Insect Biochemistry and Molecular Biology 112 (2019) 103209

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Mutation of ABC transporter *ABCA2* confers resistance to Bt toxin Cry2Ab in *Trichoplusia ni*

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ARTICLE INFO

Keywords:
Bacillus thuringiensis
Cry2Ab
ABC transporter
Bulked segregant analysis (BSA)
Amplicon sequencing (AmpSeq)
CRISPR/Cas9 mutagenesis

ABSTRACT

Insecticidal proteins from *Bacillus thuringiensis* (Bt) are the primary recombinant proteins expressed in transgenic crops (Bt-crops) to confer insect resistance. Development of resistance to Bt toxins in insect populations threatens the sustainable application of Bt-crops in agriculture. The Bt toxin Cry2Ab is a major insecticidal protein used in current Bt-crops, and resistance to Cry2Ab has been selected in several insects, including the cabbage looper, *Trichoplusia ni*. In this study, the Cry2Ab resistance gene in *T. ni* was mapped to Chromosome 17 by genetic linkage analyses using a whole genome resequencing approach, and was then finely mapped using RNA-seq-based bulked segregant analysis (BSA) and amplicon sequencing (AmpSeq)-based fine linkage mapping to a locus containing two genes, *ABCA1* and *ABCA2*. Mutations in *ABCA1* and *ABCA2* in Cry2Ab resistant *T. ni* were identified by both genomic DNA and cDNA sequencing. Analysis of the expression of *ABCA1* and *ABCA2* in *T. ni* larvae indicated that *ABCA2* is abundantly expressed in the larval midgut, but *ABCA*1 is not a midgut-expressed gene. The mutation in *ABCA2* in Cry2Ab resistant *T. ni* was identified to be an insertion of a transposon *Tntransib* in *ABCA2*. For confirmation of *ABCA2* as the Cry2Ab-resistance gene, *T. ni* mutants with frameshift mutations in *ABCA1* and *ABCA2* were generated by CRISPR/Cas9 mutagenesis. Bioassays of the *T. ni* mutants with Cry2Ab verified that the mutations of *ABCA1* did not change larval susceptibility to Cry2Ab, but the *ABCA*2 mutants were highly resistant to Cry2Ab. Genetic complementation test of the *ABCA2* allele in Cry2Ab resistant *T. ni* with an *ABCA2* mutant generated by CRISPR/Cas9 confirmed that the *ABCA2* mutation in the Cry2Ab resistant strain confers the resistance. The results from this study confirmed that *ABCA2* is essential for the toxicity of Cry2Ab in *T. ni* and mutation of *ABCA2* confers the resistance to Cry2Ab in the resistant *T. ni* strain derived from a Bt resistant greenhouse population.

1. Introduction

The soil bacterium *Bacillus thuringiensis* (Bt) was first produced as a commercial pesticide in 1938 and has been the most successfully used bioinsecticide (Bravo et al., 2011; Ibrahim et al., 2010; Tabashnik and Carriere, 2017). Bt-based biopesticides are environmentally benign (Sanahuja et al., 2011) and transgenic crops expressing insecticidal proteins from Bt have been widely adopted worldwide since 1996 (James, 2016). However, extensive use of Bt sprays and planting of Bt-crops increase the risk of evolution of resistance to Bt toxins in populations of target insects, threatening the benefits of Bt-crops (Tabashnik and Carriere, 2017). Cases of field-evolved resistance to Bt toxins have been reported in several insect pests (Tabashnik and Carriere, 2017).

The Bt toxin Cry2Ab is a major Bt toxin used in combinations with Bt Cry1A toxins in Bt-crops to increase pest control efficacy and delay development of insect resistance to Bt toxins, as Cry2Ab is known not to share the toxin binding sites with Cry1A toxins in insects (Carriere et al., 2015; Sumerford et al., 2012; Zhao et al., 2003). However, populations of several lepidopteran pests resistant to Cry2Ab have been selected under laboratory conditions or in the field (Mohan, 2017; Tabashnik and Carriere, 2017; Tabashnik et al., 2009). Increased frequencies of Cry2Ab resistant alleles in populations of the cotton pests *Helicoverpa armigera* and *H. punctigera* have been found after adoption of Bt-cotton plants in Australia (Mohan, 2017; Tabashnik and Carriere,

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https://doi.org/10.1016/j.ibmb.2019.103209
Received 25 January 2019; Received in revised form 22 July 2019; Accepted 24 July 2019
Available online 15 August 2019
0965-1748/

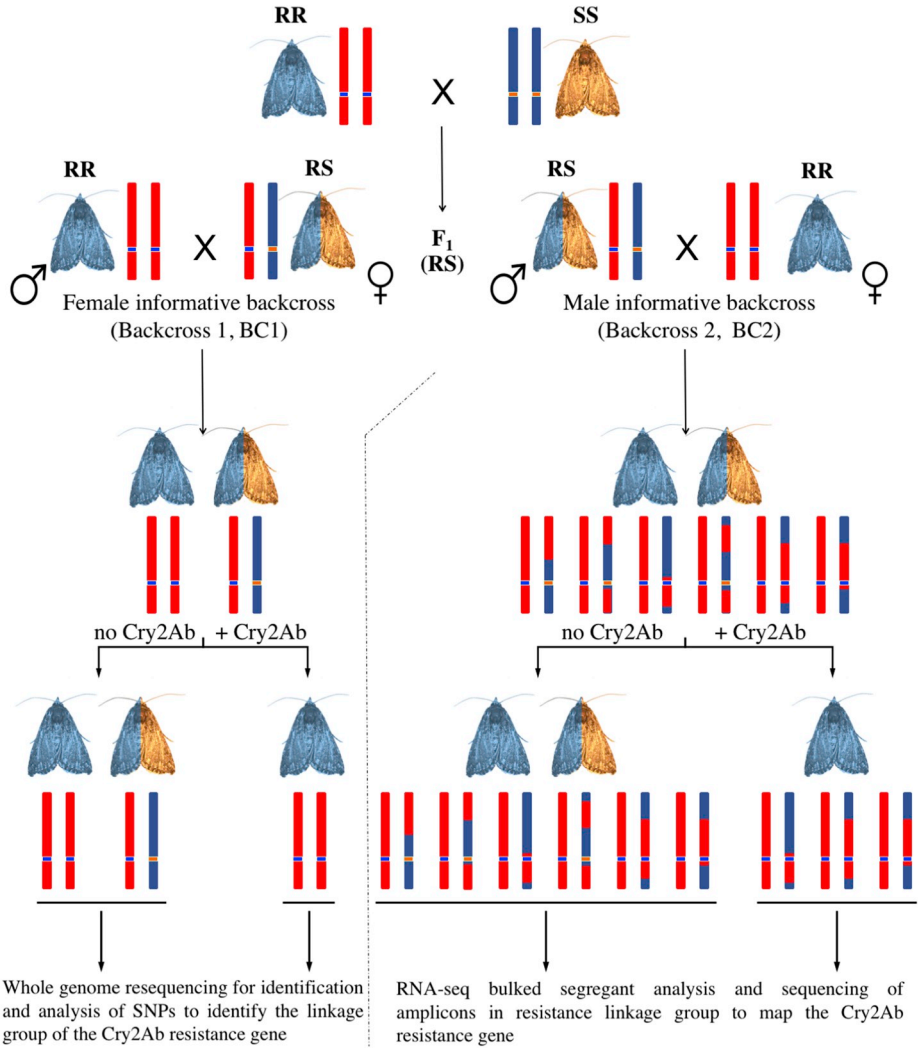


Fig. 1. Generation of male and female-informative backcross populations for genetic linkage mapping of Cry2Ab resistance gene in *T. ni*. F_1 individuals were produced by a single-pair cross between the susceptible Cornell strain (SS) and the Cry2Ab resistant GLEN-Cry2Ab-BCS strain (RR). Female-informative backcross (BC1) and male-informative backcross (BC2) populations were generated for biphasic linkage mapping of Cry2Ab resistance gene (Heckel et al., 1999). The BC1 family individuals with and without selection with Cry2Ab were used to determine the linkage group of the resistance gene. The BC2 family individuals with and without selection with Cry2Ab were used to map the resistance gene in resistance linkage group.

