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Linkage of an ABCC transporter to a single QTL that controls *Ostrinia nubilalis* larval resistance to the *Bacillus thuringiensis* Cry1Fa toxin

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ABSTRACT

Field evolved resistance of insect populations to *Bacillus thuringiensis* (Bt) crystalline (Cry) toxins expressed by crop plants has resulted in reduced control of insect feeding damage to field crops, and threatens the sustainability of Bt transgenic technologies. A single quantitative trait locus (QTL) that determines resistance in *Ostrinia nubilalis* larvae capable of surviving on reproductive stage transgenic corn that express the Bt Cry1Fa toxin was previously mapped to linkage group 12 (LG12) in a backcross pedigree. Fine mapping with high-throughput single nucleotide polymorphism (SNP) anchor markers, a candidate ABC transporter (*abcc*2) marker, and *de novo* mutations predicted from a genotyping-by-sequencing (GBS) data redefined a 268.8 cM LG12. The single QTL on LG12 spanned an approximate 46.1 cM region, in which marker 02302.286 and *abcc*2 were ≤2.81 cM, and the GBS marker 697 was an estimated 1.89 cM distant from the causal genetic factor. This positional mapping data showed that an *O. nubilalis* genome region encoding an *abcc*2 transporter is in proximity to a single QTL involved in the inheritance of Cry1F resistance, and will assist in the future identification the mutation(s) involved with this phenotype.

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1. Introduction

Mechanical damage caused by herbivorous insect feeding on cultivated crop plants lead directly to reduced commodity quality and compromised plant health, and can provide entry points for plant pathogens. Commercially available genetically engineered (GE) crop plants that express transgenic *B. thuringiensis* (Bt) crystalline (Cry) protein toxins can be effective at controlling feeding damage caused by many pest insect species. Initial releases of corn hybrids that expressed single Bt toxins suppressed several target insect populations (Hutchison et al., 2010), and have been promoted as agents of environmental stewardship that allowed a reduced reliance on broad spectrum chemical insecticide use. Despite the efficacy of early Bt crop varieties, concern was voiced that the technology would rapidly lead to resistance evolution as had be observed repeatedly with a number of species exposed to broadcast applications of an insecticides with a single mode of action (Roush and McKenzie, 1987). Furthermore, selection for survival following repeated foliar Bt sprays had already been documented in the lepidopteran insect species *Plodia interpunctella* (McGaughey, 1985) and *Plutella xylostela* (Tabashnik et al., 1990) prior to the commercialization of Bt crops in 1995. In response to these concerns, insect resistance management (IRM) plans based on the "high-dose"/refuge (HDR) strategy (Alstad and Andow, 1995; Bates et al., 2005; US EPA, 2001) were mandated in conjunction with product registrations, and were aimed at maintaining the overall Bt susceptibility of insect populations such that the efficacy of Bt toxins would be preserved.

HDR-based IRM measures have been successful in delaying or preventing the onset of resistance among a number of target insect species (Huang et al., 2011). However, field-evolved resistance, as defined by the reduced frequency of susceptible phenotypes in an insect population following exposure to an insecticidal toxin (National Research Council, 1986; Tabashnik, 1994), has been documented in an increasing number of cases. Increased resistance to corn hybrids that express the Bt Cry1Ab toxin was documented in populations of the African stem borer, *Busseola fusca* (van Rensburg, 2007). Severe damage was reported and field-evolved

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resistance was confirmed to Cry1F transgenic corn by fall armyworm, *Spodoptera frugiperda*, in Puerto Rico (Matten et al., 2008; Storer et al., 2010) and more recently in Brazil (Farias et al., 2014). Additionally, populations of the western corn rootworm, *Diabrotica virgifera virgifera* show an increased ability to survive on Cry3Bb1 and mCry3A transgenic corn hybrids (Gassmann et al., 2011, 2014). Other cropping systems have been affected by the emergence of insect pest populations that show reduced levels of Bt toxin susceptibility. For instance, the cotton pests *Helicoverpa armigera*, *H. zea*, and *Pectinophora gossypiella* show increasing levels of tolerance to transgenic plants that express the Cry1Ac toxin (Gunning et al., 2005; Dhurua and Gujar, 2011; Tabashnik and Carriere, 2010; Zhang et al., 2012; Alvi et al., 2012). Field-evolved resistance to transgenic crops have arguably developed when principles of the HDR strategy have not been fully met, such as by dominance of resistance alleles in the case of *H. armigera* (Zhang et al., 2012; Jin et al., 2013) and *B. fusca* (Campagne et al., 2013) or when a high dose is not expresses as is the case for *D. v. virgifera*, and is potentially exacerbated when resistance traits lack fitness costs (Kruger et al., 2014).

The molecular mode by which ingested Bt toxins disrupt the integrity of midgut epithelial cells among susceptible lepidopteran insects remains somewhat uncertain, but has been proposed to involve sequential binding to midgut receptors (Bravo et al., 2007). In this model, an initial transient binding to membrane-bound cadherin was suggested to facilitate the enzymatic cleavage of ingested Cry toxins, which promotes the subsequent oligimerization of cleaved toxins into unbound pre-pore structures. Secondary interactions of oligomerized toxins with GPI-anchored receptors including alkaline phosphatase and aminopeptidase N result in pre-pore localization in proximity to membrane surfaces such that the probability of insertion into the lipid bilayer is increased. This formation of ion pore channels would subsequently lead to cell disruption by ion influx or intracellular signaling (Piggot and Ellar, 2007; Soberon et al., 2009). The genetic basis of Bt toxin resistance among Lepidoptera has been linked to mutations in midgut expressed proteins, and include changes in the post-translational glycosylation of membrane-bound alkaline phosphatase (*alp*; Knowles et al., 1991; Jurat-Fuentes et al., 2002), reduced expression of aminopeptidase N (*apn*) transcripts (Herrero et al., 2005; Tiewsiri and Wang, 2011; Coates et al., 2013), and structural changes to cadherin (Gahan et al., 2001; Morin et al., 2003; Xu et al., 2005) or ABC transporter (Gahan et al., 2010; Baxter et al., 2011). Interestingly, linkage of an ABC transporter with inheritance of Cry1Ac resistance was accomplished in the *Heliothis virescens* YEE strain (Gahan et al., 2010), that was a descendent of the YHD2 strain in which Cry1Ac resistance had previously been associated with a transposon-mediated knockout of the cadherin gene (Gahan et al., 2001). Since the ABC transporter was shown to confer Cry1Ac resistance independently of cadherin (Gahan et al., 2010), a tertiary interaction of pre-pore structures with ABCC2-like proteins was proposed. In this extended model, ABCC2 transporters in the "open" state were proposed to function as receptors to which oligomerized Bt toxins bound prior to membrane insertion (Heckel, 2012).

Although resistance to Cry1F corn in field populations of the European corn borer, *O. nubilalis*, has not been shown to affect product performance (Gaspers et al., 2011; Siegfried and Hellmich, 2012), laboratory selections have resulted in increase tolerance of this insect to Bt Cry1Ac (Bolin et al., 1999), Cry1Ab (Siqueira et al., 2004; Coates et al., 2007) and Cry1Fa toxins (Pereira et al., 2008a). Traits conferring Cry1Fa resistance in *O. nubilalis* provide no significant cross resistance to Cry1Ab or Cry9C toxins, and low-levels of cross resistance to Cry1Ac (Pereira et al., 2008a). Moreover, resistance alleles conferring resistance to Cry1F have been identified in *O. nubilalis* field populations although changes in the frequency of these allele have not been detected (Siegfried et al., 2014).

Studies based on laboratory selected models have highlighted the presence of resistance alleles within natural populations (Gould, 1998; US-EPA, 1998), and have been used to identify potential modes of Bt resistance (Gahan et al., 2001, 2010). A recessively-inherited *O. nubilalis* laboratory model for Cry1Fa toxin resistance has been developed that confers larval survival on reproductive stage Cry1Fa transgenic corn plants (Pereira et al., 2008a,b). Subsequent genetic mapping showed that a single major quantitative trait locus (QTL) contributed to inheritance of Cry1Fa resistance in *O. nubilalis*, which segregated independent of genetic markers for the Bt toxin binding receptors aminopeptidase N, cadherin, and alkaline phosphatase genes (Coates et al., 2011a). In the following, fine mapping of the *O. nubilalis* Cry1Fa QTL was conducted using single locus single nucleotide polymorphism (SNP) markers (Coates et al., 2011b) and genotyping-by-sequencing (GBS) approaches (Baird et al., 2008; Elshire et al., 2011; Truong et al., 2012) in conjunction with single locus candidate Bt toxin markers. Tabashnik et al. (1997) previously demonstrated that a single genetic locus both determined Cry1A and Cry1F resistance in the diamondback moth, *Plutella xylostella*, strain NO-QA, and indirect linkage of Cry1F resistance to an ABC transporter mutation was made by QTL mapping of Cry1Ac resistant phenotypes (Baxter et al., 2011). In contrast, the current study provides direct evidence of possible ABC transporter involvement in Cry1Fa resistance in Lepidoptera, and moreover can confer Cry1Fa resistance in phenotypes with negligible cross-resistance to Cry1A toxins (Pereira et al., 2008a).

