Available online at www.sciencedirect.com
SCIENCE DIRECT®
Insect Biochemistry and Molecular Biology 34 (2004) 1225–1233
Insect Biochemistry and Molecular Biology
www.elsevier.com/locate/ibmb

SHORT COMMUNICATION

DNA-based detection of Bt resistance alleles in pink bollworm

Shai Morin[a,*], Scottie Henderson[b], Jeffrey A. Fabrick[b], Yves Carrière[b], Timothy J. Dennehy[b], Judith K. Brown[c], Bruce E. Tabashnik[b]

[a]Department of Entomology, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76100, Israel
[b]Department of Entomology, University of Arizona, Forbes 410, Tucson, AZ 85721, USA
[c]Department of Plant Sciences, University of Arizona, Forbes 303, Tucson, AZ 85721, USA

Received 7 July 2004; received in revised form 12 August 2004; accepted 13 August 2004

Abstract

Evolution of resistance by pests is the main threat to long-term insect control by transgenic crops that produce *Bacillus thuringiensis* (Bt) toxins. We previously identified three mutant alleles (*r*1, *r*2, *r*3) of a cadherin gene in pink bollworm (*Pectinophora gossypiella*) linked with recessive resistance to Bt toxin Cry1Ac and survival on transgenic Bt cotton. Here we describe a polymerase chain reaction (PCR)-based method that detects the mutation in genomic DNA of each of the three resistant alleles. Using primers that distinguish between resistant and susceptible (s) alleles, this method enables identification of 10 genotypes (*r*1*r*1, *r*1*r*2, *r*1*r*3, *r*2*r*2, *r*2*r*3, *r*3*r*3, *r*1*s*, *r*2*s*, *r*3*s*, and *ss*) at the cadherin locus. For each of the three resistant alleles, the method detected the resistance allele in a single heterozygote (*r*1*s*, *r*2*s*, or *r*3*s*) pooled with DNA from the equivalent of 19 susceptible (*ss*) individuals. The results suggest that the DNA-based detection method described here could greatly increase the efficiency of monitoring for resistance to Cry1Ac compared to bioassays that detect rare individuals with homozygous resistance.

Keywords: *Bacillus thuringiensis*; Resistance detection; Molecular monitoring; Cadherin; *Pectinophora gossypiella*; Bt cotton

1. Introduction

Transgenic crops that produce *Bacillus thuringiensis* (Bt) toxins offer safe and specific insect control, but evolution of resistance by pests could nullify their benefits. Although resistance to Bt crops in the field has not yet been reported, laboratory selection has produced Bt-resistant strains of many pests (Ferré and Van Rie, 2002; Tabashnik et al., 2003). Further, field populations of diamondback moth (*Plutella xylostella*) and greenhouse populations of cabbage looper (*Trichoplusia ni*) have evolved resistance to Bt sprays (Janmaat and Myers, 2003; Tabashnik et al., 2003).

Bioassays have been used to estimate the frequency of Bt resistance (*r*) alleles, but their failure to distinguish between heterozygotes (*rs*) and homozygous susceptible (*ss*) individuals usually necessitates huge sample sizes or multigenerational experiments to detect rare *rr* individuals. When resistance is recessive, DNA-based detection of *r* alleles directly in heterozygotes can increase efficiency relative to bioassays roughly 10- to 1000-fold as *r* allele frequency varies from 0.10 to 0.001 (Tabashnik, 1997). Because Bt resistance bioassays must be done with live larvae, months of work may be needed to rear and test insects from each field population. DNA screens can be conducted with properly preserved insects of any life stage, thereby greatly reducing labor, time, and cost. Such screening, however, requires knowledge of the molecular genetic basis of resistance to Bt toxins in field populations.

In two major lepidopteran pests of cotton, *Heliothis virescens* and pink bollworm (*Pectinophora gossypiella*), disruption of a gene in the cadherin superfamily is linked with resistance to Cry1Ac, the toxin produced by Bt cotton (Gahan et al., 2001; Morin et al., 2003). In the

*Corresponding author. Tel.: +972 8 9489365; fax: +972 8 9466768.
E-mail address: morin@agri.huji.ac.il (S. Morin).

0965-1748/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ibmb.2004.08.003

laboratory-selected YHD2 strain of *H. virescens*, insertion of an LTR-type retrotransposon in a cadherin gene (called *HevCaLP* or *BtR-4*) prematurely truncates transcription and confers >10,000-fold resistance to Cry1Ac (Gahan et al., 2001). So far, the resistance-conferring mutation in YHD2 has not been reported from other strains and has not been associated with survival on Bt cotton.

In several laboratory-selected strains of pink bollworm, three mutant alleles (*r*1, *r*2, and *r*3) of a cadherin gene (*BtR*) are associated with resistance to Cry1Ac and survival on Bt cotton (Morin et al., 2003; Tabashnik et al., 2004). Each of the three resistant cadherin alleles of pink bollworm has an mRNA deletion (*r*1: 24 bp, *r*2: 202 bp, *r*3: 126 bp) predicted to eliminate at least eight amino acids (Morin et al., 2003). All of the deletions occur upstream of the Cry1Ac-binding region of cadherin protein reported by Bulla and Candas (2001).

Here we used the identified mutations to develop a PCR-based method for detecting the *r*1, *r*2 and *r*3 alleles in pink bollworm. We isolated, cloned and sequenced the genomic region spanning each of the three mutations (*r*1, *r*2, *r*3) and designed allele-specific PCR primers for each region. To the best of our knowledge, this is the first report of DNA-based detection of individuals bearing alleles that confer resistance to a Bt crop.

