

2011

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Brad S. Coates
USDA-ARS, brad.coates@ars.uds.gov

Douglas V. Sumerford
Iowa State University

Miriam D. Lopez
Iowa State University

Haichuan Wang
University of Nebraska-Lincoln, hwang4@unl.edu

Lisa M. Fraser
Iowa State University

See next page for additional authors

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Coates, Brad S.; Sumerford, Douglas V.; Lopez, Miriam D.; Wang, Haichuan; Fraser, Lisa M.; Kroemer, Jeremy A.; Spencer, Terrence; Kim, Kyung S.; Abel, Craig A.; Hellmich, Richard L.; and Siegfried, Blair D., "A single major QTL controls expression of larval Cry1F resistance trait in *Ostrinia nubilalis* (Lepidoptera: Crambidae) and is independent of midgut receptor genes" (2011). *Faculty Publications: Department of Entomology*. 345.
<http://digitalcommons.unl.edu/entomologyfacpub/345>

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Authors

Brad S. Coates, Douglas V. Sumerford, Miriam D. Lopez, Haichuan Wang, Lisa M. Fraser, Jeremy A. Kroemer, Terrence Spencer, Kyung S. Kim, Craig A. Abel, Richard L. Hellmich, and Blair D. Siegfried

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Brad S. Coates · Douglas V. Sumerford · Miriam D. Lopez · Haichuan Wang ·
Lisa M. Fraser · Jeremy A. Kroemer · Terrence Spencer · Kyung S. Kim ·
Craig A. Abel · Richard L. Hellmich · Blair D. Siegfried

Received: 10 July 2010 / Accepted: 9 June 2011 / Published online: 6 August 2011
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Abstract The European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae), is an introduced crop pest in North America that causes major damage to corn and reduces yield of food, feed, and biofuel materials. The Cry1F toxin from *Bacillus thuringiensis* (Bt) expressed in transgenic hybrid corn is highly toxic to *O. nubilalis* larvae and effective in minimizing feeding damage. A laboratory colony of *O. nubilalis* was selected for high levels of Cry1F resistance (>12,000-fold compared to susceptible larvae) and is capable of survival on transgenic hybrid corn. Genetic linkage maps with segregating AFLP markers show that the Cry1F resistance trait is controlled by a

single quantitative trait locus (QTL) on linkage group 12. The map position of single nucleotide polymorphism (SNP) markers indicated that midgut Bt toxin-receptor genes, alkaline phosphatase, aminopeptidase N, and cadherin, are not linked with the Cry1F QTL. Evidence suggests that genes within this genome interval may give rise to a novel Bt toxin resistance trait for Lepidoptera that appears independent of known receptor-based mechanisms of resistance.

Keywords Quantitative trait locus (QTL) · Amplified fragment length polymorphism (AFLP) · Single nucleotide polymorphism (SNP)

Electronic supplementary material The online version of this article (doi:10.1007/s10709-011-9590-0) contains supplementary material, which is available to authorized users.

B. S. Coates · D. V. Sumerford · M. D. Lopez ·
J. A. Kroemer · C. A. Abel · R. L. Hellmich
USDA-ARS, Corn Insects & Crop Genetics Research Unit,
Genetics Laboratory, Iowa State University, Ames,
IA 50011, USA

B. S. Coates (✉)
USDA-ARS, Corn Insects & Crop Genetics Research,
113 Genetics Lab, Iowa State University, Ames, IA 50010, USA
e-mail: brad.coates@ars.usda.gov

D. V. Sumerford · M. D. Lopez · L. M. Fraser ·
C. A. Abel · R. L. Hellmich
Department of Entomology, Iowa State University, Ames,
IA 50011, USA

H. Wang · T. Spencer · B. D. Siegfried
Department of Entomology, University of Nebraska, Lincoln,
NE, USA

K. S. Kim
Department of Agricultural Biotechnology, Seoul National
University, Seoul 151-921, Republic of Korea

Introduction

The development of phenotypic resistance in insect populations to biopesticides is a concern for crop production efforts to meet the food, fiber and biofuel needs. Biopesticides have been developed from protein-based crystalline (*cry*) toxins endogenously expressed by the soil bacterium *Bacillus thuringiensis* (*Berliner*) (Bt) and shown to be highly toxic to larval Lepidoptera. The biological activity of *cry* toxins is specific, and shows little or no toxicity to humans, most beneficial insects, and other non-target organisms (O'Callaghan et al. 2005; Romeis et al. 2006). *Bacillus thuringiensis* spores have been used for foliar applications to crop plants to provide suppression of feeding damage by susceptible larvae, but the method is used rarely in the U.S. due to poor environmental stability of the protein toxin and difficulty timing application with peak pest populations (Lambert and Peferoen 1992). The effectiveness of Bt toxins for the control of larval feeding damage was enhanced through the development of

genetically-engineered (GE) Bt maize hybrids that express a high dose of Bt toxins throughout most of the growing season (Koziel et al. 1993). GE maize hybrids that express Cry1Ab and Cry1F were respectively released for use by crop producers in 1996 and 2002, prior to which larvae of the species *Ostrinia nubilalis*, the European corn borer, were arguably the single most destructive pest insect impacting maize production in the U.S. (Archer et al. 2000). By 2009, GE Bt maize hybrids comprised ~46% of the 35.2 million ha that were planted, with some maize production areas of the Midwestern U.S. having >50% (USDA NASS 2009). Since insect control obtained by use of Bt crops has rapidly become an integral component of maize production systems, the development of Bt toxin resistant phenotypes among target insect populations is predicted to have a high economic cost to crop producers and consumers.

Currently, GE maize hybrids provide an effective means to reduce levels of feeding damage by larval *O. nubilalis* with a concomitant reduction in use of traditional neurotoxic chemical insecticides. Although *O. nubilalis* resistance to Bt toxins is not observed in field environments, varying levels of resistance to Cry1Ab (Huang et al. 1997; Bolin et al. 1999; Chaufaux et al. 2001; Siqueira et al. 2006; Coates et al. 2007; Crespo et al. 2009) and Cry1F toxins was observed following laboratory selection (Pereira et al. 2008a, b). Additionally, resistance to Bt toxins has been reported in the lepidopteran species *Helicoverpa armigera* (Gahan et al. 2001) and *Pectinophora gossypiella* under laboratory conditions (Morin et al. 2003). Although not an equivalent representation of field conditions due to reductions in effective population sizes and exposure to initial low toxicity environments (Harshman and Hoffmann 2000), laboratory selections show that target insects have the genetic potential for developing Bt resistant phenotypes (Ferré and Van Rie 2002). Realization of this genetic potential was shown via emergence of populations that are resistant to field-exposed levels of foliar or transgenic Bt toxins, and include species *Plodia interpunctella* (McGaughey 1985), *Plutella xylostella* (Tabashnik et al. 1990), *Tricoplusia ni* (Janmaat and Myers 2003), *Busseola fusca* (van Rensburg 2007), and *Spodoptera frugiperda* (Matten et al. 2008). Although significant levels of field damage have not been reported, Tabashnik (2008) suggest resistance to GE cotton expressing Cry1Ac toxin has emerged in *Helicoverpa zea* populations based on analysis of more than a decade of phenotypic monitoring data.

