

# Novel genetic basis of field-evolved resistance to Bt toxins in *Plutella xylostella*

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## Abstract

**Insecticidal toxins from *Bacillus thuringiensis* (Bt) are widely used to control pest insects, but evolution of resistance threatens their continued efficacy. The most common type of Bt resistance ('Mode 1') is characterized by recessive inheritance, > 500-fold resistance to at least one Cry1A toxin, negligible cross-resistance to Cry1C, and reduced binding of Bt toxins to midgut membrane target sites. Mutations affecting a Cry1A-binding midgut cadherin protein are linked to laboratory-selected Mode 1 resistance in *Heliothis virescens* and *Pectinophora gossypiella*. Here we show that field-evolved Mode 1 resistance in the diamondback moth, *Plutella xylostella*, has a different genetic basis, indicating that screening for resistance in the field should not be restricted to a previously proposed DNA-based search for cadherin mutations.**

**Keywords:** diamondback moth, *Bacillus thuringiensis*, cadherin, resistance.

## Introduction

The bacterium *Bacillus thuringiensis* (Bt) produces proteins in a parasporal crystalline inclusion that are potent insecticidal toxins in widespread use as control agents for many pests of agricultural crops. Following ingestion by insect larvae, the crystal toxins are solubilized within the alkaline

gut, activated by cleavage with midgut proteases, bound by midgut receptors, and inserted into the midgut epithelial membrane; creating pores and inducing mortality (Schnepf *et al.*, 1998). The toxins are specific and environmentally benign, and can be administered to control caterpillars as foliar sprays or by expression in transgenic crops. However, the evolution of resistance to the toxins by pest populations in the field threatens the usefulness of this biological control agent (Tabashnik, 1994; Ferré & Van Rie, 2002; Janmaat & Myers, 2003). Although management programs have been implemented to delay or prevent the evolution of resistance to Bt, validation is problematical because of the difficulty of detecting low frequencies of resistant individuals in the field. Development of DNA-based screening methods has thus been a high priority. Success depends on correctly identifying the genes in the pest insects that confer resistance to Bt.

The diamondback moth, *Plutella xylostella*, is a global agricultural pest of cruciferous crops including cabbage and canola. This species has developed resistance to all synthetic insecticides (Talekar & Shelton, 1993) and is still the only pest to evolve resistance to Bt sprays in open field populations (Tabashnik, 1994). Numerous lepidopteran species have been selected for resistance in the laboratory, and many share the same resistance profile with the diamondback moth. The most common type of resistance ('Mode 1') is characterized by recessive inheritance, > 500-fold resistance to at least one Cry1A toxin, and negligible cross-resistance to Cry1C (Tabashnik *et al.*, 1998). The primary mechanism of Mode 1 resistance is reduced binding of Bt toxins to midgut membrane target sites (Tabashnik *et al.*, 1998).

The diamondback moth strain NO-QA, originally isolated from Hawaii, has been important in understanding the genetics of Mode 1 Bt resistance. There is strong evidence that a single, recessive, autosomal resistance gene is present (Tabashnik *et al.*, 1997a). Linkage mapping with AFLP markers enabled this gene to be assigned to a linkage group, and further identified a sequence tagged site (STS) 18.5 centimorgans from the predicted resistance locus (Heckel *et al.*, 1999). Bioassay data determined NO-QA to be resistant to Cry1Aa, Cry1Ab, Cry1Ac as well as cross resistant to Cry1F and Cry1Ja, when compared to a susceptible laboratory strain (Tabashnik *et al.*, 1997b).

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<sup>125</sup>I-labelled Bt toxins Cry1Aa, Cry1Ab and Cry1Ac all bound to the brush border membrane vesicles of a susceptible strain, and a reduction in binding specificity was observed for Cry1Ab and Cry1Ac in NO-QA, implying that a binding target site modification is responsible for resistance (Tabashnik *et al.*, 1994, 1996).

