Inheritance of resistance in Indian *Helicoverpa armigera* (Hübner) to Cry1Ac toxin of *Bacillus thuringiensis*


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Abstract

The mode of inheritance of resistance to Cry1Ac toxin of *Bacillus thuringiensis* in the cotton bollworm, *Helicoverpa armigera* (Hübner), was elucidated through bioassay analysis of the response of resistant, susceptible, F1 hybrid and backcross *H. armigera* progeny to Cry1Ac using semi-synthetic diet and transgenic Bt cotton plants. The dominance value estimates ranged between 0.42 and 0.57. Resistance was found to be monogenic, autosomal and inherited as a semi-dominant trait. Genetic studies of response of *H. armigera* to transgenic Bt cotton showed that the effective resistance on transgenic Bt cotton plants was also inherited as a semi-dominant trait.

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Keywords: *Helicoverpa armigera*; Bt cotton; Inheritance; Resistance; Cry1Ac

1. Introduction

Transgenic cotton incorporating *Cry1Ac* gene derived from *Bacillus thuringiensis* (Bt) is one of the most exciting advances made in cotton pest management in recent times. The cotton bollworm *Helicoverpa armigera* (Hübner) is one of the main target pests of Bt cotton technology in India. It has a history of developing resistance to almost all the insecticides used for its control (Kranthi et al., 2002). Cry1Ac is the most toxic of the *Bacillus thuringiensis* insecticidal proteins to *H. armigera* (Padidam, 1992; Akhurst and Liao, 1996; Chakraborthy et al., 1998; Akhurst et al., 2003). Laboratory experiments to select for resistance in India (Kranthi et al., 2000), Australia (Akhurst et al., 2003; Akhurst and James, 1999; Daly and Olsen, 2000) and China (Liang et al., 2000) have shown that *H. armigera* is capable of developing resistance to Cry1Ac. Bt cotton expresses the Cry1Ac toxin in all parts of the plant throughout the growth period, although to greatly varying extents (Kranthi et al., 2004). The pest would thus be exposed to a continuous selection pressure, thereby causing resistance in field populations. It is important to ensure that the technology, which represents the state of art of pest management, remains effective in controlling target pests for the longest possible time. Information on genetics of the resistant allele helps immensely in devising proactive resistance management strategies that can retard the rate of resistance development. Preliminary results published recently (Akhurst et al., 2003), showed that resistance to Cry1Ac in *H. armigera* strains from Australia was inherited as an incompletely recessive trait. The three most important pre-requisites for a successful functioning of refuge strategy are ‘recessive resistant allele’, ‘high dose’ expression of Cry1Ac in Bt cotton and ‘rare resistant allele’ in field populations. The ‘refuge’ strategy for the management of Cry1Ac resistance in *Heliothis*
virescens in the USA was developed from data, which showed that all the three criteria were adequately met. However, such data is lacking for *H. armigera* under Indian conditions. We attempt to understand the mode of inheritance of the Cry1Ac resistant allele in Indian strains of *H. armigera* with emphasis on the functional significance of effective dominance on Bt cotton.

2. Materials and methods

2.1. Insect strains

Genetic crosses were performed between a susceptible *H. armigera* strain SUS-G and two Cry1Ac resistant strains, RES-Bt and RES-Ac. We used methods described by Andow and Alstad (1998) to isolate the susceptible strain SUS-G from F₂ progeny of single pair mated isolate male moths derived from Bt susceptible populations that were collected as larvae from cotton crop in Nagpur during September 2002. The strains were maintained in the laboratory on a wheatgerm-based semi-synthetic diet (Armes et al., 1992). The resistant strain RES-Bt was derived from a field population collected as larval survivors on Bt cotton in Bharuch district of Gujarat state in October 2002. It was selected for four generations on plant parts of Bt-cotton (Var: MECH-184-Bt) and the next subsequent 11 generations on semi-synthetic diet either layered or incorporated with diluted stock solutions of MVP-II, (Dow Agrosciences San Diego, CA) a liquid formulation containing 19.7% Cry1Ac encapsulated in *Pseudomonas fluorescens* (Gilroy and Wilcox, 1992). The Cry1Ac in MVP-II is 99% identical to the active toxin based semi-synthetic diet (Armes et al., 1992). The RES-Bt strains exhibited 93 and 205-fold resistance to Cry1Ac in MVP-II is 99% identical to the active toxin

