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Inheritance of Resistance to the Cry1Ab Bacillus thuringiensis Toxin in Ostrinia nubilalis (Lepidoptera: Crambidae)

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ABSTRACT Laboratory selection with Cry1Ab, the predominant Bacillus thuringiensis (Bt) toxin in transgenic corn, Zea mays L., produced >1000-fold resistance in two laboratory strains of European corn borer, Ostrinia nubilalis (Hübner). We tested the offspring of various crosses to determine the mode of inheritance of resistance to Cry1Ab. Patterns of inheritance of resistance were similar in the two resistant strains. The progeny of reciprocal F1 crosses (resistant male × susceptible female and vice versa) responded alike in bioassays, indicating autosomal inheritance. The median lethal concentrations (LC50 values) of F1 were intermediate between the resistant and susceptible parents, indicating approximately additive inheritance. However, the dominance of resistance increased as the concentration of Cry1Ab decreased. Analysis of progeny from backcrosses (F1 × susceptible strain) suggests that resistance was controlled by more than one locus. In particular, the fit of observed to expected mortality improved as the number of putative loci increased from 1 to 10. The polygenic nature of resistance in these two laboratory strains suggests that major genes for resistance to Cry1Ab were not common in the founding populations of O. nubilalis. A low initial frequency of major genes for Cry1Ab resistance might be an important factor in delaying evolution of resistance to Bt corn in this pest.

KEY WORDS Ostrinia nubilalis, inheritance, resistance, transgenic corn

Insecticidal proteins from Bacillus thuringiensis (Bt) are useful in insect pest control, both in spray formulations and transgenic crops (Schnepf et al. 1998, Shelton et al. 2002). Despite the benefits of transgenic plants producing Bt toxins, their widespread use could cause evolution of resistance in target pests (Megahey and Whalon 1992, Tabashnik 1994, Gould 1998, ILSI/HESI 1998, Ferre and Van Rie 2002, Bates et al. 2005). Although field-evolved resistance to Bt crops has not yet been detected, resistance to the Bt toxins in foliar sprays has evolved in field populations of diamondback moth, Plutella xylostella (L.), in greenhouse populations of Trichoplusia ni (Hübner) (Janmaat and Myers 2003, Tabashnik et al. 2003).

Bt corn (Zea mays L.), which accounted for 32% of U.S. corn acreage in 2004 (ERS 2004), is useful for managing European corn borer, Ostrinia nubilalis (Hübner), a major corn pest (Mason et al. 1996). Resistance to Bt toxins in O. nubilalis has been reported in laboratory-selected strains exposed to the Bt formulation Dipel (Huang et al. 1997) and to the single toxins Cry1Ac (Bolin et al. 1999) and Cry1Ab (Chaufaux et al. 2001). Inheritance of resistance to Dipel is reported as autosomal, partially dominant, and is controlled primarily by a single gene (Huang et al. 1999). Although Cry1Ab is the most abundant Cry1 toxin in Dipel, this formulation also contains Cry1Aa, Cry1Ac, and other materials (Liu et al. 1996).

Because Cry1Ab has been the predominant toxin produced by Bt corn, we have focused on resistance of O. nubilalis to this toxin. Laboratory selection with Cry1Ab protoxin yielded significant levels of resistance in two strains after 7–10 generations (Chaufaux et al. 2001). Here, we analyzed inheritance of Cry1Ab resistance in these laboratory-selected strains after additional selection yielded >1000-fold resistance. Specific objectives were to determine sex linkage and dominance and to estimate the number of loci (one or multiple loci) involved in the resistance.

Materials and Methods

Insect Strains. We studied four strains of O. nubilalis: Europe-S, Europe-R, Nebraska-S, and RSTT-R. The parental Europe strain originated in 1993 from the Lombardia region of northern Italy and was divided into the unselected strain Europe-S and the selected strain Europe-R (Chaufaux et al. 2001). The same procedure was applied to the parental Nebraska strain, which originated in 1995 from Saunders County, Nebraska (Chaufaux et al. 2001). The RSTT-R strain was created by combining at least 200 egg masses from the

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Europe-R strain (F$_2$$_0$) with at least 200 egg masses from the Nebraska-R strain (F$_1$$^0$) (Siqueira et al. 2004b). When the inheritance experiments were initiated, the resistant strains (Europe-R and RSTT-R) had been selected for at least 45 generations (Chaufaux et al. 2001, Siqueira et al. 2004a), and the susceptible strains (Europe-S and Nebraska-S) had been reared without exposure to insecticides for at least 75 generations.