2. Materials and methods

2.1. Genotyping assays

2.1.1. Single nucleotide polymorphism (SNP) markers

A laboratory strain of *O. nubilalis* was previously described with >12,000-fold increase in larval tolerance to Cry1Fa toxin with low cross resistance to Cry1A toxins, and capable of surviving on reproductive stage transgenic corn plants (Pereira et al., 2008a), where the trait is mostly recessive and controlled by the inheritance of a single locus (Pereira et al., 2008b; Coates et al., 2011a). Two backcross families, FQ4 and FQ5, carry a segregating Cry1Fa resistance trait were previously established (Coates et al., 2011a) using a biphastic pedigree design. Briefly, the pedigree was created from a single pair mate pair of a Cry1Fa resistant female (rr♀; $P_{rr♀}$) × susceptible male (SS♂; $P_{SS♂}$). Two subsequent backcross families, FQ4 and FQ5, were respectively generated by F_1 male × Cry1Fa resistant female ($F_{1rS♂}$ × $BCP_{rr♀}$) and an F_1 female × Cry1Fa resistant male crosses ($F_{1rS♀}$ × $BCP_{rr♂}$). Backcross (F_2) progeny from FQ4 and FQ5 were reared on either non-Cry1Fa control diets or a diagnostic Cry1Fa toxin overlay that caused 100% mortality of susceptible phenotypes (see Fig. 1 in Coates et al., 2011a).

Total genomic DNA prepared from parental, untreated control and survivors of a Cry1Fa diagnostic bioassay in FQ4 and FQ5 (Coates et al., 2011a), and genotyped with single locus SNP assays on the Sequenom MassARRAY® performed at the Iowa State University Center for Plant Genomics (ISU-CPG). Preliminary genotyping of FQ5 $F_{1rS♀}$ and $BCP_{rr♂}$ parents ($n = 2$) and backcross progeny from non-Cry1Fa control diet ($n = 8$) and Cry1Fa toxin survivor groups ($n = 8$) used 756 validated *O. nubilalis* SNP markers (multiplexes W1 to W20; Coates et al., 2011b, 2013). Variation at SNP loci in aminopeptidase N (*apn*1 and *apn*3), and an ABCC2-like candidate Bt-resistance genes were detected as described by Coates

et al. (2008, 2013). PCR-RFLP data were converted to associated nucleotide data and merged with data from the Sequenom MassARRAY® multiplexes by individual.

Segregating SNP markers were identified manually which were heterozygous in the FQ5 $F_{1rS♀}$, homozygous in both the $BCP_{rr♂}$ and progeny Cry1Fa toxin survivor group, as well as homozygous and heterozygous among progeny from control diet. All other SNP markers were discarded. Deviation of segregating markers from predicted 1:1 Mendelian genotype ratios were evaluated with the Chi-square (χ^2) statistic from non-Cry1Fa control progeny group data, and non-Mendelian markers below a significance threshold of $\alpha < 0.05$ were omitted from further analyses. Sequenom MassARRAY® multiplexes that detected Mendelian SNPs inherited from the above parental genotypes (W1, W2, W3, W7, W8 and W12; see Results) were used to genotype additional FQ5 backcross progeny from non-Cry1Fa control diet ($n = 16$) and Cry1Fa toxin survivor groups ($n = 16$). Genotypes from FQ4 $F_{1rS♂}$ and $BCP_{rr♀}$ ($n = 2$) parents, and non-Cry1Fa control diet ($n = 44$) and Cry1Fa toxin survivors ($n = 72$) were determined with SNP markers in the selected set of Sequenom MassARRAY® multiplexes (W1, W2, W3, W7, W8 and W12).

2.1.2. Illumina sequencing read data

Pipelines that combine high throughput DNA sequencing methods and *de novo* SNP discovery among homologous short nucleotide read data have been reported (Baird et al., 2008; Elshire et al., 2011; Truong et al., 2012). In this experiment, PCR templates with *Eco*RI and *Mse*I adapters ligated at corresponding restriction endonuclease sites was previously generated in a standard AFLP protocol (Vos et al., 1995) for FQ4 and FQ5 backcross families (Coates et al., 2011a). This AFLP template was modified by PCR-based attachment of Illumina HiSeq2500 flow cell adapter and sequencing primer sequences to facilitate high throughput sequencing. Specifically, a set of EcoRI-GBS_bc oligonucleotides were designed to anneal AFLP *Eco*RI adapters and to destroy the remaining *Eco*RI palindrome (5′-GAC TGC CGT ACC AAT TC-3′), and also to contain 5′ overhangs with a unique barcode, P1 sequencing primer, and flow cell adapter sequences (Fig. 1; Table S1). Unique barcodes incorporated into each EcoRI-GBS_bc oligonucleotide which facilitate sample multiplexing were designed using the GBS Barcode Generator (http://www.deenabio.com/services/gbs-adapters). In contrast, a single universal oligonucleotide primer, MseI-GBS, was designed to anneal to the *Mse*I adapter end (5′-CGA TGA GTC CCT GAG TAA-3′), which also incorporated the appropriate flow cell adapter and Illumina HiSeq P2 sequencing primer sequences (Fig. 1; Table S1). All oligonucleotides were synthesized and HPLC-purified at Integrated DNA Technologies (Coralville, IA).

AFLP template for FQ4 parents ($n = 2$) and progeny from non-Cry1Fa control diet ($n = 68$; total Control + parents $n = 70$; Table 1), and FQ4 parents ($n = 2$) and Cry1Fa survivors ($n = 64$; total Survivors + parents $n = 66$; Table 1) were PCR amplified separately using 5 μM of the universal MseI-GBS primer and 5 μM of an EcoRI-GBS-bc oligonucleotide (barcode assignments shown in Table S1). Reactions were carried out in a 10 μl PCR-AFLP reaction as described by Coates et al. (2011a), except that the high fidelity LongAmp DNA polymerase was used and temperature for extension was reduced to 68 °C as defined by the manufacturer (New England Biolabs, Ipswich, MA). A total of 5 μl of each PCR amplified product was separated individually by electrophoresis on 2% agarose gels with 0.5 μg ml^{-1} ethidium bromide and visualized under UV illumination. Each successfully amplified PCR product was incubated with 0.5 U Shrimp Alkaline Phosphatase (SAP; New England Biolabs) and 0.5 Unit Exonuclease I (ExoI; New England Biolabs) at 37 °C for 45 min followed by 72 °C for 10 min. Reactions were then purified using Qiagen PCR Quick Spin columns according to manufacturer instructions (Qiagen, Valencia, CA). Purified template was quantified on a Nanodrop 2000 (Thermo Scientific, Wilmington, DE), and pooled in approximate equal proportions for controls with parents (Table 1 Control + parents $n = 70$; *Pool01*) and FQ4 Cry1Fa survivors with parents (Table 1 Control + parents $n = 66$; *Pool02*). The FQ4 $F_{1rS♂}$ and $BCP_{rr♀}$ parents were included in both *Pool01* and *Pool02*, but used different barcodes (Table S1). *Pool01* and *Pool02* template DNAs (Table 1) were submitted for single end 100-bp Illumina HiSeq2500 sequencing (with the P1 primer) in separate flow cells at the Iowa State University DNA Facility, Ames, IA.

2.2. Next generation sequence data and de novo mutation discovery

Data from *Pool01* (Contols + parents) and *Pool02* (Survivors + parents) were received as fastq-formatted files, which contain sequence and Sanger (Illumina 1.8+) quality (q) scores. Fastq files consisting of multiple barcoded samples were separated into individual-specific libraries (demultiplexed) using the PERL script bcsort_se.pl (Knaus, 2011) allowing no mismatches. Individual barcoded libraries were trimmed of adapters and sequence with a q-score < 20 using TrimmingReads.pl from the NGSQCToolkit (Patel and Jain, 2012). Processed read data from the FQ4 $BCP_{rr♀}$ parent was converted to fasta format, and then clustered using CD-HIT (Fu et al., 2012) with a 95% identity cutoff. A reference genome index was built from clustered $BCP_{rr♀}$ sequences using the Bowtie2 faidx command (Langmead and Salzberg, 2012), which allowed for comparative prediction of variant nucleotides (alleles) in the recombinant FQ4 $F_{1rS♂}$ parent as well as among segregating progeny. Processed Illumina fastq data from FQ4 parental and all progeny libraries were aligned separately to CD-HIT clusters using the Bowtie2 aligner (Langmead and Salzberg, 2012) in sensitive-local mode that allowed for soft-clipping of potential mismatches at the periphery of each read. Bowtie2 alignments were output as a sequence alignment map (SAM) file. Samtools (Li et al., 2009) was used to convert the SAM to a binary alignment (BAM) format, extract reads that mapped only once to the reference (Samtools view -bq 1), and sorted by leftmost coordinates (Samtools sort).

Sorted BAM alignments from FQ4 parents, non-Cry1Fa toxin control progeny, and Cry1Fa survivors were merged into a single file with the Samtools mpileup command using -f (fasta formatted index) and -g command (compute genotype likelihoods for each individual and output in binary variant call, .bcf, file format). Files were filtered for only variant sites using Baysian inference on a per sample basis (-vcg options) and converted to variant call format (.vcf) using BCFtools view. Resulting .vcf v 4.1 output were filtered using VCFtools vcfutils.pl (Danecek et al., 2011) varFilter options for minimum and maximum read depths respectively of 10 (-d 10) and 150 (-D 150), and for omission of *de novo* SNPs within 5-bp of any alignment gap (-w 5). Putative insertion-deletion mutations (indels) were retained. Candidate SNPs and indels were filtered to contain genotype configurations with the FQ4 $F_{1rS♂}$ heterozygous (0/1) and the $BCP_{rr♀}$ (homozygous (1/1 or 0/0)), and further to remove any locus with Phred-scaled genotype (GT) score of either parent < 20. Analogously, progeny GT scores <20 genotypes were considered missing data and significance of genotype ratios among non-Cry1Fa controls from 1:1 Mendelian genotypic proportions evaluated using the χ^2-statistic ($\alpha < 0.05$).

