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Genetic mapping of Bt-toxin binding proteins in a Cry1A-toxin resistant strain of diamondback moth *Plutella xylostella*

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Abstract

A major mechanism of resistance to *Bacillus thuringiensis* (Bt) toxins in Lepidoptera is a reduction of toxin binding to sites in the midgut membrane. Genetic studies of three different species have shown that mutations in a candidate Bt receptor, a 12-cadherin-domain protein, confer Cry1A toxin resistance. Despite a similar resistance profile in a fourth lepidopteran species, *Plutella xylostella*, we have previously shown that the cadherin orthologue maps to a different linkage group (LG8) than Cry1Ac resistance (LG22). Here we tested the hypothesis that mutations in other genes encoding candidate Bt-binding targets could be responsible for Bt resistance, by mapping eight aminopeptidases, an alkaline phosphatase (ALP), an intestinal mucin, and a P252 glycoprotein with respect to the 29 AFLP marked linkage groups in a *P. xylostella* cross segregating for Cry1Ac resistance. A homologue of the *Caenorhabditis elegans* Bt resistance gene *bre-2* was also mapped. None of the genes analysed were on the same chromosome containing the Cry1Ac resistance locus, eliminating them as candidate resistance genes in the parental resistant strain SC1. Although this finding excludes *cis*-acting mutations in these genes as causing resistance in this strain, one or more of the expressed proteins may still bind Cry1Ac toxin, and post-translational modifications could affect this binding and thereby exert a *trans*-acting effect on resistance.

Keywords: *Bacillus thuringiensis* (Bt); *Plutella xylostella*; Diamondback moth; Insecticide; Resistance; Linkage mapping

1. Introduction

Many strains of *Bacillus thuringiensis* (Bt) produce insecticidal crystal proteins (Schnepf et al., 1998) that are widely used to control agricultural insect pests by application as foliar crop sprays or by expressing the toxin genes in transgenic plants. More than 200 different Cry toxins have been identified, and can be highly specific to targeted pests while remaining harmless to beneficial insects and vertebrates. To kill the insect, the crystal protoxin must be ingested by target pests, become activated by proteases in the highly alkaline gut, cross the peritrophic membrane, and bind to targets in the midgut epithelium where it oligomerises and lyses cells.

Resistance to Bt toxins could occur if toxin is (i) not activated, (ii) immobilised in the peritrophic membrane, (iii) unable to bind its targets on the brush border membrane, (iv) unable to create pores in the epithelium or (v) ineffective due to rapid repair of cell damage (Heckel, 1994; Ferre and Van Rie, 2002; Griffitts and Aroian, 2005). Although there are no reported cases of insect resistance evolving to transgenic crops (Tabashnik et al., 2005), resistance to Bt sprays has arisen in the field multiple times in diamondback moth (Tabashnik et al., 1990, 1997b; Wright et al., 1997; Sayyed et al., 2000; Baxter

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et al., 2005). Understanding the mode of action and mechanisms of resistance in diamondback moth has been seen as crucial for long-term, sustainable Bt use.

Laboratory selection experiments on lepidopterans and EMS mutagenesis of nematodes have been used to generate Bt-resistant strains, to genetically and biochemically characterise resistance mechanisms that may be encountered in the field. A common form of Bt resistance involves reduced toxin binding to sites in the midgut epithelium. Several classes of Bt toxin-binding proteins have been identified in lepidopteran midguts including a 12-cadherin-domain protein (Gahan et al., 2001; Morin et al., 2003; Xu et al., 2005), aminopeptidases (Knight et al., 1995; Nakanishi et al., 1999), ALP (McNall and Adang, 2003; Jurat-Fuentes and Adang, 2004), and various glycoproteins (Valaitis et al., 2001; Hossain et al., 2004). In addition, membrane glycolipids in the nematode *Caenorhabditis elegans* bind to Cry5B toxin (Griffitts et al., 2005).

Mutations affecting the Cry1A-binding midgut cadherin are genetically linked to resistance in three major lepidopteran pests, *Heliothis virescens* (Gahan et al., 2001), *Pectinophora gossypiella* (Morin et al., 2003), and *Helicoverpa armigera* (Xu et al., 2005). In these species, mutations produce mRNA transcripts that differ from the wild type due to partial deletions or the presence of premature stop codons. Production of the midgut cadherin protein (HevCaLP) in *H. virescens* was necessary for Cry1Aa binding in susceptible strains, while binding was not observed in a resistant strain (YHD2-B) (Jurat-Fuentes et al., 2004).

Aminopeptidase N (APN) enzymes are bound to cell membranes by GPI anchors, and cleave N-terminal amino acids from other proteins (Knight et al., 1995). Studies have shown that Cry1Ac and Cry1C toxins are capable of binding APN targets on the brush border membrane of *Manduca sexta* (Knight et al., 1994; Sangadala et al., 1994), *H. virescens* (Gill et al., 1995; Luo et al., 1996; Oltean et al., 1999) and *Plutella xylostella* (Luo et al., 1997). *Drosophila* transformed with APN1 from *M. sexta* have an increased sensitivity to Cry1A toxins (Gill and Ellar, 2002). RNAi knockdown of a class 4 APN (slapn) in *Spodoptera litura* larvae correlated with a 70% increase in pupation success when exposed to Cry1C toxin, relative to controls, implicating slapn as a possible Bt receptor (Rajagopal et al., 2002). Recently (Herrero et al., 2005) demonstrated an association between resistance to Cry1C in *Spodoptera exigua*, and the absence of a class 1 APN mRNA from midgut tissue.

Biochemical studies have shown proteins other than APN or cadherin to bind Bt toxins. A membrane-bound ALP has been proposed as a Cry1Ac binding target in *H. virescens* (Jurat-Fuentes and Adang, 2004) and *M. sexta* based on mass spectrometry of proteins separated on 2D gel and blots using antibodies to *Bombyx mori* membrane ALP (McNall and Adang, 2003). Valaitis et al. (2001) discovered a highly anionic glycoprotein (BTR-270) in *Lymantria dispar* associated with the brush border. This protein bound Cry1Aa and Cry1Ab with higher affinity than Cry1Ac, mirroring the relative toxicity of these three toxins toward this species. Hossain et al. (2004) isolated a protein (P252) from the brush border of *B. mori* that showed specific binding to Cry1Aa, Cry1Ab, and Cry1Ac. This protein was revealed to be extensively glycosylated by probing with various lectins.

