approaches widely utilized in the field. Some clues as to the likely identities of these genes are suggested by the complex mode of action of the Cry1A-type toxins on lepidopteran larvae.

2. Cry protein mode of action

Although some aspects of Bt-toxin mechanism of action are still poorly understood and controversial, the major steps are generally agreed upon (Knowles, 1994; Pietrantonio and Gill, 1996; Rajamohan et al., 1998; Schnepf et al., 1998). The site of action is the larval midgut, and the toxin must be ingested for lethality. The toxin is packed into proteinaceous parasporal crystalline inclusions. When Bt spores are consumed, they germinate and the crystals associated with spores dissolve in the alkaline lumen of the midgut. The soluble protoxin, whether derived from crystals or the cytoplasm of a transgenic plant producing the Bt toxin, is then cleaved in stages from the carboxy- and amino-termini by insect digestive proteases to produce the active, protease-resistant toxin core protein. Some of the proteolytic processing may have occurred in the tissue of the transgenic plant as well, prior to or during ingestion. The toxin crosses the peritrophic matrix to reach the ectoperitrophic space bordered by the apical membranes of the midgut cells. Toxin molecules undergo various interactions with molecules in the epithelial membranes, including reversible and irreversible binding, which is generally accepted as being crucial to toxicity. The toxin then participates in the formation of pores in the bilayer lipid membrane. Aggregation of toxin into oligomers happens prior to or during this step. The disruption of membrane integrity eventually kills the cells; in this process the mechanism of "colloid-osmotic lysis" (Knowles and Ellar, 1987) has received the most attention. If enough toxin has been ingested, these steps may occur within minutes or hours, usually accompanied by a complete cessation of feeding behavior. Eventual death of the larva may take several hours or even days, and is generally attributed to starvation which is likely exacerbated by proliferation of Bt and other microorganisms in the damaged midgut (Broderick et al., 2006).

3. Possible mechanisms of resistance

Even if this scenario is incomplete or erroneous in some of the details, it illustrates the sequential procession of events leading to toxicity, and the fact that resistance to Bt toxin by the insect may develop by any of several mechanisms that block the sequence at any point (Heckel, 1994, 2002). Inhibition of germination could confer resistance to spores. Failure of crystal dissolution would prevent passage through the peritrophic matrix and result in eventual excretion. Formation of the active toxin could be blocked by failure to completely process the protoxin (Oppert, 1999). Even if activated toxin is provided by the transgenic plant or another source, it could be further degraded by a protease with increased activity (Shao et al., 1998), sequestered by precipitation (Milne et al., 1998) or coagulation (Ma et al., 2005) or trapped by binding sites within the peritrophic matrix. Once present in the ectoperitrophic space, modification of the binding targets or molecules that otherwise interact with the toxin could reduce or prevent the irreversible binding believed to be crucial to toxicity (van Rie et al., 1990; Ferré et al., 1991). Binding targets could be shed from the midgut epithelium (Valaitis, 1995). Pore formation could be interfered with (Shai, 2001) or pores could be plugged. Replacement of dead midgut cells could be accelerated by increased activity of stem cells (Loeb et al., 2001).

4. Genetic analysis for identification of Cry resistance genes

This diversity of potential mechanisms suggests that mutations in a large number of genes could potentially cause resistance. The goals of a genetic analysis of a given resistant strain are to determine the number of genes involved, to measure the relative potency of each gene in conferring resistance and how they interact with one another, to evaluate known genes as candidates for resistance genes, and to facilitate positional cloning of unknown genes. Genetic analysis can address these questions without making any assumptions about the mechanism of resistance, although a full understanding is only achieved when the mechanisms are also known. The basic approach relies on analysis of the patterns of toxin-induced mortality in the F_1, F_2, and backcross generations resulting from crosses between resistant and susceptible strains, and this is achievable with a good bioassay irrespective of the mechanism of resistance. Mortality bioassays producing a dose–mortality response are analyzed by probit analysis, and growth bioassays relying on the concentration-dependent growth inhibition by sublethal amounts of toxin can be employed in QTL (quantitative trait locus) analysis.

