A Polymerase Chain Reaction Screen of Field Populations of *Heliothis virescens* for a Retrotransposon Insertion Conferring Resistance to *Bacillus thuringiensis* Toxin

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ABSTRACT The evolution of pest resistance to transgenic crop plants producing insecticidal toxins from *Bacillus thuringiensis* (Bt) Berliner poses a continuing threat to their sustainable use in agriculture. One component of the U.S.-wide resistance management plan for Bt cotton, *Gossypium hirsutum* L., involves monitoring the frequency of resistance alleles in field populations. However, existing methods are expensive and may not detect recessive resistance alleles until their frequencies are too high for countermeasures to be effective; therefore, more sensitive methods are needed. The first Bt resistance-causing mutation described at the molecular level was a retrotransposon insertion into the gene encoding a 12-cadherin-domain protein expressed in the midgut of larval *Heliothis virescens* (F.). We report the first large-scale screen for this mutation using a polymerase chain reaction (PCR)-based approach on >7,000 field-collected individuals. The specific insertion was not detected in any of these samples, nor was it detected in three progeny-tested, field-caught males thought to carry a Bt resistance gene. Unlike the targets of many chemical insecticides where a limited number of resistance-causing mutations compatible with viability can occur; a very large number of such mutations seem possible for the 12-cadherin-domain gene. However, even if these mutations are viable in the laboratory, they may not threaten the effectiveness of transgenic crops because of a high fitness cost in the field. The challenge remains to detect the subset of possible resistance-conferring alleles that are still rare but are viable in the field and increasing due to selection by Bt cotton. This situation will complicate PCR-based Bt resistance monitoring strategies.

KEY WORDS DNA diagnostics, resistance monitoring, tobacco budworm, cotton

Transgenic cotton, *Gossypium hirsutum* L., producing Cry insecticidal proteins from the bacterium *Bacillus thuringiensis* (Bt) Berliner has been grown commercially in the United States since 1996, with >50% of the cotton crop expressing one or more Bt genes (James 2005). To guard against evolution of Bt resistance in tobacco budworm, *Heliothis virescens* (F.), and other cotton pests, deployment has occurred under the conditions of a resistance management plan mandated by the U.S. Environmental Protection Agency. This plan requires farmers who grow Bt transgenic cotton to also grow a certain amount of "refuge" cotton that does not produce the Bt toxin, ensuring that a fraction of the pest population each year is not exposed to selection for resistance. Matings among the selected and nonselected subpopulations should ensure that most carriers of resistance alleles in the next generation are heterozygotes and thus susceptible to the concentration of Bt toxin present in the cotton. This "refuge strategy" assumes that Bt resistance is functionally recessive under field conditions, so that only resistant homozygotes would be able to survive on Bt cotton. As long as this class of homozygotes is rare enough, resistance should not result in significant insect damage to transgenic cotton.

Unfortunately, it is very difficult to estimate the frequency of recessive resistance alleles with bioassays that only detect resistant homozygotes, because their frequency is proportional to the square of the resistance allele frequency itself. For example, if a resistance allele occurs with frequency 10^{-4}, the fraction of the population expected to consist of resistant homozygotes is 10^{-8} when there is random mating. An enormous sample size would be required even for a bioassay aimed at detecting a single resistant homozygote. If the fraction of homozygotes is high enough to be detectable by bioassay with manageable sample sizes, the refuge strategy plan may no longer be ef-

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fective in containing resistance. Thus, DNA diagnostics that can detect heterozygotes have been proposed as a more feasible methodology for detecting resistance alleles at low frequencies.

This approach requires that the genes conferring resistance are known and that resistant and susceptible alleles can be distinguished by variations in the DNA sequence. These conditions are satisfied for many cases of target site resistance to chemical insecticides, e.g., modified acetylcholinesterase (Weill et al. 2004, Cassanelli et al. 2005), *kdr* mutations in the voltage-gated sodium channel (Schuler et al. 1998, Kwon et al. 2004), and mutations in the *Rdl* gene (Daborn et al. 2004). In each of these cases, a limited number of mutations is capable of conferring resistance, these can be readily detected in DNA from field-collected individuals, and results of DNA screening correlate well with bioassays when comparisons are possible.