Toxin binding assays have identified two major classes of midgut Bt receptors in lepidopteran insects; aminopeptidases (Knight *et al.*, 1995) and a cadherin-like protein (Vadlamudi *et al.*, 1995; Nagamatsu *et al.*, 1999). So far, only the latter has been directly implicated in Bt-resistance. Ligand blotting and immunoprecipitation identified a 210 kDa cadherin-like protein (Bt-R1) in *Manduca sexta* capable of binding Cry1Ab (Vadlamudi *et al.*, 1995) as well as Cry1Aa and Cry1Ac with equal affinity (Keeton & Bulla, 1997). Transformed COS7 or *Drosophila* S2 cell lines that expressed Bt-R1 were susceptible to active Cry1A toxins, demonstrating that it is a functional receptor (Dorsch *et al.*, 2002; Hua *et al.*, 2004). The orthologue of this protein from *Bombyx mori* (BtR175) has also been identified as a binding target for Cry1Ac and Cry1Aa toxins (Nagamatsu *et al.*, 1998; Hara *et al.*, 2003). When BtR175 was expressed in Sf9 cells, Cry1Aa bound to the cadherin receptor and lysed the cells (Nagamatsu *et al.*, 1999).

Mutations affecting this Cry1A-binding midgut cadherin are tightly linked to laboratory-selected Mode 1 resistance in two major lepidopteran pests, *Heliothis virescens* and *Pectinophora gossypiella*. Insertion of an LTR-type retrotransposon in the *H. virescens* cadherin gene (*HevCaLP*) truncates the coding sequence approximately 950 amino acids before the transmembrane domain, and confers > 10 000-fold recessive resistance to Cry1Ac (Gahan *et al.*, 2001). This leads to absence of immunologically detectable HevCaLP protein and reduced toxin binding (binding to Cry1Aa absent, Cry1Ab reduced, and Cry1Ac more subtly affected; Jurat-Fuentes *et al.*, 2004). Three mutant alleles in *BtR*, the homologous cadherin of *Pectinophora gossypiella*, have deletions upstream of the transmembrane domain, conferring > 3000-fold recessive resistance to Cry1Ac (Morin *et al.*, 2003). This cadherin gene has thus been considered the prime target for DNA-based screening for resistance (Morin *et al.*, 2003). Here, we have investigated two Bt-resistant strains of diamondback moth, to determine if mutations in the cadherin orthologue are linked to Cry1A resistance in this species. Our results show that high-level resistance can be conferred by a mechanism other than mutations in the cadherin gene itself.

## Results

### *Cry1Ac and Cry1C resistance are caused by different genes*

Our previous linkage study with AFLP markers mapped a single major gene for Mode 1 Cry1A toxin resistance in the

NO-QA strain of diamondback moth from Hawaii (Heckel *et al.*, 1999). We used a similar backcross mapping design to analyse the BCS-Cry1C-1 strain (Zhao *et al.*, 2000, 2001) from South Carolina (hereafter called SC1), which unlike NO-QA was also resistant to Cry1C (Zhao *et al.*, 2000, 2001). We were interested in discriminating among two hypotheses concerning SC1: (1) that a single gene conferred both Cry1Ac and Cry1C resistance, making it qualitatively different from Mode 1 resistance; or (2) that different genes conferred resistance to Cry1Ac (possibly by a Mode 1 mechanism) and Cry1C.

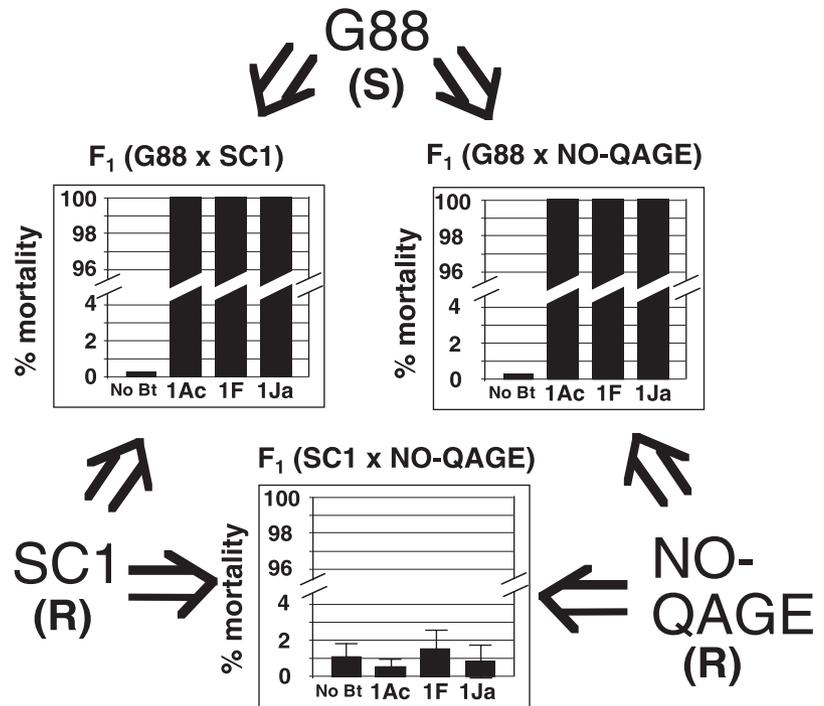
Twenty-eight of the thirty-one expected linkage groups in SC1 were marked by AFLP markers (2–18 per linkage group). Only one linkage group, LG22, was positively associated with Cry1Ac resistance in SC1 ( $\chi^2 = 15.6$ ,  $df = 1$ , Bonferroni  $P < 0.05$ ). Two other linkage groups, but not LG22, showed a positive association with Cry1C resistance ( $\chi^2 = 6.3$  and  $4.8$ ,  $df = 1$ ). Thus Cry1A and Cry1C resistance in the SC1 strain is conferred by independent genes on different chromosomes.

