Variation in the cadherin gene sequence of Cry1Ac susceptible and resistant *Helicoverpa armigera* (Lepidoptera: Noctuidae) and the identification of mutant alleles in resistant strains

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Variations in the cadherin gene sequence between Cry1Ac-resistant BH-R strain showing 227.9-fold resistance and the wild susceptible VA-S strain of *Helicoverpa armigera* were identified. Amplification of exon 26 from *H. armigera* cadherin gene identified two mutant alleles, r9 (553 bp) and r10 (717 bp) against the wild S allele (588 bp). Sequence analysis of r9 showed a deletion of 45 bp in the intron region and mutation in the 5′-splice site of exon 26 resulted in an additional amino acid. While in the r10 allele a premature stop codon resulted in the putative translation into two different proteins of 1343 and 335 amino acids relative to a single protein of 1756 amino acids of the susceptible strain. The segregation of these mutant alleles was examined in F_2 progenies derived from matings of Cry1Ac-resistant and susceptible individuals and found to be associated with Mendelian principles. Cadherin genotyping showed that resistance to Cry1Ac, survival on Cry1Ac-treated diet and frequency of mutant r allele of cadherin gene were high in the selected strain than the unselected strain and were completely absent from susceptible VA-S strain. DNA-based screening of *H. armigera* collected in India failed to detect a single r9 or r10 allele in the populations collected from vegetable-growing areas of Delhi. However, very low frequency of mutant alleles was detected in *Bt* cotton-growing areas of Anand, indicating that these mutations are likely to be rare in the field.

Keywords: Alleles, cadherin, Cry1Ac-resistance, *Helicoverpa armigera*, mutation.

THE transgenic cotton-producing Cry insecticidal proteins from a microbial pesticide *Bacillus thuringiensis* (*Bt*) have been successfully deployed in India since 2002. The area under *Bt* cotton cultivation has increased from 38,000 ha in 2002 to 10.6 million ha (m ha) in 2011, constituting 88% of cotton. One of the most significant concerns associated with the use of *Bt*-toxin-based pest management technologies is the possibility of insects evolving resistance to Cry toxins as the laboratory selection experiments have shown a high potential for resistance development in many insects[1]. This has been further validated by reports on the resistance of *Trichoplusia ni* and *Plutella xylostella* to *Bt* sprays under greenhouse and field conditions[2–4]. In addition, recent reports on the field-evolved resistance in *Helicoverpa zea*[5], *Spodoptera frugiperda*[6], *Busseola fusca*[7], *Pectinophora gossypiella*[8], *Helicoverpa armigera*[9] and *Helicoverpa punctigera*[10] pose the biggest threat to the long-term success of *Bt* transgenic crops.

The cotton bollworm *H. armigera* (Hübner) is a polyphagous and economically important pest of cotton and other crops, which is reported to cause an annual damage of US$ 1 billion. Extensive studies on the susceptibility of *H. armigera* to the Cry1Ac toxin have been made and its ability to develop resistance under selection pressure has been reported in Australia[11–13], India[14,15] and China[16,17].

The rate of development of resistance is highly influenced by the frequency of resistant alleles in the field, which is reported to be rare[18–20]. Further, resistance to *Bt* toxin is inherited differently, viz. recessive[12,13,16,21–24], semi-dominant[25,26] and dominant[27,28].

The mode of action of Cry1Ac involves a complex multistep process. These include the solubilization of the crystal to release Cry proteins in their protoxin form, activation of protoxin form to the active form by midgut proteases, binding of a protein to midgut receptor and pore formation. Resistance to Cry toxin is attained through alteration in one or more steps of the process. The best characterized mechanism of resistance is the alteration of binding of Cry proteins to their midgut receptors. Two major candidates for targets of Cry1Ac are aminopeptidase N and cadherin, both of which bind Cry1A toxins in Lepidoptera. Alkaline phosphatase (HvALP) was found as a potential receptor and a marker

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for resistance to Cry1Ac[29]. Of the different aminopeptidase N that have been reported to bind to Cry toxins, only two were shown to mediate toxin susceptibility[30,31]. A 170 kDa aminopeptidae N purified fraction was considered a functional receptor of *Heliothis virescens* for Cry1Aa, Cry1Ab and Cry1Ac toxins[32]. Experimental depression of mRNA levels for a Cry1C-binding aminopeptidase by injection of double-stranded RNA was found to confer some protection in *S. litura*[33]. These results directly implicated larval midgut aminopeptidase N as a receptor for *Bt* insecticidal proteins. Similarly, four aminopeptidase N (APN) isoforms, TnAPN1, TnAPN2, TnAPN3 and TnAPN4 were identified in *T. ni*[34]. An inactivating mutation of the ABC transporter ABCC2 was found to be genetically linked to Cry1Ac resistance and is correlated with loss of Cry1Ac binding to membrane vesicles[35].