2017; Tabashnik et al., 2009). Similarly, the Cry2Ab resistant allele was also found to be present in a high frequency in populations of the cabbage looper, *Trichoplusia ni,* that developed resistance to Bt formulations in vegetable greenhouses (Kain et al., 2015). Insects resistant to Cry2Ab are usually not cross-resistant to Cry1A toxins, due to the unshared binding sites of Cry2Ab with Cry1A toxins in insect midgut (Caccia et al., 2010; Luo et al., 2007; Pardo-Lopez et al., 2013; Song et al., 2015; Wei et al., 2015). The resistance to Cry2Ab in *H. armigera* and *H. punctigera* has been determined to be a recessive trait and conferred by reduced binding of the toxin to the insect midgut (Caccia et al., 2010; Downes et al., 2010; Mahon and Young, 2010). The genetic basis of resistance to Cry2Ab in *H. armigera* and *H. punctigera* and in another cotton pest, *Pectinophora gossypiella,* have been found to be associated with mutations of the ABC transporter ABCA2 gene (Mathew et al., 2018; Tay et al., 2015). The importance of *ABCA2* in the toxicity of Cry2Ab has been shown in *H. armigera* (Wang et al., 2017). Nevertheless, studies of the mode of action and mechanisms of resistance to Bt toxins have been mostly on Cry1A toxins. For Cry1A toxins, multiple insect midgut receptors have been identified in the mode of action and the toxicity can be rendered through different pathways of toxicity (Ocelotl et al., 2017; Pardo-Lopez et al., 2013; Wang et al., 2018), and insect resistance to Cry1A toxins may be conferred by mutations of different receptor genes, depending on the insect species (Baxter et al., 2011; Gahan et al., 2001, 2010; Pardo-Lopez et al., 2013). Although both Cry1A and Cry2A toxins share a similar 3-domain structure, the mode of action of Cry2A toxins remains unclear. Therefore, studies of midgut proteins involved in the toxicity of Cry2A toxins in different insects and in different resistance cases are needed for understanding the mode of action and mechanisms of resistance to Cry2A toxins.

T. ni is a highly polyphagous migratory agricultural pest globally distributed. Bt-resistant populations of *T. ni* have been identified in commercial greenhouses under selection by Bt sprays (Janmaat and Myers, 2003). From the Bt-resistant greenhouse *T. ni* populations, resistance to Cry1Ac and Cry2Ab has been identified, and the resistant *T. ni* shows no cross-resistance between Cry1Ac and Cry2Ab (Kain et al., 2015; Song et al., 2015). The Cry2Ab resistance in *T. ni* is a recessive trait and it does not share the same mechanism for Cry1Ac resistance and is independent of the linkage groups of the genes coding for the putative Cry1Ac receptors – the membrane bound alkaline phosphatase, aminopeptidases N, midgut cadherin and the ABC transporter ABCC2 (Kain et al., 2015; Song et al., 2015). However, the mechanism and genetic basis of Cry2Ab resistance in *T. ni* have not been previously reported. So far, resistance to Cry2Ab in the cotton pests *H. armigera*, *H. punctigera* and *P. gossypiella* has been determined to be associated with the ABCA2 gene (Mathew et al., 2018; Tay et al., 2015). It is worthwhile to understand the molecular genetic basis of resistance to Cry2Ab in other insects that have different preferred host plants and developed resistance in agriculture.

In this study, the Cry2Ab resistance gene was localized in *T. ni* genome by genome resequencing and genetic linkage mapping, using bulked segregant analysis (BSA) and fine gene mapping by amplicon sequencing (AmpSeq). The resistance-associated mutation was

determined by genomic DNA and cDNA sequencing and confirmed by functional analysis of mutant *T. ni* strains defective in the resistance gene.

2. Materials and methods

2.1. Insect strains and Cry2Ab toxin

The laboratory inbred Cornell strain of *T. ni* (Kain et al., 2004) was used as a Bt-susceptible strain. The Cry2Ab resistant strain of *T. ni* used in this study was the GLEN-Cry2Ab-BCS strain originally selected from a greenhouse-derived Bt resistant population (Song et al., 2015) and has been backcrossed with the Cornell strain 8 times. The *T. ni* strains were maintained on artificial diet as previously described (Kain et al., 2004). The Bt toxin Cry2Ab used in this study was provided by the Monsanto Company (St. Louis, MO).

2.2. Preparation of informative T. ni backcross families for mapping of the Cry2Ab resistance gene

For mapping of the Cry2Ab resistance gene, both male- and female-informative backcross families of *T. ni* were prepared for biphasic genetic linkage analysis (Heckel et al., 1999) (Fig. 1). The Cornell strain was crossed with the GLEN-Cry2Ab-BCS strain to generate F_1 progeny. Female-informative backcross populations (named backcross 1, BC1) were generated by crossing female F_1 with male GLEN-Cry2Ab-BCS individuals. Male-informative backcross populations (named backcross 2, BC2) were generated by crossing male F1 with female GLEN-Cry2Ab-BCS individuals. The BC1 populations were used to determine the linkage group (chromosome) of the resistance gene, as no crossing-over occurs during oogenesis in females, and the BC2 populations were used to map the position of the resistance gene on the chromosome, as crossing-over occurs during spermatogenesis in males and the recombinational frequencies depend on the distance of the resistance gene to markers (Heckel et al., 1999). Cry2Ab resistant F_2 individuals from both BC1 and BC2 families were selected by treatment of the F_2 individuals of the backcross families with Cry2Ab on diet at 14.4 μg/cm^2, a discriminating dose confirmed to kill 100% of the F_1 individuals in a parallel assay (n = 30). The *T. ni* groups with and without Cry2Ab treatment were used for genetic linkage analyses in this study.

2.3. RNA-seq based bulked segregant analysis (BSA)

For RNA-seq library construction, midgut tissues were isolated from mid-5th instar *T. ni* larvae and were cleaned by washing three times in cold PBS. Midgut tissues from 50 larvae were combined for each replication. In addition to midgut tissues, 3rd instar larvae from the backcross families were also used to construct RNA-seq libraries. For each treatment, 300 3rd instar larvae were pooled and ground in liquid nitrogen. Total RNA from each treatment group was extracted using the RNeasy Mini Kit from Qiagen (Germantown, MD), including an on-column DNase digestion procedure. RNA-seq libraries were prepared using the strand specific protocol as previously described (Chen et al., 2013). RNA-seq libraries were constructed from replicated BC1 and BC2 families with and without Cry2Ab selection and also from the susceptible Cornell strain, the Cry2Ab resistant GLEN-Cry2Ab-BCS strain and the F_1 families with replications. In total, 36 RNA-seq libraries were constructed (Table S1) and sequenced on an Illumina HiSeq 2000 platform in the Genomics Core Facility of the Weill Cornell Medical College (New York, NY) or in the Genomics Facility of Cornell University (Ithaca, NY).

Raw RNA-seq reads from the RNA-seq libraries were processed using Trimmomatic (Bolger et al., 2014) to remove the adaptors and low-quality sequences, and the cleaned reads obtained were aligned to the *T. ni* genome using the program STAR (Dobin et al., 2013). SNPs/Indels in RNA-seq data were identified by GATK (DePristo et al., 2011). Variants with quality scores higher than 100 and reads depth (DP) greater than 4 were considered as high confidence calls and were used for the downstream analyses. To identify the variants associated with Cry2Ab resistance, 1) homozygous variants specific in the resistant strains were identified by subtraction of the homozygous variants common in the libraries from the resistant GLEN-Cry2Ab-BCS strain by the homozygous variants common in the libraries from the susceptible Cornell strain; 2) those resistant strain-specific variants selected from above that were also present in all the libraries from the F_1 and backcross families were chosen as the marker variants from the GLEN-Cry2Ab-BCS strain for linkage analysis; and 3) the marker variants that were found to be heterozygous in all BC2 families without Cry2Ab selection but were homozygous in all BC2 families selected with Cry2Ab were identified to be the variants associated with Cry2Ab resistance.

2.4. Whole genome resequencing of GLEN-Cry2Ab-BCS larvae

Two individual 5th instar larvae from the GLEN-Cry2Ab-BCS strain were dissected to remove the gut, then the larval carcasses were used for resequencing of their genomes to identify SNPs as mapping markers from the resistance strain. Genomic DNA was prepared from individual larval carcass using the Gentra Puregene Tissue Kit (Qiagen, USA). For construction of Illumina sequencing libraries, the genomic DNA was fragmented using the NEBNext dsDNA Fragmentase Kit from New England BioLabs (Ipswich, MA) and the libraries from individual larvae were prepared using the NEBNext DNA Library Prep Master Mix (New England BioLabs). Illumina sequencing was performed on an Illumina HiSeq 2500 system at the Genomics Facility of Cornell University. The raw Illumina sequence reads were processed to remove the adaptors and low-quality sequences using Trimmomatic (Bolger et al., 2014). The *T. ni* genome assembly from the Cornell strain (Chen et al., 2019) was used as the reference to detect SNPs/Indels from the resequenced resistant *T. ni* genomes using GATK (DePristo et al., 2011).