Genetical and biochemical evidence has shown that loss-of-function mutants in a glycolipid biosynthetic pathway confer resistance to Cry5B and Cry14A Bt toxins in the nematode *C. elegans*. A glycosylation pathway consisting of four glycosyltransferase genes, (*bre-2*, *bre-3*, *bre-4* and *bre-5*) was identified by EMS mutagenesis (Griffitts et al., 2005). A knockout strain of any one of these genes failed to synthesise the gut glycolipid Bt receptor, rendering worms resistant. Moreover, reduced levels of neutral glycolipids have been associated with Cry1Ac resistance in diamondback moth (Kumaraswami et al., 2001).

The purpose of this study was to evaluate these Bt-toxin-binding targets as candidate genes for resistance in *P. xylostella*. The SC1 strain from South Carolina (also known as BCS-Cry1C-1) is resistant to Cry1A toxins as well as Cry1C (Zhao et al., 2000, 2001). We previously mapped the Cry1A resistance locus in SC1 and showed by complementation analysis that it is the same gene conferring Cry1A resistance in the NO-QA strain from Hawaii (Baxter et al., 2005). The NO-QA strain is resistant to Cry1Aa, Cry1Ab, and Cry1Ac toxins (Tabashnik et al., 1997) and shows greatly reduced binding of brush border membrane vesicles to all three (Tabashnik et al., 1994, 1996). Moreover, we have shown that the Cry1A-binding 12-cadherin-domain protein maps to a different chromosome than Cry1A resistance in both strains (Baxter et al., 2005). Thus, unlike the situation in *H. virescens*, *P. gossypiella*, and *H. armigera*, mutations in the 12-cadherin-domain protein are not responsible for Cry1A resistance in these two strains of *P. xylostella*. Here, we extend this candidate-testing approach to evaluate genes encoding putative Cry1A binding targets as candidates for Cry1A resistance in the SC1 strain. None of the 12 candidate genes tested genetically mapped to the same chromosome as Cry1Ac resistance, and these results show the genetic basis of Bt resistance in *P. xylostella* may be qualitatively different than resistance in other Lepidoptera studied to date.

2. Methods

2.1. Insect strains and crosses

Plutella xylostella strains Waite, Geneva 88, and SC1 (also known as BCS-Cry1C-1) have been described previously (Zhao et al., 2000, 2001; Baxter et al., 2005). SC1 shows incomplete recessive resistance to Cry1C (>1090-fold) and recessive resistance to Cry1Aa (395-fold), Cry1Ab (595-fold), Cry1Ac (>40,000-fold), Cry1F (7890-fold) and Cry1J (13,100-fold), relative to susceptible

control strain Geneva 88 (Zhao et al., 2001). The Cowra strain was collected from canola, *Brassica napus*, in East Cowra, NSW, Australia (37°48′S, 142°26′E) and reared for three generations in the laboratory, with no exposure to insecticide.

Plutella xylostella SC1 and G88 backcrosses were performed using single-pair matings between a resistant SC1 male and susceptible G88 female. Two F_1 female sisters were then backcrossed to two different SC1 males in single-pair matings, creating backcross BC1a and backcross BC1b. Progeny from each backcross were divided into two groups and allowed to feed on control cabbage leaf discs with no toxin, or cabbage leaf discs dipped in Cry1Ac (50 ppm AI, MVP, Dow/Mycogen). Toxin concentrations were calculated to be high enough to kill larvae that were heterozygous for recessive resistance genes, but not homozygotes (Fig. 1). Cry1Ac bioassay survivorship was recorded at 54% for BC1a and 56% BC1b. Survivors were reared to adults and snap frozen at −70 °C.

Single pair matings between the laboratory strain, Waite (>200 generation), and recent field collected strain, Cowra (three laboratory generations), were performed to generate a backcross family with greater genetic diversity than using the Waite strain alone. Cross CW1 was established for southern hybridisation analysis, from a single pair mating between Waite and Cowra, then an F_1 female backcrossed to a single Waite male.

2.2. DNA isolation

DNA isolation was carried out according to (Zraket et al., 1990). Insects were homogenised in 1.7 ml eppendorf tubes either by hand using disposable pestles or in a Mixer Mill (MEP Instruments, Retsch MM300) for 2 min at 20 rpm with a tungsten bead. DNA was extracted three times with phenol, once with chloroform, incubated with 1 µl RNase A (10 mg/ml), precipitated (two volume ethanol, 0.4 M NaCl) and resuspended in 50 µl of Tris–EDTA (TE) buffer (pH 8.0). Genomic DNA (gDNA) was assessed for quality using agarose gel electrophoresis and bands quantified with Kodak 1D software.

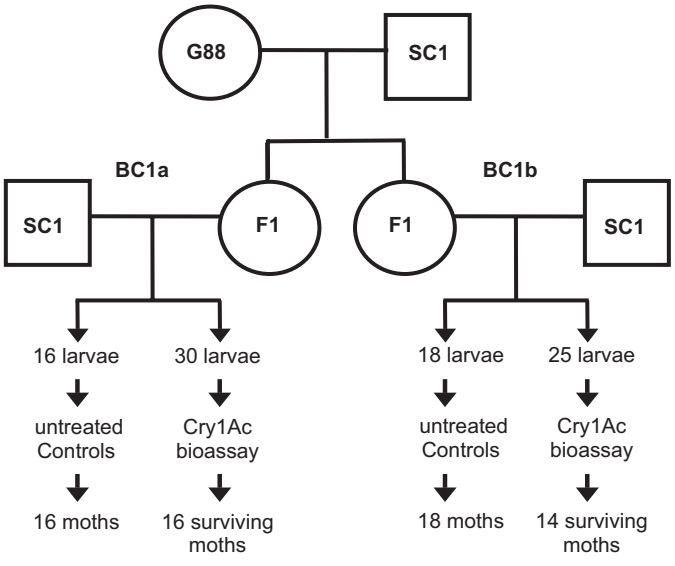

Fig. 1. Backcross and bioassay strategy. A susceptible female (Geneva 88) and resistant male (SC1) were crossed to produce F_1 progeny. Two females were backcrossed to separate SC1 males, to produce families BC1a and BC1b. Backcross progeny in untreated control groups are expected to segregate for Cry1Ac resistance or susceptibility at a 1:1 ratio. The Cry1Ac bioassay killed susceptible backcross offspring.