For genetic studies, the resistant strains were crossed with the susceptible strain in a reciprocal manner. The F₁ hybrid progeny was back-crossed to each of the parents separately. Bioassays were carried out with MVP-II on the F₁ hybrid and back cross progeny. Survival of the resistant, susceptible and hybrid *H. armigera* progeny on Bt cotton was assessed through in vitro bioassays. The RES-Bt (R), SUS-G (S), S ♀ × R ♂, and R ♀ × S ♂ F₁ hybrid larvae were released on freshly excised leaves of Bt cotton ‘MECH-184-Bt’ (75–90 days after sowing) individually in perforated plastic cups. The leaves were changed daily and mortality observations were recorded after 7 days. Larvae were also released on MECH-184 (non-Bt) leaves to assess control mortality. Median Lethal concentration (LC₅₀) values and their 95% fiducial limits (FL) were computed by probit analysis (Finney, 1971). When required, corrections for control mortality were made using Abbott’s formula (Snedecor and Cochran, 1989). Significance of differences between treatments were determined by the Student’s t-test (Snedecor and Cochran, 1989). The degree of dominance (D), dominance (DₑLC) and effective dominance (DₑML) of resistance were calculated using the methods described by Stone (1968) and Bourguet et al. (2000) as follows:

\[
D = (2X_2 - X_1 - X_3)/(X_1 - X_3),
\]

\[
DₑLC = (D + 1)/2,
\]

\[
DₑML = (MLₐₐₐₐ - MLₐₐₐₐ)/MLₐₐₐₐ - MLₐₐₐₐ,
\]

where \(X_1, X_2, X_3\) are the logarithms of the LC₅₀ values for R (resistant), F₁ hybrid and S (susceptible) strains, respectively. \(D\) values range from \(-1\) (completely recessive resistance) to +1 (completely dominant resistant). \(DₑLC\) is the estimate of dominance with 0 for completely recessive, 0.5 for semi-dominant and 1.0 for completely dominant trait. \(DₑML\) defines the effective dominance of survival where MLₐₐₐₐ, MLₐₐₐₐ and MLₐₐₐₐ are the % mortality levels of the resistant, susceptible and hybrid *H. armigera* progeny on Bt cotton respectively. The variance of \(D\left(\sigma_D^2\right)\) was estimated according to Preisler et al. (1990) as follows:

\[
\sigma_D^2 = 4/(X_1 - X_3)^2\left[\sigma_X^2 + (X_2 - X_3)^2/(X_1 - X_3)^2\sigma_X^2 + ((X_2 - X_1)^2/(X_1 - X_3)^2)\sigma_X^2\right],
\]

where \(\sigma_X^2, \sigma_X^2\) and \(\sigma_X^2\) are the variances of the LC₅₀ of the R (resistant), F₁ hybrid and S (susceptible) strains, respectively. The standard error (SE = \(\sqrt{\sigma_D^2} \)) was used to determine whether \(D\) was significantly different from +1 (completely dominant) or \(-1\) (completely recessive). \(D\) was considered to be significantly different from \(\pm 1\) when the approximate 95% confidence interval values for \(D/(D + 2SE)\) included \(\pm 1\), as described by Preisler et al. (1990). The minimum number of effective genes was calculated using methods described by Lande (1981):

\[
\eta E = (X_1 - X_3)^2/(8\sigma^2),
\]

where \(X_1\) and \(X_3\) are the logarithms of the LC₅₀ values of resistant and susceptible strains respectively, where \(\sigma^2\) is estimated as

\[
\sigma^2 = \sigma_R^2 + \sigma_B^2 - [\sigma_R^2 + 0.5(\sigma_R^2) + 0.5(\sigma_R^2)],
\]

where \(\sigma_R^2, \sigma_B^2, \sigma_F^2, \sigma_P^2, \sigma_P^2\) are the phenotypic variances of the back-cross (F₁ hybrid × susceptible parent), back-cross (F₁ hybrid × resistant parent), F₁ hybrid,
susceptible parent and resistant parent, respectively. Variance was estimated as the inverse of the slope squared (standard deviation) as described by Lande (1981). Fitness of the susceptible and F1 hybrid strains on Bt cotton plants was calculated relative to the % survival of the resistant strain on Bt cotton.

3. Results

The resistant strain RES-Ac and RES-Bt strains were found to be 93 and 205-fold resistant to Cry1Ac compared to the SUS-G strain (Table 1). The dose-mortality relationship among LC50 values of the homozygous susceptible, resistant and heterozygous individuals indicated that resistance was autosomal and inherited as a semi-dominant trait. The estimate of dominance (DLC) for reciprocal crosses was expressed at 0.42/0.43 for RES-Ac and 0.57/0.54 for the RES-Bt strains. The values obtained for the minimum number of independently segregating genes were 0.53 for the RES-Ac and 1.1 for the RES-Bt strains, indicating a single gene governed resistance.