This result suggests that it should be possible to transfer the Cry1C resistance into a strain that is susceptible to Cry1A toxins, and vice versa. The former was accomplished by Zhao *et al.* (2001) by selecting from the original South Carolina collection with Cry1C. Their BCS-Cry1C-2 strain (hereafter called SC2) was resistant to Cry1C only. The converse situation has been widely observed, and indeed the absence of Cry1C resistance had been considered part of the definition of Mode 1 resistance to the Cry1A toxins. Although Zhao *et al.* (2001) did not attempt to develop a Cry1A-only resistant strain from their field collection, we hypothesized that the South Carolina LG22 could confer Mode 1 resistance, if isolated into an otherwise susceptible strain. To test this hypothesis, we compared Cry1Ac resistance in SC1 with the previously characterized Mode 1 resistance gene in the NO-QA strain.

### *The same gene confers Cry1Ac resistance in strains from Hawaii and South Carolina*

To test whether the Cry1A resistance in SC1 occurred on the same linkage group as in NO-QA, an STS marker (sequence-tagged site, GENBANK AF149306) linked to the Mode 1 resistance gene in the latter strain (Heckel *et al.*, 1999) was PCR-amplified and mapped in an SC1 backcross family using denaturing HPLC. The STS mapped to AFLP LG22, showing that the major Cry1A resistance gene in SC1 occurs on the same chromosome as the major resistance gene of NO-QA. However, this result alone does not prove that they are the same gene.

A stronger criterion, an interstrain complementation test for allelism (Tabashnik *et al.*, 1997b), was applied to determine if mutations within the same gene caused the recessive Cry1Ac resistance in SC1 and NO-QAGE (an artificial-diet-adapted, Bt-resistant descendent of NO-QA;



**Figure 1.** Interstrain allelic complementation tests. F<sub>1</sub> progeny were produced by crossing one susceptible (S) and two resistant (R) strains in all pair-wise combinations. Larvae were tested with diagnostic doses of Cry1Ac, Cry1F, or Cry1Ja. Histograms represent mortality after 4 days. Resistance in both R strains is recessive, as the toxins killed all F<sub>1</sub> progeny from RxS crosses. If different genes caused resistance in the two R strains, complementation would restore susceptibility to their F<sub>1</sub> progeny; however, they are just as resistant as either parental R strain.

Tabashnik *et al.*, 2000). Reciprocal single-pair crosses were performed among three strains (SC1, NO-QAGE, and the susceptible strain G88). The resulting F<sub>1</sub> progeny were bioassayed on discriminating concentrations of Cry1Ac, Cry1F and Cry1Ja. Recessive resistance to these three toxins, which share a midgut binding site (Ballester *et al.*, 1999; Herrero *et al.*, 2001a), is conferred by the same gene in NO-QA and NO-QAGE (Tabashnik *et al.*, 1997b, 2000). F<sub>1</sub> hybrids produced by crossing either SC1 or NO-QAGE with G88 were fully susceptible, as expected from recessive resistance. If resistance alleles in SC1 and NO-QAGE occurred at different loci, complementation would restore susceptibility to the F<sub>1</sub> hybrids produced by intercrossing the two resistant strains. However, these hybrids were just as resistant as either parental strain (Fig. 1). This failure to complement indicates that the alleles conferring Mode 1 resistance in the two strains occur at the same locus.