Among the Cry-toxin-binding molecules, cadherin, a cell adhesion protein was first reported as binding to Cry1A toxins in *Manduca sexta*[36–38], *Bombyx mori*[39,40], *H. virescens*[41] and *P. gossypiella*[42].

Cadherins are a superfamily of transmembrane glycoproteins that are responsible for maintaining the integrity of selective cell–cell recognition and adhesion properties. The *Bt*R4 cDNA of *H. virescens*[41] has been reported to encode a predicted 1732-amino acid and pro-protein (HevCalp) with a 22-amino acid trans-endoplasmic reticulum signal peptide, 11 predicted cadherin repeats, a hydrophobic transmembrane domain and a cytoplasmic domain at the C-terminus. In a laboratory-selected strain (GY*Bt*) of *H. armigera*, a deletion mutation of the cadherin gene *Ha_Bt*R was genetically linked with high levels of Cry1Ac resistance[23]. Introgression of this *Ha_Bt*R deletion allele (r1) into the susceptible SCD strain was found to enable the SCD strain to obtain 438-fold resistance to Cry1Ac[43]. The five new resistance alleles identified for *Ha_Bt*R showed that mutational diversity of *Ha_Bt*R could impair DNA screening for *Bt* resistance allele frequency in the field[44].

In view of the association of Cry1A toxin resistance in *H. virescens*[41], *P. gossypiella*[42] and *H. armigera*[23,45,46] to mutations in the cadherin gene, there has been increasing interest in exploiting this target for monitoring resistance gene frequency in field population.

In the present study the genomic sequence of susceptible and resistant strains was compared to detect and exploit the cadherin gene mutations to develop efficient DNA-based resistance monitoring methods.

Materials and methods

Insect strains

Susceptible strain of *H. armigera* was collected from cotton and pigeon pea fields in Vadodara (VA-S) and reared in the laboratory on unselected diet. The resistant strain was collected from *Bt* cotton fields in Bharuch (BH-R) (about 77.3 km from Vadodara) and selected with Cry1Ac liquid formulation MVP II (ref. 26).

The larvae were reared on Bengal gram-based diet until pupation. The adults emerging from the pupae were kept in jars and fed with 10% honey solution. Five pairs of adults were released in each mating jar (15 × 20 cm), which was covered with a rough cloth for egg-laying. The insects were maintained at 27 ± 2°C and 60–80% RH.

Isofemale lines were developed for both the populations. The BH-R strain showed 227.9-fold resistance vis-à-vis the susceptible VA-S strain. These lines were maintained in the laboratory and further used for genetic analysis[26]. The VA-S population was reared for seven generations without any selection pressure before the initiation of crossing experiments. The isofemale line of the resistant BH-R strain was selected on Cry1Ac-treated diet (1–3 μg/g diet) and their rate of resistance development as well as the stability of resistance were monitored in different generations[26].

F_6 adults of BH-R and F_7 adults of VA-S strains were used for conducting crosses. Reciprocal F_1 crosses were performed by mating susceptible ♀ and resistant ♂ (SR) and resistant ♀ and susceptible ♂ (RS). Sibmating was also done for maintaining both the resistant and susceptible pure lines. All the crosses were conducted in masses using five pairs of adults in jars of 15 × 20 cm size with four replications. Susceptibility of F_1 neonates of *H. armigera* to Cry1Ac was evaluated using diet incorporation method. Mortality was recorded every 24 h till 4 days and the data were used to estimate the toxicity of Cry1Ac. The hybrids of each cross were reared to adults, which were further used for performing backcross with resistant parent. The progenies of all the backcrosses were subjected to bioassay and their mortality responses observed. Adults of the F_1 reciprocal crosses were further sibmated to obtain the F_2 generation.

The genomic DNA of parents, progeny of F_1 reciprocal crosses, backcrosses and F_2 of reciprocal crosses were used for estimating cadherin allele and genotype frequencies.