Despite the heavy reliance on Bt toxins for pest insect control, the genetic mechanism(s) of resistance within the insect pest populations are not well understood, such that evaluation of current insect resistance management (IRM) strategies to delay the evolution of resistance phenotypes cannot be properly evaluated (Glasser and Matten 2003).

Historically, the frequent high dose application of insecticides for insect pest control has led to the selection for individuals of a population with traits that confer resistance (Georghiou and Lagunes-Tejeda 1991). The genetic basis for chemical insecticide tolerance tends to reside in an increased ability of resistant insects to detoxify the chemical agent, or amino acid changes in receptor proteins that alter ligand binding affinities. Insecticide chemistries tend to show a high fidelity for binding specific receptor molecules that result in insect toxicity and death, but also often results in reduced toxicity when receptor mutations are encountered within insect populations (Casida and Quistad 2004). For example, the enzyme acetylcholinesterase is bound and rendered inactive by insecticides that utilize organophosphate and carbamate chemistries, but enzyme insensitivity within an insect population can be achieved by mutation of a single amino acid (Williamson et al. 1996). Similarly, a single amino acid alteration in the γ -aminobutyric acid (GABA) receptor results in resistance of *Drosophila* to cyclodiene insecticides (French-Constant et al. 1993).

In contrast to the target-specificity typical of chemical insecticides, there appears to be a heterogeneous set of protein–protein interactions that occurs within the larval midgut between an ingested Bt *cry* toxin and multiple membrane-bound glycoprotein receptors. The midgut protein receptors alkaline phosphatase, aminopeptidase N, and cadherin-like proteins have been implicated as key factors in Bt toxin modes of action for lepidopteran larvae (Vadlamudi et al. 1993; Knight et al. 1994; Francis and Bulla 1997; Jurat-Fuentes et al. 2002). Additionally, knockout of a β -1,3 galactosyltransferase from the mutagenized Bt resistant 5 (*bre5*) line of *Caenorhabditis elegans* resulted in resistance to Bt toxins (Griffitts et al. 2001), which reiterated the role that posttranslational glycosylation may play in toxin-binding of receptor proteins (Knowles et al. 1991; Jurat-Fuentes et al. 2002). Mutations in the receptors, aminopeptidase N and cadherin, were also shown to result in larval Bt toxin-resistance traits among species of Lepidoptera (Gahan et al. 2001; Morin et al. 2003; Herrero et al. 2005; Xie et al. 2005; Zhang et al. 2009). In contrast, analysis of the Cry1Ac- and Cry2Aa-resistant CP73 strain of *Heliothis virescens* indicated that neither cadherin nor aminopeptidase N receptor genes contributed to resistance traits (Gahan et al. 2005; Heckel et al. 2007). Gahan et al. (2005) further indicated that the *H. virescens* Cry2Aa resistance trait is determined by more than one genetic locus. Baxter et al. (2008) showed that a single QTL that conferred *Plutella xylostella* resistance to Cry1A toxins segregated independently of genome positions that encode the known glycoprotein receptors of Bt toxins. Similarly, single nucleotide polymorphism (SNP) markers for aminopeptidase N or cadherin were shown not

to co-segregate with Cry1Ab resistance in *O. nubilalis* (Coates et al. 2008a). These studies suggest that Bt resistance among Lepidoptera may evolve by multiple independent genetic mechanisms (Griffitts and Aroian 2005; Baxter et al. 2005; Heckel et al. 2007).

Previous investigation of *O. nubilalis* larval resistance to the Bt toxin Cry1Ab used SNP markers to track the segregation of the candidate genes aminopeptidase N, brainiac, and cadherin among full-sib F_1 s, but was unable to correlate the inheritance of alleles from resistant parents with the resistance traits (Coates et al. 2005, 2007, 2008a). These studies highlighted the limitations of investigating a small number of genomic loci, and of the need for genome-wide scans to effectively isolate quantitative trait loci (QTL). In the current study, we used an *O. nubilalis* laboratory colony with a >12,000-fold increase in resistance to the Bt toxin Cry1F (Pereira et al. 2008a), and showed recessive inheritance controlled from a single genetic locus (Pereira et al. 2008b). Moreover, the trait conferred larval survival on transgenic maize hybrids that expressed Cry1F toxin (Pereira et al. 2008a). In the following, we report the genetic analysis of *O. nubilalis* backcross progeny that survived a high dosage of Cry1F toxin and identify QTL that contribute to expression of the trait. This analysis was paired with analysis of segregating markers within genes that encode candidate Bt toxin-receptors, and include the midgut membrane-bound glycoproteins alkaline phosphatase, aminopeptidase N, and cadherin. Results are significant since the single major QTL is segregates independent

of known Bt toxin-receptor genes, and suggests that *O. nubilalis* Cry1F resistance has evolved by a novel biochemical mechanism.

Materials and methods

Larval Cry1F toxin-resistance phenotype, pedigrees, and DNA extraction

A laboratory colony with a >12,000-fold increased Cry1-toxin tolerance compared to susceptible controls and is capable of surviving on transgenic corn plants was previously selected (Pereira et al. 2008a). The trait also was shown to be controlled by a single genetic locus that is inherited in a recessive fashion (Pereira et al. 2008b). The sex determination system in *O. nubilalis*, and all Lepidoptera, is comprised of a homogametic sex chromosome pair in males (ZZ) and a heterogametic pair in females (ZW; Traut and Marec 1997), where achiasmatic females produce gametes that do not undergo meiotic recombination (Traut 1977). A biphasic linkage mapping approach was used as described by Heckel et al. (1999) to establish pedigrees initiated from a single cross between a Cry1F resistant female (rr_{rr}^{\ominus} ; $P_{rr_{rr}^{\ominus}}$) \times susceptible male (SS_{SS}^{\ominus} ; $P_{SS_{SS}^{\ominus}}$), and subsequent backcross families were derived from an F_1 male \times Bt resistant female ($F_{1rS}^{\ominus} \times BCP_{rr_{rr}^{\ominus}}$; pedigree FQ4) or reciprocal F_1 female \times resistant male ($F_{1rS}^{\ominus} \times BCP_{rr_{rr}^{\ominus}}$; pedigree FQ5; Fig. 1). Due to the recessive single locus inheritance of the Cry1F toxin