2. Materials and methods

2.1. Pink bollworm strains

We used four strains of pink bollworm: a susceptible strain (APHIS-S) and three laboratory-selected Bt-resistant strains (AZP-R, SAF97-R, and MOV97-R [previously referred to MOV97-R_{10}]; for details see Tabashnik et al., 2000, 2002; Carrière et al., 2001; Liu et al., 2001). We have found all three *r* alleles in AZP-R, *r*1 and *r*2 in SAF97-R, *r*1 and *r*3 in MOV97-R, and none of the three resistant alleles in APHIS-S (Morin et al., 2003; Tabashnik et al., 2004).

2.2. DNA extraction, PCR amplification, cloning and sequencing

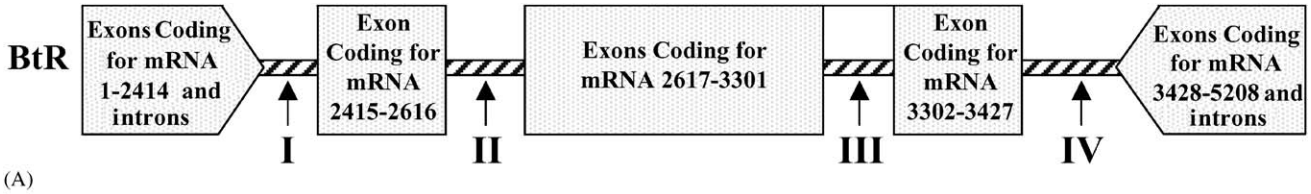
We used two DNA extraction methods: Method 1 for groups of 5–20 larvae and Method 2 for single larvae. Method 2 is simpler but cruder, and should not be used for extracting DNA from more than 1 or 2 individuals. Method 1: After homogenization in 0.5 ml of lysis buffer (100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 500 mM NaCl, 10 mM β-mecaptoethanol and 1.3% SDS), the mixture was incubated for 15 min at 65 °C, treated with phenol–chloroform–isoamylalcohol (25:24:1), chloroform-isoamylalcohol (24:1), and isopropanol precipitation. The pellet was recovered by centrifugation at 10,000 g, washed with 1 ml 75% ethanol and re-suspended in 50–100 µl of DEPC-treated water. Method 2: Each larva was placed on a section of parafilm stretched on a hard surface and ground in 150 µl of cold lysis buffer (5 mM Tris-HCl, pH 8.0, containing 0.5 mM EDTA, 0.5% Nonidet P-40, and 1 mg/ml Proteinase K). Extracts were incubated at 65 °C for 15 min and at 95 °C for 10 min before PCR amplification.

PCR amplification was conducted in 30 µl reactions containing 0.1 µg of genomic DNA, 0.8 µM of each primer and 1.5 units of Taq DNA polymerase (MasterTaq, Eppendorf). The PCR protocol for most of the work included denaturation at 94 °C for 2 min, followed by 35 cycles (94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min) and a final extension at 72 °C for 10 min. However, we eventually used a gradient cycler (MasterCycler Gradient 5331, Eppendorf) to optimize the annealing temperature for each primer set. Optimized annealing temperature was 55 °C for all PCR reactions, except for *r*2 and *r*3 specific reactions where the optimal annealing temperature was 62 °C (*r*2disback and Cad2366 for *r*2, and *r*3disback and Cad3221 for *r*3). PCR products were recovered from agarose gels using the QIAquick gel extraction kit (Qiagen), cloned using the pGEM-T easy vector system (Promega) and sequenced by the GATC sequencing facility at the University of Arizona. DNA sequence files were visualized using Chromas version 1.45 (Technelysium Pty Ltd) and analyzed using DNAMAN (Lynnon BioSoft, Montreal, Canada) software.

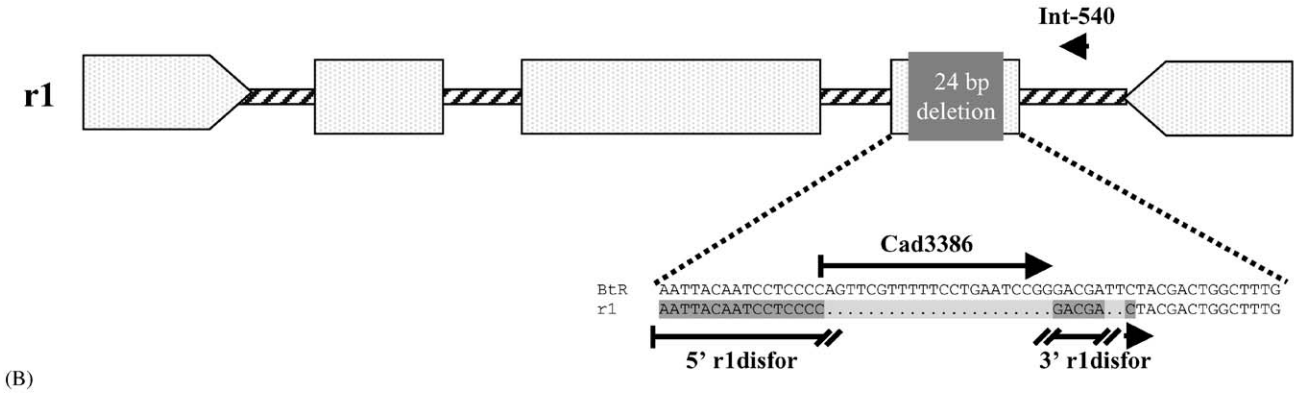
2.3. Obtaining partial genomic sequences of the r1, r2, and r3 alleles

Genomic DNA fragments (exons and flanking introns) corresponding to each of the three resistant alleles were amplified using coding sequence primers located 100–150 bp upstream and downstream from the *r*1, *r*2 and *r*3 mutations. We used primers Cad3221 and Cad3510 for *r*1, Cad2366 and Cad2741 for *r*2, and Cad3221 and Int-651 for *r*3 (see Table 2, which is published as supplementary material, see Appendix B). PCR products were cloned and sequenced as described above.