2.5. Mapping of the Cry2Ab resistance gene

To identify the linkage group of the Cry2Ab resistance gene, an Illumina sequencing library of a pool of 45 BC1 larvae without Cry2Ab selection and another library of a pool of 45 BC1 larvae selected with Cry2Ab were constructed and sequenced as described above. The sequence reads were processed and SNPs/Indels were identified and mapped to the *T. ni* genome assembly as described above. The SNPs found to be heterozygous in the non-selected group but to be homozygous in the Cry2Ab selected group were identified in each linkage group to determine the linkage group with the majority of those SNPs localized, the linkage group of the resistance gene.

For fine mapping of the Cry2Ab resistance gene in the resistance locus, a 1.58 Mb region in linkage group17 identified from the procedures described above, 44 pairs of PCR primers were designed based on the genome resequencing data from two larvae of the GLEN-Cry2Ab-BCS strain (Table S2) to amplify 44 DNA fragments of 225–686 bp to include strain specific SNPs/INDELs in this region. Genomic DNA was prepared from 664 BC2 larvae without Cry2Ab selection and 1592 BC2 larvae selected with Cry2Ab, respectively, using the EZNA Tissue DNA kit (Omega Bio-Tek, Norcross, GA), and amplicons from each DNA sample were generated by PCR with the 44 primer sets using the iProof™ High-Fidelity PCR Kit (Bio-Rad, Hercules, CA, USA). The 44 amplicons from the same group (BC2 without Cry2Ab selection or BC2 selected with Cry2Ab) were pooled and subjected to Illumina sequencing. The Illumina sequencing libraries were constructed and sequenced at the Genomics Facility of Cornell University.

The raw Illumina sequence data was processed to remove adaptor and low-quality sequences as described above. The sequence reads were then mapped to the reference *T. ni* genome assembly using BWA (Li and Durbin, 2009). SNP calling was performed using SAMtools (Li, 2011) by skipping the “mark duplicate” step, and those with the DP value greater

than 1000 were selected to use for downstream data analysis. The amplicon sequence reads were positioned in the 1.58 Mb mapping region with the known primer sequences, and SNPs in the amplicons were identified. The minor allele frequency (MAF) of SNPs in each amplicon was calculated as the susceptible allele frequency in the sequencing populations, using the VCFtools (Danecek et al., 2011). The MAFs of SNPs in the Cry2Ab selected BC2 population correlate with the recombination frequencies between the resistance gene and the amplicons, which is the genetic distance from the resistance gene to the 44 amplicon sites in the mapping region.

2.6. Sequencing of ABCA1 and ABCA2 cDNAs and genomic DNA fragments of ABCA2

Total RNA samples from the larval midgut and larval carcass (larval tissues without the gut) of the Cornell strain and the GLEN-Cry2Ab-BCS strain were prepared as described above and cDNAs were synthesized from the total RNA preparations by reverse transcription, using the ImProm-II reverse-transcription system from Promega (Madison, WI) (Tiewsiri and Wang, 2011). The cDNAs of the *ABCA1* and *ABCA2* genes were amplified by PCR using the iProof™ High-Fidelity PCR Kit (Bio-Rad) with primers specific to *ABCA1* and *ABCA2* (Table S2), and the PCR amplified cDNAs were sequenced at the Genomics Facility of Cornell University.

To determine the mutations of *ABCA2* in the genomic DNA regions where a missense mutation and a delins mutation were identified, respectively, in the GLEN-Cry2Ab-BCS strain by cDNA sequencing, the DNA fragment of the missense mutation region was amplified from *T. ni* genomic DNA by PCR with the primers ABCA2frag1F and ABCA2frag1R (Table S2) from both the susceptible and the resistant strain. The genomic DNA fragment of the delins mutation region in the Cornell strain was amplified by PCR with the primers ABCA2frag2F and ABCA2frag2R (Table S2). The PCR fragments were sequenced as described above. The genomic DNA sequence in the delins mutation region of *ABCA2* in the GLEN-Cry2Ab-BCS strain was assembled *de novo* using the resequencing data set from the GLEN-Cry2Ab-BCS strain, due to a large insertion in the region.

De novo assembly of the resequencing reads was performed to construct contigs in this delins mutation region using ABySS assembler 1.9.0 (Simpson et al., 2009). With the DNA sequence of the region assembled from the resequencing data, this *ABCA2* mutation region was amplied from the resistant *T ni* strain by PCR as two overlapping fragments (primer pair: ABCA2frag2F and ABCA2frag2R2, and primer pair: ABCA2frag2F2 and ABCA2frag2R, in Table S2). The PCR products were sequenced as described above and the sequences were assembled using the DNAStar software package (Madison, WI).

2.7. Quantitative real-time PCR analysis

The expression of *ABCA1* and *ABCA2* in *T. ni* larval midgut, in comparison with the larval carcass without the midgut, was analyzed by real-time RT-PCR analysis with the gene specific primers (Table S2). Six individual larvae from each strain were analyzed for replications. The real-time PCR reactions were prepared with the iTaq™ Universal SYBR® Green Supermix from Bio-Rad. Real-time PCR reactions of serial dilutions of cDNA samples were performed to determine amplification efficiency for each primer pair to ensure the amplification efficiency within the range 90–110%. The relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method, using the expression of the β-actin gene as the internal control (Tiewsiri and Wang, 2011).

2.8. Generation of ABCA1 and ABCA2 knockout T. ni strains by CRISPR/Cas9 mutagenesis

CRISPR/Cas9 mutagenesis in *T. ni* was performed as described by Wang et al. (2018). CRISPR/Cas9 target sites in the ABCA1 and ABCA2 genes were selected in the exons coding for the third to fifth transmembrane domains of ABCA1 and ABCA2, and the specific gRNA target sequences were designed in these regions using Cas-Designer (http://www.rgenome.net/cas-designer/) (Park et al., 2015) (Table S2). DNA templates for in vitro synthesis of gRNAs were prepared by PCR using the plasmid pRB1017 as the PCR template with target specific primer sets (Table S2) to generate the designed DNA fragments with the target sequences flanked by the T7 promoter and the gRNA scaffold sequences (Wang et al., 2018). The gRNA and Cas9 mRNA preparations were made by in vitro transcription using the same methods recently reported by Wang et al. (2018).

T. ni eggs laid within an hour were injected with 9 nL of a mixture of Cas9 mRNA (200 ng/μl) and two gRNAs (100 ng/μl each) specific to *ABCA1* or *ABCA2* in PBS with 0.1% phenol red, using a Nanoject I™ Microinjector from Drummond Scientific (Broomall, PA). G_0 larvae from the injected eggs were screened for CRISPR/Cas9 introduced mutations by PCR amplification of the target site region from hemolymph samples, collected from 5th instar G_0 larvae by piercing a proleg, with primer pairs flanking the CRISPR/Cas9 target sites (Table S2), followed by DNA sequencing to detect the presence of mutations in the target site, as described by Wang et al. (2018). The G_0 individuals detected to carry mutations were singly crossed with the Cornell strain to generate F_1 families. The F_1 individuals were screened by PCR and sequencing as described above to identify mutations in the target sites, and the F_1 individuals detected to carry the same CRISPR/Cas9 introduced mutations were crossed in single pairs to generate F_2 families. F_2 individuals were screened as described above and those identified to be homozygous for the same mutant alleles were pooled to establish mutant strains.

For complementation test of the *ABCA2* mutant generated by CRISPR/Cas9 and the *ABCA2* allele in GLEN-Cry2Ab-BCS to examine the allelic mutation in GLEN-Cry2Ab-BCS for the recessive Cry2Ab resistance, the GLEN-Cry2Ab-BCS strain was crossed with a CRISPR/Cas9 mutant strain ABCA1^{-}-1 to generate F_1 progeny. The F_1 larvae were bioassayed with Cry2Ab for their susceptibility to the toxin.

2.9. Bioassay

Susceptibility of *T. ni* larvae to Cry2Ab was determined using the toxin overlay bioassay on artificial diet as described by Kain et al. (2004). Six to 9 concentrations of Cry2Ab were included in each bioassay and larval mortality was scored after 4 days. The median lethal doses (LD_{50s}) were calculated by probit analysis, using the software PoloSuite (LeOra Software) (Robertson et al., 1980).

3. Results

3.1. Localization of the Cry2Ab resistance gene on chromosome 17 in T. ni

For the identification of the linkage group of the resistance gene, a pool of 45 T *ni* individuals from the female-informative backcross family (BC1) without Cry2Ab selection and another pool of 45 T *ni* individuals survived from selection of Cry2Ab (14.4 μg/cm^2) were subjected to whole genome resequencing to identify SNPs in their genomes. In total, 1,365,817 and 1,381,271 heterozygous SNPs were obtained from the Cry2Ab-selected and the non-selected groups of BC1, respectively. The numbers of heterozygous SNPs from the Cry2Ab-selected and non-selected *T. ni* groups were similar in 30 of the 31 linkage groups of the *T. ni* genome, but the number of heterozygous SNPs in linkage group 17 significantly decreased in the Cry2Ab-selected *T. ni* (Fig. 2A). In this linkage group (linkage group 17), 49,105 heterozygous SNPs in non-selected groups were detected to be homozygous in the selected groups. In contrast, in any one of the other 30 linkage groups, the numbers of heterozygous SNPs in non-selected groups that became homozygous in the selected groups were no more than 1794 (Fig. 2B).