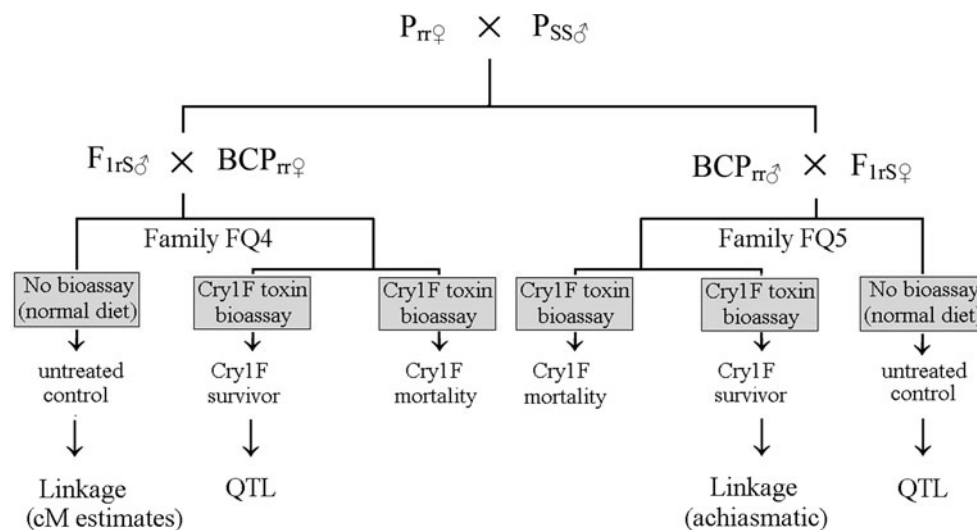


Fig. 1 Biphasic pedigree and Cry1F toxin bioassay design used to assess larval resistance traits in *O. nubilalis*. The family was initiated from a single parental (P) mate pair between a resistant female ($P_{rr_{rr}^{\ominus}}$) \times susceptible male ($P_{SS_{SS}^{\ominus}}$), followed by reciprocal backcrosses between F_1 to resistant line parents (BCP ; $F_{1rS}^{\ominus} \times BCP_{rr_{rr}^{\ominus}}$ in pedigree

family FQ4; $BCP_{rr_{rr}^{\ominus}} \times F_{1rS}^{\ominus}$ in pedigree family FQ5). A total of 44 larvae from each FQ4 and FQ5 were reared on non-Cry1F toxin diet (normal diet), and remaining backcross progeny subjected to diagnostic Cry1F toxin bioassay

Table 1 Multiplex (MP) primer pairs used for PCR amplification of AFLP markers in *O. nubilalis* FQ4 and FQ5 pedigrees (CORE1 = 5'-GAC TGC CGT ACC AAT TC-3'; CORE2 = 5'-CGA TGA GTC CCT GAG TAA-3')

MP	<i>EcoRI</i> Primer IRDye-700	<i>EcoRI</i> Primer IRDye-800	<i>MseI</i> Primer (unlabeled)
01	<i>EcoRI</i> -CORE1 + ACA-700	<i>EcoRI</i> -CORE1 + ACT-800	<i>MseI</i> -CORE2 + TG
02	<i>EcoRI</i> -CORE1-AAG700	<i>EcoRI</i> -CORE1 + AGC-800	<i>MseI</i> -CORE2 + TG
03	<i>EcoRI</i> -CORE1 + AAC-700	<i>EcoRI</i> -CORE1 + ACG-800	<i>MseI</i> -CORE2 + GA
04	<i>EcoRI</i> -CORE1 + AAG-700	<i>EcoRI</i> -CORE1 + AGC-800	<i>MseI</i> -CORE2 + GA
05	<i>EcoRI</i> -CORE1 + AAG-700	<i>EcoRI</i> -CORE1 + AGC-800	<i>MseI</i> -CORE2 + GT
06	<i>EcoRI</i> -CORE1 + AAG-700	<i>EcoRI</i> -CORE1 + AGC-800	<i>MseI</i> -CORE2 + CG
07	<i>EcoRI</i> -CORE1 + ACC-700	<i>EcoRI</i> -CORE1 + AGG-800	<i>MseI</i> -CORE2 + ACG
08	<i>EcoRI</i> -CORE1 + AAG-700	<i>EcoRI</i> -CORE1 + AGC-800	<i>MseI</i> -CORE2 + ACG
09	<i>EcoRI</i> -CORE1 + AAC-700	<i>EcoRI</i> -CORE1 + ACG-800	<i>MseI</i> -CORE2 + CAT
10	<i>EcoRI</i> -CORE1 + ACC-700	<i>EcoRI</i> -CORE1 + AGG-800	<i>MseI</i> -CORE2 + CAT
11	<i>EcoRI</i> -CORE1 + ACA-700	<i>EcoRI</i> -CORE1 + ACT-800	<i>MseI</i> -CORE2 + CAT
12	<i>EcoRI</i> -CORE1 + AAG-700	<i>EcoRI</i> -CORE1 + AGC-800	<i>MseI</i> -CORE2 + CAT
13	<i>EcoRI</i> -CORE1 + ACA-700	<i>EcoRI</i> -CORE1 + ACT-800	<i>MseI</i> -CORE2 + CTA
14	<i>EcoRI</i> -CORE1 + AAG-700	<i>EcoRI</i> -CORE1 + AGC-800	<i>MseI</i> -CORE2 + CTA
15	<i>EcoRI</i> -CORE1 + ACA-700	<i>EcoRI</i> -CORE1 + ACT-800	<i>MseI</i> -CORE2 + GAG
16	<i>EcoRI</i> -CORE1 + AAG-700	<i>EcoRI</i> -CORE1 + AGC-800	<i>MseI</i> -CORE2 + GAG

resistance trait, reciprocal backcrosses of F₁ progeny to the resistant line results in a 1:1 phenotypic ratio of resistant (rr) to susceptible (rS) larvae among backcross progeny.

A total of 48 larvae from two backcross pedigrees FQ4 and FQ5 were reared on non-Cry1F-containing diet (untreated controls), and the remaining progeny were exposed to diagnostic Cry1F concentrations that resulted in >99% mortality of SS and rS genotypes compared to 100% survival of resistant (rr) larvae (Cry1F survivors; Pereira et al. 2008a). DNA from initial parents P_{rr♀} and P_{SS♂}, FQ4 and FQ5 backcross parents, and 5th instar from respective untreated control and Cry1F-survivor groups were individually extracted using the Qiagen Blood and Tissue Extraction Kit (Qiagen, Valencia, CA) according to manufacturer instructions, and quantified on a Nanodrop ND-2000 (Thermo Scientific, Wilmington, DE).