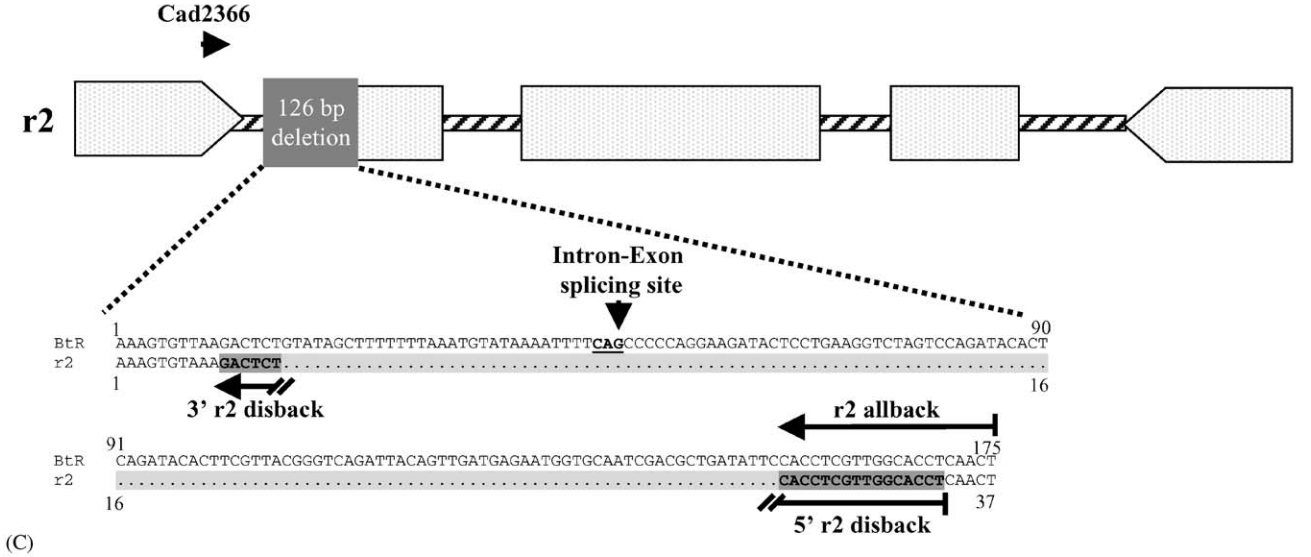
Fig. 1. PCR-based detection of pink bollworm cadherin alleles. The exons and introns (I–IV) in the genomic region between mRNA nucleotides 2414 and 3428 are represented by dotted white rectangles and striped lines, respectively. The regions surrounding the *r*1, *r*2 and *r*3 mutations are indicated by black rectangles. The alignment between the mutant (r) and wild type (s) sequences is shown below each mutation. Deleted nucleotides are indicated by dots and gray background. Inserted nucleotides are indicated by gray background only. Primers are depicted as horizontal arrows, with the direction of the arrow indicating the direction of primer extension by Taq polymerase. Vertical arrows in C and D indicate intron-exon splicing sites, which are also highlighted by bold, underlined nucleotides. (A) Primers used to isolate the genomic region harboring the *r*1, *r*2 and *r*3 mutations. (B) Detection of the *r*1 mutation using the allele-specific primer *r*1disfor with the primer Int-540. Primers Cad3386 and Int-651 discriminate between *r*1*r*1 and *r*1*s* individuals (see Fig. 2A). (C) Detection of the *r*2 mutation using allele-specific primer *r*2disback with primer Cad2366. Primers Cad2366 and *r*2allback discriminate between *r*2*r*2 and *r*2*s* individuals (see Fig. 2B). (D) Detection of the *r*3 mutation using allele-specific primer *r*3disback with primer Cad3221. Primers Cad3221 and Int-651 discriminate between *r*3*r*3 and *r*3*s* individuals (see Fig. 2C).


BtR
Exons Coding for mRNA 1-2414 and introns
Exon Coding for mRNA 2415-2616
Exons Coding for mRNA 2617-3301
Exon Coding for mRNA 3302-3427
Exons Coding for mRNA 3428-5208 and introns
I
II
III
IV

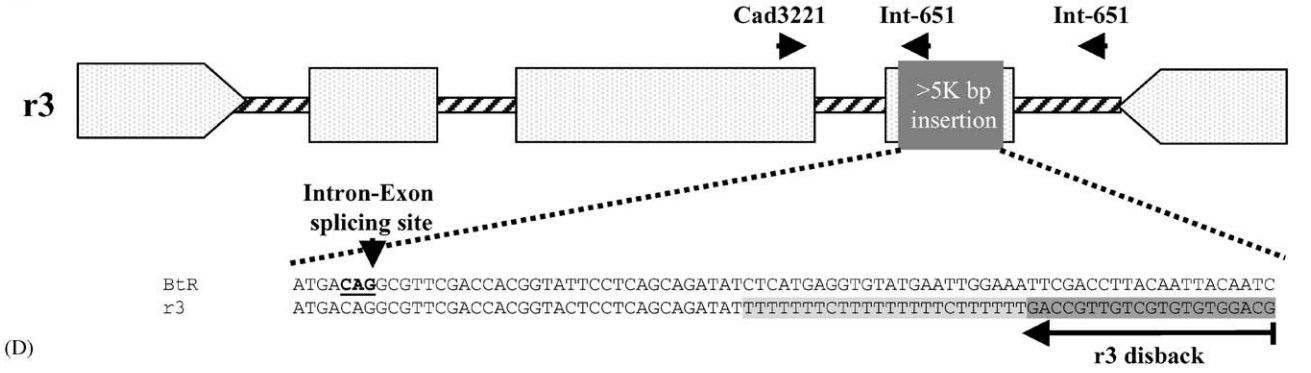
(A)


r1
Int-540
24 bp deletion
Cad3386
BtR AATTACAATCCTCCCCAGTTCGTTTTTCCTGAATCCGGGACGATTCTACGACTGGCTTTG
r1 AATTACAATCCTCCCC.....GACGA..CTACGACTGGCTTTG
5' r1disfor
3' r1disfor