Survival of the three *H. armigera* genotypes (resistant, susceptible and hybrid progeny) on transgenic Bt cotton (MECH-184-Bt), was measured to determine effective dominance (DML) levels (Table 2). The survival of the resistant RES-Bt larvae was 75% on Bt cotton, compared to 33–37% of the heterozygous hybrid progeny and 5.0% of the susceptible SUS-G strain.

The relative fitness of the susceptible SUS-G strain was 0.06, compared to that of the resistant RES-Bt (R) strain. The F1 hybrid progeny showed an intermediate relative fitness response with values of 0.44 and 0.48 in the reciprocal crosses suggesting autosomal inheritance. Effective dominance levels of 0.40–0.45 indicated semi-dominant inheritance.

4. Discussion

Our results show that Cry1Ac resistance in Indian *H. armigera* was autosomal and inherited as a semi-dominant trait. The two resistant strains used in the

Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>n</th>
<th>% Survival± SE</th>
<th>Fitness</th>
<th>DML</th>
</tr>
</thead>
<tbody>
<tr>
<td>R (R)</td>
<td>216</td>
<td>75.0±2.9c</td>
<td>1</td>
<td>0.06</td>
</tr>
<tr>
<td>S (S)</td>
<td>100</td>
<td>5.0±2.2a</td>
<td>0.07</td>
<td>0.53</td>
</tr>
<tr>
<td>S (♀)× R (♂)</td>
<td>66</td>
<td>33.3±5.8b</td>
<td>0.44</td>
<td>0.40</td>
</tr>
<tr>
<td>R (♂)× S (♀)</td>
<td>60</td>
<td>36.7±6.3b</td>
<td>0.48</td>
<td>0.45</td>
</tr>
<tr>
<td>RES-Bt (R)</td>
<td>216</td>
<td>75.0±2.9c</td>
<td>1</td>
<td>0.06</td>
</tr>
<tr>
<td>SUS-G (S)</td>
<td>100</td>
<td>5.0±2.2a</td>
<td>0.07</td>
<td>0.53</td>
</tr>
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<td>0.45</td>
</tr>
</tbody>
</table>

R and S are resistant and susceptible strains respectively. n = number of larvae tested. SE = Standard Error.

*R* and *S* are resistant and susceptible strains respectively. *n* = number of larvae tested. RF = resistance factor (LC50 of R/LC50 of S); *D* = Degree of dominance with −1 for completely recessive, 0 for semi-dominant and +1 for completely dominant; Var(D) = variance of D; DLC = Estimate of dominance at (D + 1)/2 with 0 for completely recessive, 0.5 for semi-dominant and 1.0 for completely dominant. ηE = Minimum number of independently segregating genes.
study ‘RES-Bt’ and ‘RES-Ac’ exhibited differences in resistance levels, dominance \((D_{L,C})\) values and estimates of minimum number of independently segregating genes. The different methods of selection using Bt cotton plant parts initially for the RES-Bt strain and Cry1Ac for the RES-Ac strain may have been responsible for the differences between the two strains. A recent study (Sayyed et al., 2004) suggested that different selection pressures on field and laboratory populations may produce resistant genes with different characteristics.

Maintenance of significant area of non-Bt crop, as refuge to conserve susceptible alleles, is normally considered as an important resistance management strategy for transgenic Bt crops (Tabashnik et al., 1997; Ferre et al., 2002). The strategy relies heavily on the assumption that resistance is a recessive trait. Ferre et al. (2002) surmised that, in general, resistance to Bt products or toxins was inherited as recessive or incompletely recessive in a majority of cases. Resistance to Cry1Ac was reported to be autosomal and inherited as incompletely recessive trait in Australian \(H.\ armigera\) (Akhurst et al., 2003), Plutella xylostella (Tabashnik et al., 1997), \(Heliotis virescens\) (Gould et al., 1992) and Pectinophora gossypiella (Liu et al., 1999). Dominance \((D_{L,C})\) values of 0.26 in Cry1Ac resistant \(H.\ armigera\) in Australia (Akhurst et al., 2003) and <0.31 in all the above mentioned species confirm that resistance to Cry1Ac, in general, is inherited as an incompletely recessive trait. However, there are examples of some resistance alleles being dominant or semi-dominant (Tabashnik et al., 2000). The resistance alleles in \(Ostrinia nubilalis\) to Bt-sprays (Huang et al., 1999) and Plutella xylostella to Cry1Ac (Sayyed et al., 2000) were found to be incompletely dominant. This evidence undermines the usefulness of the high-dose strategy. However, thus far, no similar results have been found in studies of resistance to transgenic plants (Tabashnik et al., 2000). Earlier reports showed that resistance of \(P.\ gossypiella\) (Liu et al., 1999) and \(P.\ xylostella\) (Tang et al., 2001) to Cry1Ac transgenic plants was inherited as a completely recessive trait. Our results indicating that resistance in \(H.\ armigera\) to Bt cotton plants being inherited as a semi-dominant trait appear to be an exception.