An integrated *O. nubilalis* AFLP- and SNP-based linkage map

In the F₁ male backcross to the resistant line, recombination among the P_{rr♀}-derived haplotypes occurs during F_{1rS♂} gametogenesis and is used to estimate cM distance between linked markers. The template for amplified fragment length polymorphism (AFLP) analysis was constructed according to Vos et al. (1995) using ~0.5 µg of genomic DNA, and included *EcoRI* and *MseI* digestion, ligation, and pre-amplification protocols described therein. AFLP markers were generated by using 32 unique pairs of selective *EcoRI* and selective *MseI* primers (Table 1) in 10 µl PCR amplification reactions described by Vos et al. (1995),

except that selective *EcoRI* primers were labeled with either IRDye-700 or IRDye-800 (LiCor, Lincoln, NE). PCR products were diluted 1:4 with deionied water, to which 8 µl stop/loading buffer was added (LiCor). Samples were heat denatured at 95°C for 10 min, followed by snap chilling on ice for 10 min, loading of 1 µl into wells of a 0.2 cm 6.5% denaturing polyacrylamide gel (including 1 µl of the LiCor 50–700 bp molecular size standard used for size comparison). Electrophoresis took place at 1,500 V, 40 mA, and 45°C for 3.5 h on a Model 4300 DNA Analyzer (LiCor). TIF images were collected from IRDye-labeled fragment migration rates by the SAGA^{MX} software suite, marker bins ±1 bp of estimated marker sizes were determined by comparison to the 50–700 bp molecular size standard, and marker data exported in tab-delimited format.

AFLP markers generated in pedigree FQ4 (F_{1rS♂} × BCP_{rr♀} mate pair) were used for linkage analyses. Markers that were “non-banding” in the BCP_{rr♀} parent and “banding” in the F_{1rS♂} parent, and also “banding” in initial parent P_{rr♀} and “non-banding” in P_{SS♂}. The genotype configuration allows tracking of “banding” markers inherited from the Cry1F resistant initial parent P_{rr♀} through the F_{1rS♂} parent. Since AFLPs are dominant markers, heterozygosity of the F_{1rS♂} parent is required to follow the segregation of “banding” markers among the FQ4 progeny and determination of the backcross progeny that inherited the “banding” marker from P_{rr♀} parent. The heterozygosity of the F_{1rS♂} “banding” marker was tested for a predicted 1:1 ratio of “banding” to “non-banding” AFLP markers among FQ4 progeny by using the Chi-square statistic (χ^2 ; *P* cutoff >0.10). Marker data from

Mendelian-inherited AFLP markers were used to create an input file for MAPMAKER 3.0 (Lincoln et al. 1992) with “banding progeny” indicated as heterozygotes (H).

Genomic DNA remaining after AFLP template preparation was diluted to ~ 10 ng/ μ l with deionized nuclease-free water. SNP markers previously developed for the candidate Bt-resistance genes *brainiac* (*brn*), cadherin (*cad*), and aminopeptidase N (*apn1* and *apn3*) were used to genotype initial parents, FQ4 and FQ5 backcross parents, and subsequent backcross progeny reared on untreated control diet, using PCR–RFLP methods described by Coates et al. (2005, 2007, 2008a, b). Additionally, locus-specific markers for a larval midgut-expressed membrane-bound alkaline phosphatase (*malp*) were PCR-amplified with mALP-F3 (5'-CCA GCG CAA CGG CCA GAC-3') and mALP-R3d1 (5'-GAT TT GA GC GT CC AG GCA ATA-3') using conditions described by Coates (personal comm.). Genotyping for cubitus interruptus (*ci*), ribosomal protein small subunit (*rpS03*) and cytochrome *b* (OnS180) were described by Coates et al. (2008a; 2009). SNP loci that show the $F_{1rS\delta}$ with the $A_{r1}A_{S2}$ genotype and the $BCP_{rr\phi}$ with a $A_{r2}A_{r2}$ genotype, and additionally showing the A_{r1} allele in the initial $P_{rr\phi}$ parent were analyzed further (A_{r2} and A_{S2} are identical by state, but respectively belong to $P_{rr\phi}$ and $P_{SS\delta}$ lineages). Analogous to the genotypic scenario from AFLP analyses, a heterozygous $F_{1rS\delta}$ ($A_{r1}A_{S2}$) and homozygous $BCP_{rr\phi}$ ($A_{r2}A_{r2}$) at a SNP locus allows tracking of the A_{r1} allele inherited by the initial parent ($P_{rr\phi}$) to the FQ4 backcross progeny. Tests for Mendelian inheritance of SNP-derived alleles among the FQ4 backcross progeny (resulting from the cross $F_{1rS\delta} A_{r1}A_{S2} \times BCP_{rr\phi} A_{r2}A_{r2}$) was tested for a predicted 1:1 genotypic ratio using the χ^2 statistic (P cutoff > 0.05). Due to a similar genotypic arrangement of the co-dominant SNP and dominant AFLP-based markers, a combined AFLP and SNP marker input file was created for MAPMAKER 3.0 (Lincoln 1992) in which SNP-based $A_{r1}A_{r2}$ heterozygotes were coded as an H. Linkage between SNP and AFLP markers was determined among haplotypes obtained from the FQ4 untreated control group using MAPMAKER as described previously with the (cutoffs: LOD ≥ 4.0 and $r \leq 0.30$).

Mapping of the larval *O. nubilalis* Cry1F resistance trait

Since diagnostic Cry1F bioassays selected for larval survivorship, AFLP and SNP marker data were collected only from backcross progeny resistant to Cry1F toxin (rr genotypes). Larval phenotypes controlled by the rS genotypes are susceptible and show 100% mortality on bioassays due to the recessive nature of the trait (Pereira et al. 2008a). During analysis of FQ4 Cry1F survivors, markers linked to QTL that

influence inheritance of the Cry1F resistance were predicted to significantly deviate from the 1:1 genotypic ratio observed for markers that were Mendelian in the untreated control. These non-Mendelian markers from the Cry1F survivor group were predicted to show a statistically-significant increase in alleles inherited from the initial Cry1F-resistant $P_{rr\phi}$ parent (“banding” AFLP or A_{r1} SNP marker). Significant deviation of markers from the 1:1 Mendelian expectation at individual loci were evaluated using the χ^2 -statistic ($P \geq 0.05$) for the untreated control and Cry1F-survivor groups. The distributions of χ^2 -statistic-derived P -values were averaged across markers on each *O. nubilalis* linkage group (LG) and the natural log of the means was plotted on the x -axis. The significance thresholds of $\alpha = 0.05$ and Bonferroni-adjusted $\alpha = 0.0016 (= 0.05 \div 32)$ were used to evaluate the influence of genome regions on the inheritance of the larval Cry1F toxin resistance trait. An F -test was used to calculate the one-sided probability of the likelihood that the variances in genotypic proportions among FQ4 untreated control and Cry1F-survivor groups were different at the SNP markers that tracked segregation of candidate Bt resistance genes.