Genomic DNA extraction and PCR amplification

Genomic DNA was extracted from parents, F_1 reciprocal cross, backcross and F_2 of reciprocal cross. Larvae weighing 25–30 mg each were used for isolating individual genomic DNA[47]. DNA isolated from each individual was treated with 25 μl of RNAase (50 μg RNAase/1000 μl) and incubated at 37°C for 1 h to remove RNA. The DNA samples were checked on 0.8% agarose electrophoretic gel and their purity determined by spectrophotometric analysis. All DNA samples were stored at –20°C till further use. The primer sequences based on the cadherin

Table 1. Primers used to amplify *Helicoverpa armigera* cadherin gene

Primer	Primer sequence(5′ > 3′)	cDNA positions (bp)
1-F (sense)	ATGGCAGTCGACGTGAGAATA	1–21
1-R (antisense)	GATACTGACTCCATGGTATTCC	136–157
2-F (sense)	CCAGGTATGCAGCAGTACATC	427–447
2-R (antisense)	CGCTGACTGTATACTTGCATG	584–604
3-F (sense)	GATGTGGTCATCATCGTGAAC	1168–1188
3-R (antisense)	CTATGTAGAACGCCTCGTGAG	1355–1375
4-F (sense)	TAACGCAGTAAGCTACCTGAG	1686–1706
4-R (antisense)	CA(AG)CGTGTCGTCAGCTCGTA	1790–1809
5-F (sense)	GCAGCCTCAGGAGTCGTTATA	2332–2352
5-R (antisense)	CGTGTTGAAGTCTATGTCCAC	2443–2463
6-F (sense)	TCAACATGATCACCATAGAGAG	3194–3215
6-R (antisense)	GTTGTAAGGTCTGATGACCAG	3349–3369
7-F (sense)	CGAGGAACATCATGTGTGAAG	3830–3850
7-R (antisense)	AGTGTAGAAGCCTGCAGGAC	4018–4037
8-F (sense)	GAACAGAACCTACAGCTAGCC	4666–4686
8-R (antisense)	AGCCTTCAACGTCTCGTTCCA	4948–4968
9-F (sense)	TGGAACGAGACGTTGAAGGCT	4948–4968
9-R (antisense)	TTATCTTCTGAACTGTGTGTTCG	5171–5193

gene[45] were custom-synthesized from SBS Genetech Co Ltd, Beijing (Table 1).

PCR amplification was conducted in 25 µl reactions containing 2.5 µl of reaction buffer with 15 mM $MgCl_2$, 1.2 unit of *Taq* polymerase (Bangalore Genei, India), 200 µM dNTPs, 5 pmol of each primer and 1.5 µl (20–30 ng of genomic DNA). PCR was performed at initial denaturation at 95°C for 3 min followed by 30 cycles (denaturation at 94°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 1 min 30 sec) and final extension at 72°C for 10 min. Samples were stored at 4°C following the amplification and the products separated on 2% agarose gel and visualized with ethidium bromide staining. Gels were documented using Alphaimager™ and analysed with AlphaEase™ software.

DNA sequencing

The resistant and susceptible alleles were amplified using PCR and the PCR products sequenced by Chromous Biotech Pvt Ltd, Delhi. Sequences were aligned and analysed using Clustal X and protein translation analysis was done using GENSCAN software. Variation in the cadherin gene sequence of *Bt*-tolerant (BH-R) and susceptible (VA-S) *H. armigera* was identified. The mutant alleles of the cadherin gene were identified in the BH-R strain and the Mendelian segregation of these mutants was analysed.

Inheritance of cadherin alleles from resistant and susceptible H. armigera

The isofemale line of the resistant BH-R strain was selected on Cry1Ac-treated diet for 11 generations[26]. The survivors were stored in –80°C for genomic DNA extraction to determine the association of cadherin gene mutation with Cry1Ac resistance. Genomic DNA of unselected individuals was compared to that of survivors of the Cry1Ac-impregnated diet. The allele frequency of BH-R strain which was collected from *Bt* cotton fields was estimated in F_1 generation. Further, the BH-R strain was selected on Cry1Ac-treated diet for six generations and thereafter the frequency of resistant allele was estimated.

DNA was extracted and PCR performed for 30 cycles. The genotypes for cadherin gene were distinguished depending upon the banding patterns. Sixty-one larvae from the backcross progeny and 55 larvae from the F_2 of reciprocal cross were used for cadherin genotyping. Further, the banding pattern was scored and a χ^2 test of goodness-of-fit was conducted and the Mendelian segregation of these mutants was characterized.

Screening of H. armigera populations for mutations conferring Cry toxin resistance

DNA-based screening method was adopted to monitor field populations of *H. armigera*. Adult male moths were collected from pheromone traps placed at different sites in vegetable-growing areas of Najafgarh in the Delhi region. All samples were stored at –80°C and DNA was extracted. To increase the efficiency of the screen, different dilutions of individual DNA were conducted. Pooled DNA samples (n =100) were used to detect a single resistance allele. Each pool of DNA contained mixed DNA extracted from ten adult moths, thereby representing 1000 individuals.