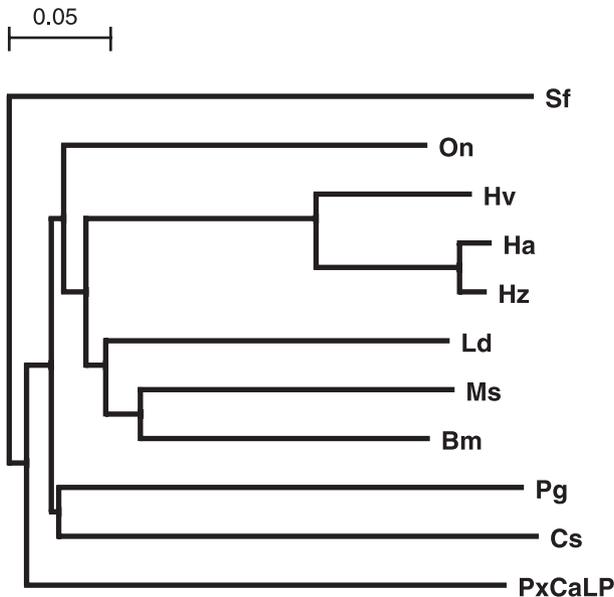
#### Identification and genetic mapping of the candidate resistance gene *PxCaLP*

In *H. virescens* and *P. gossypiella*, Mode 1 resistance is caused by mutations in a gene encoding a member of the cadherin superfamily (Gahan *et al.*, 2001; Morin *et al.*, 2003). We identified the orthologue of this cadherin (*PxCaLP*) in the diamondback moth. PCR products corresponding to 4.3 kb of *PxCaLP* mRNA were amplified from larval midgut cDNA of the Waite (Bt susceptible) strain, representing about 75% of the coding region (GENBANK accession no. AY529117). The predicted amino acid sequence displayed

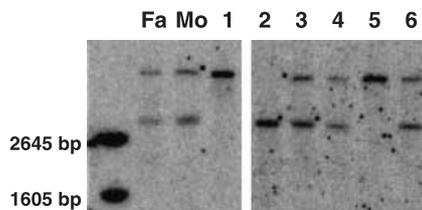
55% identity and 68% similarity to *HevCaLP* of *H. virescens*. The sequence of a 4.1 kb fragment amplified from genomic DNA (GENBANK accession no. AY529118) enabled identification of nine intron positions in diamondback moth. Seven of these occur in identical positions in *H. virescens*. A neighbor-joining tree showed that *PxCaLP* is most similar to the 12-cadherin-domain proteins of *P. gossypiella* and *Spodoptera frugiperda* (Fig. 2, and Supplementary Material).

In *H. virescens* and *P. gossypiella*, Mode 1 resistance maps to the same chromosomal location as the cadherin gene. Using intron size polymorphisms, we mapped *PxCaLP* in the SC1 strain to AFLP LG8, which was not associated with resistance to Cry1Ac ( $\chi^2 = 0.125$ ,  $P > 0.9$ ) or Cry1C ( $\chi^2 = 2.13$ ,  $P > 0.6$ ). Thus Mode 1 resistance (LG22) and *PxCaLP* (LG8) map to two different chromosomes in SC1. Confirming this result by comparing intron size polymorphisms in *PxCaLP* with AFLP markers in previously analysed pedigrees (Heckel *et al.*, 1999), we found that *PxCaLP* also mapped to a different linkage group than Mode 1 resistance in the NO-QA strain. Therefore, Mode 1 resistance is not caused by mutations in *PxCaLP* in diamondback moths strains SC1 or NO-QA.

The intron-size polymorphisms used to map *PxCaLP* were detected by PCR from genomic DNA followed by electrophoretic separation of the amplicons. This method cannot rule out the existence of a second copy of *PxCaLP* on LG22 which might have failed to amplify because its introns were too large or the PCR primers annealed less efficiently to it. We therefore used Southern blots to investigate the



**Figure 2.** Neighbor-joining tree of the 12-domain midgut cadherin proteins from eleven lepidopteran species. Sf, *Spodoptera frugiperda*; On, *Ostrinia nubilalis*; Hv, *Heliothis virescens*; Ha, *Helicoverpa armigera*; Hz, *Helicoverpa zea*; Ld, *Lymantria dispar*; Ms, *Manduca sexta*; Bm, *Bombyx mori*; Pg, *Pectinophora gossypiella*; Cs, *Chilo suppressalis*; PxCaLP, *Plutella xylostella*. Accession numbers and protein alignment are given in the Supplementary Material.