All Mendelian-inherited AFLP and SNP markers within pedigree FQ4 were used to genotype backcross parents ($BCP_{rr\delta} \times F_{1rS\phi}$ mate pair), and untreated control and Cry1F survivor-group progeny from pedigree FQ5. Linkage association between AFLP and SNP markers in FQ4 were confirmed within backcross FQ5. Statistical methods and evaluation of the initial Cry1F-resistant $P_{rr\phi}$ parent allele inheritance among FQ5 backcross progeny were performed as described for pedigree FQ4.

Results

Larval *O. nubilalis* Cry1F toxin-resistance phenotype, pedigrees, and DNA extraction

A single cross between a Cry1F-resistant female ($rr\phi$; $P_{rr\phi}$) \times susceptible male ($SS\delta$; $P_{SS\delta}$), resulted in two backcross families, FQ4 (derived from an F_1 male \times Bt resistant female; $F_{1rS\delta} \times BCP_{rr\phi}$) and FQ5 (from a reciprocal F_1 female \times resistant male; $F_{1rS\phi} \times BCP_{rr\delta}$; Fig. 1). From the 48 progeny grown on non-selective diet, 45 and 47 respectively survived to adulthood for backcross FQ4 and FQ5. The corresponding full-sib progeny from backcrosses FQ4 and FQ5 survived to adulthood when exposed to a diagnostic dose of Cry1F toxin in 102 of 234 (43.6%) and 96 of 206 instances (46.6%). Survivorship did not significantly deviate from the expected 1:1 ratio of resistant to susceptible phenotypes among backcross progeny (Pereira et al. 2008b) for FQ4 ($\chi^2 \leq 1.93$; $P \geq 0.1655$) or FQ5 survivor groups ($\chi^2 \leq 0.48$;

$P \geq 0.4904$). Additionally, the proportion of survivors among backcross progeny from FQ4 and FQ5 were not significantly different (one-tailed student's $T = 0.53$; $P = 0.3455$).

An integrated *O. nubilalis* AFLP- and SNP-based linkage map

The separation of PCR-amplified AFLP markers resulted in fragments or “bands” that represent co-dominant alleles, where parents and progeny genotypes within a pedigree that produced “bands” (i.e. are “banding”) can be heterozygous or homozygous for the “banding” allele. In contrast, an individual that shows the absence of a “band” (i.e. “non-banding”) is homozygous for a “non-banding” allele. A total of 186 markers segregated within pedigree FQ4 in the desired genotypic configuration (181 AFLPs with $F_{1rS\delta}$ “banding” and $BCP_{rr\varnothing}$ “non-banding”; 5 SNPs with $F_{1rS\delta}$ $A_{r1}A_{S2}$ and the $BCP_{rr\varnothing}$ $A_{r2}A_{r2}$). This configuration allowed the “banding” and A_{r1} alleles to be assigned to the initial Cry1F resistant female parent, $P_{rr\varnothing}$. Chi-square tests indicated that 123 AFLP and 5 SNP markers did not significantly deviate from the Mendelian-expected 1:1 genotypic ratio among FQ4 backcross progeny ($P \geq 0.05$; Online Resource ESM_1.pdf; Table 2). Genetic linkage among Mendelian-inherited markers was determined with the Kosambi mapping function, and shows an association between 93 AFLP and 5 SNP markers within 32 linkage groups (LGs; recombination fractions ≤ 0.30 in Online Resource ESM_2.txt, and LOD scores ≥ 4.00 shown in Online Resource ESM_3.txt). The resulting map contains 3.00 ± 1.34 markers per LG (range 2–6 per LG) with a total map distance of 747.7 cM (23.7 ± 24.3 cM per LG; 11.3 ± 8.8 cM between adjacent markers; Fig. 2). SNP markers for *O. nubilalis* candidate Bt resistance genes *malp*, *cad*, *apn1*, and *apn3* were segregating among FQ4 backcross progeny, and genome locations were predicted on LG4, LG27, and LG32, respectively. Results indicate

Fig. 2 An integrated AFLP and SNP marker-based genetic linkage map for *O. nubilalis* (98 markers; 32 linkage groups; total map distance = 747.65 cM) showing position of a QTL controlling the inheritance of a larval Cry1F toxin resistance trait on LG12. Genome position of SNP marker for candidate Bt resistance genes alkaline phosphatase (*malp*), cadherin, and aminopeptidase N genes (*apn1* and *apn3*) are not linked to the QTL and located on LG4, LG27, and LG32, respectively

that *O. nubilalis* *apn1* and *apn3* genes are tightly linked on LG32 ($r = 0$), and are located on a chromosome that lacks any AFLP markers. Additionally, SNP markers for *ci* and *rps03* were predicted to segregate with AFLP markers on LG1 and LG10 (Fig. 2).

Validation of the predicted genetic linkage between *apn1* and *apn3* genes in the *O. nubilalis* genome was conducted by the screening of 12,288 clones from the OnB1 BAC library by PCR ($\sim 4X$ genome coverage). The *apn3* gene fragment was PCR amplified from OnB1 clones 04G20, 50K02, 50L07, 52G07, 53A02, 55D13, 55O13, 66M16, 67O24, 72G03, 74F22, and 74N03. The *apn1* PCR reactions showed that products of predicted size were produced by OnB1 clones 04G20, 50K02, 53A02, 55D13, 72G03, and 74F22. The *apn1* and *apn3* gene fragment sizes and sequences were described by Coates et al. (2008a).

