Parallel Evolution of *Bacillus thuringiensis* Toxin Resistance in Lepidoptera

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ABSTRACT Despite the prominent and worldwide use of *Bacillus thuringiensis* (Bt) insecticidal toxins in agriculture, knowledge of the mechanism by which they kill pests remains incomplete. Here we report genetic mapping of a membrane transporter (*ABCC2*) to a locus controlling Bt Cry1Ac toxin resistance in two lepidopterans, implying that this protein plays a critical role in Bt function.

INSECTICIDE-RESISTANT phenotypes commonly arise through parallel mutations in the same gene across multiple species. However, independent resistance mechanisms can also exist within a single species. For example, resistance to dichlorodiphenyltrichloroethane (DDT) among many arthropods is caused by amino acid substitutions in the voltage-gated sodium channel (Davies *et al.* 2007), yet DDT resistance can also be achieved in *Drosophila melanogaster* through increased expression of the detoxifying enzyme cytochrome P450 CYP6G1 (Daborn *et al.* 2002). Identifying a specific insecticide resistance mutation in one organism provides candidate resistance genes to test in other species and strains.

The bacterium *Bacillus thuringiensis* (Bt) produces insecticidal toxins used for controlling agricultural pests as foliar sprays or by expressing toxin genes in transgenic plants. Insecticidal activity of the ~200 characterized Bt toxins varies considerably between insect orders (Schnepf *et al.* 1998), and they exhibit a lower impact on nontarget species than conventional pesticides do (Gatehouse *et al.* 2011). To kill lepidopteran pests, Bt toxins must be ingested by caterpillars, become activated by gut proteases, and then bind to midgut receptors (Soberon *et al.* 2009). Two mechanisms have been proposed for the subsequent steps in the toxins' mode of action: (i) pore formation in midgut epithelial cells followed by colloid-osmotic lysis or (ii) activation of a signaling cascade after binding to a primary target in the midgut (Soberon *et al.* 2009).

Numerous Bt Cry1Ac-binding proteins have been identified on the midgut brush border membrane, and some have been expressed in cell lines or in *Drosophila* to validate their function (Vadlamudi *et al.* 1995; Nagamatsu *et al.* 1998; McNall and Adang 2003). These studies have produced a suite of candidate genes for genotype–phenotype association tests on Bt-resistant and Bt-susceptible insect strains, to attempt to identify the four separate Bt resistance mutations reported in Lepidoptera (Heckel *et al.* 2007). Mutations within a 12-cadherin domain protein were found to cause Cry1Ac resistance in laboratory selected strains of *Heliothis virescens* (Gahan *et al.* 2001), *Pectinophora gossypiella* (Morin *et al.* 2003), and *Helicoverpa armigera* (Xu *et al.* 2005). However, modified Bt toxins are able to kill *P. gossypiella* that carry cadherin mutations, suggesting the presence of other major Bt-binding targets (Soberon *et al.* 2007). Most recently, genetic mapping has correlated a second and independent Cry1Ac resistance mechanism in *H. virescens* with an inactivating mutation in *ABC transporter C2*, which has not previously been associated with a Bt mode of action (Gahan *et al.* 2010).

Resistance to Bt Cry1A spray formulations has evolved in field populations of the diamondback moth, *Plutella*

