Resistance mechanisms to *Bacillus thuringiensis* subspp. *kurstaki* and *aizawai* in a multi-resistant field population of *Plutella xylostella* from Malaysia

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**Abstract**
A field population (SERD3) of *Plutella xylostella* L. collected in December 1994, and resistant to various insecticides, including *Bacillus thuringiensis* subspp. *kurstaki* (*Btk*) and *B.t.* subspp. *aizawai* (*Bta*), was selected with *Btk* (*Btk*-Sel) and *Bta* (*Bta*-Sel). Little evidence of cross-resistance was observed. Binding to midgut brush border membrane vesicles was examined for insecticidal crystal proteins specific to *Btk* [Cry1Ac], *Bta* [Cry1Ca] or to both [Cry1Aa and Cry1Ab]. In SERD3 (c. 50 and 30-fold resistance to *Btk* and *Bta*), specific binding of Cry1Aa, Cry1Ac and Cry1Ca was similar compared with a susceptible population (ROTH) but binding of Cry1Ab was minimal. The *Btk*-Sel (c. 600 and 60-fold resistance to *Btk* and *Bta*) and *Bta*-Sel (c. 80 and 300-fold resistance to *Btk* and *Bta*) populations lacked binding to Cry1Ab but in *Bta*-Sel binding of Cry1Ca was similar to ROTH. The results suggest reduced binding of Cry1Ab can partly explain resistance to *Btk* and *Bta*. However, the binding of Cry1Aa, Cry1Ac and Cry1Ca in resistant populations, and the lack of cross-resistance between *Btk* and *Bta*, also suggests additional resistance mechanisms are present.

**Key words:** *Bacillus thuringiensis*; *Plutella xylostella*; resistance; mechanisms.

**Introduction**
After several decades of usage of products based on insecticidal crystal proteins (ICPs) of *Bacillus thuringiensis* (*B.t.*), the 1990s saw an increasing number of reports of field resistance (Tabashnik, 1994), although this has only been fully substantiated in *Plutella xylostella* L. (Lepidoptera: Yponomeutidae). Field resistance in this species can be correlated with increasing usage of *B.t.* products during the 1980s (Tabashnik, 1994). Similar increases in selection pressure are likely to occur for other species with the introduction of new *B.t.* strains and transgenic crops expressing genes encoding for ICPs (e.g. Tabashnik, 1994). Characterising resistance to ICPs is thus an important component of strategies aimed at safeguarding their future use (Ferré et al., 1995).

In a laboratory-selected population of the Indian meal moth, *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae), resistance to Dipel® (*B.t.* subspp. *kurstaki*) and a constituent ICP, Cry1Ab, was correlated with reduced binding of the latter to a midgut membrane receptor (Van Rie et al., 1990a). Studies with *P. xylostella* from the Philippines, Hawaii and Florida also demonstrated reduced binding of Cry1Ab or Cry1Ac to midgut membrane receptors (Tabashnik et al., 1994; Ferré et al., 1995; Tang et al., 1996). However, there have been cases of laboratory-selected insects for which non receptor-related resistance mechanisms have been proposed (see Tabashnik, 1994; Moar et al., 1995; Forcada et al., 1996).

Products based on *B.t.* subspp. *aizawai* have been introduced relatively recently and while a few cases of low level resistance have been reported in field populations of *P. xylostella*, these have generally been attributed to cross-resistance with *B.t. kurstaki* (Tabashnik, 1994; Iqbal et al., 1996). The present paper reports studies on resistance mechanisms to *B.t. kurstaki* and *B.t. aizawai* in a field population of *P. xylostella* from Malaysia with relatively high levels of resistance to both *B.t.* subspp.

**Materials and methods.**
*Bacillus thuringiensis* products.
Test solutions were freshly prepared from Dipel® (*B.t. kurstaki* strain HD-1, containing ICPs - Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa & Cry2Ab; 16000 International Units (IU) mg⁻¹ wettable powder; Abbott Laboratories) and Florbac® (*B.t. aizawai*, containing Cry1Aa, Cry1Ab, Cry1Ca & Cry1Da; 8500 IU mg⁻¹ flowable concentrate; Novo Nordisk) in distilled water with Triton X-100 (50 µg ml⁻¹) as a surfactant.