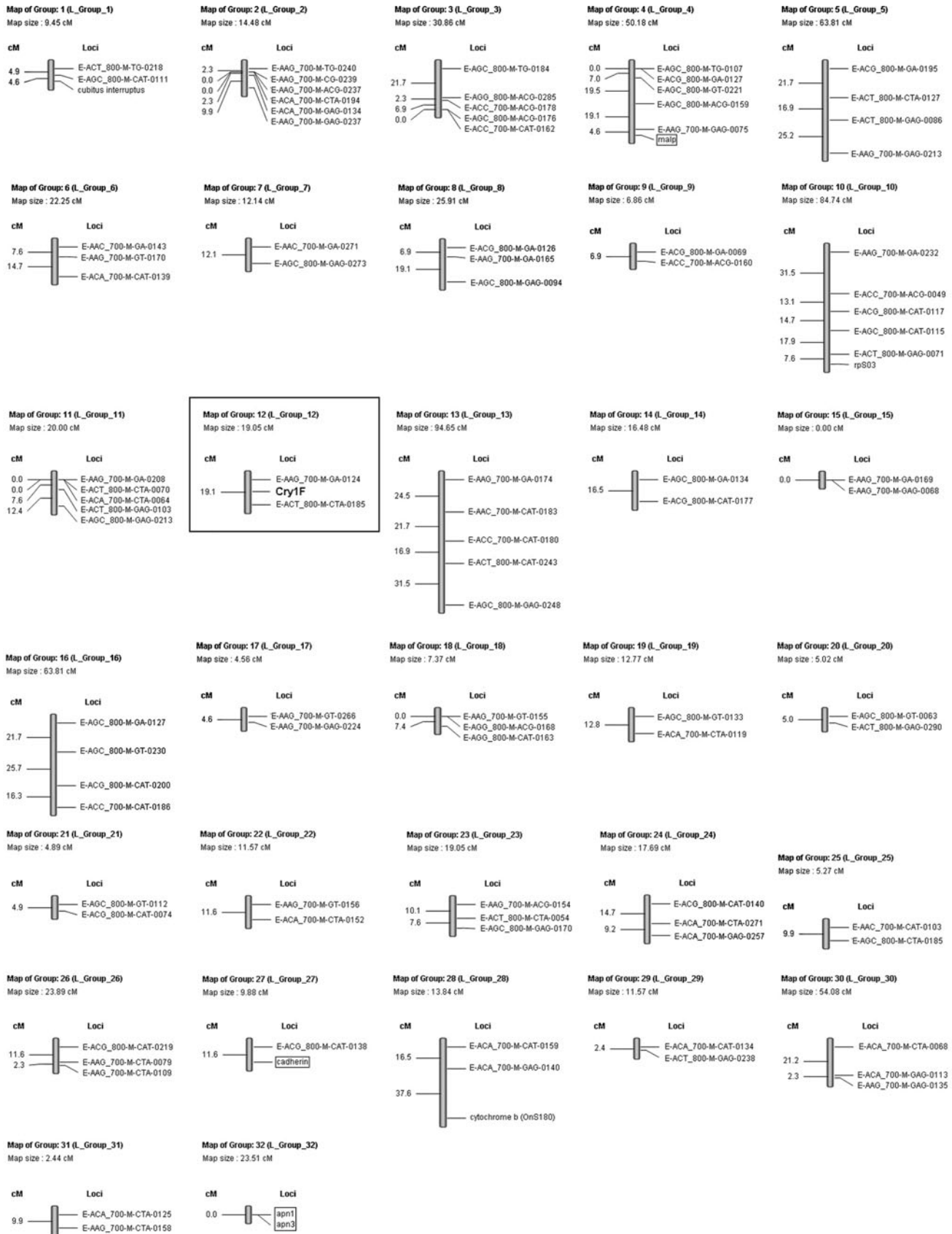
Mapping of the larval *O. nubilalis* Cry1F resistance trait

The association of Mendelian-inherited AFLP and SNP markers positioned on the FQ4 linkage map with *O. nubilalis* larval Cry1F resistance traits was determined by analysis of full-sibs from the Cry1F toxin-survivor group. Genotypes within the Cry1F toxin-survivor group that showed a significant deviation from the Mendelian expected 1:1 ratio had “banding” AFLP markers on LG12 displayed (markers E-AAAG700 M-GA-0124 $\chi^2 = 10.3$ $P = 0.0014$; marker E-ACT800 M-CTA-0185 $\chi^2 = 8.4$ $P = 0.0054$). The AFLP marker E-AAAG700 M-GA-0124 was skewed beyond the Bonferroni-adjusted significance

Table 2 Chi-square (χ^2) test statistics for adherence of SNP marker loci within candidate Bt-resistance genes (alkaline phosphatase, *malp*, cadherin, and aminopeptidase N 1 and 3 (*apn1* and *apn3*)) to

Mendelian-predicted 1:1 genotypic proportions within untreated controls and Cry1F-survivor groups (np = no observed polymorphism)

Pedigree	Group	N		<i>malp</i>	Cadherin	<i>apn1</i>	<i>apn3</i>
FQ4	Untreated control	44	χ^2 -statistic	0.401	0.000	2.227	2.227
			P -value	0.522	1.000	0.136	0.136
	Cry1F survivors	98	χ^2 -statistic	0.184	0.871	0.021	0.046
			P -value	0.668	0.351	0.884	0.829
FQ5	Untreated control	40	χ^2 -statistic	0.628	0.050	0.013	np
			P -value	0.428	0.823	0.910	np
	Cry1F survivors	85	χ^2 -statistic	1.815	0.236	0.462	np
			P -value	0.178	0.627	0.497	np



threshold of 0.0016 ($=0.05/32$) whereas marker E-ACT800 M-CTA-0185 was not. In contrast, both of these LG12 AFLP markers show Mendelian inheritance among progeny within the untreated control group to determine linkage associations ($\chi^2 \leq 0.09$; $P \geq 0.76302$; Online Resource ESM_4.pdf; Fig. 2). Genotyping results indicated that the AFLP markers E-AAG700 M-GA-0124 and E-ACT800 M-CTA-0185 were both “banding” within the initial Cry1F-resistant female parent ($P_{tr\varphi}$), and were part of Cry1F resistant haplotype inherited from the FQ4 $F_{1rS\delta}$ parent. Analysis further showed that the heterozygous “banding” E-AAG700 M-GA-0124 marker was inherited by 78% of progeny within the Cry1F survivor-group. Lack of 100% linkage likely resulted from recombination between the AFLP marker and the QTL position.

When the mean deviation of AFLP markers from expected 1:1 Mendelian ratios were plotted across LG for genotypes in the Cry1F survivor group, only LG12 showed a deviation that surpassed the significance threshold of $\alpha = 0.05$ (Fig. 3). Although the mean deviations for markers on LG12 did not surpass the Bonferroni-adjusted significance threshold of $P \leq 0.0016$, QTL analysis suggested that LG12 influences inheritance of the larval Cry1F

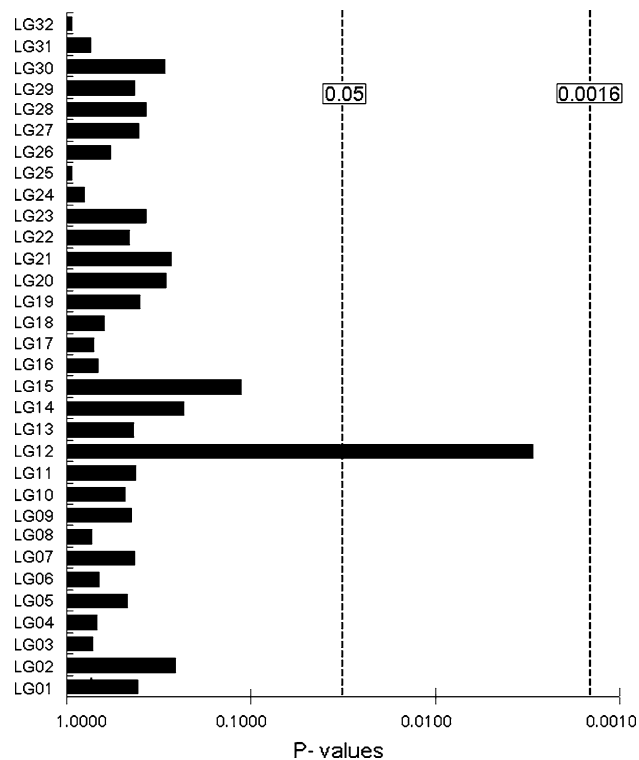


Fig. 3 Plot showing the mean contribution of AFLP markers on the inheritance of a larval Cry1F toxin resistance trait in *O. nubilalis* shown across 32 linkage groups in backcross pedigree FQ4. Significance thresholds of $\alpha = 0.05$ and Bonferroni-adjusted $\alpha = 0.0016$ are shown

toxin resistance. These results suggest that the QTL for the larval Cry1F resistance trait is likely located within a 19.1 cM genome interval and corroborates earlier findings that show the trait is controlled by a single genetic locus (Pereira et al. 2008a, b).