**Figure 3.** RFLP patterns of the *PxCaLP* gene in a mapping family of *Plutella xylostella*. Using single-pair matings, Cowra and Waite strains were crossed and the  $F_1$  backcrossed to Waite. DNA from adult moths was isolated from F<sub>1</sub> parents (Fa = father; Mo = mother) and offspring and treated with *Xba*I. Only six of the twenty-one progeny are shown. A 179 bp product, lying completely within a single exon of *PxCaLP*, with no internal *Xba*I sites was used as a probe. As expected for a single-copy gene, all individuals show one (homozygous) or two (heterozygous) bands in the expected Mendelian ratios.

possibility of gene duplication. A 179 bp PCR product lying completely within a single exon of *PxCaLP* and with no cleavage sites for the restriction enzyme *Xba*I was used to probe a Southern blot made by digesting parental and offspring DNA with *Xba*I (Fig. 3). Such a probe will hybridize to a single *Xba*I fragment produced by each allele of each *PxCaLP* gene copy present in the genome, with fragment sizes determined by the locations of the nearest *Xba*I sites in the flanking sequence. A single-copy gene with *Xba*I polymorphisms flanking the probe is expected to display single, distinct bands in homozygotes and both bands in

heterozygotes; the pattern seen in Fig. 3. The cross depicted is a mating between two heterozygotes ( $A_1A_2$ ), which produced genotypes  $A_1A_1$ ,  $A_1A_2$ , and  $A_2A_2$  in the expected 1 : 2 : 1 ratio (6 : 11 : 4 observed,  $\chi^2 = 0.43$ ,  $df = 2$ ,  $P > 0.85$ ).

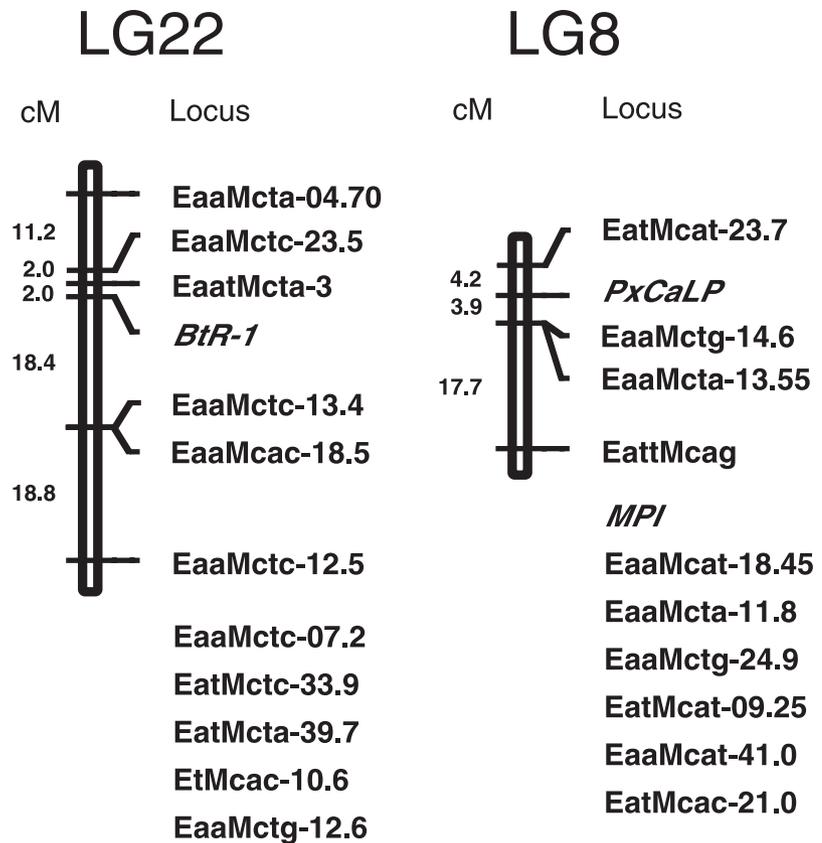
A gene duplication of *PxCaLP* with different allelic fragment sizes would produce three or more bands in some lanes, which was not observed. A gene duplication with identical allelic fragment sizes from the two copies would produce the same pattern as Fig. 3 if both copies occurred together on LG8; but then neither copy would exist on LG22 along with the Mode 1 resistance gene. If such a duplication with identical allelic fragment sizes had one copy on LG8 and the other on any other chromosome (including LG22), independent segregation of the two genes would yield a 1 : 14 : 1 ratio with an excess of the two-banded  $A_1A_2$  type, which was not observed ( $\chi^2 = 25.20$ ,  $df = 2$ ,  $P < 0.005$ ). Thus the Southern blot excludes the possibility of a duplicate copy of *PxCaLP* on LG22 or any other chromosome (with the possible exception of LG8).

