Pest Manag Sci 59:1197–1202 (online: 2003)
DOI: 10.1002/ps.754

# Inheritance of resistance to *Bt* canola in a field-derived population of *Plutella xylostella*

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**Abstract: Crops expressing *Bacillus thuringiensis* (*Bt*) insecticidal Cry proteins are grown on millions of hectares. Recommendations to delay resistance are based on a high expression/refugia strategy that aims to kill resistant heterozygotes and enable some susceptible insects to survive. Leaf-dip bioassays on F1 crosses of Malaysian populations of diamondback moth (*Plutella xylostella* (L)) showed that Cry1Ac resistance was not fully recessive. The survival of *ca* 50% of heterozygotes on *Bt* canola (*Brassica napus* L) leaves expressing low concentrations of Cry1Ac agreed with a non-fully-recessive model for resistance. Extrapolations based on log dose-logit mortality regressions for heterozygotes using leaf-dip bioassays showed that a relatively high level of expression, of *ca* 2000 ng Cry1Ac mg$^{-1}$ total leaf protein, would be required to give 90% mortality to heterozygotes. If high enough levels of expression of *Bt* toxin to kill heterozygotes cannot be achieved and maintained under field conditions, the effectiveness of the high-dose/refugia strategy would be reduced.**
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**Keywords:** *Plutella xylostella*; *Bacillus thuringiensis*; *Bt* transgenic plants; maternal influence; incomplete dominance

## 1 INTRODUCTION

Transgenic insecticidal crops expressing *Bacillus thuringiensis* Berliner (*Bt*) crystal (Cry) toxins are increasingly deployed for insect control, but their efficacy will be short-lived if pests evolve resistance quickly.[1,2] At present, there are no reports of resistance to *Bt* crops in the field[3] and *Plutella xylostella* (L) (diamondback moth) is the only field-based model for resistance to microbial *Bt* formulations.[4–6] A world-wide pest of crucifers, *P xylostella* has been subject to intense selection with Cry toxins through spray applications of microbial *Bt* products. Selection pressure is likely to increase further if brassica crops expressing Cry toxins for control of *P xylostella*[2,3] are introduced. With increased planting of transgenic cotton, maize and other crop hybrids expressing Cry toxins, many pest species could be exposed to intense selection for *Bt* resistance.[2,5,7] Current recommendations to prevent *Bt* resistance are based on a high dose and refugia strategy to enable some susceptible insects to survive and mate with resistant insects.[8,9] A critical assumption for the success of this strategy is that inheritance of resistance is recessive.[5,10]

Populations of Cry1A-resistant *P xylostella* have been shown to survive on transgenic crucifers expressing a high level of Cry1Ac and Cry1C.[3,11,12] It has been assumed that resistance to *Bt* in insects is inherited primarily as a single recessive trait, and this is true in the majority of populations examined to date.[13–15] However, non-recessive modes of inheritance to Cry1Ac involving more than one factor have been reported in two field populations of *P xylostella* from Malaysia (MEL and SERD4) collected *ca* 100 km apart in 1997 and 1998 respectively.[4,16] Some degree of dominance has also been reported in other field-derived Cry toxin resistant populations of *P xylostella* from Thailand, the Philippines and Hawaii[5,17–19] and in laboratory-selected populations of at least five other insect species.[20–23] However, the degree of dominance of resistance tended to be concentration-dependent. For example, it has been shown in some *P xylostella* populations that resistance was recessive at the highest concentrations tested, whereas it was completely dominant at the lowest concentration used.[4,17] If non-recessive inheritance and multi-factorial resistance to Cry toxins are common in field populations of insects, alternative resistance management strategies may be required.[5]

The aims of the present work were (a) to determine whether resistance to Cry1Ac in a *P xylostella* population (SERD5) collected from the Serdang

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Contract/grant sponsor: Ministry of Education, Government of Pakistan
Contract/grant sponsor: Biotechnology and Biological Sciences Research Council of the UK
(*Received 7 May 2002; revised version received 24 January 2003; accepted 6 May 2003*)
Published online 30 July 2003

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region of Malaysia (9 months later than SERD4) showed a similar pattern of mode of inheritance of resistance to Cry1Ac; and (b) to test the hypothesis that resistant heterozygotes of two field-derived populations of *P xylostella* (SERD4 and SERD5) could survive on transgenic *Bt* canola expressing Cry1Ac and thus not be functionally fully recessive.