The mode of inheritance of a resistant allele has significant implications for resistance management strategies, the most important of which would be on the rate of resistance development. The rate of change in the resistant allele frequency in field populations over time depends largely on the extent of selection pressure by Bt cotton, and is significantly influenced by the mode of inheritance. Recessive inheritance would allow effective and sustainable pest control, especially at ‘high dose’ expression levels of Cry1Ac in Bt cotton. Initially, resistant alleles are expected to be rare in field populations and would be present predominantly as heterozygous genotypes at Hardy–Weinberg equilibrium. Resistance management will be more effective if Cry1Ac levels in Bt cotton would kill all heterozygous insects. But, temporal changes resulting in a reduction in toxin expression can help heterozygous individuals to overcome the toxin and thus spread resistant alleles. The current varieties of Cry1Ac cotton being used in India (Kranthi, 2003) and Australia (Akhurst et al., 2003) cannot be considered high dose for \(H.\ armigera\) because they do not produce a sufficiently high level of toxin to kill all susceptible \(H.\ armigera\) larvae throughout the season and certainly not all heterozygous larvae at any time of the season or in all tissues attacked (Kranthi et al., 2004). Moreover, the expression of Cry1Ac in the Bt cotton varieties released for commercial cultivation in India is variable and was found to decline progressively over the crop growth (Kranthi, 2003). The semi-dominant nature of the resistant allele in \(H.\ armigera\) would allow the survival of significant numbers of homozygous resistant and heterozygous genotypes. Additionally, if heterozygotes have a fitness advantage in the field over insects homozygous for the susceptibility allele, the development of resistance would be hastened (Curtis, 1981). Hence, deployment of appropriate resistance management strategies is important for the sustainability of the technology against \(H.\ armigera\). Studies in Australia (Fitt et al., 1994) showed that insecticidal activity of Bt cotton decreased as the plants matured and some \(H.\ armigera\) were able to complete development on the transgenic plants. Thus, it was surmised that transgenic cotton in Australia does not provide adequate protection against \(H.\ armigera\) during the late maturation phase of the crop (Fitt and Wilson, 2000). Consequently, a relatively small increase in resistance by \(H.\ armigera\) would be sufficient to create an economic problem (Akhurst et al., 2003).

Considering the conditions described above, it is unlikely that maintenance of a 20% non-Bt refuge crop as prescribed in India, alone, would significantly help in delaying resistance development (Kranthi and Kranthi, 2004). Apart from conserving susceptible alleles through refuge crop maintenance, it would be useful to reduce the proportion of insect populations that survive on Bt cotton, since a majority of them are likely to harbour resistant alleles. Some additional resistant management strategies proposed for India, are 1. Use of multiple toxin-based transgenic-cotton incorporating genes that are toxic to Cry1Ac resistant \(H.\ armigera\) larvae 2. Deep-ploughing of Bt cotton fields immediately after harvest to destroy residual pupae, and 3. Spray effective bio-rational insecticides on Bt cotton at population peaks to kill heterozygous larvae surviving Bt cotton. A few such useful bio-pesticides are HaNPV (nuclear polyhedrosis virus), spinosad, and emamectin benzoate.
or insect growth regulators such as novaluron, flufenoxuron and lufenuron, all of which are known to be effective on Cry1Ac and pyrethroid resistant *H. armigera* populations (Kranthi, unpublished).

As we understand more about the nature of the resistant allele and also explore for the existence of more resistant alleles in field populations, the existing data suggest that we need to proceed cautiously, and that the issue of resistance management in *H. armigera* under the Indian conditions needs to be addressed very carefully.

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References


Akhurst, R.J., Liao, C., 1996. Possible methods of inhibiting or reversing the potential data suggest that we need to proceed cautiously, and that the issue of resistance management in *H. armigera* under the Indian conditions needs to be addressed very carefully.

Akhurst, R.J., James, W.J., Bird, L.J., Beard, C., 2003. Resistance to the Cry1Ac δ-endotoxin of *Bacillus thuringiensis* in the cotton bollworm *Helicoverpa armigera* (Lepidoptera: Noctuidae). J. Econ. Entomol. 96, 1290–1299.


Lande, R., 1981. The minimum number of genes contributing to quantitative variation between and within populations. Genetics 99, 541–553.


Paddam, M., 1992. The insecticidal crystal protein Cry1Ac from *Bacillus thuringiensis* is highly toxic to *Helicoverpa armigera*. J. Invertebr. Pathol. 59, 1649–1655.