When applicable, DNA diagnostics have several advantages compared with bioassays. Screening can be done on all life stages, including those not normally targeted by the insecticide such as eggs or pupae. Living material is not required, so long as DNA of sufficient quality has been preserved. Resistance alleles can be detected in heterozygotes, where most of them occur in the population, even if heterozygotes would seem fully susceptible by bioassay. Pooling strategies can be devised to screen DNA from several individuals at once, facilitating high-throughput approaches.

Until recently, DNA-based screening could not be applied to Bt resistance because the genetic basis of resistance was unknown. There were biochemical correlates of resistance that were experimentally measurable, such as reduced binding of membrane preparations to labeled toxins, but these measurements were more difficult to perform than feeding bioassays. However, there is now evidence from three lepidopteran species (Gahan et al. 2001, Morin et al. 2003, Xu et al. 2005) that resistance to Cry1A toxins can be caused by mutations in a gene encoding a 12-cadherin-domain protein expressed in the larval midgut. This mutation provides the opportunity to develop and test DNA diagnostic methods based on this gene.

The first reported resistance-associated mutation in this cadherin was the insertion of a partial long-terminal repeat (LTR)-type retrotransposon, Hel-1, in the coding sequence of the *HevCaLP* gene in the YHD2 strain of *Heliothis virescens* (Gahan et al. 2001). This mutation occurred in the same genomic location as the previously mapped *BtR-4* gene on linkage group 9, which accounted for >50% of the resistance levels in YHD2 (Heckel et al. 1997). The frameshift caused by this insertion introduced a premature stop codon, predicted to truncate the wild-type 1762-residue protein at position 622. Because the transmembrane domain anchoring the protein to the midgut epithelial membrane is absent from this truncated form, we hypothesized that Cry1A binding to this protein was required for toxicity and that Bt resistance in YHD2 was conferred by its absence. Subsequently, it was confirmed that the wild-type HevCaLP protein does bind to Cry1A toxins (Xie et al. 2005), as do its orthologues BtR1 in *Manduca sexta* (L.) (Vadlamudi et al. 1995) and BtR175 in *Bombyx mori* (L.) (Nagamatsu et al. 1998). Moreover, HevCaLP is not detected in membrane preparations from YHD2, by using antibodies that do detect it in Bt-susceptible strains (Jurat-Fuentes et al. 2004).

In a study conducted before the molecular basis of YHD2 resistance was known, we estimated the frequency of YHD2-like resistance alleles in field populations of *H. virescens* in 1993 (Gould et al. 1997). This estimate was accomplished by collecting males with pheromone traps, mating them to YHD2 females in single pairs in the laboratory and testing their progeny on artificial diet containing a diagnostic concentration of Cry1Ac toxin. Because Cry1Ac resistance in YHD2 is recessive, only males carrying YHD2-like resistance alleles from the field were expected to produce progeny that could grow on the Bt diet. Of 1,025 field-collected males with progeny that could be tested, three were judged to carry resistance alleles, based on the performance of their F_1 and F_2 progeny. From these three, the frequency of YHD2-type resistance alleles was estimated to be 1.5×10^{-3} in the field (Gould et al. 1997).

This estimate was surprising for two reasons. It was 2–3 orders of magnitude higher than expected by classical population genetic models of mutation–selection balance before the introduction of insecticide, when resistance alleles produced very slowly by mutation are mostly deleterious and kept at a low frequency by selection against them. Moreover, although the YHD2 strain shows typical viability and fecundity when reared on artificial diet in the laboratory, its development on nontransgenic cotton and soybean, *Glycine max* (L.) Merr., is arrested in the last instar, and it never reaches the pupal stage (F.G., unpublished observations). Progression through pupation is restored, however, if the last instar is transferred to artificial diet. Preliminary crossing experiments have determined that this developmental arrest is either caused by the resistance gene itself or by deleterious recessive alleles genetically linked to it. If the specific resistance mutation in YHD2 results in such a low fitness on cotton, it is difficult to explain why the class of YHD2-like mutations revealed by the progeny testing of field-collected males is so common. The discovery of the Hel-1 insertion in the YHD2 strain provided us with the molecular tools to address this dilemma.