(B)


r2
Cad2366
126 bp deletion
Intron-Exon splicing site
BtR AAAGTGTTAAGACTCTGTATAGCTTTTTTTAAATGTATAAAATTTTCAGCCCCCAGGAAGATACTCCTGAAGGTCTAGTCCAGATACACT
r2 AAAGTGTAAAGACTCT
3' r2 disback
r2 allback
BtR CAGATACACTTCGTTACGGGTCAGATTACAGTTGATGAGAATGGTGCAATCGACGCTGATATTCCACCTCGTTGGCACCTCAACT
r2 .....CACCTCGTTGGCACCTCAACT
5' r2 disback

(C)


r3
Cad3221
Int-651
Int-651
>5K bp insertion
Intron-Exon splicing site
BtR ATGACAGGCGTTCGACCACGGTATTCCTCAGCAGATATCTCATGAGGTGTATGAATTGGAAATTCGACCTTACAATTACAATC
r3 ATGACAGGCGTTCGACCACGGTACTCCTCAGCAGATATTTTTTTTCTTTTTTTTTCTTTTTTGACCGTTGTCGTGTGTGGACG
r3 disback

(D)

3. Results and discussion

3.1. Analysis of r1, r2, and r3 genomic DNA

Cloning and sequencing of the genomic region of the *r*1 mutation (Fig. 1B) revealed an 812 bp intron (III in Fig. 1A) upstream of the 24 bp deletion and an 721 bp intron (IV in Fig. 1A) downstream from the 24 bp deletion (GeneBank accession no. AY713482). Aside from the 24 bp deletion, we found no polymorphism in the exon coding for mRNA bases 3302–3427 (Fig. 1B), and only single bp substitutions or minor deletions in introns III and IV, compared to the corresponding genomic region of the wild type allele of *BtR* from APHIS-S (GeneBank accession no. AY707866).

The genomic region of the *r*2 deletion (Fig. 1C) contains an exon coding for mRNA bases 2415–2616 bracketed by two introns, 768 and 1329 bp long, respectively (I and II, Fig. 1A). Comparison to the wild type allele of *BtR* from APHIS-S (GeneBank accession no. AY707868) showed that *r*2 (GeneBank accession no. AY707869) is missing the last 33 bp of intron I and the first 95 bp of exon 2415–2616 (Fig. 1C). Because the deleted region contains the 3' intron-exon splice junction sequence TTTT**CAG**|CC, intron I is not spliced from the exon coding for mRNA bases 2415–2616, causing the mRNA of *r*2 to lack the entire 202 bases of the exon.

Like the *r*1 mRNA deletion, the *r*3 mRNA deletion is in exon 3302–3427 (Fig. 1D) which is bracketed by introns III and IV (Fig. 1A). We tried amplifying the genomic region of *r*3 using the PCR primers (Cad3221 and Cad3510) that successfully amplified the genomic region of *r*1. However, this approach produced no PCR products from *r*3*r*3 individuals, even though amplification reactions were repeated several times with simultaneous control reactions producing the expected products from individuals harboring the other alleles (*BtR*, *r*1, *r*2). Replacement of the primer cad3510 with primer Int-651 which is specific to intron IV generated a PCR fragment of ~1000 bp, that was smaller than expected (~1600 bp). Cloning and sequencing this fragment revealed that in the *r*3 genomic DNA (GeneBank accession no. AY707867), Int-651 recognizes an insertion of non-coding DNA in the exon coding for mRNA bases 3302–3427 (Fig. 1D). Because we could not amplify the inserted fragment using primers from its two exonic flanking regions and a high fidelity Taq polymerase (Platinum Taq DNA Polymerase High Fidelity, Invitrogen), we infer that the length of the insertion exceeds the capabilities of our PCR reactions (~5–10 kb). Although the intron splice junctions from both sides of the mutant exon are intact (ATGA-**CAG**|GC and TG|**GTAAGA**), the entire exon is missing from the *r*3 mRNA.

3.2. PCR primers for determining cadherin genotype

We used the genomic DNA sequence information described above to develop seven primer pairs that allow identification of 10 cadherin genotypes: *r*1*r*1, *r*1*s*, *r*2*r*2, *r*2*s*, *r*3*r*3, *r*3*s*, *r*1*r*2, *r*1*r*3, *r*2*r*3, and *ss* (Tables 1 and 2, which is published as supplementary material, see Appendix B). We use "*s*" here to denote alleles other than *r*1, *r*2, or *r*3. So far, alleles other than *r*1, *r*2, or *r*3 confer susceptibility to Cry1Ac, but we cannot exclude the possibility of additional *r* alleles.