2.3. AFLP template preparation and analysis

AFLP analysis was performed according to Vos et al. (1995). Genomic DNA was extracted from individual moths and 100–200 ng digested with *Mse*1 (New England Biolabs) and *Eco*R1 (Promega). Double stranded oligonucleotide adaptors were ligated to the sticky ends of digested DNA with T4 DNA ligase (Promega) and pre-amplified using primers complementary to the adaptor sequence to obtain template for AFLP analysis. To visualise AFLP products in the second selective PCR reaction, the EcoR1 primer was end-labeled with γ-^{32}P or γ-^{33}P (Perkin-Elmer) using T4 kinase (Promega) (Vos et al., 1995). Eco primers with either two or three selective bases were used in combination with Mse primers containing three selective bases. Amplification was performed with Taq polymerase in storage Buffer A (Promega) using an iCycler thermocycler (BioRad). Amplified products were separated on 6% polyacrylamide gels (Astral Scientific) under a current of 75 mA, and maximum voltage of 1750 V. Gels were dried under vacuum and were exposed on X-OMAT film (Kodak) for 1–7 days depending upon the isotope label used.

Genomic DNA was extracted and AFLP analysis performed using SC1 family BC1a which included grandparents (SC1 × G88), BC1a parents (F_1 × SC1 male) and 32 backcross progeny including 16 Cry1Ac bioassay survivors and 16 untreated controls. All AFLP bands were scored manually, by careful examination of X-OMAT film on a light box. Bands present in the F_1 female that segregated in the backcross progeny were scored using a ternary system of applying a score of "1" if the band is present in an individual or "0" if the band is absent in an individual and "2" if the result was unclear or amplification failed for an individual. Only female informative AFLP bands were used to identify linkage groups. LG1 is the sex chromosome (Z), and names LG2-29 were assigned based on the number of AFLP markers located on each linkage group.

2.4. Primer design for candidate resistance genes

Degenerate primers were designed for amplification of *P. xylostella* DNA from clustalW alignments using MacVector version 7.0 (Accelrys) for the following sequences: PxAPN-D primers were designed from *S. litura* APN, *L. dispar* aminopeptidase N3, *Helicoverpa punctigera*

aminopeptidase 2, *B. mori* APN, and *H. virescens* 110 kDa aminopeptidase (GenBank accession nos.: AAK69605, AAL26894, AAF37559, BAA33715, and AAK58066). PxAPN-E primers were designed from *Mus musculus*, *Homo sapiens*, *Drosophila melanogaster* and *Anopheles gambiae* sequences (GenBank accession nos.: AAH09653, NP_006301, AAG48733 and EAA10722). Bre2-like primers were designed from *D. melanogaster*, *A. gambiae*, *Apis mellifera* sequences (GenBank accession nos.: NP_609184, XP_310860, and XP_001122156), and conceptual translation of *B. mori* whole genome shotgun sequence (GenBank accession no.: BAAB01085195). ALP primers were designed from *B. mori*, *D. melanogaster*, and *A. gambiae* sequences (GenBank accession nos.: BAA34926, AAL13899, and XP_308522).

PCR products were generated using Taq polymerase in storage buffer A (Promega) and sequenced using BigDye Terminator (Applied Biosystems). Gene specific primer design was performed using Primer3 (Rozen and Skaletsky, 2000) or Oligo 6.4 (Molecular Biology Insights) (Table 1). DNA sequences were analysed using Sequencher version 4.2 (Gene Codes).

2.5. Southern hybridisation

One microgram of genomic DNA from parents and eight progeny of cross CW1 were digested with *Xba*I (Promega, cat. R6185) for 2 h and run on a 1% agarose gel for 16 h at 30 V. DNA was transferred to Hybond N+ membrane (Amersham Pharmacia Biotech) and cross-linked with ultraviolet light. Probes were PCR amplified from Waite fourth instar larval cDNA (primers PxAPN-C-F1 and PxAPN-C-R6), labeled with α-^{32}P using Prime-a-Gene Labeling System (Promega) and incubated overnight at 65 °C.

2.6. Phylogenetic reconstruction

The phylogenetic reconstruction implemented two methods. Phylogenetic analysis of APN proteins was performed using the neighbour-joining (NJ) method (TREECON) based on ClustalX alignments (www.ebi.ac.uk). Distance calculations were performed after Tajima & Nei and bootstrap analysis, running 1000 bootstrap samples (Felsenstein, 2004). Bayesian interference was implemented in MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). The tree was constructed using a Drosophila APN sequence as outgroup. The Marcov Chain Monte Carlo runs were carried out for 1,000,000 generations, after which log likelihood values showed that equilibrium had been reached after the first 400 generations in all cases, and those data were discarded from each run and considered as "burnin". Two runs were conducted per dataset showing agreement in topology and likelihood scores. The neighbour-joining and the Bayesian tree topologies including their general subfamily relationships were in agreement.

3. Results

3.1. Linkage group identification using AFLPs

Grandparents, parents and 32 backcross progeny from BC1a were analysed using AFLPs. The origin of each AFLP band could be traced from the progeny to the mother or father, then to the respective grandparents, to determine whether markers were inherited from the Bt resistant strain (SC1) or susceptible strain (Geneva 88). To identify linkage groups, BC1a progeny were scored for AFLP bands inherited from the F_1 mother that were absent in the SC1 father, relying on the absence of crossing over in oogenesis. In total, 247 AFLP markers were identified from 22 primer combinations, and these were grouped according to common segregation patterns to identify linkage groups. 208 AFLPs were arranged into 29 of the expected 31 linkage groups, containing between two and eighteen AFLP markers each (see Heckel et al. (1999) for method). Thirty-nine singleton AFLPs with unique banding patterns were not assigned to linkage groups. Since two of the 31 chromosomes were not marked with AFLP groups, they may be marked by singletons. However, without additional data, it is not possible to determine which are *bona fide* segregating markers and which are AFLP artefacts. Causes of such AFLP artefacts may include mispriming in some individuals, incomplete template preparation (such as partially digested DNA) or by amplification of foreign (e.g. bacterial) DNA present in the moth at the time of genomic extraction. Banding patterns from each of the 29 AFLP marked linkage groups were used to create "chromosome print" templates to enable genes to be mapped to linkage groups.