2.3. Quantitative trait locus mapping

2.3.1. Genetic linkage

Genotypes from Mendelian SNP markers from FQ4 and FQ5 pedigrees within the above sorted Sequenom Typer® output were merged with PCR-RFLP and AFLP marker data previously generated for the same individuals (Coates et al., 2011a). Genetic linkage was

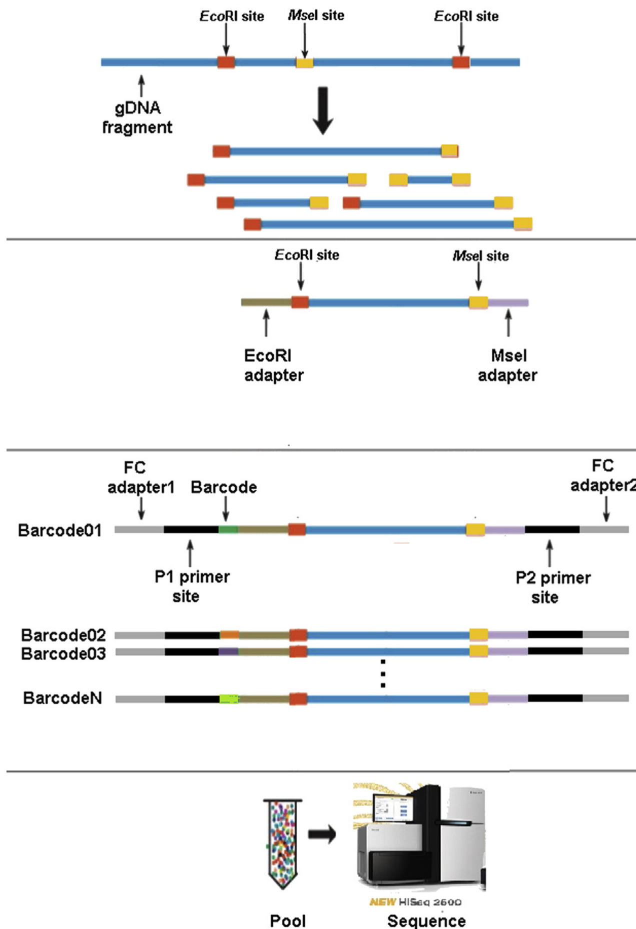

Fig. 1. Schematic showing methods used to modify amplified fragment length polymorphism (AFLP) template by PCR-based attachment of Illumina HiSeq2500 flow cell adapter, sequencing primer and unique barcode sequences in a genotyping-by-sequencing protocol.

Table 1
Illumina HiSeq 2500 reads generated from reduced representation libraries from pedigree FQ4 individuals and backcross parents; *Pool01* (non-Cry1Fa control and backcross parents: “Control + parents”) and *Pool02* (Cry1Fa survivors and backcross parents; “Survivors + parents”).

Library	Treatment	Template & raw read data				Processed read data			
		n	Reads	Mean L.	Nts	*n*	Reads	Mean L.	Nts
Pool01	Control + parents	70[a]	208.9	100	14,943	63[c]	175.1	83	11,511
Pool02	Survivors + parents	66[b]	146.8	100	11,081	60[d]	127.0	83	10,642

Number of reads (Reads) and nucleotides (Nts) reported in millions. a. PCR libraries from control ($n = 68$) and parents ($n = 2$); b. PCR libraries from Cry1Fa survivors ($n = 64$) and parents ($n = 2$); c. Successfully sequenced PCR libraries from control ($n = 61$) and parents ($n = 2$); d. PCR libraries from control ($n = 58$) and parents ($n = 2$).

initially estimated among Mendelian-inherited AFLP and Sequenom SNP markers in non-Cry1Fa control groups from pedigrees FQ4 ($n = 44$) and FQ5 ($n = 22$) using MAPMAKER 3.0 (Lincoln et al., 1992) as described by Coates et al. (2013). A merged dataset of Sequenom Typer® output and *de novo* SNP loci called from sorted BAM alignments ($n = 58$) were analogously used to construct a second linkage map with MAPMAKER 3.0 using methods as described above.

2.3.2. QTL mapping of Cry1Fa resistance

Since FQ4 Cry1Fa phenotype data were categorical (survivor only) and devoid of a susceptible group, traditional quantitative genetic interval mapping procedures could not be applied. Thus the application of the degree of deviation of Mendelian markers in the Cry1Fa survivor group from expected genotypic ratios was used as a proxy for a positional mapping statistic as previously developed by Heckel et al. (1998) as described by Coates et al. (2011a; 2013). In brief, the Chi-square (χ^2) statistic was used to assess any deviation in genotype frequency at a SNP locus among Cry1Fa survivors with a Bonferroni-adjusted significance threshold (α/N; N = number of markers on the associated LG). The relative position of a QTL was determined by plotting Bonferroni-adjusted *P*-values for each genetic marker according to the estimated centiMorgan (cM) distance along the length of each LG.

3. Results

3.1. Genotyping assays

3.1.1. Single nucleotide polymorphism (SNP) markers

Analysis of Sequenom MassARRAY® genotyping data from multiplexes W1 to W20 detected 382 segregating SNPs among

non-Cry1Fa control progeny genotypes ($n = 22$) and Cry1Fa survivors ($n = 24$) in pedigree FQ5. Of these markers, 70 SNPs exhibited both Mendelian inheritance (χ^2 associated *P*-values ≥ 0.05) and were in a configuration that allowed detection of segregating alleles from heterozygote $F_{1rS♀}$ and homozygote $BCP_{rr♂}$ parents (Table S2A). Genotyping of FQ4 $F_{1rS♂}$ and $BCP_{rr♀}$ ($n = 2$) parents, non-Cry1Fa control ($n = 44$) and Cry1Fa toxin survivors ($n = 72$) with this subset of SNP markers (Sequenom MassARRAY® multiplexes W1, W2, W3, W7, W8 and W12) detected 128 segregating markers. Among these 128 markers, 57 were inherited from a heterozygous male parent genotype and showed no deviation from a predicted 1:1 Mendelian ratio of homozygote to heterozygote genotypes among FQ4 non-Cry1Fa control individuals (χ^2 *P*-values ≥ 0.05; Table S2B).

3.1.2. Illumina sequencing read data

A total of 208.9 and 146.8 million reads were received within Illumina HiSeq 2500 raw sequence data files for *Pool01* (FQ4 Controls + parents) and *Pool02* (FQ4 Cry1Fa survivors + parents), respectively (Table S3). A total of 22.1 billion nucleotides remained after reads were trimmed to remove adapter sequences and nucleotides with a $q < 20$ from *Pool01* and *Pool02* (processed reads; Table 1).

3.2. Next generation sequence data and de novo mutation discovery

Processed read data included demultiplexing of fastq formatted sequences, which recovered barcoded libraries for multiplexed samples in the two pools; *Pool01*, 63 of 68 Controls + parents, and *Pool02*, 60 of 66 Cry1Fa toxin Survivors + parents (Table 1; Table S3). Clustering of short read data from FQ4 $BCP_{rr♀}$ parent by CD-HIT produced 67,641 sequences >82-bp (data not shown). Alignment of processed Illumina read data from parental and FQ4 progeny to the CD-HIT reference clusters resulted in mapping of 175.1 and 127.0 million reads from *Pool01* [Controls ($n = 61$) + parents ($n = 2$); Total $n = 63$] and *Pool02* [FQ4 Cry1Fa Survivors ($n = 58$) + parents ($n = 2$); Total $n = 60$] (Table 1; Table S1), but varied across libraries (*Pool01* 2.9 ± 0.4 and *Pool02* 2.2 ± 0.2 million reads per individual library; Table 2). When considering only the reads that mapped to a single position in the reference, a mean of 1.3 ± 0.3 and 1.0 ± 0.1 million reads were mapped per individual in *Pool01* and *Pool02*, respectively (e.g. putatively unique mapping reads that are putatively unaffected by repetitive DNA elements), while ≥801,297 reads from FQ4 parents were similarly mapped to the reference (Table S3).

Read depths among alignments were not taken into account within BAM files, but subsequent calling of mutations from sorted BAM files requiring a minimum read depths of 10 as well as removal of SNPs ≤5 nucleotides away from any alignment gap resulted in calling of 12,621 putative segregating mutations. Specifically, segregating mutations were defined as being inherited from heterozygous FQ4 $F_{1rS♂}$ (0/1) and homozygous $BCP_{rr♀}$ (1/1 or 0/0) parental genotypes in the VCF file output, which were further culled to 1389 after highly stringent filtering out loci with GT < 20 for any parental or progeny genotype. The number of segregating markers was further reduced to 1026 following removal of those with significant departures from a Mendelian 1:1 ratio among FQ4 control genotypes (*P*-values ≤ 0.05: Table S4).

Table 3
Significance of deviations of Mendelian-inherited SNP markers among Cry1Fa survivors using the Chi-square (χ^2) statistic in pedigrees A) FQ5 and B) FQ4.