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Sequences in this study have been deposited in GenBank Data Libraries under accession nos. JF303656, JF303657, JF825967, JN030490-JN030497, and JN383802–JN383814.
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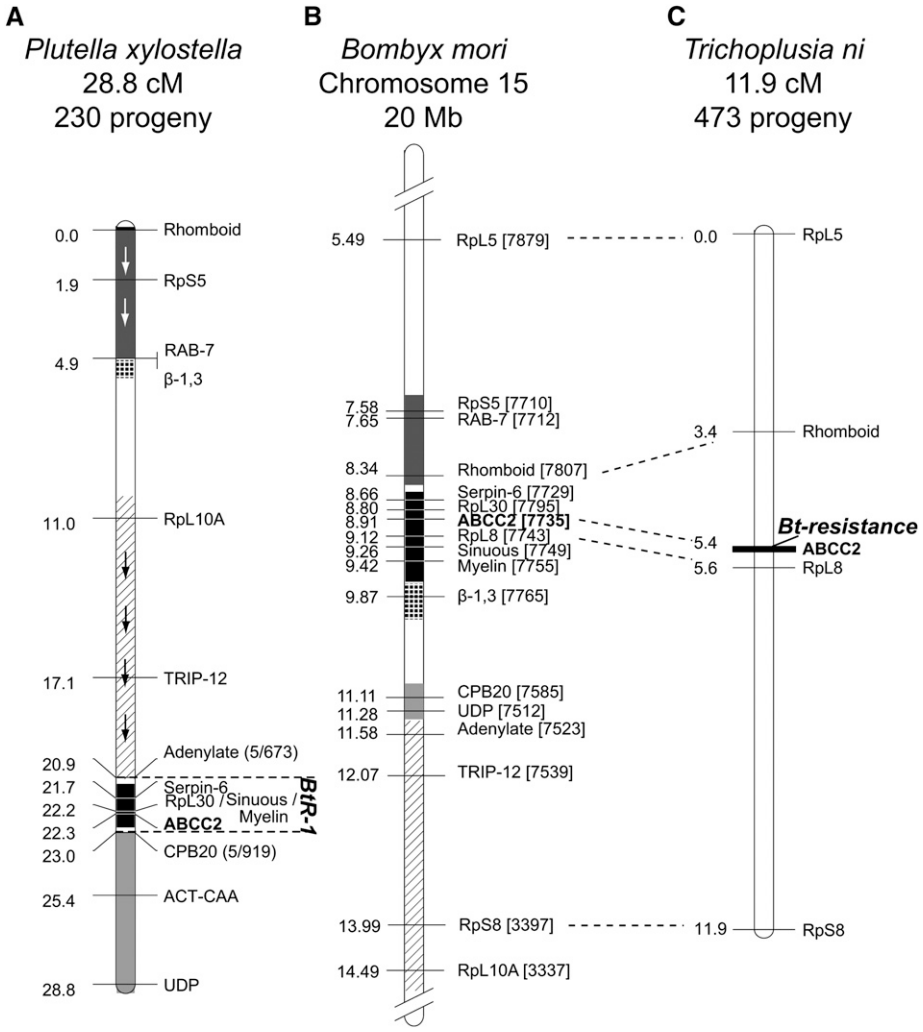

Figure 1 Bt resistance loci in Lepidoptera. Linkage maps for (A) *P. xylostella* and (C) *T. ni* in comparison with (B) *B. mori*'s sequenced chromosome 15 (partial). *P. xylostella* and *B. mori* show multiple chromosomal rearrangements while maintaining genetic synteny. Blocks of common genes are shaded and arrows depict inverted orientation in *P. xylostella*. *T. ni* and *B. mori* show a conserved gene order in this data set. Bt Cry1Ac resistance loci are in complete linkage with the *ABCC2* gene. *P. xylostella* showed low levels of recombination across this region, and five mapped genes are in complete linkage with *BtR-1*. *RpL8* also maps to *BtR-1* (see Table 1), but was not polymorphic in these mapping crosses. Both linkage maps were constructed using JoinMap 3.0 (grouping = LOD 10, Kosambi's mapping function). *P. xylostella* were fed on transgenic Bt Cry1Ac-expressing canola, and *T. ni* had purified Cry1Ac toxin incorporated into their artificial diet. Centimorgan distances may be affected by linkage disequilibrium caused by selection for resistance. *B. mori* gene identifiers refer to the final four numbers (note underlining) of the gene ID (*e.g.*, BGIBMGA003337-TA). Accession numbers are provided in Table S1.