**Rearing**. Rearing methods for *O. nubilalis* were based on those developed at the USDA-ARS Corn Insect Research Unit, Ames, IA (Guthrie et al. 1965, Lewis and Lynch 1969), as described by Siqueira et al. (2004b).

**Bioassays**. *O. nubilalis* neonates (<24 h after hatching) were exposed to toxin on the surface of artificial diet (Marçon et al. 2000). The toxin used for bioassays was the purified trypsin-resistant core (TRC) of Cry1Ab from *B. thuringiensis* Berliner subsp. *kurstaki* provided by Monsanto Co. (St. Louis, MO), referred to hereafter as Cry1Ab toxin.

The rearing diet developed for *Heliothis virescens* (F.) (King et al. 1985) and adapted for *O. nubilalis* (Marçon et al. 1999) was used in place of *O. nubilalis* diet for Bt bioassays. Bioassays were performed in 128-well trays (each well 16 mm in diameter, 16 mm in height, CD International, Pitman, NJ). Approximately 1 ml of diet was dispensed into each well and allowed to solidify. Seven concentrations of toxin were used. Dilutions were made in 0.1% Triton-X 100 non-ionic detergent to obtain uniform spreading on the diet surface. Each well was surface treated with 30 μl of the appropriate solution. Control treatments consisted of diet surface treated with 0.1% Triton X-100 only. Wells were allowed to air dry, and one neonate was randomly transferred into each well. Wells were then covered with vented lids (CD International), and trays were held in an incubator at 27°C, 24-h scotophase, and 80% RH. Mortality was recorded 7 d after treatment. Larvae that had not grown beyond the first instar and weighed ≤0.1 mg were considered dead. As a result, the criteria for "mortality" used in this study accounts for both severe growth inhibition and death. Control mortality never exceeded 10%. In each experiment, bioassays were replicated at least three times for each strain or cross, with 16 larvae per concentration (total of at least 48 larvae per concentration per strain or cross).

**Inheritance Experiments**. To evaluate sex linkage and dominance, we tested F$_1$ progeny from two sets of reciprocal mass crosses between resistant and susceptible strains: Europe-R$_{F_{2}S}$ × Nebraska-S$_{F_{77}}$ and RSTT-R$_{F_{55}}$ × Europe-S$_{F_{94}}$. For these experiments, the resistant strains were not selected for at least one generation before establishing the crosses. The pupae were sexed so that virgin males from one strain would mate with virgin females from the other, and vice versa. In all experiments, reciprocal mass crosses were established with 60–100 pairs. To estimate the number of loci influencing resistance, F$_1$ progeny from reciprocal crosses were backcrossed to the susceptible strains and tested for susceptibility to Cry1Ab as described above.

**Data Analysis**. Concentration–mortality data were analyzed by probit regression using POLO-PC (LeOra Software 1987) to calculate median lethal concentrations (LC$_{50}$ values) and their 95% fiducial limits and slopes and their standard errors. LC$_{50}$ values were considered significantly different if no overlap occurred between their 95% fiducial limits. Mortality was corrected for control mortality using the method of Abbott (1925).

We calculated degree of dominance (D) and its standard error based on LC$_{50}$ values (Lehmann 1966, Stone 1968). We calculated effective dominance (h) at specific concentrations (Liu and Tabashnik 1997, Bourguet et al. 2000) with mortality at each concentration estimated from concentration-mortality probit regression lines. D varies from −1 (recessive) to 1 (dominant), with 0 indicating codominance (additive inheritance); h varies from 0 (recessive) to 1 (dominant), with 0.5 indicating codominance. Comparisons between D and h were made using the formula $h = (D + 1)/2$ (Liu and Tabashnik 1997, Bourguet et al. 2000).

The backcross generation obtained from mating F$_1$ offspring with parental susceptible strains was tested to estimate the number of genes affecting resistance by using the method of Lande (1981) and indirect tests of models with one, two, five and 10 loci (Tabashnik et al. 1992). The minimum number of independently segregating genes with equal effect contributing to resistance was estimated according to Lande’s method as adapted by Tabashnik et al. (1992). With this method, if resistance is controlled by a single locus, a large increase in genetic variation is expected in backcross progeny relative to parental strains and their F$_1$ progeny. As the number of loci increases, decreases are expected in the extra genetic variation in backcross progeny relative to parental strains and their F$_1$ progeny. The slope of concentration–mortality lines is inversely related to variance (Tabashnik 1991, Tabashnik et al. 1992). Thus, relative to slopes for parental strains and their F$_1$ progeny, a large decrease in slope for backcross progeny indicates monogenic inheritance, whereas smaller decreases suggest that multiple loci are involved.