3.2. Associating a linkage group with Cry1Ac resistance

Progeny that survived toxin bioassays were expected to inherit resistance alleles from SC1 transmitted by the heterozygous mother, while untreated progeny may receive either resistant SC1 or susceptible G88 alleles. For each linkage group, a 2×2 χ^2 contingency table was used to compare SC1-derived vs. Geneva 88-derived homologues among untreated controls and Cry1Ac treated survivors. Over-representation of a given SC1-derived linkage group among bioassay survivors yields a positive association with resistance. In this case, a factor such as a resistance gene or genes found in the SC1 strain may be located on this chromosome, enabling larvae to survive the toxin dose. Under-representation of the SC1 homologue with concomitant over-representation of the linkage group homologue inherited from the susceptible Geneva 88 strain produces a negative association with resistance. This would indicate the presence of unexpected resistance genes in the susceptible strain (transgressive segregation) or genes improving overall fitness, but not toxin resistance per se. Since the standard χ^2 statistic is always non-negative, a sign was assigned to each χ^2 value to show whether the

Table 1
Primer sequences and GenBank accession numbers

Gene description	GenBank accession no.	Primer name	Primer sequence 5′–3′
PxAPN-A (APN Class 1)	AF020389	PxAPN-A-F1	GTGCTCGACTTCCTGTGCAACGTGGGTCA
		PxAPN-A-R2	ACCGGCGCAGTACACCCACGGCCTCATGTT
PxAPN-C (APN Class 2)	X97878	APN-2-F1	TGTCTCCACGGCAAYGARGAYTGYGT
		APN-2-R2	GCAGACACGAGAACTGAATGTC
		APN-2-F3	GCCGATAGGAGGGACTTCTTATTC
		APN-2-R4	GCATGCTCCTGAGTAGTGAGTG
		APN-2-F5	GAACATCCTCAATGCCTT
		APN-2-R6	GCAGCGTTTTGAGAGTTCCACG
PxAPN-D1	EF634236 (gDNA)	PxAPN-D-F1	GGTGCTATGGAAAACTGGGGNATGGT
		PxAPN-D-R2	GCCACAGGTTTGACCACCARAARCA
	EF634235 (cDNA)	PxAPN-D-F3	AAGGCTTTCCCNTGYTTYGAYGARCC
		APN-D1_spR	GTGGCTATGAAGATTTGGTTCTG
		PxAPN-D1-mapF	TCCAACATGGCTATCGAGAA
		PxAPN-D1-spR2	TTGAAGAATCATCACCATCAGC
		PxAPN-D1-mapR	TCAAATCTCAGGTGACTATGGAA
PxAPN-D2	EF579958 (gDNA)	PxAPN-D-F1	GGTGCTATGGAAAACTGGGGNATGGT
		PxAPN-D-R2	GCCACAGGTTTGACCACCARAARCA
	EF579956 (cDNA)	PxAPN-D-F3	AAGGCTTTCCCNTGYTTYGAYGARCC
		APN-D2_spR	GTGGCTGTGCGCAGCTTTCCCGG
		APND-D2-mapF	GGTGCTATGGAAAACTGGGGNATGGT
		APND-D2-mapR	GCCACAGGTTTGACCACCARAARCA
PxAPN-E	EF579959	PxAPN-E-F1	CTTTCGACATCGCCCTGGA
		APN-E-deg-R	GGTCACGAACTGGGTCCADATRTCRTA
PxAPN-F (APN Class 5)	AJ222699	PxAPNF-i5f	ATCAATGGACTTGGCAGACC
		PxAPNF-i5r	CCAGTCGGACAGGAAGTCAG
PxAPN-G	EF579957	PxAPN-G F1	TTCGGAGACTTGGTTACTT
		PxAPN-G R1	TATGAGTTTAGGCCATTAGC
		PxAPN-G-F2	TGGTGGAGTGATACCTGGATGA
		PxAPN-G-R2	GCCGTGTTACCAAGGAGATGGT
PxAPN-H	BP937205	PxAPN-H_F1	TTGAGAGGCGAAGACAGGACCA
		PxAPN-H_R1	GAGCCAGTTGAAGCCGGTGAT
		PxAPN-H_F2	CATACGCGCGGTCTGGCAT
		PxAPN-H_R2	GGTAGCCATAGCCCGTGAACCC
Alkaline phosphatase	EF579960	PxALP-degF	ACNGCNGAYCAYGCNCAYGTNATG
		PxALP-degR	ACRTCRTCNCCNCCRTGNGTYTC
		PxALP-spF	TGATATCCTCGGAACTGTGG
		PxALP-spR	ACCATGCCTGGGTACTCGT
Px bre-2 like	EF579961	Bre2-degF3	CCCGACTTCGCNACNGGNCCNGCNTA
		Bre2-degR4	TTNCKCCANAGRTCRAAYTGYTC
		Pxbre2_spR	GGGGTGGTACGACACCTTCTT
Insect intestinal mucin	AF545582	PxIIM-F1	AAGAAGTGCGCTGAAGGCTGCAA
		PxIIM-R2	TTCTCAGCAGTAGCCTGGGCGTT
P252-LIKE	BP937461	PxBP937461_F3	TTGTGTACAGCTGCCGTAATA
		PxBP937461_R4	CTCCAGTGATTTCGGCACATT
	BP937152	PxBP937152_F3	ATAGCTGCGTTGACTTGGGAAA
		PxBP937152_R4	CAATGTCGGGTAAATAGAAAC

homologue positively associated with bioassay survivorship was inherited from the resistant SC1 strain (+ value) or susceptible Geneva 88 strain (− value).

A single linkage group, LG22, was significantly positively associated with Cry1Ac resistance ($\chi^2 = +9.3$, d.f. = 1, $P < 0.005$). The Bonferroni correction for multiple comparisons was applied to the data set to assess the overall significance of this result, given that 29 independent tests had been conducted. None of the linkage groups were significantly associated with resistance at this level (Bonferroni correction: $0.05/29 = 0.0017$).

AFLP analysis was performed on the second female informative backcross family, BC1b, reared using the same cross design as BC1a. The F_1 females were siblings, and had a 50% chance of inheriting the same AFLP bands from their parents. BC1b parents, 18 untreated controls, and 14 Cry1Ac survivors were analysed for three *Eco*R1-*Mse*1 primer combinations, producing LG22 AFLP bands that were also present and segregating in the BC1b family (EagaMcta, EatMcta, EatMcac). Analysis of the BC1b maternal banding patterns from all three AFLP markers confirmed that 11 of 18 untreated control progeny, and 14 of 14 BC1b Cry1Ac survivors inherited LG22 from the SC1 grandparent. Combining data from the two families, 18/34 untreated controls and 29/30 Cry1Ac bioassay survivors inherited LG22 from the SC1 strain, providing a positive association with resistance that was significant at the Bonferroni level for 29 comparisons ($\chi^2 = +15.6$, d.f. = 1, $P < 0.0001$) (Fig. 2).