xylostella (Tabashnik *et al.* 1990), and greenhouse isolates of the cabbage looper, *Trichoplusia ni* (Janmaat and Myers 2003). In both species, resistance to Cry1Ab and Cry1Ac toxins is brought about by a single recessive, autosomal locus with reduced toxin binding in the midgut (Tabashnik *et al.* 1997; Wang *et al.* 2007). This evidence suggests that the likely mechanism for resistance occurs through loss or alteration of a Bt toxin receptor. Genes for the 12-cadherin domain protein, as well as for all other known Bt-binding proteins, have been mapped in *P. xylostella* and are all unlinked to the major gene responsible for field-evolved resistance (Baxter *et al.* 2005, 2008). Here we perform extensive backcrosses and genetic mapping to identify this Bt Cry1Ac resistance locus in *P. xylostella* and *T. ni*.

We used the same crossing strategy for both *P. xylostella* and *T. ni* (Heckel *et al.* 1999). Resistant and susceptible individuals were first crossed, producing F_1 progeny. Crossing over within homologous chromosomes does not occur in female Lepidoptera, so F_1 males were used in backcrosses to produce mapping families, and F_1 females were used in backcrosses for associating candidate resistance genes to a specific chromosome. Backcross progeny from each cross were either reared without insecticide (untreated controls) or exposed to Cry1Ac toxin (bioassay treated). Through this strategy, survivors of Cry1Ac were expected to be homozygous for the recessive resistance mutation, while controls were expected to be either homozygous or heterozygous.

Table 1 Percentage of Bt-susceptible alleles from 39 *P. xylostella* backcrosses with chromosomal crossing over

	Adenylate[a]	*ABCC2*	*RpL8*	*Myelin*	*CPB20*
Bioassay	5/673	0	0	0	5/919
%	0.74	0	0	0	0.54
Controls	95/182	—	—	—	170/325
%	52.2	—	—	—	52.3

P. xylostella crosses were performed using the Bt-susceptible strain Waite and Bt-resistant strains NO-QA (3 families) or NO-QAGE (36 families). Control backcross progeny inherit alleles derived from the Bt-susceptible or Bt-resistant grandparent at a 1:1 ratio.

[a] A total of 246 bioassay survivors from 14 NO-QAGE mapping families did not carry polymorphic variation in the *Adenylate* gene. In these cases, progeny were genotyped for marker *RpL8*, and no susceptible genotypes were observed. Recombinant individuals were genotyped for *ABCC2*, *RpL8*, and *Myelin Proteolipid*, and all inherited Bt-resistant genotypes.