## 2 MATERIAL AND METHODS

### 2.1 Insects

The SERD4 and SERD5 field populations of *P xylostella* were collected from Malaysia in December 1998 and August 1999, respectively, from the Serdang area that has a long history of *Bt* use.[6,16] The *Bt*-susceptible population (ROTH) was obtained from IACR-Rothamstead (Harpenden, Hertfordshire, UK) where it had been maintained for over 200 generations without exposure to pesticides. All populations were isolated from each other and maintained at 20 (±2) °C, 65 (±3)% RH under a 16:8 h light:dark photoperiod. Both the SERD4 and SERD5 populations had been reselected with Cry1Ac in the laboratory as described previously.[16]

### 2.2 *Bacillus thuringiensis* canola

Zygotic hypocotyls of canola (*Brassica napus* L) cv Oscar were transformed with a truncated synthetic *B thuringiensis* insecticidal crystal protein gene (*Cry1Ac*) under the control of the cauliflower mosaic virus 35S promoter using *Agrobacterium tumefaciens*-mediated transformation[24] and transgenic seed supplied by Professor CN Stewart. Plants were grown in plastic pots in a glasshouse. Four- to five-week-old (young) or eight- to nine-week-old (mature) leaves were used for experiments with SERD5 and SERD4, respectively.

### 2.3 *Bacillus thuringiensis* toxins

Cry1Ac toxin obtained from *B thuringiensis* as described previously[4] were supplied by Professor Juan Ferré (University of València) and stored at −20 °C. Test solutions of toxins were freshly prepared in distilled water with Triton X-100 (50 μg ml$^{-1}$) as surfactant.[4]

### 2.4 Leaf disc bioassays

Initial bioassays were conducted with $L_3$ of SERD4[16] and SERD5. Each leaf disc (4.8 cm diameter) was immersed in a test solution for 10 s and then allowed to dry with the adaxial leaf surface uppermost on a corrugated sheet of aluminum foil for 1–2 h at room temperature.[4,16] Control leaf discs were immersed in distilled water containing Triton X-100 (50 μg ml$^{-1}$). The leaf discs were then transferred to individual plastic Petri dishes (5 cm diameter) containing a single moistened Whatman No 1 filter paper. Five $L_3$ were placed on each leaf disc and each treatment was repeated for seven times. Mortality was assessed after 5 days exposure to Cry1Ac.

### 2.5 Analysis of Cry1Ac in the *Bt* canola leaves

The concentration of Cry1Ac toxin in the *Bt* canola leaves used in bioassays was established using a commercial Btk ELISA PathoScreen kit following the standard protocol provided by the manufacturer (Agdia, Elkhart, USA). Leaf samples ($n = 4$) were homogenized in PBST buffer (NaCl 140 mM, KCl 3 mM, $Na_2HPO_4$ 8 mM, $KH_2PO_4$ 1.5 mM, Tween-20 0.5 ml litre$^{-1}$; 400 μl) using a micro-homogeniser. Homogenates were centrifuged at 14 000 rev min$^{-1}$ for 10 min. Total soluble protein content was measured using the Bio-Rad protein assay (Bio-Rad, Watford, UK).

### 2.6 Maternal effects and sex linkage

The response of $F_1$ and back-cross progeny to Cry1Ac was evaluated. Mass and single-pair reciprocal crosses between Cry1Ac-SEL SERD4 and Cry1Ac-SEL SERD5 and ROTH were made as described previously.[4,16] Back-cross progeny were produced by single-pair crosses of $F_1$ with ROTH. For mass crosses, 40 females of Cry1Ac-SEL were pooled with 40 males of ROTH and 40 females of ROTH were pooled with 40 males of Cry1Ac-SEL. Mass crosses provided enough offspring for multiple concentration testing and calculation of $LC_{50}$ values.

To obtain back-cross progeny, single-pair crosses were made between $F_1$ progeny (from mass crosses between Cry1Ac-SEL and ROTH) and ROTH. The $F_2$ progeny from single-pair crosses were tested with 0.1 μg ml$^{-1}$ of Cry1Ac.

### 2.7 Estimation of degree of dominance

The degree of dominance ($D_{LC}$) was estimated from $LC_{50}$ values according to Bourguet *et al*,[25] while for the single concentration bioassays, effective dominance of resistance ($D_{ML}$) values were calculated according to Hartl[26] and Bourguet *et al*.[25]

### 2.8 Data analysis

Where necessary, bioassay data were corrected for control mortality.[27] Estimates of $LC_{50}$ values and their 95% fiducial limits (FL) were obtained by maximum-likelihood logit regression analysis in GLIM using generalized modelling techniques from which differences between sets were extracted by analysis of deviance.[28] Because of the inherent variability of bioassays, pair-wise comparisons of $LC_{50}$ values were at the 1% significance level (where individual 95% FL for two treatments did not overlap.[16]

The minimum number of effective genes was calculated using Lande's[29] method.