DNA was extracted from individuals using extraction method 2 (see 2.2 above). To avoid interactions between primers for different alleles, separate reactions were conducted for each primer pair. As detailed below, the primers detect *r* alleles, discriminate between *rr*

Table 1
Seven PCR reactions used to identify 10 cadherin genotypes of pink bollworm. +, PCR product; −, no PCR product; NA, not applicable; +/−, PCR produces product only from the *r* allele; +/+, PCR produces two products, one from the *r* allele and one from alleles other than the *r* allele

Individual	Genotype	PCR reactions with forward and reverse primers						
		*r*1 *r*1disfor Int-540	*r*2 Cad2366 *r*2disback	*r*3 Cad3221 *r*3disback	*r*1*s* Cad3386 Int-651	*r*2*s* Cad2366 *r*2allback	*r*3*s* Cad3221 Int-651	Control Cad3324 Int-651
K	*r*1*r*1	+	−	−	−	NA	NA	+
L	*r*1*s*	+	−	−	+	NA	NA	+
M	*r*2*r*2	−	+	−	NA	+/−	NA	+
N	*r*2*s*	−	+	−	NA	+/+	NA	+
P	*r*3*r*3	−	−	+	NA	NA	+/−	+
Q	*r*3*s*	−	−	+	NA	NA	+/+	+
T	*r*1*r*2	+	+	−	NA	NA	NA	+
U	*r*1*r*3	+	−	+	NA	NA	NA	+
V	*r*2*r*3	−	+	+	NA	NA	NA	+
W	*ss*	−	−	−	NA	NA	NA	+

See Fig. 2 for gel photos of individuals K–Q.

homozygotes and *rs* heterozygotes, and check for amplifiable cadherin DNA.

3.2.1. Primers for detecting r alleles

We developed three allele-specific PCR reactions, with an allele-specific primer in each pair to selectively amplify the genomic DNA of each *r* allele: *r*1disfor and Int-540 for *r*1, *r*2disback and Cad2366 for *r*2, and *r*3disback and Cad3221 for *r*3 (Fig. 1, Table 2, which is published as supplementary material, see Appendix B). For *r*1 and *r*2, the specific primers start before the deletion and end after the deletion (*r*1disfor in Fig. 1B and *r*2disback in Fig. 1C). These primers do not amplify alleles lacking the deletion because mismatches occur in the 3′ end. We used a different strategy for the *r*3 allele by designing a specific primer identical to the beginning of the inserted non-coding sequence (primer *r*3disback in Fig. 1D).

By testing for *r*1, *r*2, and *r*3, the cadherin genotype can be determined for individuals with the genotypes *r*1*r*2, *r*1*r*3, and *r*2*r*3 (Table 1, individuals T, U, and V). Consistent with the occurrence of a single locus (Morin et al., 2003), tests of >1000 individuals did not detect any that were positive for more than two alleles. For individuals testing positive for only one *r* allele, we developed primers for discriminating between resistant homozygotes and heterozygotes (see 3.2.2 below). For individuals testing negative for *r*1, *r*2, and *r*3, we developed a control reaction to check for amplifiable cadherin DNA (see 3.2.3 below).

3.2.2. Primers for discriminating between resistant homozygotes and heterozygotes

If an individual tests positive for only one type of *r* allele, it could be either homozygous for that allele (*r*1*r*1, *r*2*r*2, or *r*3*r*3) or heterozygous with one *r* allele and one *s* allele (*r*1*s*, *r*2*s*, or *r*3*s*). Thus, we developed PCR reactions to discriminate between these two possibilities for each *r* allele (Fig. 2).

To discriminate between *r*1*r*1 and *r*1*s* individuals, we use primers Cad3386 and Int-651. Cad3386 is identical to the first 22 bp of the *r*1 deletion (Fig. 1B). Because the entire primer sequence is missing from *r*1 genomic DNA, a positive PCR reaction indicates the presence of an allele other than *r*1. While an *r*1*r*1 individual (K) produces a band of ~570 bp with the *r*1 reaction and no band with the *r*1*s* reaction, an *r*1*s* individual (L) produces a band of ~570 bp for both the *r*1 reaction and the *r*1*s* reaction (Fig. 2A).

To discriminate between *r*2*r*2 and *r*2*s* individuals, we use the primers Cad2366 and *r*2allback. Because *r*2allback anneals after the *r*2 genomic deletion (Fig. 1C), the PCR product of the *r*2 allele is smaller than that of other cadherin alleles (i.e., it lacks 128 bp in the intron-exon splicing junction). Therefore, PCR with primers Cad2366 and *r*2allback produces one band of ~800 bp in an *r*2*r*2 individual (M) and two bands in an *r*2*s* individual (N), with a band of ~800 bp from *r*2 and a band of ~930 bp from alleles other than *r*2 (Fig. 2B).

To discriminate between *r*3*r*3 and *r*3*s* individuals, we use the primers Cad3221 and Int-651. This primer pair generates a PCR product of ~1000 bp for the *r*3 allele because Int-651 has an additional matching sequence at the beginning of the non-coding DNA insertion. For all other cadherin alleles, the PCR reaction generates a ~1600 bp fragment spanning intron III, the 3302–3427 exon, and the first ~650 bp of intron IV (Fig. 1D). Thus, primers Cad3221 and Int-651 generate a single band of ~1000 bp from an *r*3*r*3 individual (P) and two bands from an *r*3*s* individual (Q), with a band of ~1000 bp from *r*3 and a band of ~1600 bp from alleles other than *r*3 (Fig. 2C).

3.2.3. Primers to check for amplifiable cadherin DNA

Individuals that produce no bands when their DNA is tested for the presence of *r*1, *r*2 and *r*3 could be *ss*. However, if some technical problem is interfering with amplification of the cadherin genomic region, they could be any genotype. Thus, as a control for amplifiable cadherin DNA, we used the primers Cad3324 and Int-651, which produce an ~675 bp band from all known susceptible and resistant alleles. Individuals that test positive for the control and negative for *r*1, *r*2, and *r*3 are scored as *ss* (Table 1, individual W). Individuals that test negative for *r*1, *r*2, *r*3, and for the control reaction are excluded from further analysis. In most of our samples, >95% of individuals tested positive for the control reaction.