Further, surviving larvae collected from *Bt* as well as non-*Bt* cotton fields from Anand and from cotton fields in Nagpur and Aurangabad were also screened for

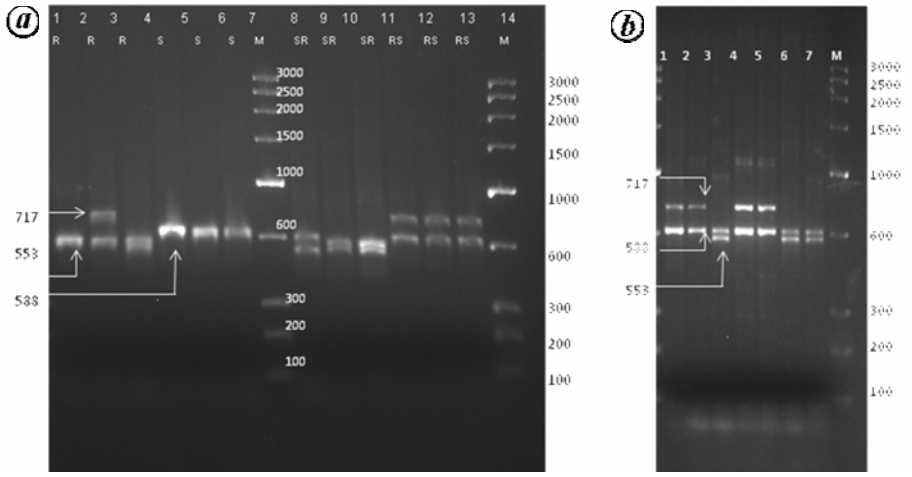

Figure 1. Agarose gel electrophoretic profile of PCR products of Cry1Ac susceptible and resistant parents and their F_1 progeny of *Helicoverpa armigera*. ***a***, Lanes 1–3 resistant BH-R(R); lanes 4–6, Susceptible VA-S(S); lanes 7 and 14, Marker (M); lanes 8–10, Progeny of cross between S (♀) and R (♂); lanes 11–13, Progeny of cross between R (♀) and S (♂). ***b***, Lanes 1–7, Progeny of cross between R(♀) and S (♂); lane 8, Marker (M).

detecting mutant cadherin alleles. As the surviving insects collected were less, DNA was not pooled and individual larvae were genotyped for identifying mutant alleles of the cadherin loci.

Results

Sequence analysis

Based on the 96 h mortality data reported in our earlier work, the BH-R population collected from *Bt* cotton fields in Bharuch area showed an LC_{50} of 0.44 µg/g in the F_1 generation. The most tolerant isofemale line of BH-R strain selected on Cry1Ac-treated diet in F_6 generation (LC_{50}, 0.638 µg/g) showed 227.9-fold higher resistance with respect to the VA-S population (LC_{50}, 0.0028 µg/g). Further, the analysis of the progeny of F_2 crosses, backcrosses of F_1 hybrid with resistant BH-R parent suggested Cry1Ac resistance as a semi-dominant trait[26].

The genomic DNA of the susceptible VA-S and resistant BH-R parents were extracted and their sequence compared to identify variation in the cadherin gene sequence. Genomic DNA of *Ha_Bt*R gene in the susceptible GY strain has been reported to span a minimum of 16.4 kb and its coding sequence is comprised of 34 exons[45]. The blast analysis of susceptible sequence showed 93% similarity over a 100% coverage area to the genomic DNA of *Ha_Bt*R gene (GenBank accession no. DQ523166). Out of the nine pairs of cadherin gene-specific primers (1F, 9R) used to amplify the DNA of susceptible parent, resistant parent, and their F_1 progeny, only one pair (7F-CGAGGAACATCATGTGTGAAG; 7R-AGTGTAGAAGCCTGCAGGAC) amplifying exon 26 and part of exon 25 showed difference between the susceptible and resistant cadherin gene sequence[45]. Further, two mutant alleles of the cadherin gene were identified in the BH-R strain. The amplification of exonic region 26 using the seventh pair of primers showed that the susceptible parent had a 588 bp fragment (Figure 1). The individuals collected from *Bt* cotton fields in Bharuch specifically had a 553 bp fragment and a 717 bp fragment (Figure 1). Thus, two alleles, r9 and r10, in resistant BH-R strain were identified. Comparison and alignment of the sequences showed that in the r9 allele (HQ453270) a deletion of 45 bp and mutation in the 5′ splice site of exon 25 (T replaced by C) was found, which did not result in protein translation. However, mutation in the 5′ splice site of exon 26 resulted in an additional amino acid. While in the r10 allele (HQ441197), a premature stop codon resulted in two different proteins of 1343 and 335 amino acids relative to a single protein of 1756 amino acids of the susceptible strain (HQ453271; Figure 2).