The goal of the current study was to apply a diagnostic polymerase chain reaction (PCR)-based screening technique to estimate the frequency of the Hel-1 insertion of the *HevCaLP* gene in field populations of *H. virescens* and to test whether this specific mutation had been sampled in the 1993 collection of males from cotton-growing areas before Bt crops were commercialized (Gould et al. 1997). Among the >7,000 field-collected individuals screened, this specific insertion was not found. Evidence from other species, however, predicts that a very large class of mutations could inactivate the HevCaLP gene and

confer resistance, providing an explanation for the high frequency estimated by the progeny testing of field-collected males. Most mutations contributing to this class, however, may have high fitness costs in field populations. Thus, specific DNA diagnostic tests for Bt resistance may be more difficult to develop and apply than for previously studied cases of chemical insecticide resistance.

Materials and Methods

Sample Collection and Storage. For the field sampling, >7,000 adult male *H. virescens* were collected from pheromone traps set in cotton-growing areas of Franklin and Bossier Parishes of Louisiana, and Burleson County of Texas from 1996 to 2002. These males were preserved in ethanol and shipped to Raleigh, NC. They were stored at room temperature or −20°C until July 2002, when subsamples were taken for DNA analysis. From each moth, one half of the abdomen was cut off with scissors and transferred to a collection tube (19560, QIAGEN, Hilden, Germany) with 2 ml of 100% ethanol and stored at 4°C. Numbers of moths analyzed for each year were as follows: 1996 (879), 1997 (1,105), 1998 (879), 1999 (861), 2000 (1,172), 2001 (1,180), and 2002 (1,028). In addition to these moths, material from the 1993 progeny test included three field-collected males (LA1, LA2, and TX1 in Gould et al. [1997]), their mates from the YHD2 colony, and 16 progeny of LA1 that survived the Bt bioassay and were reared to adulthood. These 22 individuals were kept frozen at −80°C before DNA isolation.

DNA Isolation. For ethanol-preserved specimens, the ethanol was decanted from each sample tube, and each half-abdomen was incubated in 400 μl of phosphate-buffered saline at 4°C overnight to remove excess ethanol. The saline solution was aspirated and replaced with 400 μl of DNA isolation buffer (50 mM Tris, 0.2 M NaCl, 10 mM EDTA, and 2% EDTA, pH 8.0) and one 3-mm tungsten carbide bead (69997, QIAGEN). The sample tubes, which were racked in a 96-well, 8 by 12 format, were shaken in a Mixer Mill MM300 tissue homogenizer (Retsch, Haan, Germany) at 25 strokes per s for 1 min. The sample tube racks were removed and rotated 180° and subjected to an additional 1 min of agitation. They were then centrifuged at 1680 × *g* for 5 min in an Eppendorf 5810 microplate centrifuge to disperse the foamy layer. The tungsten bead was removed from each tube, 400 μl of phenol (preequilibrated with Tris-EDTA at pH 8.0) was added, and the sample tube racks were mixed by inverting 30 times. These were then allowed to stand overnight at room temperature to facilitate separation of the phases. After centrifugation at 1680 × *g* for 20 min, the aqueous upper layer was removed to a clean sample tube, to which 300 μl of chloroform was added. These were mixed by inversion and centrifuged again for 15 min. Two hundred microliters of the aqueous layer was transferred to 0.5-ml centrifuge tubes, racked in 96-well format. DNA was precipitated by addition of 22 μl of NaCl and 450 μl of ethanol, mixed, and centrifuged at 14,000 × *g* for 15 min at 4°C. The supernatant was aspirated, and the pellet was air-dried for 10 min and 50 μl of Tris-EDTA was added. For PCR screening, pools were created by combining 5 μl of DNA solution from each of eight individuals (e.g., A1 through H1; A2 through H2, and so on in the 96-well format). DNA concentrations in the pools were 50–2,000 ng/μl.

For frozen samples, DNA was isolated using three consecutive phenol extractions followed by one chloroform extraction and precipitated in ethanol as described previously (Zraket et al. 1990).