3.3. Detecting resistant alleles in pooled samples

Because DNA-based detection of resistance is especially useful when resistance is rare in field populations, efficient processing of large samples is desirable. Thus, we tested the hypothesis that our method could detect *r* alleles in DNA from single *rs* individuals pooled with DNA from *ss* individuals. For each *r* allele, we tested a dilution series in which DNA was prepared (using DNA extraction method 1) from pools of five individuals including an *rs* individual (e.g., *r*1*s*, *r*2*s*, or *r*3*s*) and 4 *ss* individuals. We further diluted the DNA sample by mixing it 1:1 and 1:3 with DNA prepared from 5 *ss* individuals. As a result, the final dilutions for each *r* allele were: $\frac{1}{10}$ alleles in pool (original preparation), $\frac{1}{20}$ and $\frac{1}{40}$, respectively. We successfully detected each *r* allele in all three dilutions tested (see Fig. 3 for results with *r*1).

4. Conclusions

Although PCR techniques are available for monitoring insecticide resistance, their use has been limited

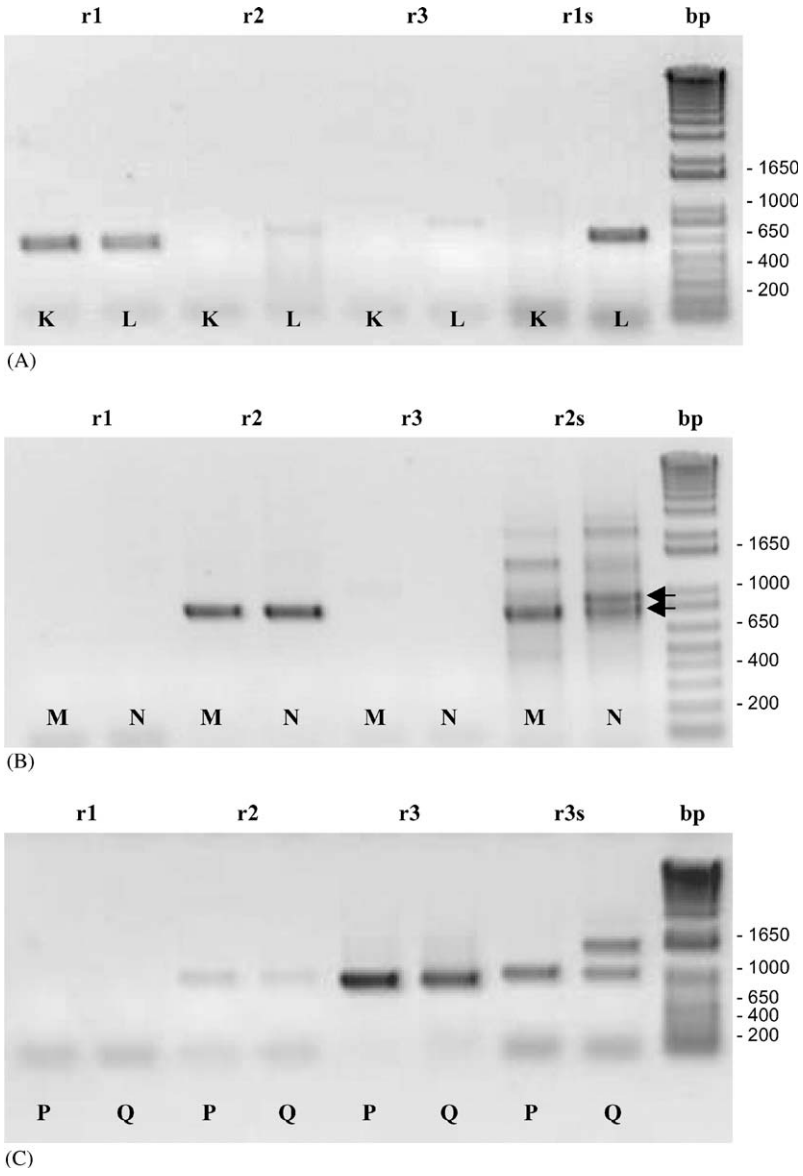


Fig. 2. Agarose gel electrophoresis of PCR products enabling discrimination between resistant homozygotes with two copies of the same *r* allele and heterozygotes with one *r* allele. (A) *r*1*r*1 (K) vs. *r*1*s* (L). (B) *r*2*r*2 (M) vs. *r*2*s* (N). (C) *r*3*r*3 (P) vs. *r*3*s* (Q). Horizontal arrows point to the two bands in an *r*2*s* individual (N), with a band of ~800 bp from *r*2 and a band of ~930 bp from an allele other than *r*2.

(Ffrench-Constant et al., 2004). We suspect this trend has occurred because such techniques for monitoring resistance to conventional insecticides have only minor advantages compared with bioassays or no monitoring at all. Molecular techniques can be expensive and time-consuming to develop. A pre-requisite for developing molecular detection technology is analysis of resistant insects. Therefore, in many cases, molecular methods are developed after resistance evolved in the field, occurring in multiple species and locations, and creating severe and sometimes irreversible problems (Denholm et al., 2002). If resistant individuals make up 1–10% of a population, bioassays of 1000 individuals will detect 10–100 resistant survivors, potentially reducing the advantages of molecular detection. Moreover, although fitness costs are often associated with resistance (Foster et al., 1999; Feyereisen, 1999; Ffrench-Constant et al., 2000), resistant alleles tend to persist in field populations even without strong insecticide selection (Ffrench-Constant, 1994).