Marker	LG	Non-Cry1Fa controls ($n = 22$)		Cry1Fa survivors ($n = 24$)	
A) Pedigree FQ5					
		χ^2 (1:1)	*P*-value	χ^2 (1:1)	*P*-value
contig00154.201	12	0.18	0.66982	24.00	<0.0001
contig00580.451	12	4.00	0.04550	24.00	<0.0001
contig02302.286	12	0.73	0.39377	24.00	<0.0001
contig05497.424	12	0.73	0.39377	24.00	<0.0001
contig05852.999	12	0.73	0.39377	24.00	<0.0001
contig06890.614	12	0.43	0.51269	24.00	<0.0001
contig07101.351	12	0.73	0.39377	24.00	<0.0001
B) Pedigree FQ4					
Marker	LG	Non-Cry1Fa controls ($n = 22$)		Cry1Fa survivors ($n = 72$)	
		χ^2 (1:1)	*P*-value	Marker	LG
contig00580.451	12	0.11	0.74560	33.82	<0.0001
contig02302.253	12	2.27	0.13167	60.50	<0.0001
contig02302.286	12	2.27	0.13167	60.50	<0.0001
contig06911.320	12	0.36	0.54649	53.39	<0.0001
contig06890.614	12	2.27	0.13167	60.50	<0.0001
contig07101.351	12	1.88	0.16991	41.00	<0.0001
ABCC2	12	1.14	0.28575	63.23	<0.0001

Progeny fed on control diet containing no Cry1Fa toxin were used to determine Mendelian inheritance among SNP markers, and linkage to Cry1Fa resistance estimated by significance of deviation from expected 1:1 genotypic proportions among Cry1Fa survivors using the Chi-square (χ^2) statistic.

3.3. Quantitative trait locus mapping

3.3.1. Mapping with single nucleotide polymorphism (SNP) markers

The aim of genotyping FQ5 progeny on the Sequenom MassARRAY® was only to identify markers on non-recombining LGs that are linked to the QTL. These results identified 54 segregating Mendelian Sequenom SNP markers among the non-Cry1Fa control genotypes in pedigree FQ5, which were assembled into 15 LGs (Table S2A) with 3.86 ± 1.95 markers per LG (range 2–7) and the greatest number assigned to LGs 6 and 12. Due to achiasmatic oogenesis in female Lepidoptera, FQ5 $F_{1rS♀}$ parent LGs were inherited as intact haplotypes without recombination such that markers on a LG collapsed to a single position between which cM distances could not be estimated (remaining data not shown). Although Mendelian inheritance was expected among all markers segregating in the non-Cry1Fa exposed control group, significant deviations among the same markers from the Cry1Fa survivor group were expected when linked to the QTL. From the set of markers that exhibited Mendelian inheritance in the control group, Chi-square tests detected deviation from 1:1 genotypic ratios below a Bonferroni-adjusted significance threshold of 0.0083 for 7 SNP markers in the Cry1Fa survivor group (*P*-values $\leq 7.1 \times 10^{-6}$; Table 3; Table S2A). Due to the absolute linkage of these 7 markers

Table 2
Illumina HiSeq 2500 read mapping statistics across individuals in reduced representation library constructed from pedigree FQ4 *Pool01* (parents and non-Cry1Fa toxin control) and *Pool02* (parents and Cry1Fa toxin survivors).

Library	Treatment	Total read mapped	Reads mapped 1X	Reads mapped >1X
Pool01	Control + parents	175.1 (2.9 ± 0.4)	78.9 (1.3 ± 0.3)	60.5 (1.0 ± 0.2)
Pool02	Survivors + parents	127.0 (2.2 ± 0.2)	58.9 (1.0 ± 0.1)	39.6 (0.7 ± 0.1)

Data partitioned across total reads, reads that mapped exactly once (1X) and greater than once to the reference sequences (>1X) (all values in millions of reads).

segregating from the FQ5 female $F_{1rS♀}$ parent, all of these SNPs mapped to a single position on LG12 (remaining data not shown).

Genetic recombination among linked markers from the FQ4 male $F_{1rS♂}$ parent genotype allowed for the estimation of cM distances among 57 Mendelian SNP markers segregating among FQ4 control individuals. Genetic linkage was detected among 36 of these segregating SNP markers and resulted in assignments to 14 LGs (2.64 ± 1.39 markers per LG; range 2–7; Table S2B). Most importantly, 7 of these SNP markers and AFLP-PCR markers E-ACT_700-M-GA-0124 and E-ACT_800-M-CTA-0185 were positioned on LG12 in FQ4 (Fig. 2; Table S2B), of which the latter AFLP markers had previously been linked to the Cry1Fa QTL (Coates et al., 2011a). Estimated recombination between these 9 markers inherited from $F_{1rS♂}$ parent haplotypes resulted in an approximate 55.0 cM distance across LG12 (11 ± 6.35 cM between adjacent markers). Furthermore, four markers on LG12 in FQ4 (contig00580.451, contig02302.286, contig06890.614, and contig07101.351) were also positioned on the same LG12 constructed from FQ5 pedigree data (Table 3), and allowed for comparison of QTL position between pedigrees.

Given that relative positions of Mendelian-inherited markers were made on the FQ4 genetic linkage map, subsequent positional mapping of the QTL for larval Cry1Fa resistance was accomplished by estimating the strength of significant statistical deviations from a 1:1 genotypic ratio among segregating markers in the Cry1Fa survivor group. Resulting Chi-square tests detected significant deviations at only 7 SNP marker loci using a Bonferroni-adjusted *P*-value threshold of 0.0055 (Table 3; Table S2B) and one AFLP marker, E-ACT_700-M-GA-0124. In contrast, the AFLP marker E-ACT_800-M-CTA-0185 was below the re-adjusted significance threshold (all AFLP data imported from Coates et al., 2011a). Furthermore, all 8 of these markers were genetically linked and the most significant deviations were predicted among 4 markers at the proximal end of LG12 (Fig. 2). The SNP marker that detected variation among an *abcc*2-like transcript previously described by Coates et al. (2013) was strongly linked to the QTL on LG12, but linkage to Cry1Fa resistance was not absolute since recombinant genotypes were detected among 2 of 71 Cry1Fa toxin survivors that were genotyped (2.8%; Table S2B).

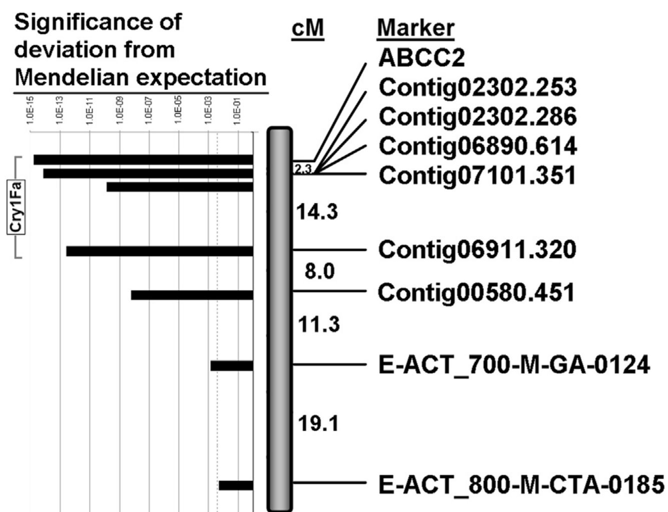

Fig. 2. Positional mapping of a single major quantitative trait locus (QTL) which determines the inheritance of a Cry1Fa resistance trait in *Ostrinia nubilalis* larvae in pedigree FQ4 which confers the ability to survive on reproductive stage transgenic corn. Markers near a single end of the 55.0 cM linkage group 12 (LG12) show the greatest deviation from expected Mendelian genotypic proportions as indicated by *P*-values from associated Chi-square (χ^2) tests (*x*-axis), and significance of departures set at a Bonferroni adjusted threshold of 0.0055 ($\alpha = 0.05/9$) is shown at the dotted line. Segregating SNP markers, including one for an *abcc*2-like gene, within a 16.6 cM interval (labeled Cry1Fa) shows the most significant deviation.

3.3.2. Mapping with SNP and NGS marker data

A genetic linkage map with higher marker density was constructed by merging Mendelian-inherited marker datasets from both Sequenom MassARRAY® and 1026 GBS-predicted mutations segregating among FQ4 non-Cry1Fa controls (Table S5). This genetic linkage map assigned 981 markers to 39 LGs (25.2 ± 20.2 markers per LG; range 4–109 markers per LG) and spanned a total of 3409.5 cM (87.4 ± 77.6 cM per LG; Table S5). Analysis of these combined data predicted that 102 GBS markers were linked to the 7 Cry1Fa QTL linked Sequenom MassARRAY® SNP markers (Fig. 2), and defined the new 268.8 cM LG12 ($n = 109$ markers; 5.9 ± 6.9 cM between adjacent markers; Fig. 3). Markers located on LG12 were inclusive of the marker for an *abcc*2-like gene. Analogous genotypes from the FQ4 Cry1Fa toxin survivor group showed significant deviation from Mendelian expectations at 37 of 109 loci on LG12 (33.9%) after Bonferroni adjustment for multiple tests (*P*-value cutoff = 0.00045). Plotting of all *P*-values from Chi-square estimated deviations from Mendelian expected genotypic proportions at GBS loci among Cry1Fa survivors ($n = 102$) showed an approximate normal distribution, such that markers within a pseudo-95% confidence interval were used to define the boundaries of the QTL. Specifically, this calculation of (102 markers × 0.05) indicated 5 markers with the most significant deviation from expected Mendelian ratios should define the QTL, and subsequently included marker clusters 697, 456, 24, 64, and 51 that were in an approximate 2.1 cM interval adjacent to the *abcc*2 marker on LG12 (Fig. 3). GBS markers 476, 304 and 302 were at the same genetic map location as 697, 456, 24, 64, and 51 (based on linkage among Control group individuals), and although outside of the Pseudo-95% confidence interval, Chi-square *P*-values estimating significance of deviation from Mendelian expectation were $\leq 3.13 \times 10^{-9}$. The male derived allele from marker 697 (cluster 697) co-segregated with the Cry1Fa resistance trait at a greater frequency (98.0%) compared to the *abcc*2 marker (97.1%) as well as compared to all other markers within the QTL (≤95.7%).