**Insects**
An insecticide susceptible, laboratory strain of *P. xylostella* (ROTH) was obtained from IACR Rothamsted (Harpenden, UK). A field population (SERD3) was collected from crucifer crops in Serdang, Selangor, Malaysia in December 1994 and imported into the UK at the F6 generation (c. 200 insects). Insect larvae were reared and tested on organically-grown 4–6 week-old Chinese cabbage, *Brassica chinensis* var. *pekinesis* cv. Tip Top, at 20 °C, c. 65% r.h. and 16 h
photophase (Iqbal et al., 1996). The SERD3 population was divided into three sub-populations at F7. Two were selected with B.t. kurstaki (Btk-Sel) and B.t. aizawai (Bta-Sel) from F7 to F9 (mean survival to adult = 58 and 46% for Btk and Bta respectively), the third was left unselected (SERD3).

Toxicity bioassays
Third instar larvae (at F7 and F10) were tested using a leaf-dip bioassay (Iqbal et al., 1996). Each leaf disc (4.8 cm dia.) was immersed in test solution for 10 s. Controls were immersed in distilled water with Triton X-100. Five larvae were placed on each leaf disc and each treatment was replicated 10 times. After 5 days, the remains of the leaf discs were removed and replaced by fresh, untreated leaves. Mortality was assessed after 9 days (Iqbal et al., 1996). Where necessary, bioassay data were corrected for control mortality (Abbott, 1925). Estimates of LC\textsubscript{50} values and their 95% fiducial limits (FL) were obtained by maximum likelihood logit regression analysis in GLIM 3.77 (Numerical Algorithms Group, Oxford, 1985) using generalised linear modelling techniques from which differences between data sets were extracted by analysis of deviance (Crawley, 1993). LC\textsubscript{50} values were compared using individual 95% FL for two parameters (p = 0.01). An estimate of 'realised heritability' (h^2), the proportion of phenotypic variance accounted for by additive genetic variation, was calculated (Tabashnik, 1992) to compare the rate of selection in different populations.

*Bacillus thuringiensis* ICPs and iodination procedure
Trypsin-activated Cry1Aa, Cry1Ab and Cry1Ac were kindly provided by Luke Masson (National Research Council of Canada, Montreal) and Cry1Ca by Jeroen Van Rie (Plant Genetic Systems, Gent, Belgium). Cry1A ICPs were obtained as recombinant proteins, cloned from the NRD-12 strain of *B.t. kurstaki* and expressed in *Escherichia coli* (Masson et al., 1989). Cry1Ca was a recombinant protein, cloned from *B. thuringiensis* var. entomocidus HD-110, and expressed in *E. coli* (Ferré et al., 1991). The protein concentration of purified trypsin-activated toxins was determined using the method of Bradford (1976) with bovine serum albumin (BSA) as standard. Iodination of Cry1A ICPs was carried out by the chloramine-T method (Van Rie et al., 1990b). Cry1Ca toxin was labelled by means of the Iodogen method (Hoffman et al., 1988).

Preparation of brush border membrane vesicles and binding assays
Brush border membrane vesicles (BBMV) from each population of *P. xylostella* were prepared (Esriche et al., 1995) from whole last instar larvae (c. 1 500 larvae from each population), frozen in liquid nitrogen and stored at -80°C. Protein concentrations in the BBMV preparations were determined by the method of Bradford (1976). The conditions used for the binding assays were essentially the same as previously published (Ferré et al., 1995). Duplicate samples of BBMVs and labelled ICPs were incubated in PBS/0.1% BSA (0.1 ml final volume) at room temperature. After 30 min (for Cry1A's) or 90 min. (for Cry1Ca), the reaction was stopped by filtration through Whatman GF/F glass-fibre filters (previously soaked with PBS/0.5% BSA) in a Millipore manifold sample filtration unit. Filters were washed rapidly with 5 ml of ice-cold PBS/0.1% BSA and transferred to microtubes. The radioactivity retained in the filters was measured in a 1282 Compugamma CS gamma-counter (LKB). Non-specific binding was determined by adding a 100-fold excess of the corresponding unlabelled ICP. For competition experiments, 9–10 µg of BBMV proteins were incubated with labelled ICPs at increasing concentrations of the corresponding unlabelled ICP. Quantitative binding data were obtained from competition experiments using the LIGAND computer programme (Ferré et al., 1991).