Inheritance of cadherin alleles from resistant and susceptible H. armigera

All resistant individuals had two mutant alleles (r9r9 and r9r10) and the susceptible VA-S had SS genotypes. PCR analysis of cadherin genotype showed that all the survivors collected from *Bt* cotton fields in Bharuch at F_1 generation were r9r9, r9r10, r9s (genotypic frequencies of 0.7, 0.13 and 0.16 respectively) and at F_6 generation selected at 1 ppm dose of Cry1Ac were rr (r9r9 with genotypic frequency of 0.47 and r9r10 with genotypic frequency of 0.53). While in the susceptible strain VA-S both mutant alleles r9 and r10 were absent. Thus resistance to Cry1Ac, survival on Cry1Ac-treated diet and the frequency of mutant r allele of cadherin gene

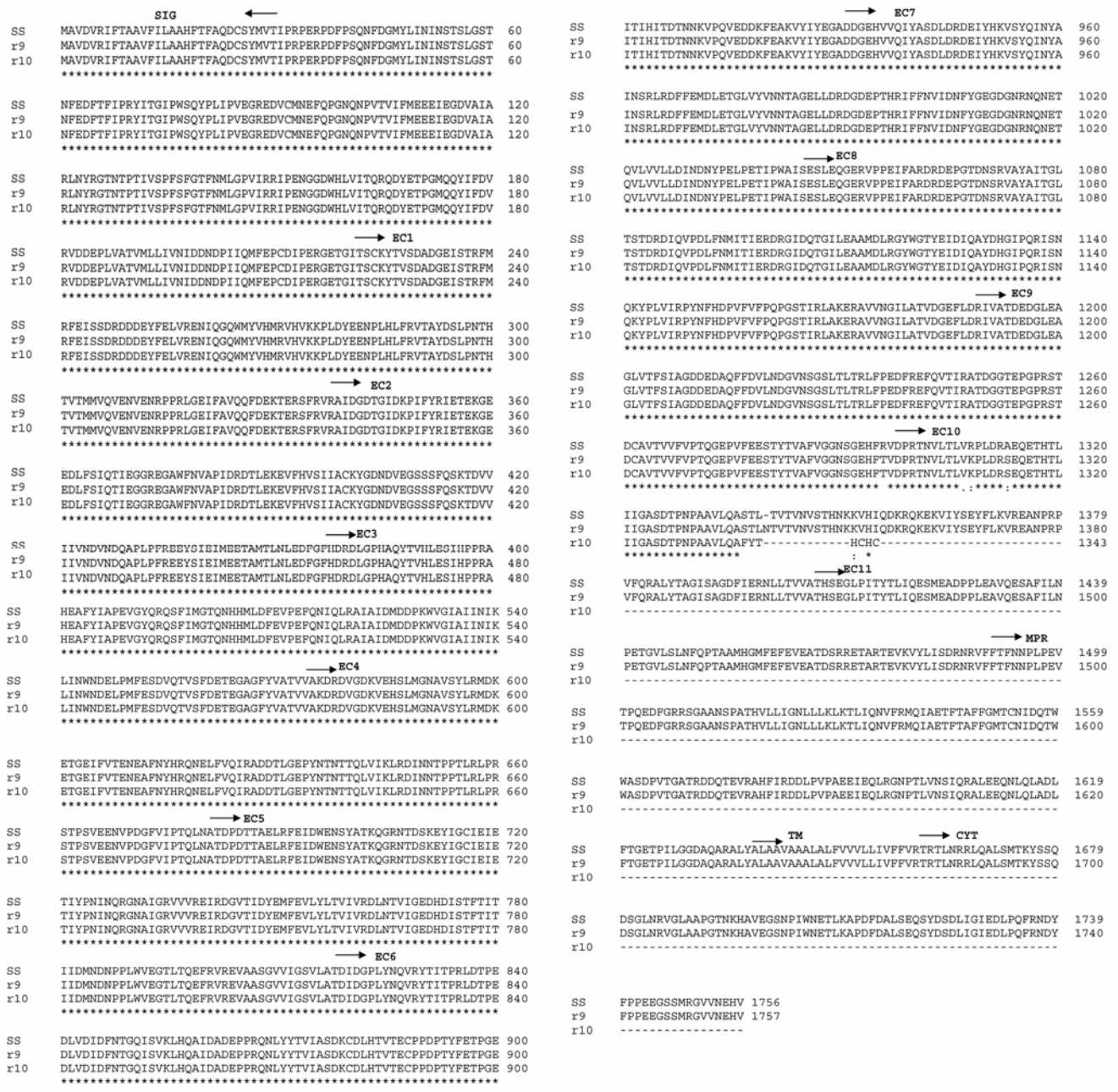

Figure 2. Amino acid sequence predicted using GENSCAN software. Resistance alleles r9 (HQ453270) and r10 (HQ441197) and susceptible allele (HQ453271). Horizontal arrows specify start sites of putative domains. SIG, Signal peptide; CR, Cadherin repeat; MPR, Membrane-proximal region; TM, Transmembrane domain; CYT, Cytoplasmic domain. *Shows the premature stop codon in r10.

were high in the selected strain than the unselected strain as well as the susceptible VA-S strain.