Another major weakness of any molecular detection method is reliance on known resistance alleles and mechanisms. A narrow focus on one or a small set of resistance mechanisms is another inherent limitation of most molecular techniques for monitoring resistance. In many cases, pests have evolved more than one

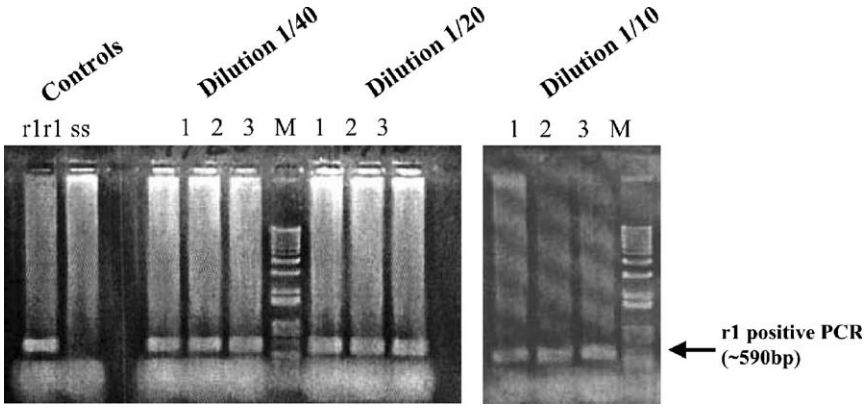


Fig. 3. Detecting the *r*1 allele in pooled samples with *ss* individuals. PCR products were generated with the allele-specific primer *r*1disfor and primer Int-540. The *r*1 allele was detected in DNA preparations from one *r*1*s* individual and four *ss* individuals (i.e., one *r*1 allele plus nine *s* alleles representing a dilution of $\frac{1}{10}$). The *r*1 allele was also detected in DNA preparations in which the original preparation ($\frac{1}{10}$ dilution) was further diluted with DNA from 5 *ss* individuals in mixtures of 1:1 ($\frac{1}{20}$ dilution) and 1:3 ($\frac{1}{40}$ dilution). In the controls, DNA was extracted from one *r*1*r*1 individual and one *ss* individual.

mechanism of resistance against the same insecticide (Field et al., 1997), which requires the development and interpretation of complex molecular assays.

Some of these drawbacks are less relevant in molecular monitoring for resistance to Bt crops. Pest resistance to Bt crops in the field has not been documented yet, despite large scale planting of Bt crops since 1996 (Tabashnik et al., 2003). Therefore, we have the opportunity to develop potentially useful monitoring tools before resistance is widespread in the field. Tracking the field frequency of resistance alleles identified from laboratory-selected strains could provide sufficiently early warning to take appropriate remedial action. However, one cannot determine if resistance alleles identified in laboratory-selected strains are important in field-evolved resistance until after resistance evolves in the field.

Unlike some conventional bioassays that do not detect heterozygous individuals, DNA-based detection can identify individuals with single *r* alleles. This is especially important in the crucial early stages of a resistance episode. Thus, DNA-based monitoring could be useful for testing the efficacy of the refuge strategy for managing resistance to Bt crops that is mandated by the US, Environmental Protection Agency (2001). With the growing need for data to satisfy regulatory requirements for transgenic crops, molecular monitoring of Bt resistance might be the only feasible approach.

In addition to monitoring Bt resistance in field populations of pink bollworm, the DNA-based detection method is useful for: (1) determining the association between genotypes and fitness, (2) characterizing laboratory-selected resistant strains, and (3) determining if field-evolved resistance is caused by known or novel resistance alleles.

DNA-based identification of genotypes is useful for determining the association between genotypes and performance in different environments. For example, DNA-based identification of cadherin genotype can be used in conjunction with plant bioassays to assess genotype-specific fitness costs (e.g., disadvantages suffered by resistant insects relative to susceptible insects on non-Bt cotton), as well as incomplete resistance (e.g., disadvantages suffered by resistant insects on Bt cotton relative to non-Bt cotton, Morin et al., 2003). A major advantage is that effects of specific alleles can be tested in heterogeneous strains in which alleles for resistance and susceptibility segregate in a common genetic background. Allele frequencies can be tracked over time, for example before and after overwintering, to test for genotype-specific effects on life history traits. This overcomes a limitation of comparisons between resistant and susceptible strains, which can be confounded by inter-strain differences unrelated to resistance.

DNA-based genotyping enables comparison of resistance allele frequencies among resistant strains. For example, application of the methods described here revealed that two independently derived resistant strains of pink bollworm, AZP-R (started in 1997 from pink bollworm collected in 10 Arizona cotton fields) and APHIS-98R (derived by selection from a long-term susceptible laboratory strain), each have the *r*1, *r*2, and *r*3 alleles but in different frequencies (Tabashnik et al., 2004). Although Bt-resistant strains of pink bollworm have been reported only from Arizona so far, Bt-resistant pink bollworm from anywhere in the world can be tested by the method described here to discover if *r*1, *r*2, and *r*3 are present.

In conjunction with bioassays, DNA-based detection can help to determine if field-evolved resistance is caused

by known or novel resistance alleles. Resistant survivors from bioassays of field-derived strains can be tested for *r*1, *r*2, and *r*3. If genetically based resistance not associated with *r*1, *r*2, or *r*3 is found, other portions of the *BtR* gene can be sequenced to determine if other *r* alleles occur at this locus. If such alternative cadherin mutants are not found, the search could be expanded to other loci and resistance mechanisms not related to cadherin.

In conclusion, the PCR method described here is useful for detecting the three known Bt resistance alleles of pink bollworm (*r*1, *r*2, and *r*3). If additional resistance alleles are discovered, the method might be readily modified to include them. Meanwhile, in all laboratory-selected Bt-resistant strains of pink bollworm tested so far (APHIS-98R, AZP-R, MOV97-R, and SAF97-R), some combination of *r*1, *r*2, and *r*3 is associated with resistance to Cry1Ac and no other resistant alleles have been detected (Morin et al., 2003; Tabashnik et al., 2004). Thus, results to date suggest that DNA-based detection of *r*1, *r*2, and *r*3 may help to monitor and manage pink bollworm resistance to Bt cotton.