Results
Toxicity of *B. t. products*
The F7 generation of the SERD3 population was c. 330-fold resistant to *B.t. kurstaki* and 160-fold resistant to *B.t. aizawai* (at LC\textsubscript{50} level) compared with the control, ROTH population (Table 1). Selection of sub-populations of SERD3 with *B.t. kurstaki* and *B.t. aizawai* increased resistance c. 2-fold in the F10 generation compared with the F7 generation while resistance to these *B.t.* products declined c. 7- and 5-fold respectively in the unselected sub-population of SERD3. The slope of the regression for *B.t. kurstaki* against the F7 generation of SERD3 was markedly greater (P<0.05) compared with the F10 generations of SERD3 and *Btk-Sel* (Table 1).

In the laboratory generation (F10) of SERD3 examined in subsequent binding assays, the level of resistance to *B.t. kurstaki* (c. 600) and *B.t. aizawai* (c. 300) was thus an order of magnitude greater in both the *Btk-Sel* and *Bta-Sel* sub-populations respectively compared with the concurrent generation (F10) of the unselected SERD3 sub-population (Table 1). The corresponding level of cross-resistance to *B.t. aizawai* and *B.t. kurstaki* in *Btk-Sel* and *Bta-Sel* respectively was < 2-fold greater (P>0.01) compared with unselected SERD3 at F10. Estimations of realised heritability (h^2) of resistance gave an intermediate value for *B.t. kurstaki* and a relatively low value for *B.t. aizawai* (Table 1).

Binding of iodinated ICPs to BBMVs.
BBMV from unselected (SERD3) and insecticide susceptible, control (ROTH) insects were tested for binding with ^125^I-labelled. Saturable specific binding was found for all ICPs tested with BBMV from ROTH. In contrast, BBMV from SERD3 showed strongly reduced binding of ^125^I-labelled Cry1Ab (maximum binding was 0.6 % compared with 3.8 % for ROTH). Saturation experiments with SERD3 and ROTH did not appear to differ appreciably for the other three
**Table 1. Toxicity of *B.t.* subsp. *kurstaki* (*Btk*) and *B.t.* subsp. *aizawai* (*Bta*) against a *B.t.* susceptible (ROTH) and a field population (SERD3) of *Plutella xylostella*, and to laboratory-selected sub-populations of SERD3 (*Btk*-Sel and *Bta*-Sel).**

<table>
<thead>
<tr>
<th>Population (generation)</th>
<th>Test product</th>
<th>LC$_{50}$ (95% FL)$^3$</th>
<th>SLOPE ± SE</th>
<th>Resistance ratio$^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROTH</td>
<td><em>Btk</em></td>
<td>0.018 (0.013–0.022)</td>
<td>2.92 ± 0.46</td>
<td>–</td>
</tr>
<tr>
<td>SERD3 (F7)</td>
<td><em>Btk</em></td>
<td>5.89 (4.66–7.60)</td>
<td>3.85 ± 0.52</td>
<td>330</td>
</tr>
<tr>
<td>SERD3 (F10)</td>
<td><em>Btk</em></td>
<td>0.87 (0.64–1.24)</td>
<td>2.16 ± 0.25</td>
<td>50</td>
</tr>
<tr>
<td><em>Btk</em>-Sel (F10)</td>
<td><em>Btk</em></td>
<td>10.7 (7.65–15.9)</td>
<td>2.00 ± 0.25</td>
<td>600 (13)</td>
</tr>
<tr>
<td><em>Bta</em>-Sel (F10)</td>
<td><em>Bta</em></td>
<td>1.40 (1.01–2.08)</td>
<td>2.10 ± 0.24</td>
<td>80 (2)</td>
</tr>
<tr>
<td>ROTH</td>
<td><em>Bta</em></td>
<td>0.012 (0.009–0.017)</td>
<td>2.20 ± 0.24</td>
<td>–</td>
</tr>
<tr>
<td>SERD3 (F7)</td>
<td><em>Bta</em></td>
<td>1.92 (1.33–2.93)</td>
<td>1.77 ± 0.21</td>
<td>160</td>
</tr>
<tr>
<td>SERD3 (F10)</td>
<td><em>Bta</em></td>
<td>0.38 (0.24–0.59)</td>
<td>1.38 ± 0.18</td>
<td>30</td>
</tr>
<tr>
<td><em>Btk</em>-Sel (F10)</td>
<td><em>Bta</em></td>
<td>0.67 (0.49–0.91)</td>
<td>2.42 ± 0.29</td>
<td>60 (2)</td>
</tr>
<tr>
<td><em>Bta</em>-Sel (F10)</td>
<td><em>Bta</em></td>
<td>3.64 (2.74–5.01)</td>
<td>2.33 ± 0.21</td>
<td>300 (10)</td>
</tr>
</tbody>
</table>

$^1$Leaf-dip assay against third instar larvae; mortality assessed after 9 days.