Genetic analysis is even more powerful when marker loci and a genetic linkage map are used. Genetic linkage between marker loci and resistance genes causes a correlation between resistance level and marker genotype that can be measured. Complete coverage of a species' genome with marker genes ensures that all potential resistance genes will be near a marker and hence detectable by linkage. Probit analysis measures the combined action of different resistance genes but lacks the ability to distinguish among them. However, once localized on a linkage map, map position becomes an identifying property of a resistance gene independent of the mechanism it encodes. Candidate genes, encoding putative receptors for example, can be localized on the map and tested for linkage to resistance genes. Rejection of candidates is useful as it focuses future effort on a subset of genes; and acceptance of a candidate by linkage is the first step to more definitive studies including positional cloning. Finally, identification by linkage map locations enables strain or species comparisons even if the identities of the resistance genes are unknown.

The optimal linkage mapping strategy for species of Lepidoptera is dictated by two important genetic properties shared by most or all species in this insect Order: a very large number of chromosomes and the absence of crossing-over during meiosis in females (Heckel et al., 1999). When the linkage map consists largely of dominant markers such as RAPDs or AFLPs, backcrosses are more useful than F_2 mapping populations. Backcrosses using F_1 mothers enable straightforward assignment of genes to the many linkage groups, and backcrosses using F_1 fathers allow estimation of marker order and distances due to the pattern of crossing-over.

This approach, coupled with QTL analysis and resulting in the discovery of linkage, led us to one particular gene among many candidates in the analysis of Cry1A-toxin resistance in the YHD2 strain of *Heliothis virescens* (Gould et al., 1995). We mapped the *BtR-4* gene accounting for the majority of the Cry1Ac resistance in that strain (Heckel et al., 1997) to the same position as the gene encoding a 12-cadherin-domain protein (HevCaLP) expressed in the larval midgut (Gahan et al., 2001). We found that the gene in the YHD2 strain is interrupted by a portion of a retrotransposon, preventing the translation of a full-length protein and resulting in the lack of any immunologically detectable HevCaLP protein in the midgut membrane (Jurat-Fuentes et al., 2004). Subsequently, lesions in the 12-cadherin-domain gene were found to be genetically linked to high levels of Cry1Ac resistance in two other species, the pink bollworm *Pectinophora gossypiella* (Morin et al., 2003) and the cotton bollworm *Helicoverpa armigera* (Xu et al., 2005). From these studies it appeared that there was a common genetic basis to the "Mode 1" pattern of Bt resistance, characterized by the properties of recessive inheritance, >500-fold resistance to at least one Cry1A toxin, negligible cross-resistance to Cry1C, and reduced binding of membrane preparations to at least one Cry1A toxin (Tabashnik et al., 1998).

5. Cadherin-mediated resistance

The role of the cadherin in resistance was surprising to many because most research up to that time had focused on membrane-bound aminopeptidases as the most important binding target of the Cry1A toxins (Sangadala et al., 1994; Knight et al., 1995). Studies with the 12-cadherin-domain proteins from susceptible strains of *Manduca sexta* (Vadlamudi et al., 1995) and *Bombyx mori* (Nagamatsu et al., 1999) showed that they also bound Cry1A toxins, whether present in the native brush border membrane or on the surface of cells heterologously expressing them. These studies made it plausible that removal of the 12-cadherin-domain protein would remove a toxin binding site but did not explain how this could confer resistance in the presence of toxin-binding aminopeptidases that remained. Nor was it anticipated that the absence of this protein could be tolerated by the organism and not result in lethality, even though it has been found in the midgut of every lepidopteran investigated so far (despite being absent from the fully sequenced genomes of *Drosophila melanogaster* and *Caenorhabditis elegans*).

Indeed, the precise role of the 12-cadherin-domain protein in the mechanism of toxicity is still controversial. Binding sites for Cry1A toxins have been mapped to the membrane-proximal region of the protein (Nagamatsu et al., 1999; Dorsch et al., 2002; Hua et al., 2004) suggesting a role in increasing toxin concentrations at the membrane surface which may promote oligomerization and pore formation. By using alanine-scanning mutagenesis on peptide fragments overlapping this region, Xie et al. (2005) have shown that single amino acid substitutions can reduce toxin binding, although whether this could confer resistance *in vivo* is unknown. Bravo et al. (2004) have hypothesized that cadherin binding by the toxin results in an additional processing step whereby two alpha-helices from Domain 1 are clipped off, promoting toxin oligomerization and subsequent binding to aminopeptidases eventually resulting in pore formation. This model would be consistent with the finding of Adang et al. (2005) that peptide fragments from the membrane-proximal region of the cadherin protein unexpectedly increase the toxicity of Cry1A, if these fragments could accelerate the hypothesized additional toxin processing step. Zhang et al. (2006) argue that the cell-killing mechanism in cultured cells heterologously expressing the cadherin is not pore formation but rather a signaling pathway initiated by toxin binding to the cadherin and mediated by adenylyl cyclase and Protein Kinase A, although data on this mechanism in midgut cells is lacking.