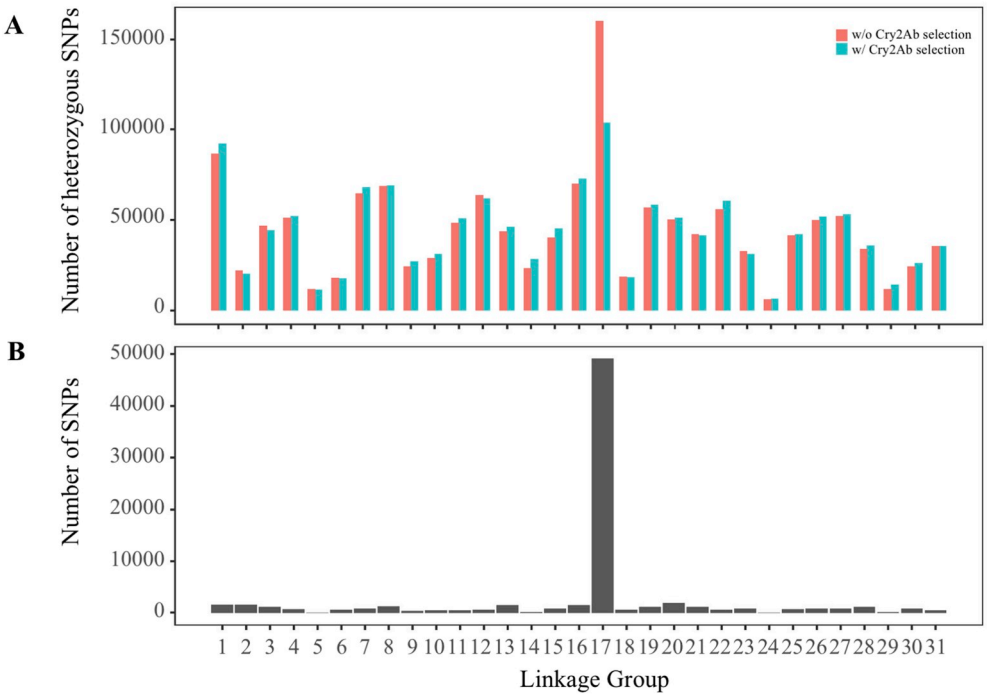


Fig. 2. Distribution of the Cry2Ab resistance associated SNPs in the 31 linkage groups in *T. ni*. (A) SNPs in the 31 T *ni* linkage groups (chromosomes) identified from BC1 family larvae with and without Cry2Ab selection by whole genome resequencing. (B) The SNPs present to be heterozygous in the non-selected larval group but to be homozygous in the selected group.

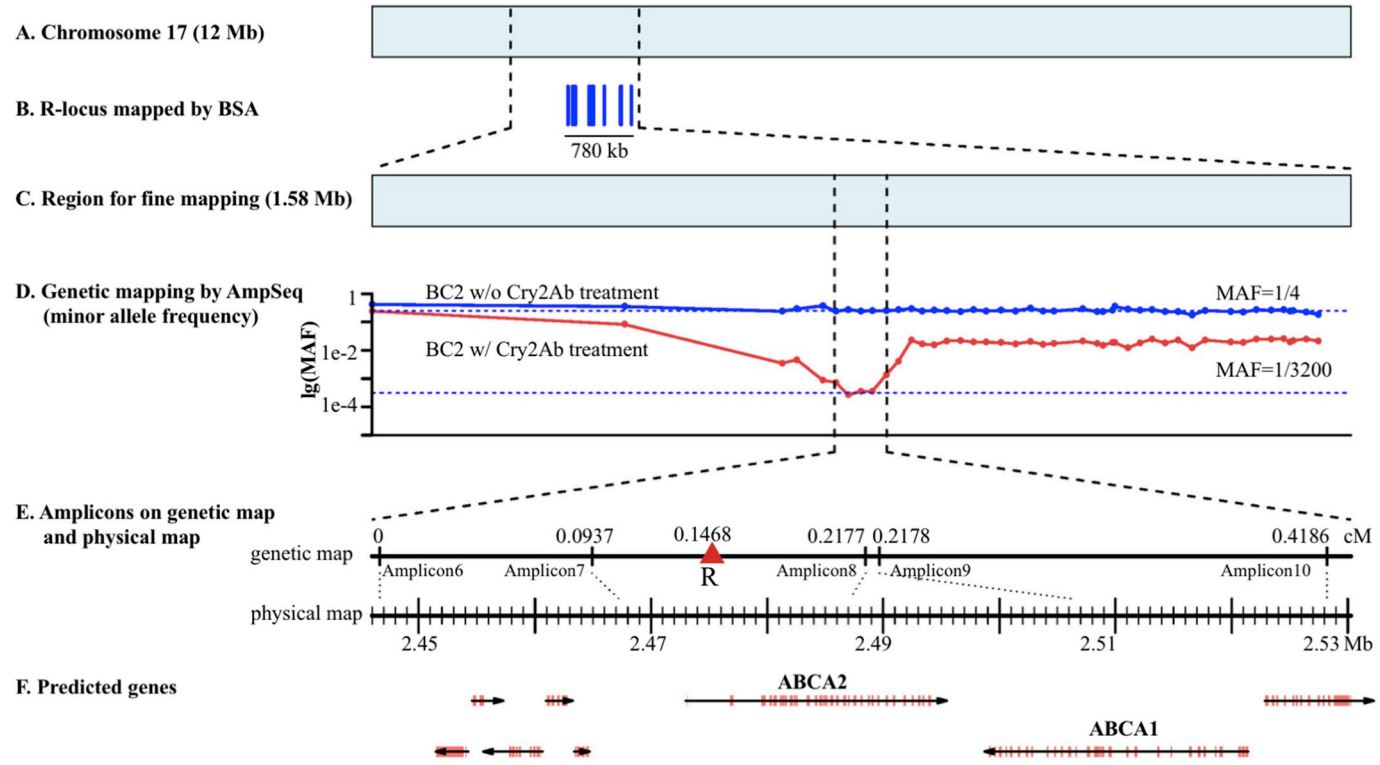


Fig. 3. Mapping of the Cry2Ab resistance gene on Chromosome 17 by genetic linkage analysis using BSA and AmpSeq. (A) The resistance to Cry2Ab was mapped to linkage group 17 (chromosome 17) in *T. ni*; (B) The Cry2Ab resistance gene was mapped to a 780 kb locus by BSA; (C) A 1.58 Mb region with a high density of SNPs/Indels in Cry2Ab resistant *T. ni* was identified to be overlapping with the 780 kb Cry2Ab resistance locus mapped by BSA; (D) The Cry2Ab resistance gene was finely mapped in the 1.58 Mb region by AmpSeq; (E) Genetic map of the finely mapped Cry2Ab-resistance locus was constructed with the corresponding physical map of 81 kb; and (F) Eight genes were predicted in the 81 kb region in chromosome 17.

Therefore, the Cry2Ab-resistance gene is localized in linkage group 17. Linkage group 17 (Chromosome 17) is 12 Mb in length in the *T. ni* genome assembly (Fig. 3A).