$^2$Sub-populations selected from F7 to F9.

$^3$ICP Population Resistance ratio compared with equivalent LC$_{50}$ value for ROTH population.

$^4$Values in parentheses = resistance ratio compared with unselected SERD3 at F10.

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**Table 2. Equilibrium dissociation constants (K$_d$) and concentration of receptors (R$_q$) of *B.t.* ICPs on BBMV for a control (ROTH) and two resistant (unselected SERD3 and *Bta*-Sel) populations of *P. xylostella*.**

<table>
<thead>
<tr>
<th>ICP</th>
<th>Population</th>
<th>K$_d$ nM</th>
<th>R$_q$ pmol/mg BBMV protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry1Ac</td>
<td>ROTH</td>
<td>22.4 (0.1)</td>
<td>2.7 (0.1)</td>
</tr>
<tr>
<td></td>
<td>SERD3 (F10)</td>
<td>27.3 (0.3)</td>
<td>3.3 (0.1)</td>
</tr>
<tr>
<td>Cry1Ca</td>
<td>ROTH</td>
<td>8.9 (0.1)</td>
<td>9.2 (1.0)</td>
</tr>
<tr>
<td></td>
<td><em>Bta</em>-Sel (F10)</td>
<td>8.7 (0.1)</td>
<td>9.0 (0.9)</td>
</tr>
</tbody>
</table>

$^1$K$_d$ and R$_q$ values (mean ±SE) were calculated from homologous competition experiments for Cry1Ac (n = 2) and Cry1Ca (n = 2) performed on the same batch of BBMV.

ICPs. Maximum specific binding was 4.6%, 1.7% and 5.4% for SERD3 and 3.6%, 1.7% and 6.9% for ROTH, with Cry1Aa, Cry1Ac and Cry1Ca respectively.

Because of the low specific binding obtained with Cry1Ac, competition experiments were conducted to confirm the specificity of binding and so discard the possibility of an artifact in the saturation experiment. Results showed (Table 2) that binding of $^{125}$I-labelled Cry1Ac was competed by non-labelled Cry1Ac, giving typical curves for this type of experiment. Neither the equilibrium dissociation constant (K$_d$) nor the receptor concentration (R$_q$) were noticeably different for Cry1Ac between the two insect populations (Table 2).

Analysis of BBMV from the two selected resistant populations (*Btk*-Sel and *Bta*-Sel) with $^{125}$I-labelled Cry1Ab also showed strongly reduced binding to this ICP (0.6% and 0.3% respectively). Unfortunately, it was not possible to conduct binding assays for these populations with either Cry1Aa or Cry1Ac.

Since Cry1Ca is a component of FlorbacR but is not present in DipelR, binding experiments were conducted with BBMV from the *Btu*-Sel population to test whether selection had changed the affinity for this ICP. No reduction in specific binding of Cry1Ca was detected in saturation experiments (maximum binding = 8.8%). Moreover, competition experiments with *Bta*-Sel and ROTH did not show any noticeable differences in binding affinity or receptor concentration (Table 2).

**Discussion**

In contrast to previous field populations of *P. xylostella* examined (e.g. Tabashnik, 1994; Tang et al., 1996; Iqbal et al., 1996), SERD3 appeared to show appreciable levels of resistance to *B. t. aizawai* in addition to resistance to *B. t. kurstaki*. The selection experiments confirmed resistance to both *B.t.* subssp. and showed that selection of resistance occurred at a greater rate to *B.t. kurstaki* compared with *B.t. aizawai* under our laboratory conditions. The lack of cross-resistance in the *Btk*-Sel and *Bta*-Sel populations suggests the presence of gene(s) for resistance to toxic components in *B.t. kurstaki* and *B.t. aizawai* which segregate independently. Liu et al. (1996) have recently provided evidence that gene(s) conferring resistance to Cry1Ab and Cry1Ca segregate independently in a field population of *P. xylostella* from Hawaii.

The binding experiments appear to indicate that the only major biochemical difference between the ROTH and SERD3 populations of *P. xylostella* is reduced binding of the latter to Cry1Ab. However, it is noteworthy that binding to Cry1Aa and Cry1Ac was not affected, despite their close structural relationship with Cry1Ab. Furthermore, it seems to be a common feature that these three ICPs share common binding sites in many lepidopteran species, including *P. xylostella* (Escriche et al., 1996). This feature has helped promote the assumption that insects resistant to commercial products of *B.t. kurstaki*, for which
reduced binding to Cry1Ab or Cry1Ac has been shown, also have reduced binding to other Cry1A ICPs.