3.3. Mapping genes encoding Bt-toxin binding proteins to linkage groups

Candidate Bt resistance genes were identified using degenerate PCR or database searching, and mapped to linkage group by matching a segregating polymorphism in BC1a to one of the 29 "chromosome prints". Numerous strategies were implemented to identify genetic polymorphisms and map genes to linkage groups, including PCR-RFLP, direct sequencing, denaturing HPLC, PCR size polymorphism, and southern hybridisation (supplementary figures 1–4).

3.3.1. PxAPN-A (assigned to APN Class 1 in Herrero et al. (2005))

Chang et al. (1999) identified the APN gene PxAPN-A (GenBank accession no.: AF020389) from a *P. xylostella* midgut cDNA library probed with *M. sexta* aminopeptidase, MsAPN-1. An imperfect "GT" dinucleotide

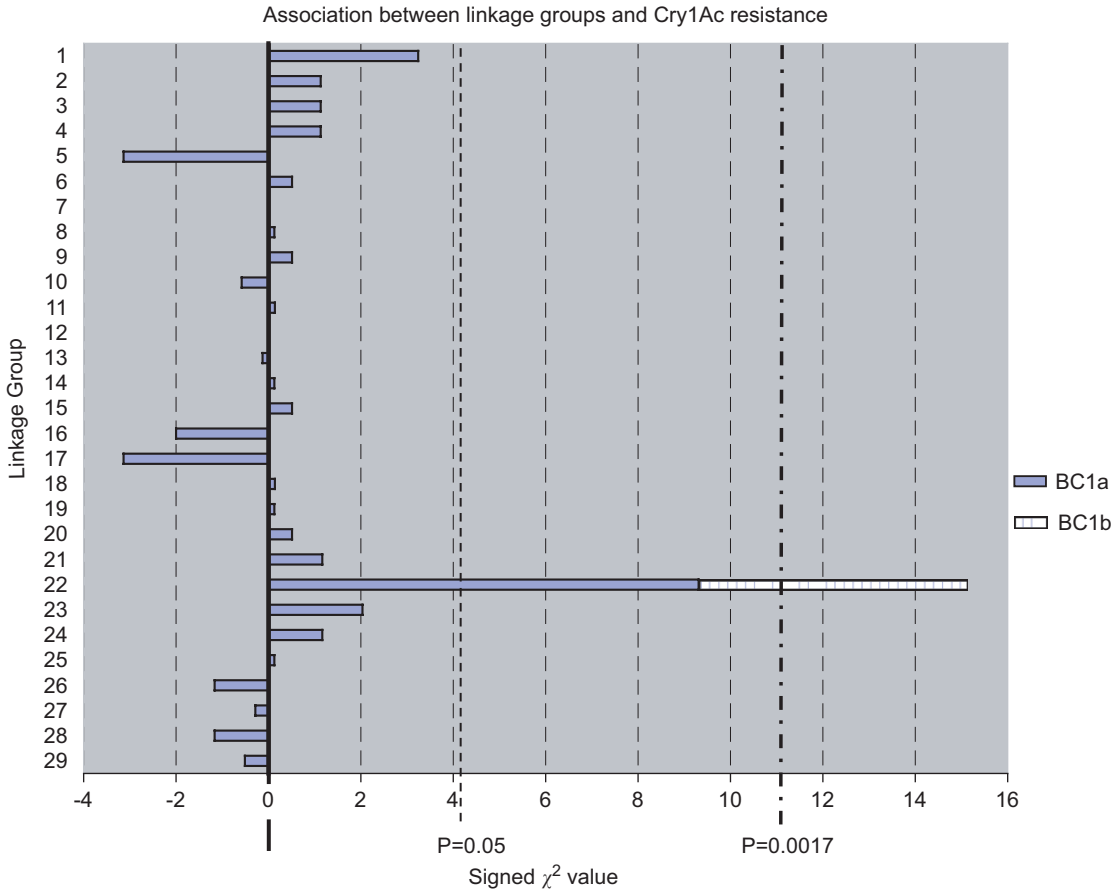

Fig. 2. Association between linkage groups 1–29 and resistance to Cry1Ac. The χ^2 values were calculated using 2×2 contingency tables that compare untreated controls to Cry1Ac bioassay survivors. A sign (+ or −) was assigned to the numerical value to indicate the direction of the deviation from the null hypothesis. Positive χ^2 values indicate over-representation of chromosomes inherited from the SC1 grandparent via the F_1 female in bioassay survivors relative to untreated controls; negative χ^2 values indicate their under-representation. Tests were conducted for BC1a linkage groups 1–29, and a single linkage group (LG22) was associated with resistance (solid bars ($\chi^2 = +9.3$, d.f. = 1, $P < 0.005$)). LG22 was then tested for an association with resistance in BC1b using AFLP markers. The combined BC1a and BC1b association with resistance was significant at the Bonferroni level (striped bar ($\chi^2 = +15.6$, d.f. = 1, $P < 0.0001$)).

microsatellite repeat [$(TG)_3TA(TG)_8$] within the tenth intron of PxAPN-A (GenBank accession no.: AY699799) was resolved using acrylamide gel electrophoresis segregating in BC1a progeny in an identical pattern to AFLP linkage group 11. No association with that linkage group and Cry1Ac resistance was observed.

3.3.2. *PxAPN-F (assigned to APN Class 5 in Herrero et al. (2005))*

PCR primers were designed for PxAPN-F based upon published sequence (GenBank accession no.: AJ222699). Genomic DNA from the SC1 BC1a mapping family was PCR amplified with α^{32}P-dATP and samples run on 6% denaturing polyacrylamide gel, dried, and exposed to film. A segregating size polymorphism was observed, and PxAPN-F was mapped to LG11, the same linkage group as PxAPN-A, which was not associated with resistance.