Diagnostic PCR. The PCR was carried out on pooled samples in 10-μl reaction volumes consisting of 1 μl of DNA template, 2 μl of 5× GoTaq reaction buffer (Promega, Madison, WI), 0.8 μl of 2.5 mM dNTPs, 0.6 μl of 25 mM $MgCl_2$, 0.2 μl each of three oligonucleotide primer solutions at 10 μM, 0.05 μl of GoTaq polymerase (5 U/μl), and 4.95 μl of water. The initial assay used PCR primers SF1 (5′-ATA CGA GCT GAC GAC ACG CTG GGA GA), SR2 (5′-TCT GAG CGT AGG AGG TGT GTT GTT GAT GTC), and RR3 (5′-GCG CGA TGT GAC AGT CCG GAA CAG). The cycle conditions for the Eppendorf Mastercycler (Hamburg, Germany) were 95°C for 2 min and then 30 cycles at 95°C for 30 s, 50°C for 30 s, and 72°C for 40 s, followed by incubation at 4°C. The optimized assay used PCR primers SF1, SR2, and RF5 (5′-CGC AAC GCG CGA TCT ACT CTT GTC ACC). The PCR cycle conditions were the same as described above except the annealing step was 58°C instead of 50°C for 30 s. Reaction products were electrophoresed on agarose gels consisting of 2.5% Metaphor (FMC, Rockland, ME) in Tris borate-EDTA buffer, for 2 h 15 min at 70 V, stained in ethidium bromide, destained for 60 min, and photographed under UV illumination. Gel images were inverted for publication so that fluorescing, ethidium-bromide-stained DNA bands look dark against a light background.

DNA Sequencing. The r_1 allele was amplified using PCR in 30 segments from genomic DNA of YHD2 individuals, and the PCR products cloned in the pCR4-TOPO vector (Invitrogen, Carlsbad, CA) for sequencing on a Li-Cor (Lincoln, NE) apparatus. Sequences were aligned and analyzed using Sequencher 4.1 (GeneCodes, Oxford, United Kingdom). PCR errors in the cloned products were detected as unique single-nucleotide polymorphisms in the alignment of replicate clones. Problematic regions were reamplified using different primers, cloned, and sequenced.

Allele Frequency Estimation. A Bayesian estimate of the population frequency q of HevCaLP alleles carrying the Hel-1 insertion was calculated by using equation 1 of Stodola and Andow (2004), with N taken as the number of individuals contributing to a pool or individual sample that supported PCR amplification, and S = u = v = 0 representing the observed number of r_1 alleles. Equation 3 was used to calculate the 95% credibility interval.

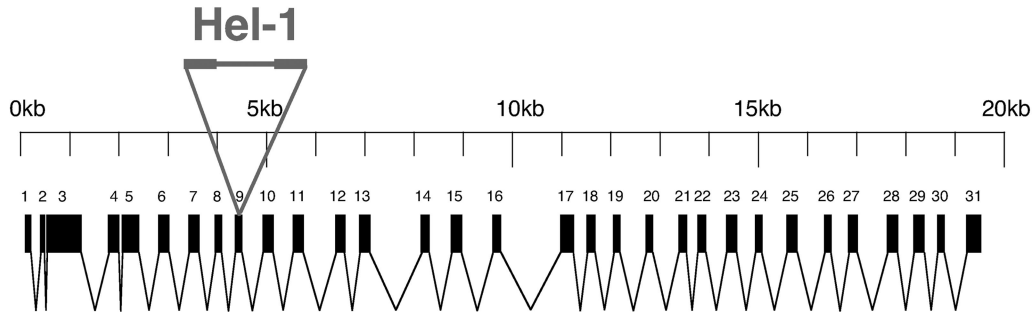

Fig. 1. Intron-exon structure of the r_1 allele of the HevCaLP gene.

Results

Identification of Region for Diagnostic Screening. We previously reported that resistance to Cry1Ac toxin in the YHD2 strain of *H. virescens* was correlated with the disruption of the HevCaLP gene by a portion of an LTR-type retrotransposon, Hel-1 (Gahan et al. 2001). Apart from the premature stop codon provided by the Hel-1 insertion, the cDNA sequence of the r_1 allele from YHD2 (GenBank accession no. AF367363) showed no evidence of frameshifts, premature stop codons, or other lesions that could result in the absence of the protein product. However, the possibility remained that additional mutation(s) in intron splice sites also could be responsible for failure to produce a full-length protein. Therefore, we determined the genomic sequence of the r_1 allele from YHD2, by sequencing overlapping PCR products from genomic DNA. This reveals 30 introns ranging in size from 91 bp (intron 4) to 1,272 bp (intron 16) (Fig. 1). All introns possessed the canonical splice sites, with GT- at the 5′ ends and -AG at the 3′ ends, in agreement with the splice sites of HevCaLP alleles from Cry1Ac susceptible strains (data not shown).