Additional linkage tests were performed with another marker, *MPI*, which encodes the enzyme mannose-6-phosphate isomerase (EC 5.3.1.8). In *H. virescens*, *MPI* is linked to *HevCaLP* and to Mode 1 resistance (Gahan *et al.*, 2001). A PCR product amplified with degenerate primers from diamondback moth DNA (GENBANK accession no. AY529119), with 54% predicted coding similarity to *MPI* from *Drosophila melanogaster*, was mapped in SC1 and NO-QA. In both strains, *MPI* and *PxCaLP* are linked to each other, but not to Mode 1 resistance (Fig. 4). Thus, genetic linkage between the cadherin and *MPI* is conserved in *H. virescens* and diamondback moth, but genetic linkage between the cadherin and Mode 1 resistance is not.

## Discussion

These results show that, in contrast to findings from *H. virescens* and *P. gossypiella*, Mode 1 resistance in the SC1 and NO-QA strains of diamondback moth is not caused by mutations in the gene encoding the 12-cadherin-domain protein. This gene has been proposed as the prime target for DNA-based screening tests for resistance in field populations (Morin *et al.*, 2003). Although this approach might be valid for the other species, it would not detect the Mode 1 resistance in the two strains of diamondback moth.

The results reported here do not rule out involvement of the *PxCaLP* protein in Cry1A resistance. The Mode 1 resistance gene may affect its expression, post-translational modification, or localization. If any of these changes prevented protein expression on the midgut epithelial membrane, resistance might occur for the same ultimate reason (absence or modification of the cadherin binding target) in diamondback moth as in the other species. This possibility is currently under investigation; however, whether or not it



**Figure 4.** Linkage groups corresponding to two chromosomes in the diamondback moth. Linkage groups were detected in female-informative families as groups of markers showing no recombinants, since crossing-over does not occur in females (see Experimental procedures). The linkage map for male-informative markers was estimated from the patterns of crossing-over in males using maximum likelihood. Markers that were not male-informative are listed below the respective maps, since their relative positions cannot be determined without additional data. The gene for Mode 1 resistance (*BtR-1*) occurs on one chromosome (LG22) and the genes encoding the 12-cadherin-domain protein (*PxCaLP*) and mannose-6-phosphate isomerase (*MPI*) occur on another (LG8).

is true, our results imply that Mode 1 resistance does not have the same genetic basis in all Lepidoptera.

It may not even have the same genetic basis in all strains of diamondback moth. Two other Mode 1 strains have also been tested by complementation with NO-QA (Tabashnik *et al.*, 1997b). The PEN strain from Pennsylvania, like NO-QA, showed recessive resistance to Cry1Aa, Cry1Ab, Cry1Ac, and Cry1F. F<sub>1</sub> hybrids between PEN and NO-QA were also resistant to all four toxins, indicating that the same resistance gene occurs in both strains. The PHI strain from the Philippines, on the other hand, was only partially resistant to the Cry1A toxins and not resistant to Cry1F. Resistance to Cry1Aa and Cry1Ac was partially dominant, and only resistance to Cry1Ab was recessive, satisfying the precondition for complementation testing. F<sub>1</sub> hybrids between PHI and either PEN or NO-QA showed variable results for Cry1Ab, with 2/3 of the F<sub>1</sub> families showing high resistance as expected, but 1/3 of the families showing susceptibility (Tabashnik *et al.*, 1997b). Thus, mutations in the same gene cause resistance in PEN and NO-QA. In PHI, this shared gene accounts for Cry1Ab resistance in some families, but additional genes must be invoked to account for Cry1Ab resistance in other F<sub>1</sub> families as well as resistance to the other toxins.

A correlation between *MPI* and Cry1Ab resistance has been demonstrated in the PHI strain. A change in

mannose-6-phosphate isomerase allozyme frequencies and an increase in resistance were observed after laboratory selection with Cry1Ab (Herrero *et al.*, 2001b). These results with PHI are consistent with involvement of mutations in *PxCaLP* or other genes on the same chromosome, although linkage tests were not performed and the cadherin gene was not investigated in that study.