The F_1 reciprocal crosses tested individuals were found to be heterozygotes, r10S and r9S (Figure 1). The genotype frequency of r9S was 0.73 and r10s was 0.27. The allele frequency of the mutant r10 allele was low (0.13) compared with mutant allele r9 (0.37) in 41 individuals tested from the progeny of F_1 reciprocal crosses.

Sixty-one larvae from the backcross progeny and 55 larvae from the F_2 progeny were chosen randomly for cadherin genotyping. As expected in the backcross nearly 57.4% individuals (35 out of 61) were carrying the resistant alleles (either r9r9 or r9r10) and nearly 42.6% (26 out of 61) were heterozygous, either r9S or r10S (Figure 3). The χ^2 test of the goodness-of-fit between the observations and predictions under the assumption of monofactorial inheritance did not significantly differ from the expected 1 : 1 ratio ($\chi^2 = 1.328$, d$f = 1$ at $P = 0.05$).

Similarly, F_2 of the reciprocal cross individuals segregated in the ratio of 18 : 21 : 16 (RR : RS : SS), where

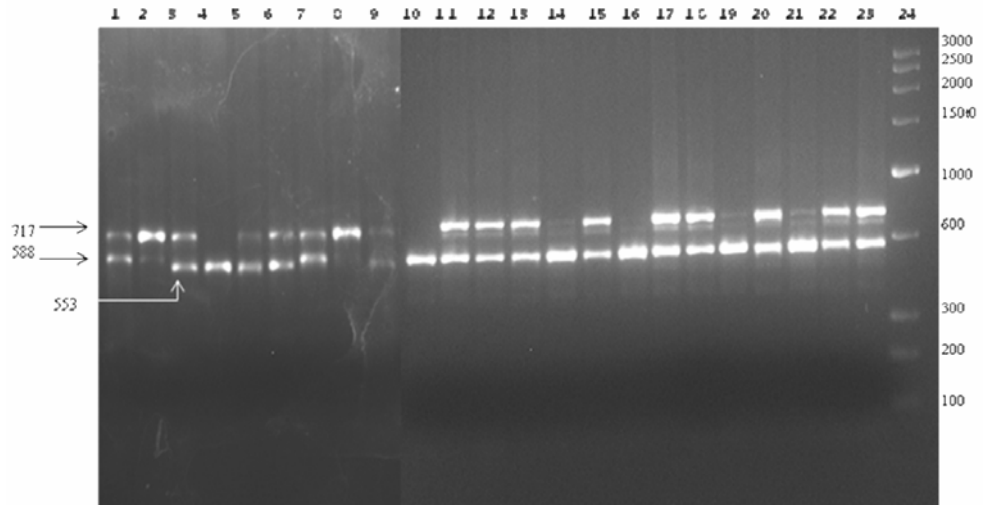

Figure 3. Agarose gel electrophoretic profile of PCR products of individuals representing backcross progeny obtained by crossing the resistant parent and the F_1 adults. Lanes 1–6, R (♀) × RS (♂); lanes 7–12, R (♂) × RS (♀); lanes 13–17, R (♂) × SR (♀); lanes 18–23, R (♀) × SR (♂); lane 24, Marker.