Because the Hel-1 insertion is the only identifiable DNA sequence feature of the r_1 allele to which the failure to produce a functional HevCaLP protein can be attributed, we focused on PCR-based assays for the detection of this specific mutation. We had previously developed an assay using three primers (SF1, RR3, and SR2), that produced a 71-bp SF1-RR3 product from the 5′ end of the Hel-1 element of the r_1 allele (Fig. 2), and a 99-bp SF1-SR2 product from any other amplifiable allele lacking an insertion at that point. These primers produced only the expected products when the YHD2 and other laboratory strains were being screened, but they often gave additional products when the more variable DNA from field samples were being screened. Because the Hel-1 element occurs in 15–20 copies per genome (Gahan et al. 2001), we suspected that these products could be generated by RR3 alone, from another one of the Hel-1 insertion sites and its flanking region. Substitution of RR3 with the RF5 primer produced a 175-bp RF5-SR2 product from the 3′ end of Hel-1 in the r_1 allele (Fig. 2), and it did not yield additional products from the field samples, other than the 99-bp SF1-SR2 product that was always generated.

PCR Screening Results. To increase the efficiency of the screen, we pooled DNA from several individuals in each PCR reaction. Preliminary experiments with a dilution series of DNA from the YHD2 strain and a susceptible strain established that the 71-bp r_1-specific band could still be detected when YHD2 DNA was diluted 64-fold with non-YHD2 DNA (Fig. 3). This is equivalent to detecting one heterozygote in a pool of 32 individuals. To ensure reliable detection, we decided to pool DNA from groups of eight individuals; moreover, this was convenient in the 8 by 12 96-well format of the DNA isolation apparatus.

DNA was isolated from 7,104 field-collected *H. virescens* males in 74 groups of 96. A subset of samples was analyzed by agarose gel electrophoresis (Fig. 4).

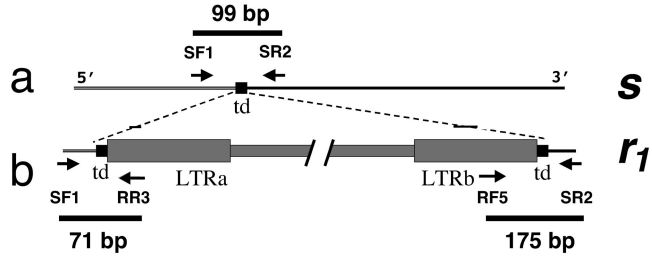

Fig. 2. Orientation of PCR primers and origin of the 71-, 99-, and 175-bp diagnostic products used in screening for the Hel-1 insertion in the HevCaLP gene. (a) Representative wild-type *s* allele. (b) Expanded view of the region flanking the Hel-1 insertion in the r_1 allele. td, target-site duplication; LTRa and LTRb, left and right long terminal repeats. Features are not to scale.

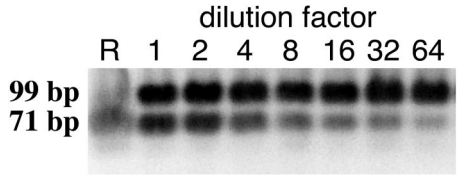

Fig. 3. Dilution experiment to estimate maximum pool size for detection of the Hel-1 element. YHD2 DNA was diluted with increasing amounts of non-YHD2 DNA before diagnostic PCR with the SF1, SR2, and RR3 primers. R represents undiluted YHD2 DNA after PCR.

As has been noted previously for samples stored in ethanol for prolonged periods, the DNA seemed highly fragmented, with an average fragment size <300 bp as estimated by comigration of size markers. This was judged to be adequate for the amplification of the PCR products expected from the primer design.