## 3 RESULTS AND DISCUSSION

### 3.1 Concentration of Cry1Ac toxin in *Bt* canola leaves

The mean concentrations of Cry1Ac in mature (7- to 8-week-old) and young (4- to 5-week-old) *Bt* canola

leaves were 131 (range 69–154) and 32 (range 10–60) ng Cry1Ac $mg^{-1}$ total soluble leaf protein, respectively, for Cry1Ac-SEL SERD4 and SERD5 bioassays.

### 3.2 Maternal effects and sex linkage in leaf dip bioassays

On Cry1Ac-treated Chinese cabbage the median lethal concentration ($LC_{50}$) values for $F_1$ progeny of Cry1Ac-SEL SERD5 females × ROTH males were significantly ($P < 0.01$) different from those of $F_1$ progeny of Cry1Ac-SEL SERD5 males × ROTH females, based on 95% fiducial limits (FL) (Table 1), suggesting that resistance to Cry1Ac had some maternal influence. However, there was no significant ($P > 0.05$) difference in the number of male and female survivors, a finding consistent with an autosomal mode of inheritance.[13]

### 3.3 Degree of dominance in leaf dip bioassays

On Cry1Ac-treated Chinese cabbage, the $LC_{50}$ for $F_1$ progenies from mass crosses between the Cry1Ac-selected SERD5 and the susceptible ROTH population yielded $D_{LC}$ values of 0.67 and 0.47 respectively (Table 1). This suggested that resistance to Cry1Ac in this population was inherited as an incompletely dominant trait; the extent of dominance of resistance to Cry1Ac depended upon the concentration of the toxin used (Table 2). Resistance was completely recessive at the highest dose while almost completely dominant at the lowest dose for $F_1$ (Cry1Ac-SEL SERD5 female × ROTH male) and incompletely dominant for $F_1$ progeny (Cry1Ac-SEL SERD5 male × ROTH female). These results are in broad agreement with previously reported work with two populations of *P xylostella* from Malaysia.[4,13] The resistance to Cry1Ac in the SERD4 population has been shown to be incompletely dominant in $F_1$ progeny of Cry1Ac-SEL SERD5 females × ROTH males ($D_{LC} = 0.56$) while it was incompletely recessive in $F_1$ progeny of Cry1Ac-SEL SERD5 males × ROTH females ($D_{LC} = 0.38$; Reference 16).

**Table 1.** Estimates for the degree of dominance (h) of resistance to Cry1Ac in the $F_1$ progeny of Cry1Ac-selected SERD5 and susceptible ROTH populations of *Plutella xylostella* using leaf-dip bioassays with untransformed plant material[a]

| Strain | $LC_{50}$ (μg $ml^{-1}$) | 95% FL | Slope (±SE) | $D_{LC}$ |
|---|---|---|---|---|
| Cry1Ac-SEL SERD5 | 241 | 133–1230 | 1.61 (±0.49) | |
| Cry1Ac-selected female × ROTH male | 4.36 | 2.08–13.02 | 1.28 (±0.32) | 0.67 |
| Cry1Ac-selected male × ROTH female | 0.34 | 0.07–0.75 | 1.28 (±0.31) | 0.47 |

[a] The Cry1Ac $LC_{50}$ (95% FL) values (μg $ml^{-1}$) for ROTH were 0.001 ($1 \times 10^{-4}$–$2 \times 10^{-3}$; Reference 16).

**Table 2.** Dominance (h) of resistance to Cry1Ab in the SERD5 population of *Plutella xylostella* as a function of the concentration of Cry1Ac

| Conc (μg $ml^{-1}$) | Strain | Number of larvae | Mortality (%) | $D_{ML}$[a] |
|---|---|---|---|---|
| 0.15 | Cry1Ac-SEL | 25 | 0 | |
| | ROTH | 25 | 100 | |
| | $F_1$[b] | 25 | 40 | 0.60 |
| | $F_1$[c] | 25 | 12 | 0.98 |
| 0.75 | Cry1Ac-SEL | 25 | 0 | |
| | ROTH | 25 | 100 | |
| | $F_1$[b] | 25 | 60 | 0.40 |
| | $F_1$[c] | 25 | 28 | 0.72 |
| 3.1 | Cry1Ac-SEL | 25 | 0 | |
| | ROTH | 25 | 100 | |
| | $F_1$[b] | 25 | 76 | 0.24 |
| | $F_1$[c] | 26 | 50 | 0.50 |
| 6.2 | Cry1Ac-SEL | 25 | 0 | |
| | ROTH | 25 | 100 | |
| | $F_1$[b] | 25 | 80 | 0.20 |
| | $F_1$[c] | 25 | 56 | 0.44 |
| 12.4 | Cry1Ac-SEL | 25 | 1 | |
| | ROTH | 26 | 100 | |
| | $F_1$[b] | 25 | 92 | 0.08 |
| | $F_1$[c] | 26 | 58 | 0.42 |