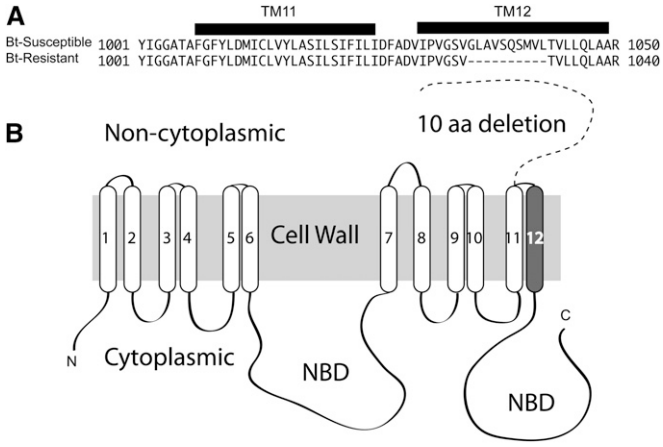

Figure 2 *P. xylostella* ABCC2 schematic. A *P. xylostella* BAC library was screened with partial gene sequence from *ABCC2*, which was identified from an EST library, and two clones were sequenced (25L19 and 11G15). *P. xylostella ABCC2* genomic sequence was predicted on the basis of similarity with the *H. virescens* homolog and then confirmed through amplification from midgut cDNA, using backcross progeny that survived a Cry1Ac bioassay (Bt-resistant) or from the control strain Geneva88 (Bt-susceptible). (A) Partial amino acid alignment of ABCC2 of Bt-susceptible (JN030490) and Bt-resistant (JN030491) *P. xylostella*. A 30-bp, 10-amino-acid deletion is predicted within transmembrane (TM) domain 12. (B) Schematic of ABCC2 displaying 12 transmembrane domains predicted using Phobius (http://phobius.sbc.su.se/) and two nucleotide-binding domains (NBD). The 10-amino-acid deletion in Bt-resistant individuals is predicted to remove TM12, leaving the carboxyl terminus in a noncytoplasmic region (dashed line).

The Bt Cry1Ac resistance locus (*BtR-1*) in the NO-QA strain of *P. xylostella* from Hawaii was originally identified using anonymous AFLP markers (Heckel *et al.* 1999). A sequenced AFLP marker linked to *BtR-1* contained coding sequence for the predicted gene *Thyroid Hormone Receptor Interactor 12* (GenBank JN030496), which has an ortholog located on chromosome 15 of *Bombyx mori* (Baxter *et al.* 2005). As Lepidoptera commonly show conserved chromosomal synteny, predicted proteins from *B. mori* chromosome 15 were compared using BLAST against a *P. xylostella* transcriptome (454-ESTs) to design specific primers for linkage mapping. Sixteen genes (Supporting Information, Table S1) were mapped in most progeny in backcrosses to NO-QA (3 families, 184 bioassay survivors, 46 controls), and a linkage map was generated to identify the resistance locus. Multiple rearrangements and inversions of macro-chromosomal regions were observed when compared with *B. mori*, yet blocks of genes were clearly clustered (Figure 1 A and B). Five gene markers—*Serpin-6*, *RpL30*, *Sinuous*, *Myelin Proteolipid*, and the resistance candidate gene *ABCC2*—were in perfect association with the *BtR-1* locus, suggesting that this chromosomal region may be gene dense or have a low recombination rate.

Attempts were made to further resolve the *BtR-1* locus by creating 36 additional backcross families between the susceptible strain Waite and Cry1Ac-resistant NO-QAGE, a descendant of NO-QA (Tabashnik *et al.* 2000). Despite genotyping >900 progeny, mapping resolution of the resistance locus was not improved (Table 1).

As *ABCC2* is correlated with Cry1Ac resistance in *H. virescens*, the ortholog was cloned and sequenced using a genomic BAC library constructed with susceptible strain Geneva88 (Figure S1) (Baxter *et al.* 2010). The gene contains 26 exons, and these were verified through PCR amplification from Bt-susceptible (Geneva88) and Bt-resistant (NO-QAGE × Waite backcross progeny) midgut cDNA. The resistant strain NO-QAGE contains a 30-bp deletion in exon 20, which is predicted to remove the 12th and final transmembrane domain and aberrantly position the carboxyl-terminal outside the cell. If this gene is translated and inserted into the midgut membrane, a core ATP-binding loop is expected to be nonfunctional (Figure 2).

Like *P. xylostella*, Bt resistance in *T. ni* is autosomal, recessive, and predicted to be a single major gene. First, we performed a series of backcrosses using F_1 females to associate Bt resistance candidate genes with chromosomes. The cadherin-like protein (Zhang 2007), aminopeptidase N5 (APN5), and alkaline phosphatase (ALP) all mapped to separate chromosomes and were all unlinked to Cry1Ac resistance. In both *B. mori* and *P. xylostella*, APN5 is located on the same chromosome as other known APN genes, suggesting that none of these carry the resistance mutation (Crava *et al.* 2010). The gene *ribosomal protein L8*, however, was located

Table 2 Percentage of Bt-susceptible alleles from two *T. ni* backcrosses with no chromosomal crossing over