Initially, 888 pools of DNA from eight individuals were screened with the diagnostic PCR primers, representing 7,104 individuals in the screening attempt (Table 1). Of these pools, 861 produced a PCR product and 27 failed to amplify any products. Of the 861 pools that amplified, all produced a single band of 99 bp. None of them produced a band diagnostic for the presence of the r_1 allele (71 bp for the SF1 + SR2 + RR3 primers; 175 bp for the SF1 + SR2 + RF5 primers). Thus, there was no evidence for an r_1 allele in the DNA of these 861 pools, representing DNA from 6,888 different individuals.

Various substances in tissues or environmental samples are known to inhibit PCR amplification by *Taq* polymerase (e.g., Abu Al-Soud et al. 2005, Jiang et al. 2005). PCR inhibitors present in one or more of the samples contributing to the 27 nonamplifying pools may have masked the presence of r_1 alleles in the pools. Therefore we repeated the PCR screen with each of the 216 individuals contributing to these 27 pools. From 162 of these, only the 99-bp PCR product characteristic of the non-r_1 allele was produced; the remaining 54 failed to produce any products. Thus each of the 27 nonamplifying pools had one or more nonamplifying individuals as well as other individuals that did amplify, consistent with the inhibitor hypothesis. The total number of individuals with an inconclusive PCR result (nonamplifying, probably due to PCR inhibitors) was 54; and 7,050 individuals gave a conclusive PCR result in bulks or individually (Table 1).

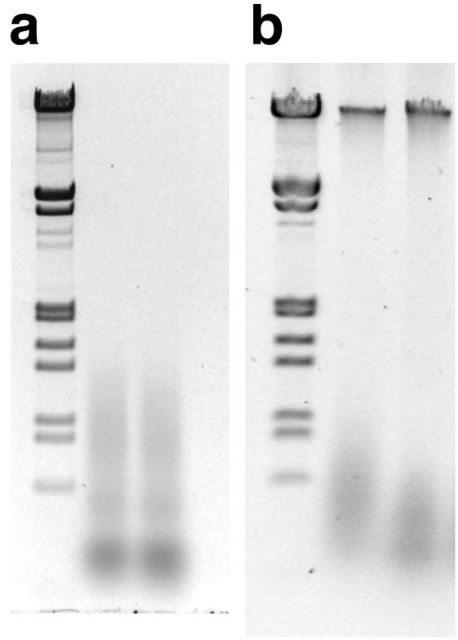

Fig. 4. Genomic DNA isolated from representative samples. (a) Two field-collected males preserved in ethanol. (b) Two progeny of male LA1 frozen at −80°C.

Screening of Field-Derived, Putative Resistance Alleles. In a previous study, field-collected males had been mated to YHD2 females and the progeny screened for resistance on artificial diet containing Cry1Ac toxin (Gould et al. 1997). DNA from one of the males (LA1) producing resistant progeny, his YHD2 mate, and 16 of their offspring was isolated and tested for presence of the Hel-1 insert. DNA from two additional males (TX1 and LA2) producing resistant progeny and their YHD2 mates was examined as well (progeny were not available for analysis from these crosses). As expected, the YHD2 females showed only the 71-bp r_1-diagnostic band (Fig. 5). The field-collected males showed only a 99-bp non-r_1 band. All progeny of LA1 had both bands, corresponding to one r_1 allele inherited from the YHD2 mother and one of the two non-r_1 alleles from the father LA1. When PCR using specific primers from neighboring exons was used to amplify selected introns of the cadherin gene, it was evident that the LA1 male carried two different alleles with different intron sizes (data not shown). Use of the same exon-specific primers showed that two types of progeny could be identified, depending on the particular paternal allele they received; but neither paternal allele carried the Hel-1 element in the characteristic position.

Discussion

The field samples analyzed here represent a long-term, sustained collecting effort and provide a valuable resource for DNA-based screening efforts. The preservation conditions were not optimal for DNA quality, but average fragment sizes were still larger than the PCR products required for diagnosis. Most of the pooled samples did in fact produce PCR products, and the presence of PCR inhibitors is the likely cause of failure to amplify from some of the pools. This was confirmed by successful amplification from the majority of individual samples making up those pools. Of 7,104 samples tested in pools or individually, 54 failed to amplify, giving a fraction of 0.75% of samples in which the PCR diagnostic test failed to produce any result.