The lepidopteran midgut 12-cadherin-domain proteins form a distinct group within the cadherin superfamily, with no orthologues in the fully sequenced human, mouse, rat, *Drosophila melanogaster* or *D. pseudoobscura* genomes. To date, a single form has been found in each of eleven lepidopteran species (Fig. 2). Apparent loss-of-function alleles in *H. virescens* and *P. gossypiella* are not lethal when homozygous; and it is plausible that Bt-resistance in other Lepidoptera may arise by similar mutations. However, our results, taken together with previous work, show that such mutations are not the only genetic mechanism of Mode 1 resistance. Diamondback moth is still the only species to have evolved Bt resistance in open field populations, yet several resistant strains studied so far would not be diagnosed as such from their *PxCaLP* sequence. Diagnostic tests based on screening cadherin genes may thus fail to detect resistance in field populations of diamondback moth and other lepidopteran species.

## Experimental procedures

### *Insect strains and crosses*

Diamondback moth strains NO-QA, NO-QAGE, SC1 (also known as BCS-Cry1C-1) and G88 (Geneva 88) have been described previously (Tabashnik *et al.*, 2000; Zhao *et al.*, 2000, 2001). The Bt-susceptible Australian strains Waite (South Australia) and Cowra (New South Wales) had been reared on fresh cabbage for > 200 and three generations, respectively, without exposure to any insecticide. For linkage analysis, single-pair matings between SC1 and G88 were performed, and F<sub>1</sub> females were backcrossed to SC1 males. Larvae from large backcross families were divided into three groups and allowed to feed on cabbage leaf disks dipped in Cry1Ac (5 µg Al/ml, MVP, Dow Agro Sciences, IN, USA/ Mycogen, IN, USA), Cry1C (10 µg Al/ml, M-C, Mycogen), or no toxin (Zhao *et al.*, 2001). Survivors were reared to adulthood for DNA isolation and AFLP analysis. Crosses with NO-QA have been described previously (Heckel *et al.*, 1999). Single-pair matings between Waite and Cowra were performed to generate diamondback moth backcross families for Southern hybridization analysis. For interstrain allelic complementation tests, sixty single-pair reciprocal crosses between NO-QAGE and SC1 were conducted. F<sub>1</sub> larvae from each family were reared separately on artificial diet in 30-ml plastic cups. We obtained sufficient numbers of second-instar larvae from thirty-one families to test using an artificial diet overlay with no toxin or with 10 µg Al/ml Cry1Ac (MVP-II freeze-dried powder from Monsanto, MO, USA), 200 µg Al/ml Cry1F (M-press liquid formulation from Dow AgroSciences), or 20 µg Al/ml Cry1Ja (powder formulation, Ecogen, PA, USA). Preliminary tests proved that these doses killed 100% of G88 larvae but caused minimal mortality for SC1 or NO-QAGE. Mortality was recorded after 4 days at 27° C.

### *AFLP methods*

Genomic DNA was isolated using a modified phenol-chloroform method (Zraket *et al.*, 1990) and AFLP analysis was performed (Vos *et al.*, 1995). The Eco AFLP primer was end-labelled with  $\gamma$ -<sup>32</sup>P or  $\gamma$ -<sup>33</sup>P and used in the second, selective PCR reaction (Vos *et al.*, 1995) with Taq polymerase (Promega, WI, USA) using an iCycler (Bio-Rad, CA, USA) thermocycler. Amplified products were separated on 6% polyacrylamide gels (Astral Scientific NSW, Australia), and the dried gels were exposed on X-OMAT film (Kodak, NY, USA).