	Individuals genotyped	% progeny that inherit a chromosome from the susceptible strain			
		RpL8	*Cadherin*	*APN5*	*ALP*
Chromosome[a]		15	6	9	3
Cry1Ac bioassay	57	0.0	38.6	47.4	64.9
Untreated control	65	50.8	43.1	43.1	46.2
χ-Squared		39.67	0.25	0.23	4.31
P-value		<<0.0001	0.62	0.63	0.037

T. ni crosses were performed using Bt-resistant strain GLEN-Cry1Ac-BCS (Wang *et al.* 2007) and a Bt-susceptible strain purchased from Benzon Research (Carlisle, PA). F_1 females inherited one chromosome set from the susceptible grandmother and one chromosome set from the resistant grandfather. As there is no crossing over in females, backcross progeny inherit one complete maternal chromosome derived from a susceptible or resistant origin. Data set combines two related families: cross A (31 bioassay survivors, 35 untreated controls) and cross B (35 bioassay survivors, 30 untreated controls). Bioassay survivors did not inherit any *RpL8* alleles derived from the susceptible grandmother, indicating that this gene is on the same chromosome as the Bt resistance locus. The *ALP* allele from the Bt-susceptible strain shows a significant overrepresentation in bioassay survivors; however, this is probably due to small sample sizes.

[a] Indicates the *B. mori* chromosome containing this gene ortholog.

Table 3 Percentage of Bt-susceptible alleles inherited from eight *T. ni* backcrosses crosses with chromosomal crossing over

	RpL5	*Rhomboid*	*ABCC2*	*RpL8*	*RpS8*
Bioassay	15/307	9/323	0/325	1/322	18/323
%	4.9	2.8	0.0	0.3	5.6
Controls	65/142	70/146	72/147	72/147	78/147
%	45.8	47.9	49.0	49.0	53.1

Eight backcrosses between F_1 males and GLEN-Cry1Ac-BCS-resistant females produced 326 bioassay progeny and 147 untreated controls for linkage mapping. Approximately 50% of control progeny inherited paternal alleles from the Bt-susceptible strain, confirming Mendelian segregation. *ABCC2* genotypes from bioassay survivors show a perfect association with the Bt resistance locus in *T. ni*.

on the same chromosome as the mutation causing Cry1Ac resistance, as in *P. xylostella* (Table 2).

Eight male informative backcrosses produced 326 bioassay survivors and 147 untreated controls to resolve the precise Bt Cry1Ac resistance locus in *T. ni*. Gene fragments from *Rhomboid* and *Ribosomal protein genes L5*, *L8*, and *S8*, as well as the candidate resistance gene *ABCC2*, were PCR amplified and mapped in backcross progeny. *ABCC2* was in complete linkage with the resistance mutation (Table 3), demonstrating that this chromosomal region contains a genetic mutation that ultimately causes Bt Cry1Ac resistance in *H. virescens*, *P. xylostella*, and *T. ni* (Figure 1C).

Here we have demonstrated that a single homologous locus controls recessive resistance to Bt Cry1Ac toxins in widely divergent Lepidoptera, suggesting independent, parallel evolutionary responses to this strong selective agent. Although functional evidence is still required to confirm that a mutation in *ABCC2* directly causes Bt resistance, the frameshifting 22-bp deletion in *H. virescens* (Gahan *et al.* 2010) and the 30-bp deletion in the NO-QAGE strain of *P. xylostella* provide strong circumstantial evidence. Complementation tests showing a common genetic basis of resistance in *P. xylostella* strains from Hawaii, Pennsylvania, South Carolina, and the Philippines implicate the *ABCC2* gene (Tabashnik *et al.* 1997; Baxter *et al.* 2005); however, additional unlinked resistance genes are evident in the Philippine strain and in some populations from Malaysia (Sayyed *et al.* 2000). The association of *ABCC2* with Bt resistance in a third species, *T. ni*, further supports the hypothesis that this gene is functionally implicated in resistance. It remains to be seen whether resistant strains of *T. ni* from Mexico (Tamez-Guerra *et al.* 2006) and Canada (Estada and Ferre 1994; Janmaat and Myers 2003) also have *ABCC2* mutations. Like the previous work with *H. virescens*, the results here provide evidence of resistance-conferring mutations in an ABC transporter gene that has not previously been associated with Bt toxin interaction. Future functional analysis of ABCC2, and sequencing of the corresponding genome regions of *T. ni* and *P. xylostella*, will be needed to fully elucidate the role of ABCC2 in field-evolved resistance to Bt Cry1Ac toxins and how ABCC2 interacts with the other genes affecting the complex genetic basis of Bt resistance in Lepidoptera (Tabashnik *et al.* 1998; Heckel *et al.* 2007).