3.2. Mapping of the Cry2Ab resistance locus on chromosome 17 by bulked segregant analysis (BSA)

The Cry2Ab resistance was mapped in the *T. ni* genome by BSA using RNA-seq of the susceptible and resistant parental *T. ni* strains,

their F_1 progeny and the Cry2Ab selected and non-selected *T. ni* groups of the backcross families (Fig. 1). A total of 36 RNA-seq libraries from multiple biological replications for each treatment were prepared and sequenced (Table S1). With the genome assembly of the *T. ni* Cornell strain as the reference genome (Chen et al., 2019), SNPs/Indels in the experimental *T. ni* populations (Table S1) were identified from the pooled RNA-seq data by GATK (DePristo et al., 2011) as molecular markers for mapping of the recessive resistance gene. A total of 1308 SNPs/Indels were identified to be homozygous in all 6 replicates of the GLEN-Cry2Ab-BCS strain. Among these 1308 SNPs/Indels, 206 remained after removal of the same SNPs/Indels that were also found to be homozygous in the 8 replicates of the midgut samples of the Cornell strain. The 206 remaining SNPs/Indels were further filtered by removal of those that appeared to be homozygous in 6 replicates of the F_1 and 7 replicates of BC2 without Cry2Ab selection, resulting in 41 SNPs/Indels potentially associated with the Cry2Ab resistance in *T. ni*. As the resistance to Cry2Ab in *T. ni* was recessive, the SNPs/Indels at the locus of the resistance gene were expected to be homozygous in the backcross families selected with Cry2Ab, while both of the two alleles of the SNPs/Indels could be detected in the pooled backcross family samples without Cry2Ab selection (Fig. 1). Therefore, the SNPs/Indels that were homozygous in Cry2Ab-selected larvae from the backcross families but were heterozygous in non-selected larvae from the same backcross families were further examined for their association with resistance. Thirty of these SNPs/Indels were found to be homozygous in Cry2Ab selected larvae but were found to be heterozygous in non-selected larvae in all 8 replicate backcross families (Table S3), and so were identified to be tightly linked to the resistance. These 30 SNPs/Indels were assigned to three scaffolds in a block of about 780 kb in Chromosome 17 of the *T. ni* genome assembly (Fig. 3B). In other words, the resistance gene was localized in a locus of 780 kb in chromosome 17.

3.3. Whole genome resequencing of Cry2Ab resistant T. ni

For identification of SNPs/Indels as markers for fine mapping of the Cry2Ab resistance gene, whole genome resequencing was performed in two *T. ni* individuals of the GLEN-Cry2Ab-BCS strain, and SNPs/Indels were identified in genomes of the two individuals from the resequencing data. In total, 1,289,000 SNPs/Indels were found homozygous in both of the two *T. ni* individuals. Among these homozygous SNPs/Indels, 77,961 were located on Chromosome 17. The SNPs/Indels on Chromosome 17 were not evenly distributed across the chromosome and one region (1.58 Mb) with a high density of SNPs/Indels contains the locus of the Cry2Ab resistance gene mapped by RNA-seq based BSA (Fig. 3C). The SNPs/Indels in this region were used as markers for fine mapping of the resistance gene in this 1.58 Mb region by AmpSeq.

3.4. Fine mapping of Cry2Ab resistance gene by AmpSeq

For fine mapping of the Cry2Ab resistance gene in Chromosome 17, DNA samples were prepared from a pool of 664 larvae of male-informative backcross without Cry2Ab selection and a pool of 1592 larvae survived from a treatment of 7031 larvae with Cry2Ab at 14.4 μg/cm^2 on diet (Fig. 1). PCR amplicons from the DNA samples were generated for 44 loci distributed in the 1.58 Mb region in Chromosome 17 (Fig. 3D and Table S2). Illumina sequencing of the amplicons detected two alleles for each amplicon with the major allele originated from the Cry2Ab resistant GLEN-Cry2Ab-BCS strain and the minor allele originated from the susceptible Cornell strain. The minor allele frequency (MAF) of the 44 amplicons from the non-Cry2Ab selected 664 larvae were or were very close to ¼ (Fig. 3D), as expected for alleles from the susceptible strain in the BCS populations (Fig. 1). The MAF of the amplicons from the Cry2Ab selected 1592 larvae indicates the map distance of the amplicons to the Cry2Ab-resistant gene. The MAF of the 44 amplicons from the Cry2Ab selected larvae ranged from 24% to 2.1% at the two ends of the 1.58 Mb region to a locus with the MAF in 3 amplicons to 0.03% (Fig. 3D). The extended region to include the 3 amplicons (amplicons 7 to 9) with the lowest MAF (between amplicon 6 and amplicon 10) (Fig. 3E) is only 81 kb (Chromosome 17: 2,446,963–2528,100), including 8 predicted genes (Fig. 3F and Table S4). The 3 amplicons with the lowest MAF (amplicons 7 to 9) were localized in 2 genes, *ABCA1* and *ABCA2*. The PCR amplicon 8 was in the ABCA2 gene, and the amplicon 9 was in the *ABCA1* gene (Fig. 3F). Therefore, this fine mapping localized the Cry2Ab resistance locus of 39.4 Kb (from amplicons 7 to 9) with the calculated genetic distance to the resistance gene within 0.05–0.07 CM (Fig. 3E) by VCFtools (Danecek et al., 2011). The Cry2Ab resistance locus only contains the genes *ABCA1* and *ABCA2* (Fig. 3F).

3.5. cDNA sequencing and expression of ABCA1 and ABCA2 genes in T. ni larval midgut

The ABCA1 and ABCA2 genes in the Cry2Ab resistance locus are predicted to be 22,677 bp and 22,479 bp in length in the *T. ni* genome with 29 and 31 introns, respectively (Tni17G01250 and Tni17G01240 in *T. ni* genome, http://www.tnibase.org). The predicted coding sequences (CDS) of the *T. ni* ABCA1 and ABCA2 genes code for the proteins of 1695 and 1747 amino acid residues, respectively (Table S4). cDNA sequencing of the ABCA1 and ABCA2 genes from the Cornell strain confirmed that the cDNA of the ABCA1 gene contains an open reading frame (ORF) of 5088 bp, coding for the ABCA1 with 1695 amino acid residues (Genbank acc. no. MK125333) and the cDNA of the ABCA2 gene has an ORF of 5304 bp, coding for the ABCA2 with 1767 amino acid residues (GenBank acc. no. MK125335). Analysis of *ABCA1* and *ABCA2* transcription by real-time RT-PCR indicates that the *ABCA2* transcript is more abundant within the *T. ni* larval midgut than in the larval carcass (Fig. 4). In contrast, *ABCA1* was expressed in the carcass, but the expression in the midgut was only minimally detectable (Fig. 4). The levels of expression of both *ABCA1* and *ABCA2* in the Cry2Ab resistant strain GLEN-Cry2Ab-BCS were similar to the susceptible Cornell strain without statistically significant difference (Fig. 4).

3.6. Mutations of ABCA1 and ABCA2 genes in GLEN-Cry2Ab-BCS

Sequencing of the cDNAs of the ABCA1 and ABCA2 genes from the GLEN-Cry2Ab-BCS strain identified three mutations in the ABCA1 gene and two mutations in the ABCA2 genes of the GLEN-Cry2Ab-BCS strain (GenBank acc. nos. MK125334 and MK125336). The mutations in the ABCA1 gene resulted in 3 single amino acid substitutions N788K, S988P and H1371R (Fig. S1A). The mutations in the ABCA2 gene resulted in a substitution mutation L1056V and a deletion-insertion (delins) mutation that changed 1551VETLAHALGFLRHLDKR1567 to 1551AHWGKLYGSNTQN1563 (Fig. 5; Fig. S1B).

Sequencing of the genomic DNA fragment of *ABCA2* in the L1056V mutation region from the susceptible and resistant strains (GenBank acc. nos. MN125702 and MN125703) showed that the L1056V mutation in the resistant strain resulted from a missense substitution mutation among other silent mutations and indels in introns (Fig. S2). The delins mutation at 1551VETLAHALGFLRHLDKR1567 resulted from an 2581 bp insertion. The genomic sequence at this mutation site region in the GLEN-Cry2Ab-BCS strain was assembled *de novo*, using the resequencing data (Genbank acc. no. MN088391). To confirm the sequence of this insertion, two overlapping PCR fragments of 2.3 kb and 2.1 kb were generated. Sequencing of the PCR products to assemble the 3425 bp fragment of this genomic DNA region (Genbank acc. no. MN088393) confirmed the sequence of the 2581 bp insertion in the GLEN-Cry2Ab-BCS strain, compared to the sequence from the Cornell strain (Genbank acc. no. MN088392) (Fig. S3). Sequencing of the genomic DNA in the two mutation regions from 3 Cornell strain individuals and 3 GLEN-Cry2Ab-BCS individuals showed no sequence variations among the individuals within the same strains.