One of the first and most widely used Bt kurstaki products, DipelR, is based on strain HD-1 which produces Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab toxins. However, Cry2Aa is reported to be scarcely toxic to P. xylostella (Tang et al., 1996) and this also likely to be the case for Cry2Ab. Therefore, a change in a common receptor for the three Cry1A ICPs would probably be sufficient to confer to P. xylostella resistance to B. kurstaki HD-1 products. This is clearly not the case in the SERD3 population of P. xylostella. Thus, despite Cry1A ICPs sharing common binding sites, Cry1Ab at least must have another, independent binding site(s) susceptible to change without affecting binding to the other ICPs. Lack of cross-resistance to other Cry1A toxins in a population of P. xylostella from the Philippines highly resistant to Cry1Ab also suggests the presence of independent binding sites (Ballester et al., 1994) although in the latter there was no resistance to Bt kurstaki.

How can reduced binding of Cry1Ab confer resistance to Bt kurstaki and Bt aizawai products? The composition of ICPs in a DipelR formulation has been reported to be 28% Cry1Aa, 53% Cry1Ab, 19% Cry1Ac, while another Bt aizawai product, XenTariR, contains 32% Cry1Aa, 38% Cry1Ab, 26% Cry1Ca and 5% Cry1Da (Liu et al., 1996). The toxicities of these ICPs in a susceptible colony of P. xylostella (Tang et al., 1996) were found to be 0.3 for Cry1Aa, 0.6 for Cry1Ab, 1.1 for Cry1Ac, 4.3 for Cry1Ca and 0.2 for Cry1Da (LC50 in µl/ml). If both parameters are combined, assuming similar joint action of the components (Tang et al., 1996), the contribution of Cry1Ab to the toxicity of both formulations is considerable (c. 40% and 30% for Cry toxins in DipelR and XenTariR respectively). Thus, reduced binding of Cry1Ab alone could confer some degree of resistance to both Bt kurstaki and aizawai formulations. A similar calculation would suggest that Cry1Ca contributes relatively little to the toxicity of, and thus resistance to, Bt aizawai (XenTariR), an observation supported by the much greater degree of resistance observed to Cry1Ca compared with Bt aizawai in a field population of P. xylostella from Hawaii (Liu et al., 1996).

However, while a modification in the binding site, specific for Cry1Ab, can explain increased resistance to Bt kurstaki and aizawai formulations, it cannot explain the differences in susceptibility of the three resistant populations (SERD3, Btk-Sel and Bta-Sel) after selection in the laboratory for three generations. Our results show that selection with Bt kurstaki did not noticeably decrease binding of Cry1Ab, although this was minimal anyway in SERD3 at F10, nor selection with Bt aizawai change binding parameters for Cry1Ca. A possible explanation, although unlikely, is that selection acted upon binding affinities of ICPs not tested with the selected populations (Cry1Aa, Cry1Ac and Cry1Da). Alternatively, SERD3 had other, independent mechanisms of resistance that potentiated the effect of the modified receptor for Cry1Ab. If the latter were true, resistance due to these non-receptor related mechanisms seems to have been unstable, and only selection with the other Bt formulations maintained or increased them.

Interestingly, studies on P. interpunctella have shown that laboratory-selection with Bt kurstaki causes relatively narrow-spectrum resistance to Cry1Ab and Cry1Ac, while selection with Bt aizawai causes broader-spectrum resistance to Cry1A ICPs, Cry1B, Cry1C and Cry2A ICPs (McGaughey & Johnson, 1994). The presence of independent, unstable resistance mechanisms could account for the apparent lack of cross-resistance observed in the bioassays with the Btk-Sel and Bta-Sel populations. Such mechanisms may be related to any other step in the mode of action of ICPs (Knowles, 1994).

Finally, we must not forget another component of B. thuringiensis formulations: the spore. Besides its intrinsic toxic effect, synergism between spores and ICPs has been reported recently in P. xylostella (Tang et al., 1996) and the beet armyworm, Spodoptera exigua Hübner (Lepidoptera; Noctuidae) (Moar et al., 1995), although its synergistic effect with B. thuringiensis crystals is long known (Li et al., 1987). It is logical to think that there must be genes controlling sensitivity to the spore and to its synergistic effects. This possibility has just started to be explored (Tang et al., 1996).

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References