3.3.3. *PxAPN-E*

Degenerate PCR amplified a 599 bp fragment from genomic DNA, containing no predicted introns (GenBank accession no.: EF579959). The gene fragment was similar

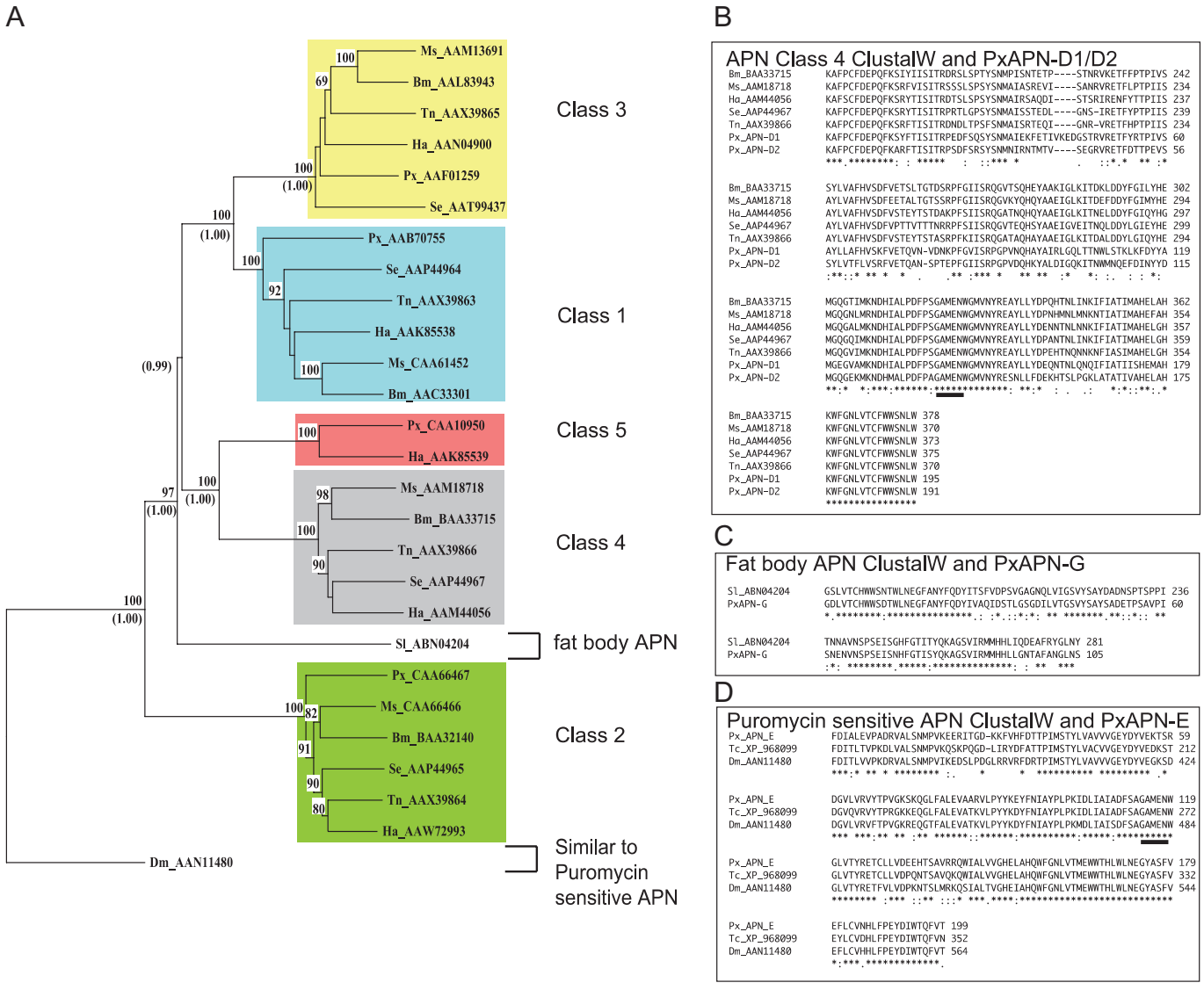

Fig. 3. Gene phylogeny of aminopeptidase N protein sequences. (A) Neighbour-joining (NJ) consensus tree of APN sequences from *Spodoptera exigua* (Se), *Spodoptera littoralis* (Sl), *Helicoverpa armigera* (Ha), *Manduca sexta* (Ms), *Bombyx mori* (Bm), *Plodia interpuctella* (Pi), *Trichoplusia ni* (Tn), *Plutella xylostella* (Px), and *Drosophila melanogaster* (Dm). Bootstrap values from NJ analyses are shown as percentages, and Bayesian posterior probabilities are shown for all major nodes in parenthesis. Partial sequence information was available for Sl_ABN04204 (766 amino acids) hence sequence gaps were trimmed for tree construction. (B) PxAPN-D1 and PxAPN-D2 ClustalW alignment with APN Class 4 proteins listed in the phylogeny. A conserved GAMEN motif is underlined. (C) Alignment of *P. xylostella* midgut EST fragment PxAPN-G (GenBank accession no.: EF579957) with a fat body aminopeptidase from *Spodoptera litura* (GenBank accession no.: ABN04204). Percentage identity from a pairwise alignment is 70.5%. (D) Alignment of *D. melanogaster* puromycin sensitive aminopeptidase N (GenBank accession no.: AAN11480), and partial protein sequences of *P. xylostella* PxAPN-E and *T. castaneum* (Tc) aminopeptidase N (GenBank accession no.: XP_968099). Percentage identity from pairwise alignment of this region: Px-Tc 82.9%, Px-Dm 80.5%, Dm-Tc 82.1%.

to a *D. melanogaster* APN (GenBank accession no.: AAN11480) and had no homology to known lepidopteran APN proteins and did not fall into a known APN class (Fig. 3). Fifteen progeny from BC1a were sequenced and PxAPN-E was mapped to LG4, which had no association with Cry1Ac resistance.

3.3.4. *PxAPN-D1 and PxAPN-D2*

Two products with similarity to class 4 APN proteins (Herrero et al., 2005) were amplified from genomic DNA using one pair of degenerate oligonucleotides, PxAPN-D1 (GenBank accession no.: EF634236) and PxAPN-D2 (GenBank accession no.: EF579958). Anti-sense gene specific primers were designed for both products and paired with an additional common degenerate primer. Additional sequence was then obtained for PxAPN-D1 (GenBank accession no.: EF634235) and PxAPN-D2 (GenBank accession no.: EF579956) using total larval cDNA (Waite strain) (Fig. 3), confirming gene expression of both products.

Specific primers for PxAPN-D2 (APN-D2-mapF and APN-D2-mapR) were designed across an intron to scan for polymorphisms in the SC1 BC1a mapping family. An intron size polymorphism was identified and scored using denaturing HPLC. An informative polymorphism was identified and PxAPN-D2 was mapped to linkage group 11.