The insertion of 2581 bp in *ABCA2* in the GLEN-Cry2Ab-BCS strain

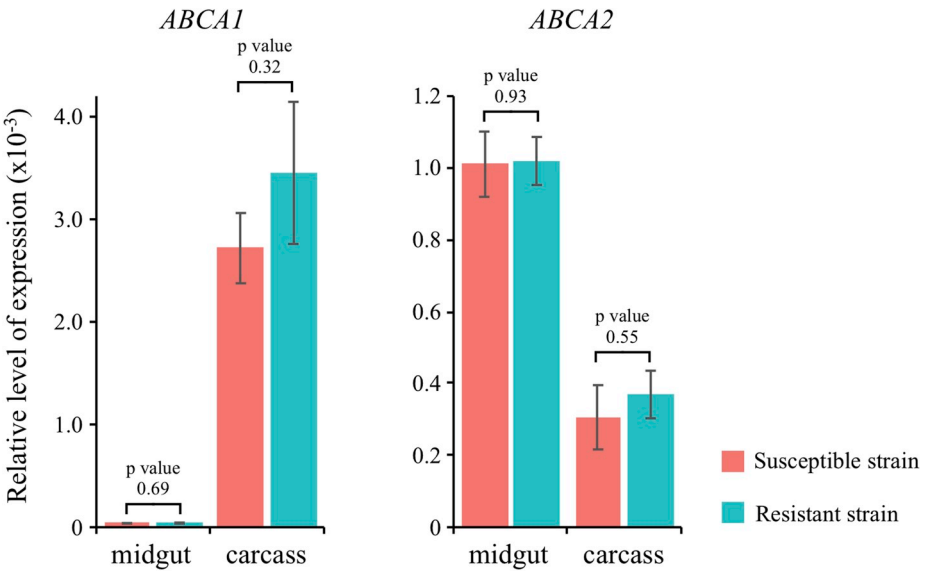
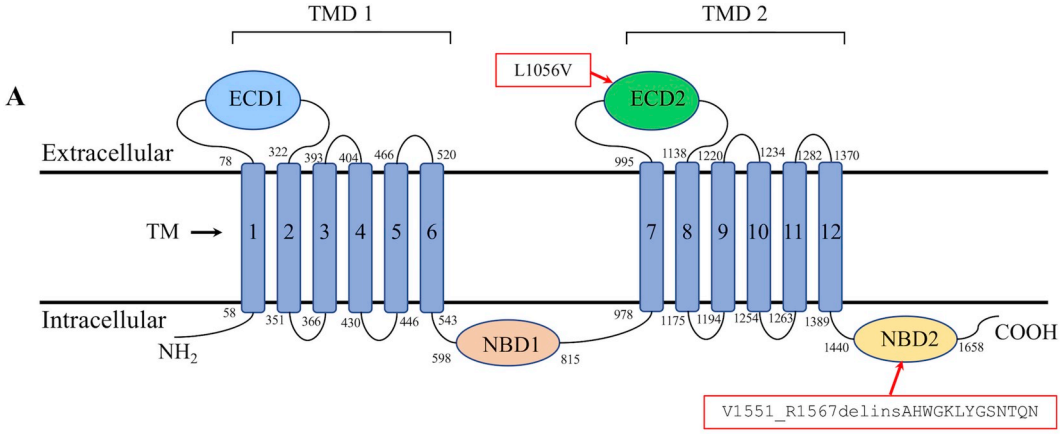


Fig. 4. Relative expression levels of the ABCA1 and ABCA2 genes in the midgut and carcass of *T. ni* larvae. Relative levels of expression of *ABCA1* and *ABCA2* in the midgut and carcass of *T. ni* larvae from the susceptible and Cry2Ab resistant strains. The expression levels were normalized to the expression of β-actin gene as the internal reference. Error bars indicate standard errors of the means. The p values shown in the figure were from *t*-tests of the expression levels between the susceptible and resistant strains with six individual larvae analyzed for each strain.

changed splicing sites in the transcript, leading to the delins mutation (Fig. S3, Fig. S1B). This insertion sequence could be found in multiple other regions in Chromosome 7, 16 and 24 in the *T. ni* genome (http://www.tnibase.org) (data not shown). By searching the repetitive DNA elements databases of Dfam (Hubley et al., 2015) and Repbase (Bao et al., 2015), this insertion sequence was identified to be a DNA transposon, named *Tntransib* hereafter, which encodes a 650 aa transposase, contains 38 bp terminal inverted repeats (TIRs) and 5 bp target site duplications (TSDs) (Fig. S3, Fig. 6). The *Tntransib* shares significant similarity with the Transib elements (Kapitonov and Jurka, 2003) and was identified to contain five repeats including a Transib-1_Sin, using the CENSOR software tool (https://www.girinst.org/censor/index.php) (Fig. 6B).

In the ABCA2, two transmembrane domains were predicted using CCTOP (http://cctop.enzim.ttk.mta.hu/) (Dobson et al., 2015) and structure of human ABCA1 (Qian et al., 2017), and the two nucleotide-



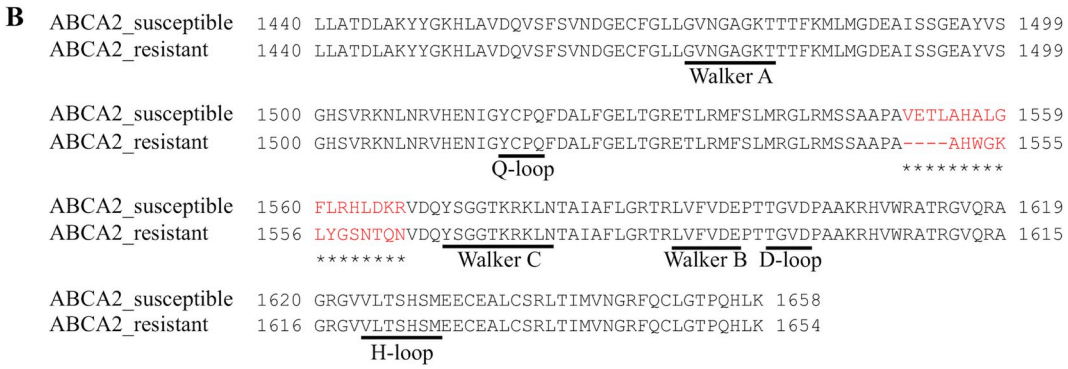


Fig. 5. Schematic structure of the *T. ni* ABCA2 protein and mutations in the Cry2Ab resistant strain. (A) The ABCA2 protein is predicted to contain two transmembrane domains (TMD 1 and 2) with 6 transmembrane helices (TM) in each domain, two nucleotide-binding domains (NBD1 and NBD2) and two extracellular domains (ECD1 and ECD2). Two mutations identified in the ABCA2 from the Cry2Ab resistant *T. ni* include a substitution mutation L1056V in ECD2 and a delins mutation from 1551VETLAHALGFLRHLDKR1567 to 1551AHWGKLYGSNTQN1563 in NBD2. (B) The delins mutation in NBD2 of ABCA2 in the Cry2Ab resistant strain is between the Q-loop motif and Walker C motif without disrupting the conserved motifs for the NBD.

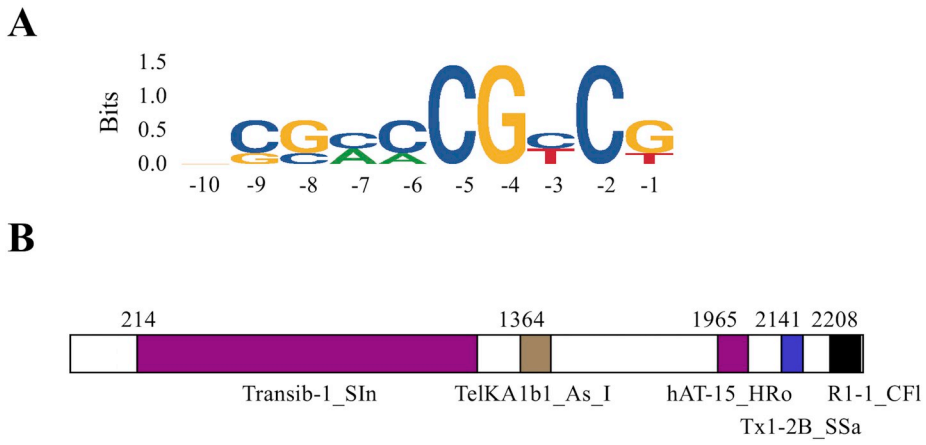


Fig. 6. Structure of *Tntransib*. (A) The *Tntransib* target site consensus sequence. Consensus sequence logo was created with four *Tntransib* insertions found in *T. ni* genome (http://www.tnibase.org). The relative height of each letter (nucleotide) represents the frequency of the nucleotide at each position and the height of a stack of letters represent the degree of sequence conservation at each position, indicated in arbitrary units (bits). The nucleotide position (the x-axis) indicates nucleotide position upstream of the *Tntransib* insertion. (B) Schematic structure of *Tntransib*. Five transposable elements were found in *Tntranib*, including two DNA transposons (Transib and hAT), one LTR retrotransposon (TelKA1b1) and two Non-LTR retrotransposon (R1 and Tx1).

binding domains, NBD1 and NBD2, were identified as ATP-binding cassette domains of the lipid transporters subfamily A (cd03263) by Conserved Domain Database (CDD) search (Marchler-Bauer et al., 2017) (Fig. 5A). The ABCA2 protein has the two extracellular domains, ECD1 between TM1 and TM2 and ECD2 between TM7 and TM8, which are unique to the ABCA subfamily (Biswas-Fiss et al., 2017; Qian et al., 2017). The substitution mutation L1056V in the Cry2Ab resistant strain was localized in ECD2. The 1551VETLAHALGFLRHLDKR1567 to 1551AHWGKLYGSNTQN1563 delins mutation was in NBD2, localized between Q-loop motif and Walker C motif (Fig. 5B).