### *Inheritance of AFLP bands and linkage analysis*

The genetic system of *P. xylostella* has been described (Heckel *et al.*, 1999) and applies generally to Lepidoptera. Briefly, chromosomal crossing-over during meiosis and gamete formation occurs in males, but not in females. Female-informative pairs of marker genes (inherited from an F<sub>1</sub> mother) show absolute linkage when they occur on the same chromosome, no matter how distant from one another, and free association only when they occur on different chromosomes. Observation of recombinants provides definitive proof that markers or genes occur on different chromosomes. In Fig. 4 for example, within each of the two linkage groups, no recombinants were observed in the female-informative family. Between the two linkage groups, twenty-three out of fifty progeny from the female-informative family were recombinants, demonstrating that the linkage groups correspond to two independently segregating chromosomes. Male-informative pairs of marker genes (inherited

from an F<sub>1</sub> father) show linkage only when they occur close together on the same chromosome; the recombination fraction being related to physical distance as in other organisms. Backcross progeny from the SC1 crosses were scored for AFLP bands inherited from the F<sub>1</sub> mother that were absent in the SC1 father. These were analysed using a Pascal program DBM3Lnk.p written by DGH, which groups markers with the same segregation patterns into linkage groups, allowing for minor scoring errors. These segregation patterns were then used as templates to assign STS AF149306, *PxCaLP* and *MPI* to linkage groups based on female-informative polymorphisms within these sequences. Each linkage group was analysed for an association with resistance, separately for treated Cry1Ac survivors and Cry1C survivors, by performing  $\chi^2$  tests on contingency tables with the untreated controls, applying the appropriate Bonferroni correction for multiple comparisons (Heckel *et al.*, 1999). Male-informative markers were analysed using MapMaker 2.0 for Macintosh to calculate maximum-likelihood estimates of order and spacing in Haldane centimorgans.

### *Identification of gene orthologues*

Partially degenerate primers for *PxCaLP* (sense: ATC CGC GCT ACC GAY GGN GGN AC, antisense: GCC AAT GAG GTC AGA GTT GTC CGA RTC YTC) were designed from amino acid alignments of Lepidopteran orthologues (*Helicoverpa armigera*, *H. virescens*, *P. gossypiella*, *M. sexta* and *B. mori*). A 4.1 kb product (GENBANK AY529118) was amplified using the Expand 20 kb<sup>PLUS</sup> PCR System (Roche, IN, USA) from Waite strain genomic DNA. RT-PCR (Invitrogen, CA, USA) and 3' RACE (BD Biosciences, CA, USA) was then performed on total larval RNA to generate products accounting for 75% of the coding sequence (GENBANK AY529117). Partially degenerate primers (sense ATG TAC AAG GCA CCC AAC CAY AAR CCN GA, antisense CCG AAG CCA CCA ACG TCA CCN GGR AAR TC) for *MPI* (GENBANK AY529119) were designed from protein alignments of *D. melanogaster*, *C. elegans*, human and yeast.

Specific primers for the STS (GENBANK AF149306) were CCG TGC TGA GCA TTG GAC AGT GAG and TTA ACT ATA TTT GTT GGT GAC GAT AAG GTG. Polymorphisms were scored by separating PCR amplicons on a Helix System denaturing HPLC (Varian, Victoria, Australia) at 57.5 C on a gradient from 11.25% to 17% acetonitrile over 7 min.

### *PxCaLP Southern blot*

Using single-pair matings, Cowra and Waite strains were crossed and the F<sub>1</sub> backcrossed to Waite. DNA was isolated from F<sub>1</sub> parents and offspring and treated with *Xba*I (Promega). Restriction fragments were separated on a 1% agarose gel and transferred to a Hybond N + nylon membrane (Amersham, NJ, USA). A probe was made using PCR primers (sense: GCA ACC CAC ACA GAG AAC CT, antisense: ACG ACC TCG AAC TCG AAC AT) amplifying a 179 bp product lying completely within a single exon of *PxCaLP*, with no internal *Xba*I sites. After overnight hybridization, the filters were washed at low stringency (2× SSC, 0.1% SDS). RFLP patterns were scored for the two parents and twenty-one offspring. The observed frequencies were examined for goodness-of-fit under two segregation hypotheses using the chi-square test: (1) single-copy gene or tandem duplication, with expected ratios of 1 : 2 : 1; (2) independent segregation of two genes on different chromosomes, with expected ratios of 1 : 14 : 1.

#### GENBANK accession numbers

The following nucleotide sequences have been deposited in GENBANK: AY529117 *Plutella xylostella* cadherin-like protein mRNA, AY529118 *Plutella xylostella* cadherin-like protein gene, AY529119 *Plutella xylostella* mannose-6-phosphate isomerase gene.

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#### Supplementary Material

The following material is available from <http://www.blackwellpublishing.com/products/journals/suppmat/IMB/IMB563/IMB563sm.htm>

**Figure S1.** Sequence alignment of the 12-domain midgut cadherin from eleven lepidopteran species.

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