Table 1. Results of screening pools and individuals for ethanol-preserved specimens

	No. pools	No. individuals in pool	Amplification from entire pool	No. individuals/ pool that amplified individually	99-bp S band present	71-bp or 175-bp R band present	Total no. individuals screened, producing S band but not R band	No. unscreenable individuals in group	Grand total of individuals in screening attempt
	861	8	Yes		Yes	No	6,888	0	
	12	8	No	7	Yes	No	84	12	
	7	8	No	6	Yes	No	42	14	
	4	8	No	5	Yes	No	20	12	
	4	8	No	4	Yes	No	16	16	
Total	888	8					7,050	54	7,104

Our PCR-based screen showed that the specific Hel-1 insertion defining the r_1 resistance allele of HevCaLP, which occurred at 100% frequency in the highly Cry1Ac-resistant YHD2 strain, did not occur in 7,050 field-collected *H. virescens* males of unknown resistance status from Louisiana and Texas nor in the field-collected males from Louisiana (LA1 and LA2) and Texas (TX1) that produced resistant progeny when mated with YHD2 females. Considering this as zero successes among the 14,106 ($=2 \times [7{,}050 + 3]$) field-sampled alleles screened by PCR, we calculate the Bayesian estimate (Stodola and Andow 2004) of the population allele frequency as 7.0×10^{-5}, with the 95% confidence interval ranging from 0 to 2.1×10^{-4}.

Using the same PCR diagnostic test, the Hel-1 insertion was detected in the CxC resistant strain (Gahan et al. 2005), in which it played a minor role in the overall resistance because of its low frequency (the major resistance gene in that strain being *BtR-5* on linkage group 10). In addition, the Hel-1 insertion frequency in other strains was estimated at 8% in YDK, 100% in YHD2-B, 3% in CxC, and 51% in KCBhyb (Jurat-Fuentes et al. 2004). All of the latter strains are derived from North Carolina collections, all have been maintained in the same laboratory for several years, and all but YDK have been maintained with selection by using Cry1Ac and other Bt toxins. Gould et al. (1997) suggested that the frequency of the YHD2-type resistance allele was 1.02×10^{-3} in field populations from North Carolina, based on the assumption of a single resistance allele among 490 eggs contributing to the initial YHD2 strain before Bt selection.

At the moment, two scenarios are consistent with the data: 1) the Hel-1 insertion in *HevCaLP* occurs in the field, with geographic variation in its frequency higher in North Carolina than in Texas and Louisiana; or 2) the Hel-1 insertion in *HevCaLP* does not exist in the field but instead occurred first in the laboratory, after which it was rapidly selected to a high frequency by mortality caused by Bt toxins in artificial diet (Gould et al. 1995). Detection of the Hel-1 insertion in new field samples from North Carolina would confirm the first scenario. Evidence for a full-length actively transposing Hel-1 element in the founding population of YHD2 would support the second scenario.

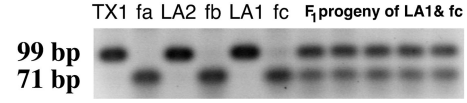

Fig. 5. Diagnostic PCR with SF1, SR2, and RR3 primers on TX1, LA2, and LA1 field-collected males putatively carrying resistance alleles, their YHD2 mates fa, fb, and fc, and five of the F_1 progeny of LA1 and fc.

Even if the Hel-1 insertion in *HevCaLP* does not exist in field populations, its occurrence in the laboratory strains is biologically relevant and provides an important insight into a crucial difference between target site resistance to Bt versus chemical insecticides. The widely used organophosphates, cyclodienes, and pyrethroids have targets in the nervous system (acetylcholinesterase, GABA-gated chloride channel, and voltage-gated sodium channel) that are essential for life. Only a few resistance-causing mutations are compatible with viability (ffrench-Constant et al. 2004). However, the HevCaLP protein expressed in the larval midgut is not essential for life. The protein is immunologically indetectable in membrane preparations from the YHD2 strain, which is viable and fertile in the laboratory. This predicts that *any* mutation disrupting the formation of a full-length HevCaLP protein attached to the midgut membrane could confer Cry1A toxin resistance compatible with viability.