Homology modeling of the ABCA2 using Swiss-Model (Waterhouse et al., 2018) showed that L1056V is localized in a loop in ECD2 (Fig. 7). The 1551VETLAHALGFLRHLDKR1567 to 1551AHWGKLYGSNTQN1563 mutation in NBD2 does not cause change of any of the known conserved motifs for the NBD in ABC transporters (Fig. 5B), but is predicted to cause change of the secondary structure in the region (Fig. 7).

3.7. Generation of T. ni strains with frameshift mutations in ABCA1 and ABCA2 by CRISPR/Cas9 mutagenesis and response of the mutant strains to Cry2Ab

Injection of gRNAs specific to the ABCA1 and ABCA2 genes, respectively, together with the Cas9 mRNA successfully introduced mutations in the ABCA1 and ABCA2 genes, respectively. After backcrossing of the mutant *T. ni* individuals with the wild type Cornell strain and selection of homozygous mutant individuals from the F_2 families, two *ABCA1* frameshift mutant strains and four *ABCA2* frameshift mutant strains were obtained. The two *ABCA1* mutant strains, Tn-ABCA1^{-}-1 and Tn-ABCA1^{-}-2, have a 22 bp and 11 bp deletion, respectively, in the coding region (Fig. S4A), leading to premature termination of the translation of ABCA1 (Fig. S4B). Of the four *ABCA2* mutant strains, Tn-ABCA2^{-}-1 and Tn-ABCA2^{-}-2 have a 395 bp and 394 bp deletion in genomic DNA, respectively, resulting in a deletion of 239 bp and 238 bp, respectively, in the cDNA (Fig. S5A). Tn-ABCA2^{-}-3 has a 2 bp deletion and Tn-ABCA2^{-}-4 has a 1 bp insertion in the coding region (Fig. S5A). The mutations in all four ABCA2 mutant strains lead to premature termination of translation of the ABCA2 protein (Fig. S5B).

Bioassays of the ABCA1 mutant strains, Tn-ABCA1^{-}-1 and Tn-ABCA1^{-}-2, with Cry2Ab showed that both ABCA1 mutant strains were similarly susceptible to Cry2Ab as the Cornell strain (Table 1). However, the 4 ABCA2 mutant strains were all highly resistant to Cry2Ab compared with the susceptible Cornell strain (Table 1). The F_1 progeny from the cross of the Cry2Ab resistant strain GLEN-Cry2Ab-BCS and the CRISPR/Cas9 mutant strain ABCA1^{-}-1 was also resistant to Cry2Ab (Table 1).

4. Discussion

In this study, the Cry2Ab resistance gene in *T. ni* was localized by

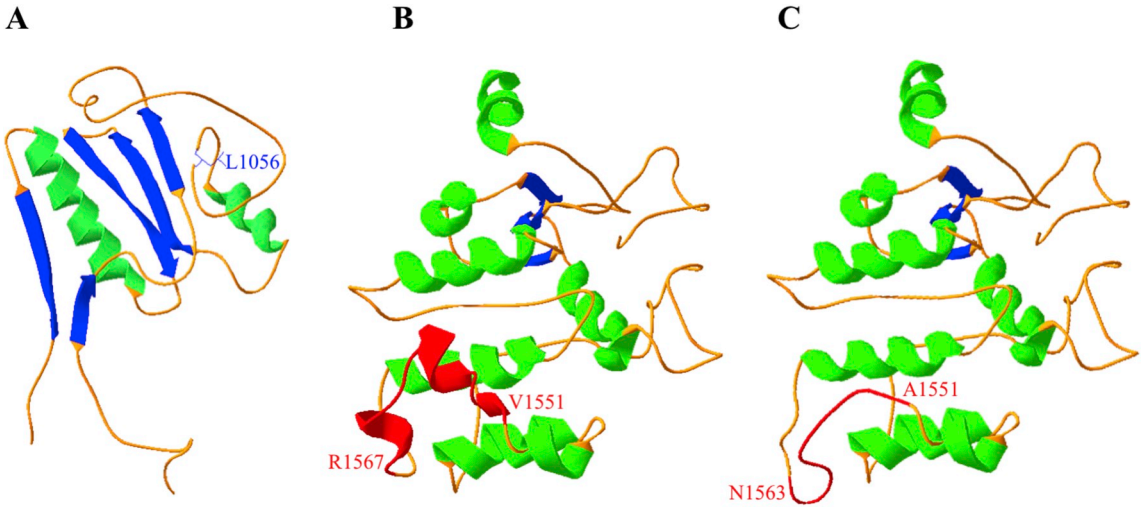


Fig. 7. Structural models of the domains ECD2 and NBD2 of *T. ni* ABCA2. (A) In the ECB2 domain, the mutation site L1056 is predicted to be in a loop of the extracellular domain. (B) The NBD2 domain of ABCA2 from Cornell strain. (C) The delins mutation in NBD2 from 1551VETLAHALGFLRHLDKR1567 in the susceptible strain to 1551AHWGKLYGSNTQN1563 in the Cry2Ab resistant strain is predicted to change of the secondary structure in the region.

Table 1
Susceptibility of *T. ni* strains to Bt toxin Cry2Ab.

T. ni strain	n	Slope (SE)	LC_{50} (μg/ml) (95% CI)	RR (95% CI)[a]
Cornell strain	400	2.73 (0.27)	0.40 (0.26–0.62)	1
GLEN-Cry2Ab-BCS	400	0.82 (0.13)	91.87 (41.03–181.05)	228 (125–333)
Tn-ABCA1^{-}-1	400	2.22 (0.21)	0.38 (0.18–0.88)	0.95 (0.73–1.23)
Tn-ABCA1^{-}-2	400	2.52 (0.23)	0.29 (0.15–0.59)	0.73 (0.57–0.94)
Tn-ABCA2^{-}-1	400	NA	> 360[b]	> 893
Tn-ABCA2^{-}-2	400	NA	> 360[b]	> 893
Tn-ABCA2^{-}-3	400	NA	> 360[b]	> 893
Tn-ABCA2^{-}-4	400	NA	> 360[b]	> 893
F_1 (Tn-ABCA2^{-}-1 x GLEN-Cry2Ab-BCS)	400	NA	> 360[b]	> 893

[a] RR = resistance ratio (LC_{50} of resistant strain/LC_{50} of Cornell strain).
[b] LC_{50} was not reached at the highest Cry2Ab concentration 360 μg/ml.

genetic linkage mapping, using high-throughput sequencing strategies (Figs. 2 and 3). Using an AmpSeq approach designed based on the genome resequencing data from the Cry2Ab resistant *T. ni*, the Cry2Ab resistance gene was finely mapped to a locus within 0.05–0.07 CM (Fig. 3E). The mapped resistance locus, a 39.4 kb region, only covers 2 genes, *ABCA1* and *ABCA2* (Fig. 3F). The expression of *ABCA1* in the larval midgut was only minimally detectable in *T. ni* larvae (Fig. 4), leaving *ABCA2* to be the only midgut gene in the resistance locus, which is similar to the ABCA1 gene expression in other lepidopteran larvae (Tay et al., 2015). The identified mutations in *ABCA2* from the Cry2Ab resistant strain lead to changes of the ABCA2 protein in two sites – a single amino acid substitution mutation in the extracellular domain ECD2 and a delins mutation of 17 aa fragment with a different 13 aa fragment in the intracellular nucleotide binding domain NBD2 (Fig. 5). The delins mutation in the ABCA2 domain NBD2 resulted from an insertion of *Tntransib*, a newly identified *T. ni* transposon in this study. The *Tntransib* insertion resulted in a change of an RNA splicing site (Fig. S3). Therefore, the Cry2Ab resistance in *T. ni* is associated with mutations of the same *ABCA2* gene that is also found to be associated with Cry2Ab resistance in the cotton pests *H. armigera*, *H. punctigera* and *P. gossypiella* (Mathew et al., 2018; Tay et al., 2015).