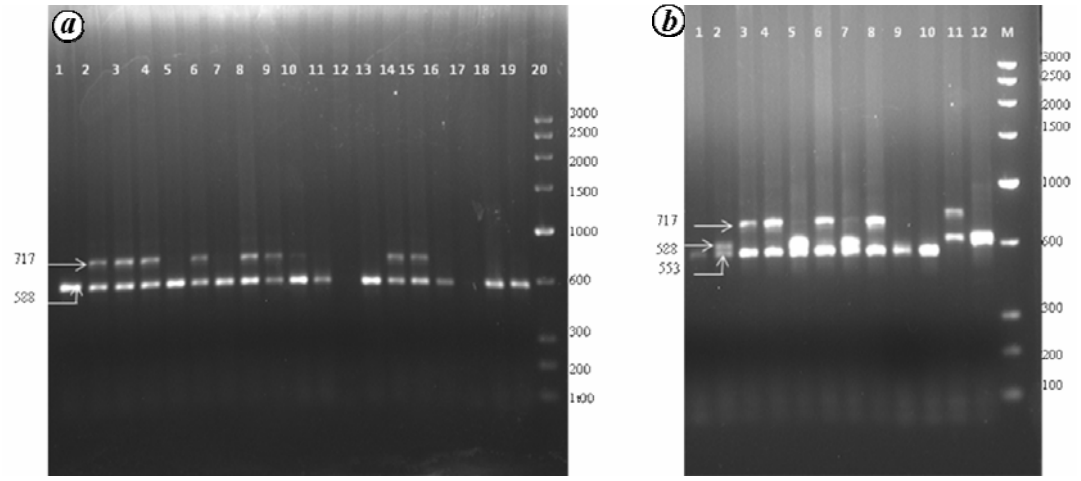

Figure 4. Agarose gel electrophoretic profile of PCR products of individuals belonging to F_2 of R (♀) × S (♂) hybrid (***a***) and S (♀) × R (♂) hybrid (***b***).

one-fourth individuals carried the resistant-specific alleles (r9r10 or r9r9), one-half were heterozygous (r9S or r10S) and the remaining one-fourth with susceptible specific alleles (SS) (Figure 4). The χ^2 test of the goodness-of-fit did not significantly differ from the expected 1 : 2 : 1 ratio ($\chi^2 = 3.209$, d$f = 2$ at $P = 0.05$).

Screening of field populations of H. armigera

The sensitivity test of PCR showed that a pool of nine wild and one resistant individual showed the presence of either homozygote SS or a heterozygote r9S. Only one heterozygote r10S was detected in a pool of 40 samples of 10 individuals each. Hence DNA was pooled from groups of 10 individuals for reliable detection. In the screening of 1000 male adults collected from vegetable fields of Delhi region, scorable bands were produced by 920 individuals. The banding pattern showed the presence of 588 bp susceptible-specific fragment (Figure 5). Thus, there was no evidence of any deletion mutation in any of the individuals collected from the field.

Populations of *H. armigera* collected from non-*Bt* cotton fields in Anand, Aurangabad, Nagpur and *Bt* cotton field in Anand were assayed for their sensitivity to Cry1Ac toxin. Perusal of the data showed that all populations collected from non-*Bt* cotton fields were susceptible to Cry1Ac toxin, whereas population collected from *Bt* cotton fields in Anand was the most tolerant, showing an LC_{50} of 4.3 µg/g diet. Insects surviving on *Bt* cotton in Anand had developed 204.5-fold resistance to Cry1Ac compared to the most susceptible strain from Nagpur and 43-fold resistance over susceptible strain collected from non-*Bt* cotton fields in Anand (Table 2).

Table 2. Toxicity of Cry1Ac to neonates of *Helicoverpa armigera*

Place of insect collection	LC_{50} µg/g of diet (4 days) (FL at 95%)	χ^2	Resistance ratio
Nagpur (n-*Bt* cotton)	0.021 (0.019–0.033)	5.46	1
Aurangabad (n-*Bt* cotton)	0.085 (0.042–0.171)	7.09	4.0
Anand (n-*Bt* cotton)	0.100 (0.049–0.218)	5.59	4.8
Anand (*Bt* cotton)	4.295 (1.027–17.15)	0.611	204.5

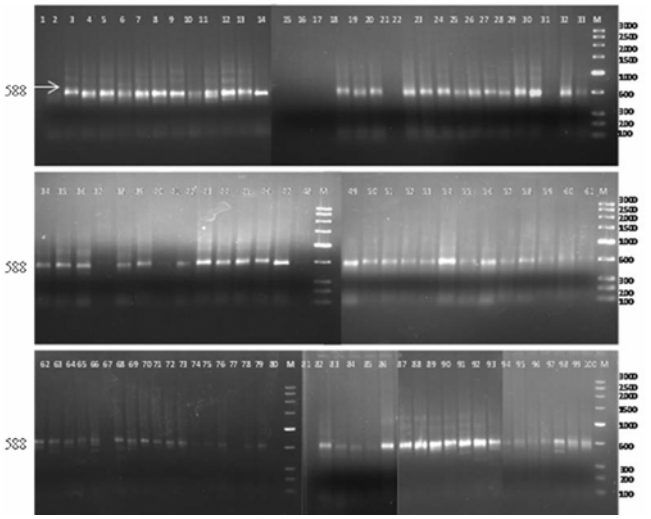

Figure 5. Agarose gel electrophoretic profile of PCR products of male adult moths of *Helicoverpa armigera* collected from different locations using pheromone traps (n = 100; each containing pooled DNA of ten individuals, thus representing 1000 individuals).