Primers specific for *PxAPN-D1* (PxAPN-D1-mapF and PxAPN-D1-spR2) were used to amplify from the SC1 BC1a parents. PCR products were cloned and sequenced to search for suitable polymorphism to enable gene mapping in the backcross progeny. A single base polymorphism was identified, and PxAPN-D1 was mapped to linkage group 11 by direct sequencing using primer pair PxAPN-D1-mapF and PxAPN-D1-mapR.

3.3.5. *PxAPN-C (assigned to APN Class 2 in Herrero et al. (2005))*

Lack of detectable polymorphism in regions tested prevented APN2 (GenBank accession no.: X97878.1) from being directly mapped to a linkage group in BC1a (Primers APN-C-F1x-R2; -F3x-R4; -F5x-R6). A southern blot created with parents and progeny from a different mapping family, CW1, showed the same female informative segregating pattern when probed with PxAPN-D2 and PxAPN-C, enabling this gene to be assigned to linkage group 11.

3.3.6. *PxAPN-G*

EST clones sequenced from a *P. xylostella* larval midgut cDNA library were analysed for homology to aminopeptidase like proteins (library constructed and clones identified by HV). Pairwise sequence identity of 70.5% (74/105 amino acids) was observed between a fat body aminopeptidase identified from *S. litura* (GenBank accession no.: ABN04204) and PxAPN-G (GenBank accession no.: EF579957; Fig. 3). Specific primers were designed (APN-G-F1 and APN-G-R1) and a 319 bp product was amplified from Waite larval cDNA, confirming gene expression. A second primer set (APN-G-F2 and APN-G-R2) was designed within this region for SC1 BC1a gene mapping, as existing primers failed to amplify from genomic DNA. Eleven SC1 BC1a progeny were amplified and analysed using denaturing HPLC. A polymorphic pattern was detected, mapping this gene to LG11. This APN gene did not fall into any of the known APN classes (Fig. 3) (Herrero et al., 2005).

3.3.7. *PxAPN-H*

An EST clone from *P. xylostella* whole body third instar larvae (GenBank accession no.: BP937205) with homology to APN proteins was identified. Conceptual translation of bases 18–455 produces a 145 amino acid sequence with identity to APN Class 3 proteins from *B. mori* (68%), *H. armigera* (66%), *M. sexta* (65%), and *P. xylostella* (65%) over the homologous region (GenBank accession nos.: AAL83943, AAN04900, AAM13691, AAF01259). Segregating size polymorphism mapped PxAPN-H to LG11, in the BC1a progeny.

3.3.8. *PxAPN-B (assigned to APN Class 3 in Herrero et al. (2005))*

A PxAPN-B (GenBank accession no.: AAF01259) was not identified using PCR amplification in susceptible or resistant *P. xylostella* strains analysed in this study. Multiple attempts to amplify different regions of this gene failed and consequently PxAPN-B could not be assigned to a linkage group.

3.3.9. *Alkaline phosphatase (ALP: EC 3.1.3.1)*

A gene fragment encoding ~85 amino acids was amplified using degenerate primers, with 48% identity with *B. mori* membrane bound ALP (GenBank accession no.: BAB62745) and 56% identity to *B. mori* soluble ALP (GenBank accession no.: BAB62746). Genetic mapping of PxALP (GenBank accession no.: EF579960) was accomplished using an intron size polymorphism identified with specific *P. xylostella* ALP primers (PxALP-spF and PxALP-spR) and ALP was mapped to linkage group 14. There is no association between this linkage group and Cry1Ac resistance ($\chi^2 = 0.13$, d.f. = 1, $P > 0.7$).

3.3.10. *Bre-2-like*

BLAST searches of *C. elegans* gene *bre-2* (GenBank accession no.: AY533304) identified low levels of sequence similarity to *D. melanogaster* galactosyltransferase gene CG8668. Degenerate PCR amplified a *P. xylostella* product (GenBank accession no.: EF579961) with 48% identity and 74% similarity to CG8668. A degenerate forward primer (PxBre2-degF3) and specific reverse primer (PxBre2_spR) were used for PCR-based mapping. An SNP was identified in the BC1a F_1 female, and sequence analysis mapped this gene to LG4, the same linkage group as

PxAPN-E. There was no association between Cry1Ac for LG4.

3.3.11. Insect intestinal Mucin

The peritrophic matrix (PM) protects the larval midgut epithelium from damage by food or pathogens. The proteinaceous gel structure of the matrix contains a mesh of chitin fibrils cross-linked with proteins that vary in size from 15 to 220 kDa. Microorganisms can become entrapped within the PM through binding to carbohydrate groups on these proteins. (Sarauer et al., 2003) identified 12 associated and 18 loosely associated PM proteins in *P. xylostella* including an insect intestinal mucin (IIM) (GenBank accession no.: AF545582). IIMs are glycosylated, providing elasticity as well as protection against pathogen invasion. Although unlikely to be a major mechanism for Cry1A resistance, altered glycosylation patterns or mutations within the IIM gene could potentially bind toxin and prevent passage through the PM.

The mucin sequence encodes a high percentage of threonine, alanine, and proline residues, often in repeated stretches. Primers were designed to avoid repeat regions. A 197 bp product was amplified and PCR-RFLP mapped this IIM to LG26, which is not associated with resistance.

3.3.12. P252-like glycoproteins

A 252 kDa protein (P252) isolated from the brush border membrane of *B. mori* larvae bound to Cry1A toxins and could potentially be involved in Bt resistance (Hossain et al., 2004). Hossain et al. (2004) sequenced two internal peptides, ATYLAGSGGVVPVCN and ATYTLNSDNTITVFN, which share 12/15 and 15/15 amino acid matches with the same, recently identified *B. mori* 15-lipocalin domain protein, *chbp* (GenBank accession no.: NP_001037071) (Mauchamp et al., 2006). Two lipocalin-like sequences were identified from published *P. xylostella* ESTs, BP937152 and BP937461, whose top BLAST hits were the *B. mori chbp* gene (expect values 2e-28 and 6e-48, respectively).

Primers designed for BP937152 amplified a 313 bp product from BC1a genomic DNA and contained two introns. Polymorphic variation was observed using denaturing HPLC analysis (with an oven temperature of 54 °C), which mapped to LG10, which is not associated with resistance to Cry1Ac. A 441 bp product was amplified within the EST BP937461, spanning 246 bp of coding and 195 bp of intronic sequence; however, a segregating polymorphism was not detected in the SC1 BC1a mapping family.