The essential role of ABCA2 in Cry2Ab toxicity in *T. ni* was confirmed by generation of *ABCA2* mutant strains using CRISPR/Cas9 mutagenesis in this study. Four different *ABCA2* mutant *T. ni* strains generated had frameshift mutations in the ABCA2 gene and were all resistant to Cry2Ab (Table 1). However, introduction of frameshift mutations in the ABCA1 gene, which is paralogous and adjacent to the ABCA2 gene, did not result in a change of susceptibility of the larvae to Cry2Ab (Table 1), confirming the accurate mapping of *ABCA2* to be the resistance gene. Genetic complementation test of the Cry2Ab resistance in GLEN-Cry2Ab-BCS strain with the *ABCA2* mutation in the ABCA2^{-}-1 strain indicated that the ABCA2 gene allele in the GLEN-Cry2Ab-BCS strain confers the recessive resistance to Cry2Ab (Table 1). However, the *ABCA2* CRISPR mutant strains all showed a significantly higher level of resistance to Cry2Ab than the GLEN-Cry2Ab-BCS strain and so did the F_1 progeny from the cross of an *ABCA2* CRISPR mutant with the GLEN-Cry2Ab-BCS strain (Table 1). Considering the mutations in the GLEN-Cry2Ab-BCS strain and the mutations introduced in the four *ABCA2* CRISPR mutant strains, it is not surprising to observe a higher level of resistance in the CRISPR mutant strains. The mutations in the GLEN-Cry2Ab-BCS strain cause one amino acid residue change in ECD2 and a replacement of a 17-aa fragment with a 13-aa fragment in NBD2, but the ABCA2 protein is expected to be intact in the midgut brush border. The mutant ABCA2 may partly retain the role mediating the toxicity of Cry2Ab in *T. ni*. In contrast, in the four CRISPR mutant strains translation of the ABCA2 protein was terminated in the transmembrane domain 1 between TM3 and TM5. The truncated ABCA2 protein is likely not to retain any function to mediate the toxicity of Cry2Ab or may even not be present in the midgut brush border. Therefore, the *ABCA2* allele in the GLEN-Cry2Ab-BCS strain is associated with the resistance at a level not as high as that of the alleles in CRISPR mutant strains.

Association of ABCA2 gene mutations with Cry2Ab resistance has been identified in three cotton pests *H. armigera*, *H. punctigera* and *P. gossypiella* (Mathew et al., 2018; Tay et al., 2015). The high level resistance to Cry2Ab observed in *T. ni* (Table 1) and *H. armigera* (Wang et al., 2017) with the ABCA2 gene disrupted by CRISPR/Cas9 mutagenesis definitively confirmed that ABCA2 is essential in the mode of action of Cry2Ab. However, how ABCA2 is involved in the pathway of toxicity of Cry2Ab remains unknown. The ABCA2 in the Cry2Ab resistant *T. ni* differs from the susceptible strain by a substitution mutation L1056V in the extracellular ECD2 and the change of 1551VETLAHALGFLRHLDKR1567 to 1551AHWGKLYGSNTQN1563 in the intracellular domain NBD2. L1056V is localized in an exposed loop in ECD2 (Fig. 7), so it could potentially be involved in interaction with Cry2Ab and change of this residue might lead to a change in interaction with the toxin. Binding of Cry2Ab to ABCA2 from *H. armigera* has been indicated (Wang et al., 2017). The deduced protein sequence of the *T. ni* ABCA2 from its cDNA and protein modeling indicated that the L1056V substitution is the only difference in the extracellular regions of ABCA2 between the susceptible and Cry2Ab resistant strains (Fig. 7). If the resistance to Cry2Ab in *T. ni* is due to loss of interaction of Cry2Ab with ABCA2 at the surface of the midgut brush border membrane, then the loop with L1056 in *T. ni* might be directly involved in interacting with Cry2Ab and the L1056V mutation may lead to change of the interaction. However, the L1056V mutation is a substitution of L to the structurally and biochemically very similar V in a loop, and this substitution is also present in the ABCA2 gene in the *T. ni* cell line HighFive (XP_026,725,288.1) (Fu et al., 2018). As the HighFive cell line (Granados et al., 1994) was established from a *T. ni* laboratory colony that had never been selected with Bt, it is not expected that the HighFive cell line would be established from a *T. ni* colony carrying the Cry2Ab resistant allele of the ABCA2 gene. Whether the L1056V mutation confers the resistance requires to be experimentally tested. The protein region 1551VETLAHALGFLRHLDKR1567 is localized in the intracellular NBD2, so it is not expected to be directly involved in binding with the toxin at the cell surface. The change of 1551VETLAHALGFLRHLDKR1567 to 1551AHWGKLYGSNTQN1563 is not predicted to cause any changes of the known conserved motifs for an NBD in ABC transporters (Fig. 5), but is expected to disrupt the secondary structures to become a loop in this region (Fig. 7). Whether this structural change leads to a functional change of ABCA2 as an ABC transporter is unknown. Interestingly, a mutation in the NBD2 of ABCA2 was also found in Cry2Ab resistant *H. punctigera* (Tay et al., 2015). Therefore, the mutations in the NBD2 of ABCA2 found in *T. ni* and *H. punctigera* indicate that Cry2Ab resistance might be conferred by a mutation in the intracellular NBD domain, in addition to disruptive mutations in an extracellular domain at the cell surface (Mathew et al., 2018; Tay et al., 2015). Further studies are needed to understand whether the *ABCA2* mutation mediated resistance to Cry2Ab in *T. ni* is

conferred by alteration of binding of ABCA2 with the toxin at the cell surface or by a functional change of ABCA2 as a transporter.

Cry2Ab is known not to share binding sites with Cry1A toxins and there is no cross-resistance between Cry2Ab and Cry1A toxins (Caccia et al., 2010; Hernandez-Rodriguez et al., 2008, 2013; Song et al., 2015; Wang et al., 2018). Therefore, major midgut proteins involved in the mode of action of Cry2Ab are not expected to be shared with the major proteins involved in the pathways of toxicity of Cry1A toxins (Wang et al., 2018). Studies on Cry2Ab resistance in *T. ni* and three other lepidopterans have consistently identified the association of ABCA2 with the toxicity of Cry2Ab (Mathew et al., 2018; Tay et al., 2015; Wang et al., 2017) and demonstrated that mutation of *ABCA2* alone is sufficient to confer resistance to Cry2Ab (Table 1) (Wang et al., 2017). The Cry2Ab resistant *H. armigera*, *H. punctigera, P. gossypiella* and *T. ni* studied were derived populations that had been exposed to Bt in the field or greenhouses (Kain et al., 2015; Mathew et al., 2018; Tay et al., 2015). These four insects belong to three different families and their resistant populations were from three different continents, and were exposed to Bt toxins in cotton plants for *H. armigera*, *H. punctigera* and *P. gossypiella* and to Bt sprays on vegetable plants (Kain et al., 2015; Mathew et al., 2018; Tay et al., 2015). However, the resistance gene selected was the same, the ABCA2 gene, in all four species. It is interesting that resistance of lepidopterans to different groups of Cry toxins has been found to be associated with an ABC transporter from different subfamilies. Cry1Ac resistance has been found to be associated with *ABCC2*, Cry2Ab resistance has been found to be associated with *ABCA2*, and Cry3Aa resistance has been found to be associated with *ABCB1* (Baxter et al., 2011; Gahan et al., 2010; Mathew et al., 2018; Pauchet et al., 2016; Tay et al., 2015) (Fig. 2). Studies on mode of action of Cry toxins has been mainly from Cry1A toxins. For Cry1Ac, multiple insect midgut receptors for Cry1Ac have been identified and functionally confirmed (Baxter et al., 2011; Bretschneider et al., 2016; Gahan et al., 2001, 2010; Morin et al., 2003; Xu et al., 2005; Zhang et al., 2017). The midgut cadherin and ABCC2 as functional receptors for Cry1Ac are involved in two independent pathways of toxicity of Cry1Ac in *T. ni* (Wang et al., 2018). Depending on the pathways of toxicity effective in insects, high level resistance to Cry1Ac can be conferred by mutations of the cadherin gene (Morin et al., 2003; Xu et al., 2005), the ABCC2 gene (Baxter et al., 2011) or both genes (Gahan et al., 2001, 2010). So far, ABCA2 is the only protein that has been functionally confirmed to be an essential midgut protein involved in the toxicity of Cry2Ab (Table 1) (Wang et al., 2017), and all cases of Cry2Ab resistance have been found to be associated with mutations in the ABCA2 gene (Mathew et al., 2018; Tay et al., 2015). The finding that ABCA2 mutation is the common genetic basis of resistance to Cry2Ab indicates that F_1 screening with an ABCA2 knockout mutant strain could be an effective approach for detection of Cry2Ab resistant alleles in field insect populations.

Acknowledgements

We thank Dr. Te-Wen Lo for providing us with plasmid pTY2631 for Cas9 mRNA preparation and Dr. Qi Sun for advice on bioinformatic analysis. This work was supported by the USDA Biotechnology Risk Assessment Grant Program Competitive Grant no. 2012-33522-19791, the USDA AFRI Foundational Program Competitive Grants no 2016-67013-24754 and no. 2019-67013-29349.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.103209.

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