[a] Dominance can vary from 0 (completely recessive resistance) to 1 (completely dominant resistance) $D = (ML_{RS} - ML_{SS})/(ML_{RR} - ML_{SS})$ where $ML_{RR}$, $ML_{RS}$ and $ML_{SS}$ are the resistant homozygotes, heterozygotes and susceptible homozygotes respectively.
[b] $F_1$ progeny of cross between Cry1Ac-SEL male and ROTH female.
[c] $F_1$ progeny of cross between Cry1Ac-SEL female and ROTH male.

### 3.4 Maternal effects and degree of dominance with *Bt* canola assays

When larvae of Cry1Ac-selected SERD4 and Cry1Ac-SEL SERD5 populations were tested on *Bt* canola expressing averages of 131 and 32 ng Cry1Ac $mg^{-1}$ total soluble protein, respectively, 0% mortality was found, whereas there was 100% mortality in the ROTH population. $F_1$ progeny of SERD4 females × ROTH males and SERD4 males × ROTH females, and the equivalent crosses for SERD5 gave 45% and 77%, and 48% and 72% mortality, respectively. Using the single concentration method[25,26] the effective dominance of resistance ($D_{ML}$) values were 0.55 and 0.23, and 0.52 and 0.28 respectively for the above-mentioned crosses. These results suggest that Cry1Ac resistance in both Cry1Ac-selected strains was incompletely dominant in resistant female crosses while it was incompletely recessive in resistant male crosses. There again appeared to be some maternal influence. As in the equivalent wild-type Chinese cabbage experiment, the number of female and male survivors was not significantly ($P > 0.05$) different, suggesting an autosomal mode of inheritance.

Since the ROTH population has been shown to be exceptionally susceptible to Cry1Ac,[30] the $LC_{50}$ values of $F_1$ progeny may have been affected. In this case, the dose required to kill 100% heterozygotes would be much higher.

When the $F_1$ progeny of Cry1Ac-selected SERD5 were backcrossed in single pairs with ROTH parents and were exposed to a discriminating dose of Cry1Ac (0.1 μg ml$^{-1}$; the dose found to kill 100% of susceptible insects and 0% of the resistant SERD4[16] and SERD5 populations), seven families out of 10 tested showed 60–80% mortality and three showed 100% mortality. When the minimum number of effective factors calculated from dose-response data using Lande's[29] method yielded a number of factors $n_E \geq 1$. These results suggest that resistance to Cry1Ac in the SERD5 population is controlled by more than one allele. Resistance to Cry1Ac in the SERD4 population also appears to be controlled by more than one allele.[16] For SERD4, loss of binding of Cry1Ac has been demonstrated as a resistance mechanism (J Ferré, pers comm). For SERD5, biochemical studies have shown that reduced activation of toxin is a major resistance mechanism.[31] The presence of the latter mechanism of resistance implies that the truncated Cry1Ac toxin[24] expressed in *Bt* Oscar still requires some activation. An alternate explanation for the above dose–response data is that the parental resistant population had some heterozygotes. This would have given $F_1$ progeny of RS (heterozygotes) and SS (homogeneous susceptible). In the backcross the former would give *ca* 50% mortality and the latter 100%.

The genetic basis for resistance to *Bt* toxins appears to vary in different insects and even for different toxins.[3,4,5,17,23] Our results using *Bt* canola with two field populations of *P xylostella* provide the first experimental evidence for non-recessive resistance on *Bt* plants. In a previous study with *P xylostella*, a population showing incompletely recessive resistance to Cry1C when the toxin was applied exogenously in leaf-dip bioassays became fully recessive when tested with *Bt* broccoli expressing high levels of Cry1C.[3]