Evidence from two other lepidopteran pest species supports this prediction. In *Pectinophora gossypiella* (Saunders), three different mutants causing lesions in the gene encoding the homologous BtR protein have been found; and survivorship on Bt cotton can be conferred by any combination of the three mutants (Morin et al. 2003, 2004). Moreover, different combinations of these resistance alleles were found in strains derived from different regions of Arizona (Tabashnik et al. 2005). In *Helicoverpa armigera* (Hübner) from Hebei Province, China, an allele with a premature stop codon at position 429 of the homologous Ha-BtR protein was linked to >500-fold resistance to Cry1Ac in the GYBT strain (GenBank accession no. AY647975; Xu et al. 2005). Another allele from a Chinese population has a large internal deletion removing several exons from the genomic DNA sequence (GenBank accession no. AY714875).

Whether such mutations pose a threat to the continued efficacy of Bt cotton will depend on their fitness costs in the field. The fitness costs of disruption of the 12-cadherin-domain gene are best known in *P. gossypiella*, where there is evidence for reduced survival on non-Bt cotton (Carrière et al. 2001a), lower overwintering success (Carrière et al. 2001b) and re-

duced sperm precedence (Higginson et al. 2005). No published information on fitness costs in *H. armigera* is available. The inability of YHD2 to complete development on cotton would imply a very high fitness cost associated with the Hel-1 insertion in *H. virescens.* There is evidence that other, less severe resistance-conferring mutations could have a much lower fitness cost. Recent studies on HevCaLP have shown that in vitro binding to Cry1A toxins can be reduced by substituting amino acids in a number of different positions in the toxin-binding region (Xie et al. 2005); these substitutions would be compatible with production of the full-length protein and insertion into the membrane. One might predict that such mutations would replace gene disruption mutants as resistance develops in field populations.

The very wide spectrum of viable, resistance-causing mutations provided by inactivating the HevCaLP gene provides an explanation for the puzzlingly high estimate of the YHD2-like resistance frequency of 0.0015 (Gould et al. 1997). Because absence of the HevCaLP protein is not lethal, resistance conferred by this absence can be detected in viable organisms, at least under laboratory conditions. Thus, any allele creating a lesion in HevCaLP protein, shielded from selection in the wild in a heterozygous carrier, could yield a positive response in the progeny test in the laboratory when homozygous. The summed frequencies of a class of rare, deleterious alleles contributed to the quantity estimated in the progeny test experiment. The size of the subset of these alleles that are also viable as homozygotes under field conditions remains unknown. This subset would be expected to increase due to selection by Bt cotton and may already exist at a higher frequency than the others because of the lower fitness cost in the absence of Bt.

Therefore, for the case of cadherin-mediated Cry1A resistance, any strategy for DNA-assisted resistance monitoring faces the problem of deciding which mutations should be monitored. For chemical insecticides, most mutations were identified from populations that had already developed resistance in the field. The challenge for Bt resistance monitoring is to detect the most common, highest viability set of resistance alleles in field populations while they are still moderately rare. The methodology we previously used (Gould et al. 1997) could be used to identify these by progeny bioassay, and then specific diagnostic tests could be developed for any new alleles found by sequencing. Toward this goal, DNA sequencing of offspring the LA1 male identified in the 1993 screen is currently in progress.

In a recent study published after the submission of our manuscript, Tabashnik et al. (2006) conducted PCR screens of >5,500 *P. gossypiella* collected from cotton fields in Arizona, California, and Texas from 2001 to 2005. They did not detect any of the previously characterized r_1, r_2, or r_3 alleles of the 12-cadherin-domain homologue *BtR* of that species. They concluded that the resistance management strategy based on non-Bt refuges may be helping to delay pest resistance to Bt crops. Each of these alleles is predicted to truncate the protein upstream of the transmembrane domain, and the high fitness costs entailed by the absence of the cadherin protein from the membrane have been previously documented in this species. This negative selection pressure would act as a potent force in keeping these allele frequencies low, especially in the context of the refuge strategy. What remains to be determined, in both *P. gossypiella* and *H. virescens*, is whether other alleles producing nontoxin-binding, nontruncated versions of the cadherin such as suggested by the experiments of Xie et al. (2005) might pose a bigger threat to the durability of the refuge strategy. The difficult task of screening field populations for such alleles should play an increasing role in DNA-based diagnostic strategies for detecting Bt resistance in field populations.

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