Co-dominant SNP markers were segregating within backcross pedigree FQ4 for lepidopteran candidate Bt resistance genes cadherin, *apn1*, *apn3*, and *malp*, as well as SNP markers for genes *ci*, *rpS03*, and OnS180. All SNPs show polymorphism between backcross parents, with the $BCP_{tr\varphi}$ homozygous ($A_{r2}A_{r2}$) and the $F_{1rS\delta}$ being heterozygous ($A_{r1}A_S$; A_{r1} derived from initial $P_{tr\varphi}$; Fig. 1). SNP-based genotypes among FQ4 backcross progeny within the untreated control group did not deviate significantly from the 1:1 proportion of the $A_{r1}A_{r2}$: $A_S A_{r2}$ Mendelian prediction (χ^2 -derived $P > 0.05$; Table 2). Additionally, no significant deviation from the 1:1 proportion was observed at the same SNP marker loci among the FQ4 Cry1F-survivor group (χ^2 -derived $P > 0.05$; Table 2). When comparing the two groups, an *F*-test indicated that the variance in genotypic proportions at *cad*, *apn1*, *apn3*, and *malp* loci from FQ4 untreated-control and Cry1F-survivor groups are not significantly different (*F*-statistic = 0.101, $P = 0.954$, $df_{numerator} = 3$, $df_{denominator} = 3$). Construction of an AFLP- and SNP-based linkage map indicates that the LG positions of known Bt-toxin receptor protein genes did not co-localize with the QTL that determines *O. nubilalis* Cry1F resistance traits on LG12 (Fig. 2).

Despite the association of AFLP makers E-AAG700 M-GA-0124 and E-ACT800 M-CTA-0185 with a major Cry1F QTL on LG12 for FQ4, neither marker was segregating within the FQ5 backcross pedigree. In this FQ5 backcross, the $F_{1rS\varphi}$ parent exhibited homozygous “banding” and homozygous “non-banding” at E-AAG700 M-GA-0124 and E-ACT800 M-CTA-0185 markers, respectively, such that segregation of these markers could not be documented among FQ5 progeny. As a consequence, the backcross pedigree could not be used to corroborate the QTL location on LG12 of the AFLP-based FQ4 linkage map. SNP-based genotyping results indicated that *malp* and *apn1* loci are segregating within the FQ5 backcross pedigree, with the genotypes of $F_{1rS\varphi}$ $A_{r1}A_{S2}$ and $BCP_{tr\delta}$ $A_{r2}A_{r2}$. The FQ5 backcross parents $F_{1rS\varphi} \times BCP_{tr\delta}$ showed *cad* alleles of $A_{r1}A_{r1}$ and $A_{r1}A_{r2}$, respectively. Genotypes defined at *malp*, *apn1*, and *cad* SNP loci were inherited by FQ5 backcross progeny within the untreated control and Cry1F survivor groups in ratios that did not significantly deviate from the Mendelian-predicted proportions (χ^2 -derived $P > 0.05$; Table 2). Moreover, the proportions of SNP-based genotypes within the untreated control and Cry1F survivor groups of the FQ5 backcross were not significantly different (*F*-statistic = 0.706, $P = 0.586$, $df_{numerator} = 2$, $df_{denominator} = 2$).

Discussion

An integrated *O. nubilalis* AFLP- and SNP-based linkage map

Linkage maps are genetic tools used to investigate genome structure and organization. For species of Lepidoptera, linkage maps have relied mainly upon the use of AFLP markers with inclusion of a few single locus markers (Dopman et al. 2004; Heckel et al. 1999; Baxter et al. 2005; Gahan et al. 2005). Recently, linkage maps composed exclusively of locus-specific co-dominant markers have been constructed for species of Lepidoptera (Miao et al. 2005; Yamamoto et al. 2006; Beldade et al. 2009). The 123 markers within our *O. nubilalis* backcross pedigree FQ4 was relatively low compared to that within other segregating populations. Some markers were excluded from analyses due to the limitations of dominant AFLP markers, where the “band” from the F_{1RS3} parent needs to represent a heterozygous genotype in order to be mapped. Use of a LOD score cutoff ≥ 4.0 and $r \leq 0.30$ for the estimation of linkage between markers also contributed to our inclusion of only 93 of 123 Mendelian-inherited markers (75.6%) within the final map, but also may have reduced the number of erroneous predictions. AFLP genotypes can be prone to scoring errors (Meudt and Clarke 2007), where subsequent recombination fractions may result in the omission of some LGs or inaccurate distance estimates (Göring and Terwilliger 2000). In contrast, the segregation of Mendelian-inherited co-dominant genetic markers within pedigree FQ4 showed a higher proportion that were incorporated into the final map (5 of 5; 100%), and may be related to the accuracy of SNP detection by RFLP assay (Urban et al. 1993). Although intriguing, the focus of the current study was not to compare of AFLP- and SNP-based markers for LG determination, but could be important to future studies designed to refine the linkage map.

Analysis of segregating AFLP and SNP marker data indicated that 32 *O. nubilalis* LGs are present which corroborated prior cytological (Guthrie et al. 1965) and genetic analyses (Dopman et al. 2004; map BC1 M) that suggested 32 chromosome pairs are present. The FQ4-based map spans 747.7 cM, is $\sim 50\%$ of the 1,697.3 cM within a previous AFLP-based linkage map, BC1 M, constructed for *O. nubilalis* (Dopman et al. 2004). However, FQ4 shows a similar marker interval (11.3 ± 8.8 cM) compared to BC1 M (8.8 cM). AFLP makers are not amenable to direct comparison across pedigrees due to the uncertain genome origin, such that specious comparisons of map position often arises when similar-sized fragments are assumed to be homologous (Meudt and Clarke 2007). For these reasons, we cannot make use of the AFLP linkage map developed by Dopman et al. (2004) to increase marker