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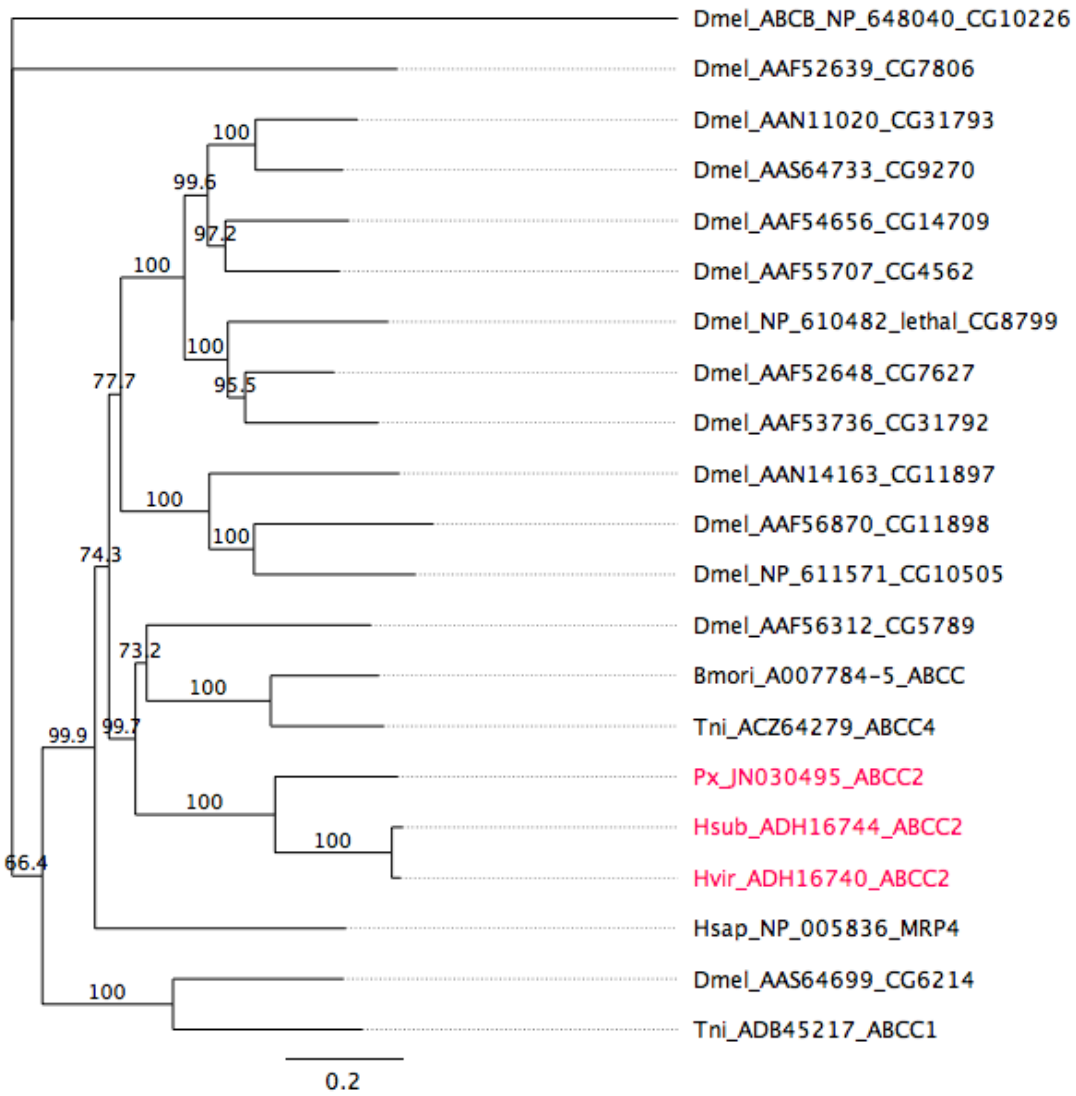