4. Discussion

Field-evolved resistance to transgenic Bt toxins expressed by GE crop plants has been documented among a few important species of Lepidoptera (see Introduction), as well as by the coleopteran, the western corn rootworm, *D. virgifera virgifera*, which exhibits resistance to Bt Cry3Bb1 and mCry3A toxins (Gassmann et al., 2011, 2014). Such instances of resistance highlight potential shortcomings in our scientific understanding of biochemical and ecological factors that lead to selection within natural populations. Biological factors have lead to an increasing proportion of functionally resistant individuals in these target insect populations such that economically-significant levels of crop damage have been observed. Methods to remediate inherent phenotypic and underlying genotypic changes that occur in response to Bt toxin exposure (e.g. selection) through the HDR strategy are based on the premise that homozygous resistant individuals that develop on transgenic crops are "rare" ($q \leq 10^{-3}$; Onstad and Guse, 1999) and more likely to mate at random with an overwhelming excess of susceptible individuals the emerge from non-Bt refuge. Secondly, the "high-dose" component of this strategy assumes that toxins expressed by Bt crops are sufficient to cause mortality among 100% of any heterozygous insects (Alstad and Andow, 1995; Tabashnik, 2008). Studies challenging these tenants have shown that resistance alleles are not always rare (Huang et al., 2014; Siegfried et al., 2014) or

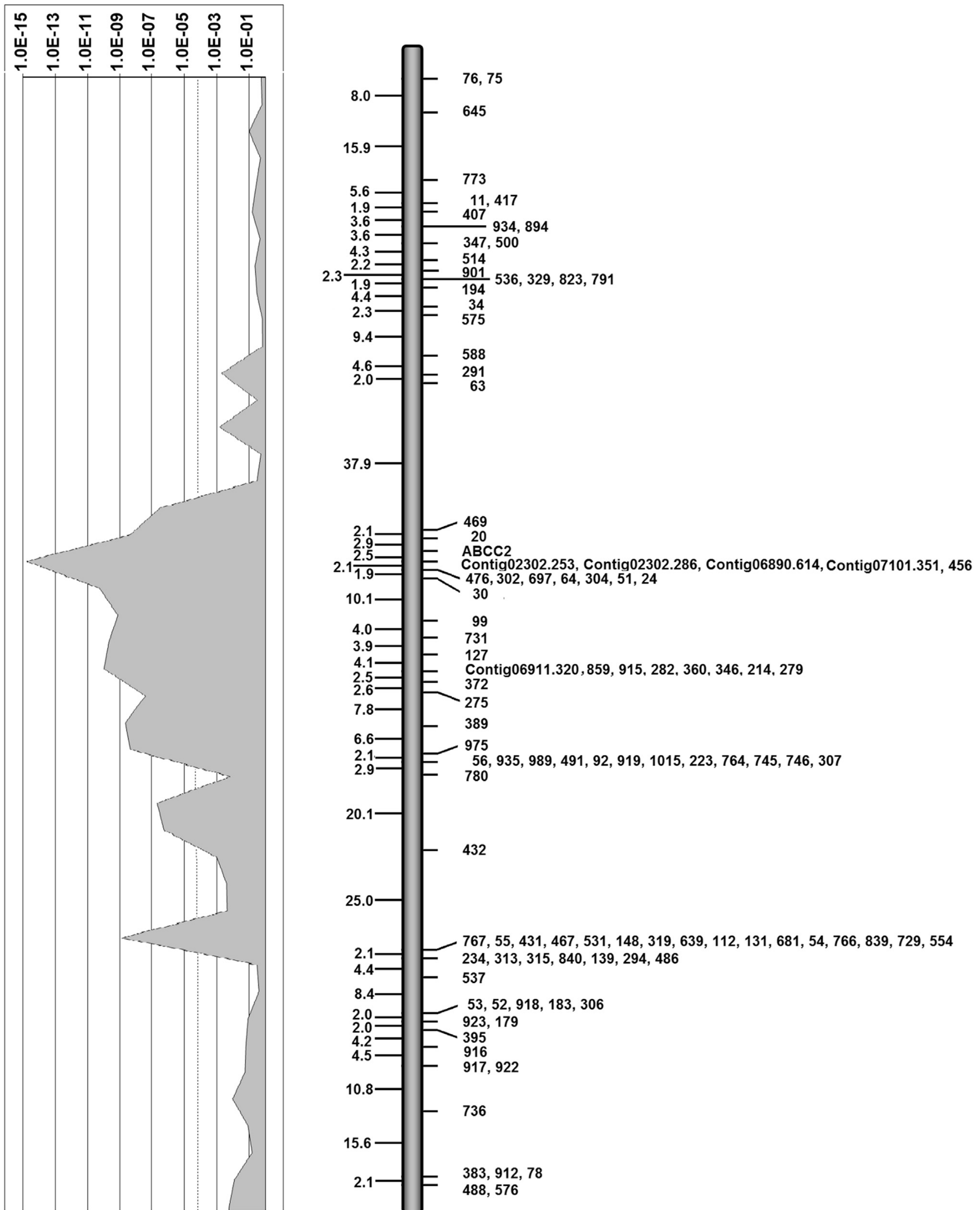

Fig. 3. Integrated genetic linkage map constructed from 109 segregating *de novo* genotyping-by-sequencing (GBS) and Sequenom MassARRAY®-based SNP markers on the 268.8 cM LG12. Markers linked to a QTL that determines Cry1Fa toxin resistance among *Ostrinia nubilalis* larvae in backcross pedigree FQ4 are shown as significance of Chi-square (χ^2) estimated deviations from Mendelian expectation in the Cry1Fa toxin survivor group. Markers in an estimated 46.5 cM region (markers 469 thru 389, and including *abcc*2) show departures below a Bonferroni adjusted significance threshold of 0.00045 ($\alpha = 0.05/109$) as shown at the dotted line.

functionally recessive in insect populations (Zhang et al., 2012; Jin et al., 2013; Campagne et al., 2013). Furthermore, the level of Bt toxin varies across plant tissues (Siegfried et al., 2001) and decreases with increasing plant maturities (Greenplate et al., 2003). Possible lower dose exposures may be encountered in situations where plants senesce during their life cycles, and was demonstrated by in survival of the *O. nubilalis* Cry1Fa strain only on reproductive stage transgenic corn that likely would be encountered by larvae produced the second generation bivoltine moths (Pereira et al., 2008a).

Understanding the genetic mechanisms that increase the survivorship of target insects when exposed to pesticides, as well as biological and ecological factors that influence persistence of causal alleles in a population, may reveal means by which to better predict and manage the evolution of resistance (Siegfried et al., 2007; Tabashnik et al., 2009). The isolation of mutations directly linked to Bt resistance traits have remained elusive due to difficulties in dissecting complex biochemical interactions and the realization that independent modes of resistance were possible (Griffitts and Aroian, 2005). For instance, previous ligand blotting results indicated that both Cry1Fa and Cry1Ab toxins could bind strongly to the *O. nubilalis* midgut receptors cadherin, and/or *apn* protein isoforms (Hua et al., 2001; Crava et al., 2013; Tan et al., 2013). Although Bt toxin binding to aminopeptidase N and cadherin is likely important within the context of the sequential binding model (Bravo et al., 2007), studies have failed to show co-segregation of molecular genetic markers within these gene coding regions with Bt resistance traits in *Plutalla xylostella* (Baxter et al., 2005), *H. virescens* (Gahan et al., 2005), *Trichoplusia ni* (Zhang et al., 2012), or the *O. nubilalis* Cry1Fa resistance investigated in this study (Coates et al., 2011a). These reports suggest that Bt resistance is not ubiquitously linked to mutations that cause protein structural changes or *cis*-acting mutations. Indeed, the involvement of gene regulatory mutations that act in *trans*- to affect the expression of midgut receptors were implicated in the inheritance of resistance to Cry1Ac in *T. ni* (Tiewsiri and Wang, 2011), Cry1Ab in *O. nubilalis* (Coates et al., 2013), and Cry1Ac in *P. xylostella* (Guo et al., 2015) which highlight the possible importance of genetic background and epistatic effects on the evolution of Bt resistance.

Traditional genetic mapping aims to associate the segregation of anonymous molecular markers with the inheritance of a phenotype, and operates independent of *a priori* assumptions regarding mode of action such that background genetic affects can be detected. Application of GBS for high throughput marker discovery and subsequent QTL mapping, such as that used in the current study, provide methods to detect thousands of segregating mutations and to improve the resolution of QTL position. Analogously, the co-segregation of anonymous molecular genetic markers was successful in identifying a novel genome region that determined Cry1Ac resistance in the *H. virescens* strain YEE, and led to the discovery of a previously unknown candidate Bt binding receptor, the *abcc*2 transporter (Gahan et al., 2010), and was crucial for a proposed extension of the sequential binding model (Heckel, 2012). Subsequent genetic mapping approaches have implicated *abcc*2 gene family members in Cry1Ac resistance in *P. xylostella* and *T. ni* (Baxter et al., 2011) as well as *Bombyx mori* (Atsumi et al., 2012). Most recently, a bulk segregate analysis associated different members of the *abcc*2 transporter gene family with Cry1Ac and Cry1Ca resistance in *Spodoptera exigua* (Park et al., 2014). Mutations in alleles at the *abcc*2 locus in resistant individuals, compared to susceptible counterparts, include a truncation due to a premature stop codon preceding transmembrane domain 1 in *H. virescens* (Gahan et al., 2010), deletion of all or part of the cytoplasmic tail for *P. xylostella* (Baxter et al., 2011) and *S. exigua* (Park et al., 2014), and a single point mutation in the second extracellular loop among Cry1Ac resistant *B. mori* strains (Atsumi et al., 2012). In the current study, fine mapping with 109 segregating GBS and SNP markers positioned on the 268.8 cM *O. nubilalis* LG12 defined a 46.5 cM QTL for Cry1Fa resistance that included a marker for an *abcc*2-like gene (Fig. 3). Incorporation of GBS markers significantly increased the marker density on this LG compared to use of single base extension SNP assays alone (Fig. 2). Interestingly, the *abcc*2 genetic marker was mapped to a position $\leq$2.8 cM from the QTL. Even though the GBS marker 697 was estimated to be within 2.1 cM of the QTL and closer compared to the *abcc*2 marker, estimates of map distances are relative and potentially subject to error incurred during sampling of progeny. Furthermore, tight but lack of absolute linkage between the *abcc*2-like transporter and Cry1Fa resistance in *O. nubilalis* might be affected by minor errors incurred during bioassays, or the possibility that another member of the *abcc*2 transporter gene family may be directly involved in the inheritance of this resistance trait. Specifically, the members of the *abcc*2 gene family members are clustered within a narrow interval in the *H. virescens* (Gahan et al., 2010) and similarly shown for their othologous *B. mori* genomes (Xie et al., 2012), which suggests that another gene within the *O. nubilalis* QTL may potentially show greater or absolute linkage with Cry1Fa resistance. This latter assumption may be partially confirmed by a recent study that demonstrated a mitogen-activated protein kinase (*mapk*4) gene in the same *P. xylostella* region genome as *abcc*2 may function in a *trans*-regulatory pathway to control the expression of the *alp* binding receptor and *abcc*2 (Guo et al., 2015). The position of the *mapk*4 ortholog in the *O. nubilalis* genome remains unknown, but given the high degree of synteny between lepidopteran genomes at this locus (Baxter et al., 2011), our GBS mapping results might be considered in agreement with finding by Guo et al. (2015) that implicate a gene in proximity to *abcc*2 as the causal genetic factor of Cry1 toxin resistance.