densities on the LGs of FQ4. Reference points or “anchor” loci comprising locus-specific co-dominant markers allows LG assignment across pedigrees (Heckel et al. 1998), and was accomplished for 6 of 32 *O. nubilalis* LGs (18.8%). Although modest in scope, the anchored positions constitute receptor protein genes, alkaline phosphatase, cadherin, and aminopeptidase N, which previously were shown to function in the expression of Bt toxin-resistant traits in other species of Lepidoptera (Jurat-Fuentes and Adang 2004; Gahan et al. 2001; Herrero et al. 2005; Fig. 2). Analysis showed that these candidate loci segregate independently of the larval *O. nubilalis* Cry1F resistance trait (see section “Mapping of the larval *O. nubilalis* Cry1F resistance trait”), and indicate a tight linkage ($r = 0$) between *apn1* and *apn3* in the *O. nubilalis* genome on LG32. Proximity of *apn1* and *apn3* on the *O. nubilalis* linkage map was corroborated by our identification of single OnB1 BAC library clones that contain both genes. Linkage of aminopeptidase N genes was previously shown in *Helicoverpa armigera* and *Bombyx mori* genomes (Chang et al. 1999; Crava et al. 2010), which suggests that species of Lepidoptera may share synteny and potential co-linearity in the tandemly duplicated aminopeptidase N gene family.

Mapping of the larval *O. nubilalis* Cry1F resistance trait

Resistance to Bt toxins has developed among species of Lepidoptera by multiple independent mechanisms (Griffitts and Aroian 2005; Heckel et al. 2007) and has been suggested to involve modified expression of midgut protein receptor genes such as alkaline phosphatase, aminopeptidase N and cadherin (Jurat-Fuentes and Adang 2004; Gahan et al. 2001; Morin et al. 2003; Herrero et al. 2005). Assays show that gut epithelia of *Heliothis virescens* have a common binding site for Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa and Cry1Ja toxins (receptor A), whereas Cry1Ab and Cry1Ac have an additional binding site (receptor B) and Cry1Ac is the only toxin that recognizes a third binding site (receptor C) (Van Rie et al. 1989; Jurat-Fuentes and Adang 2004). Protein–protein interactions between Cry1F and *O. nubilalis* aminopeptidase N 1 (*apn1*)- and cadherin-like proteins were shown by ligand blot assays, which appeared to be receptors shared with the Cry1Ab toxin (Hua et al. 2001). In contrast, interaction was shown between Cry1Ab and cadherin for both resistant and susceptible *O. nubilalis* (Siqueira et al. 2006; Flannagan et al. 2005). Despite the possibility that Cry1F and Cry1Ab toxins share receptor sites on *O. nubilalis* *cad* and *apn1* proteins, the laboratory-selected Cry1Ab-resistant strains shows low cross-resistance to Cry1F toxin (Siqueira et al. 2004) as did the *O. furnicalis* ACB-AbR strain (Xu et al.

2010). Furthermore, *cad* and *apn1* loci do not co-segregate with an *O. nubilalis* Cry1Ab trait from a colony that shows >2,500-fold increase in resistance (Coates et al. 2008a), which that alleles at these loci do not contribute to larval Cry1Ab resistance traits.

Similarly, we show that the larval Cry1F resistance in *O. nubilalis* does not co-segregate with known protein receptor genes. Specifically, SNP assays showed that *malp* is positioned on LG4, cadherin on LG17, and *apn1* and *apn3* are linked on LG32, which all independent of the Cry1F QTL on LG12. Tests for significance using the *F*-statistic indicated that there is no difference in the proportion of alleles at *malp*, *apn*, or *cad* loci inherited from the initial resistant female (allele A_{r1} from $P_{rr\varnothing}$) among untreated control and Cry1F survivor groups from backcross pedigrees FQ4 and FQ5. In terms of susceptibility, there was also no difference in the frequency of backcross progeny in untreated control and Cry1F survivor-group pedigrees that inherited the susceptible allele from the $F_{1rS\varnothing}$ or $F_{1rS\sigma}$ parents. Since the Cry1F-resistance trait is recessive, any backcross progeny that inherit an allele from the susceptible initial male parent ($P_{SS\sigma}$) would have an $A_S A_{r2}$ genotype and have a recessive phenotype (Pereira et al. 2008a). Our evidence suggests that the Cry1F-resistance trait has evolved by a mechanism that does not directly involve genes to encode known Bt-binding receptors in the larval midgut, and that further steps are required to decipher the gene(s) involved in expression of this resistance trait.

Compared to candidate gene approaches that have investigated the inheritance of Bt toxin-resistance traits (Coates et al. 2005, 2007, 2008a), QTL analysis detect the co-segregation of anonymous markers. Candidate gene studies are limited in scope due to a focus upon one to few loci, and assume that similar traits evolve by analogous biochemical mechanisms. Multiple modes of Bt toxin-resistance have evolved among arthropod species (Griffitts and Aroian 2005; Heckel et al. 2007), which suggests novel mechanisms likely remain uncharacterized and that candidate gene approaches may not provide an appropriate avenue for trait characterization in Lepidoptera. Results also highlight the need for genome-wide approaches for identifying novel QTL involved in trait evolution of crop pest species, and suggest that the application of genomic technologies may rapidly dissect these complex phenotypic traits.

The larval *O. nubilalis* Cry1F-resistance trait is determined by a single QTL on *O. nubilalis* LG12, and the ~19.1 cM genome interval likely may encode ≥ 1 linked gene(s) that influence expression of the trait. Using the *Bombyx mori* estimate of 0.25 Mb per cM distance, the 19.1 cM Cry1F QTL interval could be ~4.8 Mb of physical sequence. The use of clones from the *O. nubilalis* BAC

library, OnB1 BAC (average insert size = 0.125 Mb; Coates et al. 2009), would necessitate isolation of >40 overlapping clones to reconstruct a physical map of this genome interval. Undoubtedly, an increase in the single locus marker density on LG12 will assist in narrowing the QTL interval for eventual isolation of gene(s) involved in inheritance of the trait from OnB1 (Coates et al. 2009). Moreover, compared to AFLPs, single locus markers offer greater flexibility for downstream BAC library-screening applications. Single locus SNP markers are also more amendable to high-throughput genotyping methods (Tang et al. 1999), and are genetic markers with stable genome position, which allows for direct comparison between linkage maps within species.

Acknowledgments This research was a joint contribution from the USDA, Agricultural Research Service (CRIS Project 3625-22000-017-00), the Iowa Agriculture and Home Economics Experiment Station, Ames, IA (Project 3543), and the University of Nebraska. Funding also was provided by a USDA, Biotechnology and Risk Assessment Grant (BRAG) “Quantifying risk factors for evolution of European corn borer resistance to Cry1F expressing corn hybrids: Contribution to a framework for managing insect resistance to transgenic crop” (2006-03697). This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or a recommendation by USDA, Iowa State University or the University of Nebraska for its use.

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