Figure S1 ABCC neighbour-joining tree based on Labbé *et al.* (2011) suggests orthology between *Heliothis virescens* and *Plutella xylostella* ABCC2 proteins. Geneious Pro v5.4.5 software produced a protein alignment with MUSCLE and a neighbour-joining consensus tree with the Jukes-Cantor distance model and 5000 bootstrap replicates. *P. xylostella* ABCC2 clusters with 100% bootstrap support with other lepidopteran ABCC2 proteins (red text). Sample names contain species, GenBank accession number and *Drosophila* CG number or description. Dmel, *Drosophila melanogaster*; Bmori, *Bombxy mori*; Px, *Plutella xylostella*; Hvir, *Heliothis virescens*; Hsub, *Heliothis subflexa*; Tni, *Trichoplusia ni*; Hsap, *Homo sapien*. The outgroup is *D. melanogaster* ABCB (NP_648040). A 1268 amino acid *B. mori* ABCC protein sequence (Labbe *et al.* 2011) was generated by joining BGIBMGA007784-TA and BGIBMGA007785-TA on chromosome 15 (http://silkworm.genomics.org.cn).

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Table S1 PCR primers for gene mapping and sequencing

Species	Marker	Accession	Forward (5'-3')	Reverse (5'-3')	Mapping Method	
					NO-QA*	NO-QAGE**
P. xylostella	UDP	JN383802	TACGTGGTGGACCTGAGGAG	GCCTTGATCTCCTCGTCGTA	LPA	-
P. xylostella	ACTxCAA	JN030494	GCTCTGTCTGACACAATAAGAAGAA	GAACGTAGCGCCAAGTCGTACC	LPA	-
P. xylostella	CPB20	JN383803	GGCTCCAGAAACGAACAAGA	ATCCCAGTCACACCGGATTA	-	RFLP *Pvu*II
P. xylostella	CPB20	JN383803	AAATAAGTTCCTACCGCGATCA	AAAGCCGCAAGGAGTTTTCT	LPA	-
P. xylostella	ABCC2	JN030495	GGACGTGATCCCGGTGGGCAGC	TCCTCGTGCGGCAGCTTAGTGTACTCG	LPA	LPA
P. xylostella	Myelin	JN383805	AACCCTCATCGCCACAATTA	GGGTTGGTCAGGTACTGCAA	RFLP *Not*I	RFLP *Bst*Y1
P. xylostella	sinuous	JN383806	AACTGCGGCATTGGTGTATT	GAGGATCTGGAAGCGATCTG	LPA	-
P. xylostella	RpL8	JN383814	GACACGCAAGGAGTTGTTCA	CTGGAGGGCAGGACTTTCTT	-	RFLP *Alw*NI
P. xylostella	RpL30	JN383804	TACTGCCTTGGCTACAAACAAA	TCAATGTTGTTTCCGCTGTAGT	RFLP *Mbo*II	-
P. xylostella	Serpin-6	JN383808	CACAAGGCCAAGATCACACT	ACAGAGTTGGTGGGTCGTTC	LPA	-