Monitoring remains the prescribed tactic for detecting the onset of resistance within insect populations, and long duration studies are necessitated to detect changes in phenotypic frequencies over time to document field-evolved resistance (Tabashnik et al., 2008). After nearly two decades since initial commercialization of Bt corn *O. nubilalis* remains susceptible to those toxins deployed for its control (Hutchison et al., 2010; Siegfried and Hellmich, 2012) the HDR strategy may be credited for circumventing any potential onset of Cry1Ab or Cry1Fa resistance (Bourguet et al., 2002; Gaspers et al., 2011; Siegfried et al., 2014), even though the estimated frequencies of Cry1Fa resistance alleles ($q \leq 0.0286$) are beyond modeling thresholds (Siegfried et al., 2014). Similarly, initial F_2 screens have estimated Cry2Ab and Vip3A toxin resistance alleles at 0.021 and 0.027, respectively, in populations of *H. armigera* and *H. punctigera* in Australia prior to commercial Bt cotton expressing either toxin (Downes and Mahon, 2012a,b; Mahon et al., 2012), but cannot be predictive of any future field failures given the above case with *O. nubilalis*. Determining the allelic variants that give rise to functional resistance, and how biological or ecological factors modulate the frequency of those alleles in an insect population to levels that overcome the suppressive effects of the HDR strategy remains an important unknown for the formulation of sustainable IRM tactics.

Bioassay-based monitoring aimed to detect changes in resistant phenotype frequencies in target insect populations are oftentimes insufficient in scope to support a realistic assumption that increased proportions of resistant individuals can be readily detected. Cases of field-evolved resistance have thus far been documented only after observation of significant crop damage, and arguably after the point at which prescribed remediation plans have limited effectiveness. One-to-one correlations have been made between a single mutation in the *Anopheles funestus*

glutathione S-transferase epsilon 2 (*GSTe2*) gene with pyrethroid and DDT resistance (Riveron et al., 2014), as well as between an gamma-aminobutryic acid (GABA) receptor mutation and organochlorine resistance (ffrench-Constant et al., 2000; Wang et al., 2013) such that opportunities are now present to directly monitor changes in the frequency of resistance in field populations using molecular genetic markers (Siegfried et al., 2007; Black and Vontas, 2007; Riveron et al., 2014). Incorporating this principle of monitoring direct genotype–phenotype associations into crop pest management strategies has the potential to help evaluate resistance management programs and guide regulatory and management decisions. Defining genome regions and implicating candidate genes as causal genetic factors as done in this study is a crucial step in realizing a molecular assay based methods for field monitoring. Ultra-fine mapping of Bt resistance traits using thousands of GBS markers provide improved resolution of QTL position as done for Cry1Fa resistance in this study, and may provide a mechanism for identifying unknown mutations that are causal of Cry1A resistance (Tiewsiri and Wang, 2011; Coates et al., 2013), any number of other insecticide resistance traits or phenotypes that evolved from ecological diversification. Furthermore, GBS-based methods and genome wide association studies (GWAS) of population data may provide future paths for direct analyses of field-evolved resistance that forego the need for establishing laboratory populations. Most importantly, identification of specific resistance conferring mutations may also enhance our understanding of Bt toxin mode of action and the ability to track changes in allele frequencies in response to selection.

5. Conclusions

Mutations in genes encoding putative midgut-expressed Cry toxin binding receptors are known to result in reduced susceptibilities among larvae, and is one of the principle modes by which Bt toxin resistance is expected to develop. Alterations in genes and transcripts encoding ABCC2 transporter proteins had previously been implicated in lepidopteran resistance to Cry1Ac (Gahan et al., 2010; Baxter et al., 2011; Atsumi et al., 2012) and Cry1Ca (Park et al., 2014). The current study is the first to report direct linkage of an *abcc*2 transporter to Cry1Fa resistance, which suggests it may represent a major pathway by which Cry1F toxin resistance may develop. In contrast, lack of cross resistance to Cry1A by Cry1Fa resistant *O. nubilalis* likely demonstrates independent modes of action for these groups of toxins, and is supported by independence of QTL positions for these two traits (Coates et al., 2013). Although both Cry1Fa and Cry1A toxins may share common midgut binding sites (Hua et al., 2001; Crava et al., 2013; Tan et al., 2013), differences in ABCC2 transporter involvement in these two traits may assist in future research to elucidate the participation of this receptor in the Bt mode of action. Alternatively, a unified underlying mechanism may exist that controls the expression of one or more Bt receptors and be actualized through mutations in yet undescribed *trans*-regulatory factors.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2015.06.003.

References

Alstad, D.N., Andow, D.A., 1995. Managing the evolution of insect resistance to transgenic plants. Science 268, 1894–1896.

Alvi, A.H.K., Sayyed, A.H., Naeem, M., Ali, M., 2012. Field evolved resistance in *Helicoverpa armigera* (Lepidoptera: Noctuidae) to *Bacillus thuringiensis* toxin Cry1Ac in Pakistan. PLoS One 7, e47309.

Atsumi, S., Miyamoto, K., Yamamoto, K., Narukawa, J., Kawai, S., Sezutsu, H., Kobayashi, I., et al., 2012. Single amino acid mutation in an ATP-binding cassette transporter gene causes resistance to Bt toxin Cry1Ab in the silkworm, *Bombyx mori*. Proc. Natl. Acad. Sci. U. S. A. 109, E1591–E1598.

Baird, N.A., Etter, P.D., Atwood, T.S., Currey, M.C., Shiver, A.L., et al., 2008. Rapid SNP discovery and genetic mapping using sequenced RAD markers. PLoS ONE 3, e3376.

Bates, S.L., Zhao, J.Z., Roush, R.T., Shelton, A.M., 2005. Insect resistance management in GM crops: past, present and future. Nat. Biotechnol. 23, 57–62.

Baxter, S., Zhao, J.Z., Gahan, L.J., Shelton, A.M., Tabashnik, B.E., Heckel, D.G., 2005. Novel genetic basis of field-evolved resistance to Bt toxins in *Plutella xylostella*. Insect Mol. Biol. 14, 327–334.

Baxter, S.W., Badenes-Perez, F.R., Morrison, A., Vogel, H., Crickmore, N., Kain, W., Wang, P., Heckel, D.G., Jiggins, C.D., 2011. Parallel evolution of *Bacillus thuringiensis* toxin resistance in Lepidoptera. Genetics 189, 675–679.

Black, W.C., Vontas, J., 2007. Affordable assays for genotyping single nucleotide polymorphism in insects. Insect Mol. Biol. 16, 377–387.

Bolin, P.C., Hutchison, W.D., Andow, D.A., 1999. Long-term selection for resistance to *Bacillus thuringiensis* Cry1Ac endotoxin in a Minnesota population of European corn borer (Lepidoptera: Crambidae). J. Econ. Entomol. 92, 1021–1030.

Bourguet, D., Chaufaux, J., Seguin, J.M., Buisson, C., Hinton, J.L., Stodola, T.J., et al., 2002. Frequency of alleles conferring resistance to *Bt* maize in French and US corn belt populations of the European corn borer. Theor. Appl. Genet. 106, 1225–1233.

Bravo, A., Gill, S.S., Soberon, M., 2007. Mode of action of *Bacillus thuringiensis* Cry1 and Cyt toxins and thir potential for insect control. Toxicon 49, 423–435.

Campagne, P., Kruger, M., Pasquet, R., Le Ru, B., Van den Berg, J., 2013. Dominant inheritance of field-evolved resistance to *Bt* corn in *Busseola fusca*. PLoS ONE 8, e69675.

Coates, B.S., Sumerford, D.V., Lewis, L.C., 2007. A (β-1,3-galactosyltransferase and brainiac/bre5 homolog expressed in the midgut did not contribute to a Cry1Ab toxin resistance trait in *Ostrinia nubilalis*. Insect Biochem. Mol. Biol. 37, 346–355.