Extrapolation of the bioassay data on *Bt* canola using data obtained from the leaf-dip bioassay log dose-logit mortality regressions (Fig 1) showed that, to achieve 90% mortality in $F_1$ progeny of SERD5, relatively high levels of toxin expression would have been required. In leaf-dip bioassays, the ratio between the $LC_{48}$ (% mortality of $F_1$ progeny of SERD5 female × ROTH male on *Bt* canola leaves expressing an average of 32 ng toxin mg$^{-1}$ soluble protein) and the $LC_{90}$ was 1:79 (based on a slope of 1.28). On this basis, to achieve 90% mortality of $F_1$ heterozygotes in *Bt* canola leaf bioassays, the expression level of Cry1Ac in *Bt* canola leaves was estimated to be 2528 ng mg$^{-1}$ total soluble leaf protein. Similarly, in leaf-dip bioassays the ratio between $LC_{72}$ (% mortality of $F_1$ progeny of SERD5 male × ROTH female on *Bt* canola leaves) and $LC_{90}$ was 1:59 and the expression level in *Bt* canola leaves required to give 90% mortality was estimated to be 1888 ng mg$^{-1}$ total leaf protein. Stewart *et al*[24] have reported high levels of expression of *Bt* toxins in canola leaves to be ≥1300 ng mg$^{-1}$ leaf protein. The present results therefore suggest that even with relatively high levels of expression of toxin in crucifers, some survival of heterozygotes might occur.

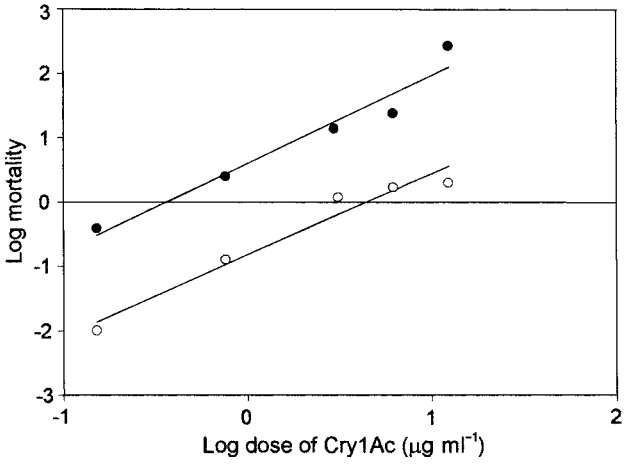


**Figure 1.** Dose mortality response for $F_1$ heterozygotes (Cry1Ac-SEL × ROTH) of *Plutella xylostella* using a leaf-dip bioassay with Cry1Ac: (•) Cry1Ac-SEL SERD5 male × ROTH; (○) Cry1Ac-SEL SERD5 female × ROTH.

A criticism of the method used to estimate the expression levels required to give 90% mortality is that the slope of the log dose–mortality regression from leaf-dip bioassays, where the toxin is on the leaf surface, might be very different from the slope obtained with a transgenic plant, where the toxin would be within the leaf tissue. This would be difficult to test experimentally. However, the slope observed for the leaf dip bioassay in the present study (slope = 1.28) was similar to the values for slopes obtained with *P xylostella* using diet-based assays with *B thuringiensis* var *kurstaki* (average slope *ca* 1.4).[32] While estimates of $LC_{90}$ values are also subject to much larger variances than estimates of $LC_{50}$ values, the above calculations suggest that relatively high levels of toxin expression would have to be achieved to kill all heterozygotes.

High levels of toxin expression (*ca* 4000 ng mg$^{-1}$ total leaf protein) have been reported in crucifers.[33] However, these levels would have to be maintained in both young and mature foliage. The present study with *Bt* canola suggests that this is not necessarily the case; toxin expression in young leaves being low (32 ng mg$^{-1}$) compared with mature leaves (131 ng mg$^{-1}$). There are also reports that the effectiveness of *Bt* cotton Cry toxin against bollworm declines in more mature plants.[34]

The results presented here could have important implications for resistance management. The high-dose/refugia strategy requires that heterozygotes must be killed on *Bt* plants. If survival of heterozygotes can occur under field conditions for this and other pest species, the usefulness of this strategy for single toxin *Bt* crops would be greatly reduced. Simulation models show that a two-toxin expression strategy could markedly delay resistance when there is 50–70% mortality of heterozygotes.[35] However, the low-dose toxin strategy combined with natural enemies could also be of long-term benefit because insects would not

adapt to such a system as rapidly as they would do to the high-dose strategy.[36,37] In general, if pest mortality due to natural enemies decreases the differential in fitness of resistant and susceptible populations, it will reduce the resistance spreads.

## ACKNOWLEDGEMENTS

We thank D Omar and A Martinou for collecting the field populations of *Plutella xylostella* (SERD4 and SERD5), AJ Clark for measuring Cry1Ac expression levels, Professor J Ferré for supplying Cry1Ac toxin and Professor CN Stewart Jr for providing the *Bt* canola line. AHS was supported by the Ministry of Education, Government of Pakistan. Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the UK. This work was conducted under MAFF licences PHL17A/3057(5/1999) and PHL 17B/3479(06/2000).

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