P. xylostella	Adenylate	JN383807	GCTTCAGGAAAAGGCACAAT	GTCCGGCCTTCAAACTCTTT	RFLP *Mse*I	RFLP *Pfl*FI
P. xylostella	TRIP-12	JN030497	GCATGGACGTGGCKGARCA	CGCGTTGATCGAGAAGAAGT	LPA	-
P. xylostella	RpL10A	JN383809	GGAGACCAGCAGCACTGTGA	AACTTGATGGTGGCCTTGAC	RFLP *Bam*HI	-
P. xylostella	β-1,3	JN383811	TTCCCGCAGTATTGTGGATT	CCTCTGGACCTCCATTGTGT	RFLP *Tsp*509I	-
P. xylostella	RAB7	JN383810	TCCTCACGAAGGAAGTTATCGT	TTTTTGCTTTGACACCACTGTT	RFLP *Hae*III	-
P. xylostella	RpS5	JN383812	ATCCCCGAAATCAAGCTGTT	CCGATTCTGGTCGAGTCTTC	RFLP *Hpy*CH4IV	-
P. xylostella	Rhomboid	JN383813	CGCCAACATGTATGTGCTGT	CTAAGTCCGGGTTGTTTGGA	LPA	-
T. ni	Cadherin	JF303656	TTTCTAACGACTCGGACCTGA	GGCTGGCGTTGAACTTGA	RFLP *Nde*I	
T. ni	APN5	JF303657	CCGTGTCAAGGAGACTTTCG	GATGCCCAGTAAGGAGATGC	RFLP *Sau*AI	
T. ni	ALP	JF825967	TGCGTGAACTCTCAAGTTGC	CAGGCGTTGATGTCTTGGTT	RFLP *Msp*I	
T. ni	RpL8	FF374781	TGCAACCTTGAGGAGAAAATG	GTTACCACCACCGTGAGGAT	RFLP *Bst*UI	
T. ni	RpL5	FF377076	GAGCCATGAAAGGTGCAGTT	TCAGCCACGTGTAAACCAAA	RFLP *Msp*I	
T. ni	Rhomboid	JN030492	GCTTTCGACATTCAGCCATT	CCAAGACTTAGGCCAGGTTG	RFLP *Acc*I	

T. ni	ABCC2	JN030493	TGGAGTTGAAAGACGGAATACA	ACGAATCGTCTTTTGGATCA	RFLP *Hha*I
T. ni	RpS8	CF258242	GGAGGCAGAAGATGCCATTAT	CTTCCAGAATGTAGCCGTCAG	RFLP *Sty*I

ABCC2 gene sequencing primers

P. xylostella	Exons 1-6	CGGAAAGAGTCGGAAGAGAA	CTCCGTGAACAGCATGAACC
P. xylostella	Exons 4-10	GCGTTCATGTTCCTGCACTA	AGACACCGGAAGCTCTTTCA
P. xylostella	Exons 8-14	TCCAAGAAGGAGGACGACAC	GAGTCCACGGAGACCAGGTA
P. xylostella	Exons 12-19	TATTCGATGACCCCCTATCG	CCCGATGTAAGTGTGGAAGG
P. xylostella	Exons 18-26	TCCAGTGTTTGGAATGATCG	CATGTTGTCTCCGGTCTCCT

LPA: Length polymorphism using agarose gel electrophoresis to separate PCR products. RFLP: Restriction Fragment Length Polymorphism observed using specified enzyme. NO-QA* refers to 3 backcross families [F_1 male (NO-QA x Waite) x NO-QA female] and NO-QAGE** refers to 36 backcross families [F_1 male (NO-QAGE x Waite) x NO-QAGE female]. *P. xylostella* ABCC2 (exons 1-26) was amplified from midgut tissue (cDNA) of Bt-susceptible Geneva88 (JN030490) and Bt-resistant backcross progeny (JN030491).