Acknowledgements

We thank R. Biggs, D. Unnithan, and the staff of the Extension Arthropod Resistance Management Laboratory for technical assistance. Xianchun Li, Dawn Higginson and Iris Karunker provided helpful comments on the manuscript. This work was supported by Vaadia-BARD Postdoctoral Fellowship FI-300-2000, the University of Arizona, Cotton Incorporated, the Arizona Cotton Research & Protection Council, Monsanto, the Cotton Foundation, USDA-NRI grants 99-35302-8300, 2001-35302-09976 and 2003-01649, and USDA Biotechnology Risk Assessment Grants 2001-33120-11213 and 2003-04371. S. Henderson was funded by the Center for Insect Science through NIH training grant 1K12GM00708.

Appendix A. Supplementary analysis tools

A collection of macros that can be applied to sequence batches of up to 5000 sequences can be obtained from our web site (http://igm.ccc.uab.edu/~schroeder/publications/).

Appendix B. Supplementary data

The online version of this article contains additional supplementary data. Please visit doi:10.1016/j.ibmb.2004.08.003.

References

Bulla Jr., L.A., Candas, M., 2001. *Pectinophora gossypiella* (*Pink Bollworm*) *Bacillus thuringiensis Toxin Receptor* BT-R_2. World Intellectual Property Organization, Geneva Publication no. WO 01/34807 A2.

Carrière, Y., Ellers-Kirk, C., Liu, Y.B., Sims, M.A., Patin, A.L., Dennehy, T.J., Tabashnik, B.E., 2001. Fitness costs and maternal effects associated with resistance to transgenic cotton in the pink bollworm (Lepidoptera : Gelechiidae). J. Econ. Entomol. 94, 1571–1576.

Denholm, I., Devine, G.J., Williamson, M.S., 2002. Evolutionary genetics—Insecticide resistance on the move. Science 297, 2222–2223.

Ferré, J., Van Rie, J., 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Ann. Rev. Entomol. 47, 501–533.

Feyereisen, R., 1999. Insect P450 enzymes. Ann. Rev. Entomol. 44, 507–533.

Ffrench-Constant, R.H., 1994. The molecular and population genetics of cyclodiene insecticide resistance. Insect Biochem. Mol. Biol. 24, 335–345.

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.

Ffrench-Constant, R.H., Daborn, P.J., Le Goff, G., 2004. The genetics and genomics of insecticide resistance. Trends Genet. 20, 163–170.

Field, L.M., Anderson, A.P., Denholm, I., Foster, S.P., Harling, Z.K., Javed, N., Martinez-Torres, D., Moores, G.D., Williamson, M.S., Devonshire, A.L., 1997. Use of biochemical and DNA diagnostics for characterising multiple mechanisms of insecticide resistance in the peach-potato aphid, *Myzus persicae* (Sulzer). Pestic. Sci. 51, 283–289.

Foster, S.P., Woodcock, C.M., Williamson, M.S., Devonshire, A.L., Denholm, I., Thompson, R., 1999. Reduced alarm response by peach-potato aphids, *Myzus persicae* (Hemiptera: Aphididae), with knock-down resistance to insecticides (kdr) may impose a fitness cost through increased vulnerability to natural enemies. Bull. Entomol. Res. 89, 133–138.

Gahan, L.J., Gould, F., Heckel, D.G., 2001. Identification of a gene associated with Bt resistance in *Heliothis virescens*. Science 293, 857–860.

Janmaat, A.F., Myers, J.H., 2003. Rapid evolution and the cost of resistance to *Bacillus thuringiensis* in greenhouse populations of cabbage loopers, *Trichoplusia ni*. Proceedings of the Royal Society of London, Series B 270, 2263–2270.

Liu, Y.B., Tabashnik, B.E., Meyer, S.K., Carrière, Y., Bartlett, A.C., 2001. Genetics of pink bollworm resistance to *Bacillus thuringiensis* toxin Cry1Ac. J. Econ. Entomol. 94, 248–252.

Morin, S., Biggs, R.W., Sisteron, 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. Proceedings of the National Academy of Sciences, USA, vol. 100, pp. 5004–5009.

Tabashnik, B.E., 1997. Seeking the root of insect resistance to transgenic plants. Proceedings of the National Academy of Sciences, USA, vol. 94, pp. 3488–3490.

Tabashnik, B.E., Patin, A.L., Dennehy, T.J., Liu, Y.B., Carrière, Y., Sims, M.A., Antilla, L., 2000. Frequency of resistance to *Bacillus thuringiensis* in field populations of pink bollworm. Proceedings of the National Academy of Sciences, USA, vol. 97, pp. 12980–12984.

Tabashnik, B.E., Dennehy, T.J., Sims, M.A., Larkin, K., Head, G.P., Moar, W.J., Carrière, Y., 2002. Control of resistant pink bollworm by transgenic cotton with *Bacillus thuringiensis* toxin Cry2Ab. Appl. Environ. Microbiol. 68, 3790–3794.

Tabashnik, B.E., Carrière, Y., Dennehy, T.J., Morin, S., Sisterson, M.S., Roush, R.T., Shelton, A.M., Zhao, J.Z., 2003. Insect resistance to transgenic Bt crops: lessons from the laboratory and field. J. Econ. Entomol. 96, 1031–1038.

Tabashnik, B.E., Liu, Y.B., Unnithan, D.C., Carrière, Y., Dennehy, T.J., Morin, S., 2004. Shared genetic basis of resistance to Bt toxin Cry1Ac in independent strains of pink bollworm. J. Econ. Entomol. 97, 721–726.

US Environmental Protection Agency, 2001. Biopesticides Registration Action Document *Bacillus thuringiensis* Plant-Incorporated Protectants, http://www.epa.gov/pesticides/biopesticides/pips/bt_brad.htm 2001.