The population collected from Aurangabad showed complete absence of any mutant allele of the cadherin locus. However, in the population collected from non-*Bt* cotton fields in Nagpur and Anand, the frequency of r9 ranged from 0.06 to 0.08 and frequency of r10 ranged from 0 to 0.04 respectively. While in the population collected from *Bt* cotton field in Anand, the frequency of mutant alleles r9 and r10 was 0.09 and 0.04 respectively.

Discussion

Although laboratory selection results show that many pests have the genetic potential to evolve resistance to *Bt* toxins, the molecular basis of resistance had remained elusive for many years. *Bt* resistance causing mutation at the molecular level was first reported in *H. virescens*[41], where a retrotransposon-mediated insertion into a gene encoding a 12-cadherin domain protein expressed in midgut of larvae was found to be linked to high levels of resistance. Three mutant alleles of an encoding gene linked with resistance to Cry1Ac in *P. gossypiella* were identified[42]. Deletions were found in all the three resistant alleles, which could eliminate at least 8 amino acids upstream of the putative toxin-binding region of cadherin protein. Xu *et al.*[23] confirmed the use of cadherin gene for development of DNA-based monitoring of *Bt* resistance. They found that high levels of resistance was associated with disruption of cadherin gene by a premature stop codon. A deletion from exon 8 to exon 25 was reported to be responsible for truncated protein in Cry1Ac-resistant GY*Bt* insect strain[45]. Further, two new cadherin alleles were identified[46], which were created by insertion of a long repeat of transposons at same position in exon 8. All three alleles were found to be associated with a mutation in exon 8 of *Ha_Bt*R, which was regarded as a hotspot for mutation. Another allele from a Chinese population has been reported to have a large deletion involving several exons from genomic DNA sequences (GenBank Accession no. AY714875)[48]. Similar observations were found in Cry1Ab-selected *Ostrinia nubilalis*, where several mutations in cadherin gene introduced premature termination codons[49].

In the present study, comparison of the genomic organization of *Ha_Bt*R from both susceptible and resistant strains identified two mutant alleles in the Cry1Ac-resistant BH-R strain. Further, the survival on Cry1Ac-treated diet and frequency of mutant r allele of cadherin gene were high in the selected strain than the unselected strain as well as the susceptible VA-S strain. The use of these mutant alleles as molecular markers in screening field populations will aid in exploiting the cadherin gene mutation to develop efficient DNA-based resistance monitoring methods.

Further, the segregation of these mutant alleles examined in F_2 families derived from group matings of Cry1Ac-resistant and susceptible individuals showed that the genotypic frequencies for these mutant alleles did not deviate from Mendelian expectations. Similar observations were made using backcross progeny and as expected it was found that nearly 53% of individual larvae (16 in 30) were homozygous for the r1 allele and nearly 50% (14 in 30) were heterozygous for the r1 allele (*r1s*)[23]. Further, mutation in the coding region was found to be responsible for a truncated cadherin protein in the resistant strain. Mutation in exon 26 resulted in the disruption of the cadherin gene leading to the failure to produce full-length protein. A premature stop codon resulted in translation of two different proteins (1343 and 335 amino acids) relative to the 1756 amino acids of the susceptible strains. The stop codon is expected to block the production of the

binding region, thereby the *Bt* toxin loses specific binding receptors in the midgut of the resistant BH-R strain. We used these variations for the cadherin loci in resistance monitoring studies. The screening of *H. armigera* field populations from vegetable-growing areas in Najafgarh, Delhi did not detect any mutants in the 1000 individuals tested. However, low frequency of the mutant alleles r9 and r10 was found in populations collected from *Bt* cotton-growing areas in Anand as well as non-*Bt* cotton-growing areas in Anand and Nagpur. Our results are consistent with the findings where retrotransposon insertion into cadherin gene was not detected in any of the 7000 field-collected individuals of *H. virescens* screened for the *Bt* resistant gene[48]. Further, screening of 2250 individuals of *H. armigera* also could not detect mutation of aminopeptidase N gene associated with resistance to Cry1Ac toxin[50]. However, using biphasic strategy two cadherin alleles associated with Cry1Ac resistance were detected from a field population of *H. armigera* collected from China in 2005 (ref. 46).

The progeny of the F_2 crosses showed a Mendelian inheritance of the mutant alleles indicating the appropriateness of the markers into linkage mapping studies and field surveillance of wild populations of *H. armigera.*

Development of allele-specific molecular markers based on the mutation in exon 26 could be a useful tool in screening and estimating the frequency of rare resistance allele in field populations in India, which are increasing in view of the intense selection pressure imposed due to the extensive transgenic cotton cultivation in the country.

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