6. Resistance in *Plutella* and other species

Thus, in spite of disagreement about the precise role of the 12-cadherin-domain protein in the Cry1A toxin mode of action, it is clear that mutations in the gene encoding it are somehow responsible for Cry1A resistance in three different lepidopteran species. This, however, is not the case for "Mode 1" resistance in the diamondback moth, *Plutella xylostella*, in which the major Cry1A resistance gene has been mapped to AFLP Linkage Group 22 (LG22; Heckel et al., 1999; Baxter et al., 2005). The gene for the 12-cadherin-domain protein maps to LG8 in that species (Baxter et al., 2005), and so any and all mutations in that gene are genetically unlinked to resistance in all of the strains we have investigated so far. Further candidate gene testing has rejected eight aminopeptidases, one alkaline phosphatase (Jurat-Fuentes and Adang, 2004), an intestinal mucin (Sarauer et al., 2003), one glycosyltransferase (Griffitts et al., 2003), and a homologue of a Cry1A-binding protein from *B. mori* (Hossain et al., 2005) by linkage analysis, as none of these genes map to LG22 (Baxter, 2005). Midgut membranes from the NO-QA strain show a nearly complete absence of binding to Cry1Aa, Cry1Ab, and Cry1Ac (Tabashnik et al., 1994,1997a) and it has been widely anticipated that loss or modification of a major binding target

would be the mechanism of resistance. In NO-QA and the other *Plutella* strains, although the genetic approach has so far failed to reveal the identity of the resistance gene, it has conclusively eliminated 13 known genes as candidates, including the cadherin. The resistance gene in *Plutella* has increased to high frequencies in populations worldwide due to selection by overuse of Bt sprays (Tabashnik et al., 1997a,b). If the homologous gene exists in the cotton pests (tobacco budworm, pink bollworm, and cotton bollworm) it could pose a greater threat to the durability of Cry1Ac-expressing cotton than lesions in the cadherin gene, which appear to incur a high fitness cost (Carrière et al., 2006).

The genetic approach has also revealed the existence of a Cry1Ac resistance gene distinct from both the 12-domain cadherin and the *Plutella* gene. Linkage mapping has identified a gene (*BtR-5*) on LG10 of *H. virescens* that is the Cry1Ac major resistance factor in the CxC strain (Gahan et al., 2005). Various combinations of resistant and susceptible genotypes at *BtR-5* and the cadherin gene indicate that the two genes act in an approximately additive fashion in the growth inhibition bioassay. The CxC strain exhibited about 60-fold resistance to Cry1Ac and about 50-fold resistance to Cry2Aa in the mortality bioassay, but *BtR-5* makes no contribution to the Cry2Aa resistance which appears to be polygenic in CxC (Gahan et al., 2005). None of the seven aminopeptidases mapped in *H. virescens* so far map to LG10, eliminating them as candidates for *BtR-5*.

Although midgut aminopeptidases have been widely studied for their ability to bind the Cry1A toxins, the evidence linking them to resistance comes from studies with the Cry1C toxins. Species in the genus *Spodoptera* are generally less sensitive to Cry1A toxins and more sensitive to Cry1C than are the heliothines. In Cry1C-susceptible cotton leafworm *Spodoptera litura*, experimental depression of mRNA levels for a Cry1C-binding aminopeptidase by injection of double-stranded RNA confers some protection from Cry1C toxicity (Rajagopal et al., 2002). This suggests that a strain of this species with reduced or absent aminopeptidase levels might display resistance against Cry1C, although such a strain has not yet been reported. In the beet armyworm *Spodoptera exigua*, however, there is a Cry1C-resistant strain (Moar et al., 1995) in which mRNA for APN1, one of four aminopeptidases studied, could not be detected by Northern blotting (Herrero et al., 2005). This suggests that low APN1 expression would co-segregate with Cry1C resistance in a linkage analysis, although such a study was not reported.