Coates, B.S., Sumerford, D.V., Lewis, L.C., 2008. Segregation of *Ostrinia nubilalis* aminopeptidase 1 (APN1), cadherin, and bre5-like alleles from a Cry1Ab resistant colony are not associated with F_2 larval weights when fed on toxin-containing diet. J. Insect Sci. 8, 21.

Coates, B.S., Sumerford, D.V., Lopez, M.D., Wang, H., Fraser, L.M., Kroemer, J.A., Spencer, T., Kim, K.S., Abel, C.A., Hellmich, R.L., Siegfried, B.D., 2011a. A single major QTL controls the expression of a larval Cry1F resistance trait in *Ostrinia nubilalis* (Lepidoptera: Crambidae). Genetica 139, 961–972.

Coates, B.S., Bayles, D.O., Wanner, K.W., Robertson, H.M., Hellmich, R.L., Sappington, T.W., 2011b. The application and performance of single nucleotide polymorphism (SNP) markers for population genetic analyses of Lepidoptera. Front. Genet. 2, 38.

Coates, B.S., Sumerford, D.V., Hellmich, R.L., Abel, C.A., 2013. Unlinked genetic loci control the reduced transcription of aminopeptidase N 1 and 3 in the European corn borer and determine tolerance to *Bacillus thuringiensis* Cry1Ab toxin. Insect Biochem. Mol. Biol. 43, 1152–1160.

Crava, C.M., Bel, Y., Jakubowska, A.K., Ferre, J., Escriche, B., 2013. Midgut aminopeptidase N isoforms from *Ostrinia nubilalis*: activity characterization and differential binding to Cry1Ab and Cry1Fa proteins from *Bacillus thuringiensis*. Insect Biochem. Mol. Biol. 42, 924–935.

Danecek, P., Auton, A., Abecasis, G., Albers, C.A., Banks, E., DePristo, M.A., Handsaker, R., Lunter, G., Marth, G., Sherry, S.T., McVean, G., Durbin, R., 1000 Genomes Project Analysis Group, 2011. The variant call format and VCFtools. Bioinformatics 27, 2156–2158.

Dhurua, S., Gujar, G.T., 2011. Field-evolved resistance to *Bt* toxin Cry1Ac in the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), from India. Pest. Manag. Sci. 67, 898–903.

Downes, S., Mahon, R., 2012a. Evolution, ecology and management of resistance in *Helicoverpa* spp. to Bt cotton in Australia. J. Invertebr. Pathol. 110, 281–286.

Downes, S., Mahon, R., 2012b. Successes and challenges of managing resistance in *Helicoverpa armigera* Bt cotton in Australia. Gm. Crops Food 3, 228–234.

Elshire, R.J., Glaubitz, J.C., Sun, Q., Poland, J.A., Kawamoto, K., Buckler, E.S., Mitchell, S.E., 2011. A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PloS One 6, e19379.
Farias, J.R., Andow, D.A., Horiskoshi, R.J., Sorgatto, R.J., Fresia, P.F., Santos, A.C., Omoto, C., 2014. Field-evolved resistance to Cry1F maize by *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Brazil. Crop Prot. 64, 150–158.
ffrench-Constant, R.H., Anthony, N., Aronstein, K., Rocheleau, T., Stilwell, G., 2000. Cyclodiene insecticide resistance: from molecular to population genetics. Ann. Rev. Entomol. 45, 449–466.
Fu, L., Niu, B., Zhu, Z., Wu, S., Li, W., 2012. CD-HIT: accelerated for clustering the next-generation sequencing data. Bioinformatics 28, 3150–3152.
Gahan, L.J., Gould, F., Heckel, D.G., 2001. Identification of a gene associated with Bt resistance in *Heliothis virescens*. Science 293, 857–860.
Gahan, L.J., Ma, Y.T., Coble, M.L.M., Gould, F., Moar, W.J., Heckel, D.G., 2005. Genetic basis of resistance to Cry1Ac and Cry2Aa in *Heliothis virescens* (Lepidoptera: Noctuidae). J. Econ. Entomol. 98, 1357–1368.
Gahan, L.J., Pauchet, Y., Vogel, H., Heckel, D.G., 2010. An ABC transporter mutation is correlated with insect resistance to *Bacillus thuringiensis* Cry1Ac toxin. PLoS Genet. 6, e1001248.
Gaspers, C., Siegfried, B.D., Spencer, T., Alves, A.P., Storer, N.P., Schuphan, I., Eber, S., 2011. Susceptibility of European and North American populations of the European corn borer to the Cry1F insecticidal protein. J. Appl. Entomol. 135, 7–16.
Gassmann, A.J., Petzold-Maxwell, J.L., Keweshan, R.S., Dunbar, M.W., 2011. Field-evolved resistance to Bt maize by western corn rootworm. PLoS One 6, e22629.
Gassmann, A.J., Petzold-Maxwell, J.L., Clifton, E.H., Dunbar, M.W., Hoffmann, A.M., Ingber, D.A., Keweshan, R.S., 2014. Field-evolved resistance by western corn rootworm to multiple Bacillus thuringiensis toxins in transgenic maize. Proc. Natl. Acad. Sci. U. S. A. 111, 5141–5146.
Gould, F., 1998. Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. Annu. Rev. Entomol. 43, 701–726.
Greenplate, J.T., Mullins, J.W., Penn, S.R., Dahm, A., Reich, B.J., Osborn, J.A., Rahn, P.R., Ruschke, L., Shappley, Z.W., 2003. Partial characterization of cotton plants expressing two toxin proteins from *Bacillus thuringiensis*: relative toxin contribution, toxin interaction, and resistance management. Appl. Entomol. 127, 340–347.
Griffitts, J.S., Aroian, R.V., 2005. Many roads to resistance: how invertebrates adapt to Bt toxins. Bioessays 27, 614–624.
Gunning, R.V., Dang, H.T., Kemp, F.C., Nicholson, I.C., Moores, G.D., 2005. New resistance mechanism in *Helicoverpa armigera* threatens transgenic crops expressing *Bacillus thuringiensis* Cry1Ac toxin. Appl. Environ. Microbiol. 71, 2558–2563.
Guo, Z., Kang, S., Chen, D., Wu, Q., Wang, S., Xie, W., Zhu, X., Baxter, S.W., Zhou, X., Jurat-Fuentes, J.L., Zhang, Y., 2015. MAPK signaling pathway alters expression of midgut ALP and ABCC genes and causes resistance to *Bacillus thuringiensis* Cry1Ac toxin in Diamondback Moth. PLoS Genet. 11, e1005124.
Heckel, D.G., Gahan, L.J., Daly, J.C., Trowell, S., 1998. A genomic approach to understanding *Heliothis* and *Helicoverpa* resistance to chemical and biological insecticides. Phil. Trans. R. Soc. Lond. B 353, 1713–1722.
Heckel, D.G., 2012. Learning the ABCs of Bt: ABC transporters and insect resistance to *Bacillus thuringiensis* provide clues to a crucial step in toxin mode of action. Pestic. Biochem. Physiol. 104, 103–110.
Herrero, S., Gechev, T., Bakker, P.L., Moar, W.J., de Maagd, R.A., 2005. *Bacillus thuringiensis* Cry1Ca-resistant *Spodoptera exigua* lacks expression of one of four aminopeptidase N genes. BMC Genom. 6, 96.
Hua, G., Masson, L., Jurat-Fuentes, J.L., Schwab, G., Adang, M.J., 2001. Binding analyses of *Bacillus thuringiensis* Cry-endotoxins using brush border membrane vesicles of *Ostrinia nubilalis*. Appl. Environ. Microbiol. 67, 872–879.
Huang, F., Andow, D.A., Buschman, L.L., 2011. Success of the high-dose/refuge resistance management strategy after 15 years of Bt crop use in North America. Entomol. Exp. Appl. 140, 1–16.
Huang, F., Qureshi, J.A., Meagher Jr., R.L., Reisig, D.D., Head, G.P., et al., 2014. Cry1F resistance in Fall armyworm *Spodoptera frugiperda*: single gene versus pyramided Bt maize. PLoS One 9, e112958.
Hutchison, W.D., Burkness, E.C., Mitchell, P.D., Moon, R.D., Leslie, T.W., Fleischer, S.J., Abrahamson, M., Hamilton, K.L., Steffey, K.L., Gray, M.E., Hellmich II, R.L., Kaster, V., Hunt, T.E., Wright, R.J., Pecinovsky, K.T., Rabaey, T.L., Flood, B.R., Raun, E.S., 2010. Areawide suppression of European corn borer with Bt maize reaps savings to non-Bt maize growers. Science 330, 222–225.
Jin, L., Wei, Y., Zhang, L., Yang, Y., Tabashnik, B.E., Wu, Y., 2013. Dominant resistance to Bt cotton and minor cross-resistance to Bt toxin Cry2Ab in cotton bollworm from China. Evol. Appl. 6, 1222–1235.
Jurat-Fuentes, J.L., Gould, F.L., Adang, M.J., 2002. Altered glycosylation of 63- and 68-kilodalton microvillar proteins in *Heliothis virescens* correlates with reduced Cry1 toxin binding, decreased pore formation, and increased resistance to *Bacillus thuringiensis* Cry1 toxins. Appl. Environ. Microbiol. 68, 5711–5717.
Knaus, B., 2011. Short Read Toolbox. http://brianknaus.comlsoftware/srtoolboxlshortread.html.
Knowles, B.H., Knight, P.J.K., Ellar, D.J., 1991. N-acetylgalactosamine is part of the receptor in insect gut epithelia that recognizes an insecticidal protein from *Bacillus thuringiensis*. Proc. R. Soc. Lond. Biol. 245, 31–35.
Kruger, M., Van Rensburg, J.B.J., Van den Berg, J., 2014. No fitness costs associated with resistance of *Busseola fusca* (Lepidoptera: Noctuidae) to genetically modified Bt maize. Crop Prot. 55, 1–6.
Langmead, B., Salzberg, S., 2012. Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357–359.
Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., 2009. The sequence alignment/map format and SAMtools. Bioinformatics 25, 2078–2079.
Lincoln, S., Daly, M., Lander, E., 1992. Constructing Genetic Maps with MAPMAKER/EXP 3.0, third ed. (Whitehead Institute Technical Report).
Mahon, R.J., Downes, S.J., James, B., 2012. Vip3A resistance alleles exist at high levels in Australian targets before release of cotton expressing this toxin. PLoS ONE 7, e39192.
Matten, R.S., Head, G.P., MacIntoch, S.C., 2008. How government regulation can help or hinder the integration of Bt crops within IPM programs. In: Romeis, J., Shelton, A.M., Kennedy, G.G. (Eds.), Integration of Insect Resistant Genetically Modified Crops with IPM Programs. Springer Science þ Business Media B. V, New York, USA, pp. 27–39.
McGaughey, W.H., 1985. Insect resistance to the biological insecticide *Bacillus thuringiensis*. Science 229, 193–195.
Morin, S., Biggs, R.W., Sisterson, M.S., Shriver, L., Ellers-Kirk, C., Higginson, D., Holley, D., Gahan, L.J., Heckel, D.G., Carrière, Y., Dennehy, T.J., Brown, J.K., Tabashnik, B.E., 2003. Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. Proc. Natl. Acad. Sci. U. S. A. 100, 5004–5009.
National Research Council, 1986. Pesticide Resistance: Strategies and Tactics for Management. National Academy Press, Washington, DC.
Onstad, D.W., Guse, C.A., 1999. Economic analysis of transgenic maize and nontransgenic refuges for managing European corn borer (Lepidoptera: Pyralidae). J. Econ. Entomol. 92, 1256–1265.
Park, Y., González-Martínez, R.M., Navarro-Cerrillo, G., Chakroun, M., Kim, Y., Ziarsolo, P., Blanca, J., Canizares, J., Ferre, J., Herrero, S., 2014. ABCC transporters mediate insect resistance to multiple Bt toxins revealed by bulk segregant analysis. BMC Biol. 12, 46.
Pereira, E.J.G., Lang, B.A., Storer, N.P., Siegfried, B.D., 2008a. Selection for Cry1F resistance in the European corn borer and cross resistance to other Cry toxins. Entomol. Exper. Appl. 126, 115–121.
Pereira, E.J.G., Storer, N.P., Siegfried, B.D., 2008b. Inheritance of Cry1F resistance in laboratory-selected European corn borer and its survival on transgenic corn expressing the Cry1F toxin. Bull. Entomol. Res. 98, 621–629.
Piggot, C.R., Ellar, D.J., 2007. Role of receptors in *Bacillus thuringiensis* crystal toxin activity. Microbiol. Mol. Biol. Rev. 71, 255–281.
Riveron, J.M., Yunta, C., Ibrahim, S.S., Djouaka, R., Irving, H., Menze, B.D., Ismail, H.M., Hemingway, J., Ranson, H., Albert, A., Wondji, C.S., 2014. The detailed molecular and structural basis through which candidate metabolic resistance genes confer resistance remains poorly characterized. Genome Biol. 15, R27.
Roush, R.T., McKenzie, J.A., 1987. Ecological genetics of insecticide and acaricide resistance. Ann. Rev. Entomol. 32, 361–380.
Siegfried, B.D., Hellmich, R.L., 2012. Understanding successful resistance management: the European corn borer and Bt corn in the United States. Spec. Issue GM Crops Foods 3, 184–193.
Siegfried, B.D., Zoerb, A., Spencer, T., 2001. Effect of Event 176 transgenic maize on susceptibility and fitness of the European corn borer. Entomol. Exp. Appl. 100, 15–20.
Siegfried, B.D., Spencer, T., Crespo, A.L., Storer, N.P., Head, G.P., Owens, E.D., Guyer, D., 2007. Ten years of Bt resistance monitoring in the European corn borer: what we know, what we don't know, and what we can do better. Am. Entomol. 53, 208–214.
Siegfried, B.D., Rangasamy, M., Wang, H., Spencer, T., Haridas, C.V., Tenhumberg, B., Sumerford, D.V., Storer, N.P., 2014. Estimating the frequency of Cry1F resistance in field populations of the European corn borer (Lepidoptera: Crambidae). Pest Manag. Sci. 70, 725–733.
Siqueira, H.A.A., Moellenbeck, D., Spencer, T., Siegfried, B.D., 2004. Cross-resistance of CrylAb-selected *Ostrinia nubilalis* (Lepidoptera: Crambidae) to *Bacillus thuringiensis* delta-endotoxins. J. Econ. Entomol. 97, 1049–1057.
Soberon, M., Gill, S., Bravo, A., 2009. Signaling versus punching hole: how do *Bacillus thuringiensis* toxins kill insect midgut cells. Cell. Mol. Life Sci. 66, 1337–1349.
Storer, N.P., Babcock, J.M., Schlenz, M., Meade, T., Thompson, G.D., Bing, J.W., Huckaba, R.M., 2010. Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. J. Econ. Entomol. 03, 1031–1038.
Tabashnik, B.E., 1994. Evolution of resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. 39, 47–79.
Tabashnik, B.E., 2008. Delaying insect resistance to transgenic crops. Proc. Natl. Acad. Sci. U. S. A. 105, 19029–19030.
Tabashnik, B.E., Carriere, Y., 2010. Field-evolved resistance to Bt cotton: bollworm in the U.S. and pink bollworm in India. Southwest. Entomol. 35, 417–424.
Tabashnik, B.E., Cushing, N.L., Finson, N., Johnson, M.W., 1990. Field development of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. 83, 1671–1676.
Tabashnik, B.E., Liu, Y.B., Finson, N., Masson, L., Heckel, D.G., 1997. One gene in diamondback moth confers resistance to four *Bacillus thuringiensis* toxins. Proc. Natl. Acad. Sci. U. S. A. 94, 1640–1644.
Tabashnik, B.E., Gassmann, A.J., Crowder, D.W., Carriere, Y., 2008. Insect resistance to *Bt* crops: evidence versus theory. Nat. Biotechnol. 26, 199–202.