4. Discussion

Mode 1 type Bt resistance in Lepidoptera has recessive inheritance, shows reduced Cry1A toxin binding to receptors, has little or no cross-resistance to Cry1C and 500-fold resistance to at least one Cry1A toxin (Tabashnik et al., 1998). We have used AFLP linkage mapping to assess the Cry1A resistance contribution from 29 of the 31 linkage groups in a *P. xylostella* strain (SC1) with mode 1 resistance. Here, we have shown a recessive factor on LG22 in the SC1 strain controls mode 1 type resistance. A candidate gene approach was undertaken to determine whether the genes encoding Bt binding proteins identified from biochemical studies are on the same chromosomes as the Cry1A resistance factor.

Aminopeptidase N proteins have been identified as Bt receptors in many insects (Knight et al., 1995, Nakanishi et al., 1999) and five distinct APN classes have been identified in Lepidoptera (Herrero et al., 2005). Here, APN genes from four of the five known classes were mapped to linkage groups in the SC1 strain including PxAPN-A (Class 1), PxAPN-C (Class 2), PxAPN-D1 plus PxAPN-D2 (Class4) and PxAPN-F (Class5). In addition, other means were used to identify partial sequence for three APN genes, two of which, PxAPN-E, PxAPN-G, did not fall into any of these five classes and PxAPN-H which has 68% identity with a *B. mori* Class 3 APN protein. Of the eight aminopeptidase genes analysed, seven mapped to the same linkage group (LG 11), and PxAPN-E mapped to LG4. All these APN genes have therefore been rejected as Bt resistance genes as none map to LG22. Despite repeated attempts, one published *P. xylostella* APN gene, PxAPN-B, was not PCR-amplified from genomic DNA or larval cDNA in resistant or susceptible strains. In this case, intron size may have been too large to amplify across using PCR or larval expression may have been absent from strains analysed here. With this exception, and unless additional, previously unpublished classes become available, APNs can be ruled out as the major Cry1A resistance gene in *P. xylostella*. Although Luo et al. (1997) found that Cry1Ac could bind a 120 kDa APN on ligand blots from preparations of Bt susceptible larvae and Cry1A resistant larvae (in the NO-QA strain, which carries the same resistance mechanism as SC1), binding is unlikely to occur in vivo, as direct analysis of NO-QA BBMVs fail to bind the same toxin (Ballester et al., 1999).

Of the eight APN genes mapped to linkage groups here, seven mapped to linkage group 11. Although the physical distance between these genes remains unknown, BLAST searches of *B. mori* whole genome sequence identifies APN class 1 (GenBank accession no.: AF084257) and APN class 4 (GenBank accession no.: AB013400) genes on the same 40,950 bp contig (GenBank accession no.: AADK 01000258) providing some evidence for APN gene clustering in this species.

Griffitts et al. (2005) identified four Bt resistance genes (*bre-2*, *bre-3*, *bre-4*, and *bre-5*) in the same biosynthetic pathway for a membrane glycolipid in *C. elegans*. Additionally, they demonstrated that glycolipids from *M. sexta* midguts could bind Bt toxin (Cry1Aa, Cry1Ab, and Cry1Ac). It is possible a similar mechanism for resistance exists in the SC1 strain—lesion of a glycosyltransferase enzyme that would normally assemble a target for Bt toxin to bind. Only one *P. xylostella* gene with

similarity to *bre-2* was successfully mapped, and that to LG4, the same linkage group as PxAPN-E. Many more glycosyltransferases are likely to be expressed in *P. xylostella*; however, degenerate PCR has failed to identify other homologues at this stage, probably due to high evolutionary divergence between moths and nematodes. Griffitts et al. (2005) proposed monitoring glycolipid-mediated resistance using methods such as thin layer chromatography in field and lab resistant populations of insects and nematodes. We are currently analysing midgut glycolipid extracts from *P. xylostella* Cry1A resistant NO-QA and susceptible strains to determine whether any glycolipid differences can be visually observed.

Bombyx mori has two ALP genes, encoding a membrane bound and soluble form, respectively, that are expressed in the midgut and share 60% amino acid identity (GenBank accession no.: AB055428). Both genes lie within a 12 kb region, are in the same transcriptional orientation, and have been mapped to *B. mori* chromosome 3 (Itoh et al., 2003). If ALP is a functional Bt receptor, the membrane bound form would be the predicted toxin target, rather than the soluble form. The ALP gene fragment amplified from *P. xylostella* is more similar to the *B. mori* soluble form of alkaline phosphatase; however, if both membrane and soluble forms of ALP are found in *P. xylostella*, we speculate they will also be linked and map to the same linkage group.

Although mutations in the genes encoding the candidate Bt receptors described here are rejected from playing a direct role in Cry1Ac resistance, the proteins themselves may still play a role in the mode of action and the resistance pathway. For example, a transcription factor or DNA binding protein may prevent expression of an APN gene, or post-translational modifications of wild type proteins could still be required for Bt binding. Quantifying expression levels of these genes in susceptible and resistant strains will be of interest, to determine whether a trans-acting transcription factor regulating the expression of an aminopeptidase (for example) is responsible for Cry1A resistance. Herrero et al. (2005) demonstrated the lack of APN-1 expression in an *S. exigua* strain resistant to Cry1C when compared to a susceptible strain. An indepth linkage analysis using an existing *P. xylostella* strain that is only resistant to Cry1C (BCS-Cry1C-2) will be necessary to identify the genetic loci encoding Cry1C resistance (Zhao et al., 2001).

Here we have ruled out eight APN genes, a *bre-2* like gene, a P252-like gene, an IIM and an ALP gene as Cry1A type resistance genes in the SC1 strain. Thus far, a candidate gene approach has failed to identify genes responsible for Bt resistance in *P. xylostella* and positional cloning of the locus may be required. Fine scale mapping and positional cloning of quantitative trait loci in non-model organisms is becoming possible with the increasing ability to develop species specific genomic resources, as shown recently in three-spined sticklebacks (Colosimo et al., 2005). Improving the genomic tools available for *P. xylostella*, such as creating a bacterial artificial chromosome (BAC) library of the genome and sequencing tissue-specific cDNA libraries, will facilitate the discovery of genes involved with quantitative traits in this agricultural pest.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ibmb.2007.09.014.

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