7. Protease-mediated resistance in *Plodia*

Apart from the 12-cadherin-domain protein, the only toxin-interacting protein with genetic evidence of linkage to resistance is a protease in the Indianmeal moth *Plodia interpunctella* (Oppert et al., 1997). The 198-r strain is resistant to *Bt entomocidus* and the 688-s strain is susceptible. When midgut proteins are separated by native polyacrylamide gel electrophoresis and stained with the artificial protease substrate BApNA, different isozymes can be resolved, and the assay is sensitive enough to be performed on homogenates from individual insects. The BApNA-hydrolyzing isozyme T1 was absent from midguts of all 198-r individuals and present in some but not all 688-s individuals examined. An isofemale line derived from 688-s was established in which all individuals expressed the T1 isozyme and this was used in crosses to 198-r. The electrophoretic pattern of F_1 hybrid individuals showed the T1 form in all progeny, indicating its inheritance from the susceptible parent. When F_1 progeny were backcrossed to the 198-r strain, T1 was present in about half the backcross progeny on the gels, and the presence of T1 was shown to co-segregate with susceptibility to a discriminating dose of Bt by a combination of bulked segregant analysis and progeny testing (Oppert et al., 1997). This genetic analysis and additional biochemical work showing that T1 is a protoxin-hydrolyzing enzyme support the authors' hypothesis that failure to produce the active toxin is one of the resistance mechanisms possessed by the 198-r strain. Although there is no direct genetic evidence to rule out the possibility that this gene is the same as the *BtR-5* gene in the CxC strain of *H. virescens* or the LG22 gene in the NO-QA strain of *P. xylostella*, failure of protoxin activation does not seem to be a major resistance mechanism in either strain.

8. Carboxylesterases and resistance?

Another class of hydrolyzing enzymes studied by electrophoresis, the carboxylesterases, has recently been proposed to be involved in Cry1Ac resistance in *H. armigera* (Gunning et al., 2005). Overproduced "nonspecific esterases" were hypothesized to bind to and sequester Cry1Ac toxin, and this novel resistance mechanism was invoked to explain the decreased mortality of the "silver selected" strain on Cry1Ac-expressing Ingard cotton. Cry1Ac toxin was shown to exert a concentration-dependent inhibitory effect on total esterase activity as measured by hydrolysis of the artificial substrate 1-naphthyl acetate, and the same substrate was used to stain native polyacrylamide gels, revealing a complex spectrum of isozymes. Both techniques used entire larval homogenates, thus esterases that are not expressed in the midgut would also have been detected. The zymograms show a greatly increased number and activity of bands from the "silver selected" strain compared to a Bt-susceptible strain reared in the lab for many generations and other recent field collections. A gel of 7 individual progeny each with a different isozyme pattern was shown, labelled "F_1 backcross". This inexact designation implies that F_1 hybrids were backcrossed to one of the parental strains for investigation of co-segregation of esterase patterns and resistance among backcross progeny, but the paper provides no evidence of such an experiment. Instead, it is stated that "the F_1 backcross [sic] had a level of esterase that was intermediate between the levels for the suscep-

tible and resistant parents, proving the link between resistance and increased levels of esterase'' (p. 2560). This is not a valid conclusion and thus despite the authors' claims that ''there is inherited increased esterase activity which genetically segregates with resistance'' (p. 2563), the paper provides no evidence for linkage or co-segregation of any of the esterase isozymes, or any factor controlling their activity level, to Bt resistance.

9. Summary

The genetic approaches taken in the several studies reviewed here have resulted in the enumeration of four different genes conferring resistance to a Cry1A toxin among different species of Lepidoptera. The products of only two of these are known, and only mutations in the cadherin gene have been shown to confer resistance in more than one species. Clearly, much work remains to be done in elucidating the identity and mechanisms of the other two, and there are likely many other resistance genes to be discovered as the number of potential resistance mechanisms is much greater than four. At this point it is not clear how useful extrapolation of results from work on species outside the Lepidoptera will be in this endeavor. The importance of the 12-cadherin-domain protein in Lepidopteran resistance to Cry1A proteins could not have been deduced from studies of model systems such as *Drosophila* and *C. elegans* because both lack an orthologue of this protein and neither is sensitive to Cry1A Bt toxins. Yet *C. elegans* offers an excellent example of the power of the genetic approach when it can be fully utilized (Marroquin et al., 2000; Griffitts et al., 2001, 2003, 2005) and other groups are now investigating the glycolipid synthesis pathway involved in *C. elegans* resistance to Cry5B to see whether homologous genes could confer Bt-toxin resistance in insects.

Acknowledgements

This work was supported by NSF Grant MCB-0517107, ARC-SPIRT Grant C00106849, and CESAR, a Special Research Centre of the Australian Research Council.

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