Tabashnik, B.E., Ban Rensburg, J.B.J., Carriere, Y., 2009. Field-evolved insect resistance to Bt crops: definition, theory and data. J. Econ. Entomol. 102, 2011–2025.

Tan, S.Y., Cayabyab, B.F., Alcantara, E.P., Huang, F., He, K., Nickerson, K.W., Siegfried, B.D., 2013. Comparative binding of Cry1Ab and Cry1F Bacillus thuringiensis toxins to brush border membrane proteins from *Ostrinia nubilalis, Ostrinia furnacalis* and *Diatraea saccharalis* (Lepidoptera: Crambidae) midgut tissue. J. Invert. Pathol. 114, 234–240.

Tiewsiri, K., Wang, P., 2011. Differential alteration of two aminopeptidases N associated with resistance to *Bacillus thuringiensis* toxin Cry1Ac in cabbage looper. Proc. Natl. Acad. Sci. USA 108, 14037–14042.

Truong, H.T., Ramos, A.M., Yalcin, F., de Ruiter, M., van der Poel, H.J.A., Huvenaars, K.H.J., Hogers, R.C.J., van Enckevort, L.J.G., Janssen, A., van Orsouw, N.J., van Eijk, M.J.T., 2012. Sequence-based genotyping for marker discovery and co-dominant scoring in germplasm and populations. PLoS One 7, e37565.

US EPA (United States Environmental Protection Agency, Washington, DC, USA), 1998. The Environmental Protection Agency's White Paper on Bt Plant-pesticide Resistance Management (EPA Publication 739-S-98–001). Available at: http://www.epa.gov/EPA-PEST/1998/January/Day-14/paper.pdf.

US EPA (United States Environmental Protection Agency, Washington, DC, USA), 2001. Biopesticides Registration Action Document - *Bacillus Thuringiensis* Plant-incorporated Protestants. E: Benefits Assessment. Available at: http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad2/5-benefits.pdf.

van Rensburg, J.B.J., 2007. First field report of field resistance by the stem borer, *Busseola fusca* (Fuller) to transgenic maize. S. Afr. J. Plant. Soil 24, 147–151.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., et al., 1995. AFLP: a new technique for DNA fingerprinting. Nucl. Acids Res. 23, 4407–4414.

Wang, H., Coates, B.S., Chen, H., Sappington, T.W., Guillemaud, T., Siegfried, B.D., 2013. Role of a gamma-aminobutryic acid (GABA) receptor mutation in the evolution and spread of *Diabrotica virgifera virgifera* resistance to cyclodiene insecticides. Insect Mol. Biol. 22, 473–484.

Xie, X., Cheng, T., Wang, G., Duan, J., Niu, W., Xia, Q., 2012. Genome-wide analysis of the ATP-binding cassette (ABC) transporter gene family in the silkworm, *Bombyx mori*. Mol. Biol. Rep. 39, 7281–7291.

Xu, X., Yu, L., Wu, Y., 2005. Disruption of a cadherin gene associated with resistance of Cry1Ac δ-endotoxins of *Bacillus thuringiensis* in *Helicoverpa armigera*. Appl. Environ. Microbiol. 71, 948–954.

Zhang, X., Tiewsiri, K., Kain, W., Huang, L., Wang, P., 2012. Resistance of *Trichoplusia ni* to *Bacillus thuringiensis* toxin Cry1Ac is independent of alteration of the cadherin-like receptor for Cry toxins. PLoS ONE 7, e35991.