In the indirect tests of monogenic and polygenic models, we tested the fit between observed mortality and mortality predicted by models with one, two, five, and 10 loci. This approach follows the one described by Tabashnik et al. (1992, 2002), except that backcrosses were done with the susceptible parental strains. The slopes of concentration–response lines for the parental strains were calculated as the mean of the slopes experimentally determined for each parental strain. For example, we used a slope of 2.0 for indirect tests when the slopes of the parental strains in the backcross were 2.2 (susceptible) and 1.8 (F$_1$). We assumed that each locus had one allele conferring resistance (R) and the other conferring susceptibility (S). We also assumed equal and additive effects of loci in polygenic models. The expected mortality for each
concentration under each hypothesis was estimated as the cumulative probability from the appropriate tolerance distributions for each model (PROBNORM, SAS Institute 2001). The average absolute difference between observed and expected mortality was calculated as the mean of the absolute values of expected percentage mortality minus the observed percentage mortality for each concentration (Tabashnik et al. 1992).

**Results**

Resistance Levels, Maternal Effects, Sex Linkage, and Dominance. Relative to the susceptible Europe-S strain, LC$_{50}$ values of Cry1Ab toxin were 2,000-fold higher for Europe-R and 1,300-fold higher for RSTT-R (Table 1; Fig. 1). The LC$_{50}$ values did not differ significantly between the two resistant strains (Table 1).

For F$_1$ progeny from Europe-R × Nebraska-S and RSTT-R × Europe-S, the LC$_{50}$ values did not differ significantly between the F$_1$ progeny of the two reciprocal crosses (Table 1). Thus, inheritance was autosomal, and maternal effects and sex linkage were not evident. For each resistant strain, the LC$_{50}$ for the F$_1$ progeny pooled from reciprocal crosses was significantly greater than the LC$_{50}$ for the susceptible parental strain and significantly less than the LC$_{50}$ for the resistant parental strain (Table 1). Based on LC$_{50}$ values for the parental strains and the pooled F$_1$ progeny, degree of dominance ($D$ ± SE) was $-0.12 ± 0.49$ ($h^2 = 0.45$) for Europe-R and 0.19 ± 0.38 ($h^2 = 0.59$) for RSTT-R, which are both close to additive inheritance. For both resistant strains, effective dominance ($h$) varied widely with concentration, from dominant inheritance at low concentrations to recessive inheritance at high concentrations (Table 2).

Number of Genes. Results from two different methods to test for the number of genes responsible for resistance suggest that resistance was not controlled by a single major gene in either resistant strain (Figs. 2–4; Table 3). For both resistant strains, analysis by Lande’s method implies that many loci affected resistance. Lande’s method yielded an estimate of 20 for the minimum number of independently segregating loci with equal effect contributing to the difference in susceptibility between the RSTT-R and Europe-S strains. For the Europe-R strain, a specific estimate for the minimum number of genes could not be calculated because the slope for the backcross progeny (2.4) was not lower than the slopes for the parental and F$_1$ strains (mean = 2.0; Table 1). Because slope is inversely related to variance, this indicates no extra
genetic variation was seen in the backcross progeny relative to the parental and F1 strains, which suggests resistance in Europe-R was controlled by many loci (see Materials and Methods).

For both resistant strains, results of indirect tests show that the difference between observed and expected mortality decreased as the number of loci in the model increased (Table 3; Figs. 3 and 4). The average absolute difference between observed and expected mortality was highest for the one-locus model and lowest for the 10-locus model (Europe-R/H11005 9.5% for one locus versus 3.6% for 10 loci, RSTT-R/H11005 16.5% for one locus versus 3.4% for 10 loci; Table 3). Consistent with results from the Lande’s test described above, results from indirect tests of the monogenic model show poor fit with the data. With indirect tests of the monogenic model, significant deviation occurred at three of seven concentrations for Europe-R and six of seven concentrations for RSTT-R (Table 3). In contrast, significant deviation from the 10-locus model occurred at only one concentration for Europe-R and none for RSTT-R (Table 3).

Discussion

As far as we know, the levels of resistance to Cry1Ab in the laboratory-selected strains studied here (2,000-fold for Europe-R and 1,300-fold for RSTT-R; Table 1) are the highest levels of resistance to a Cry1A toxin ever reported for O. nubilalis. Previous reports from other laboratory-selected strains of this pest include up to 160-fold resistance to Cry1Ac in the S-I strain from Minnesota (Bolin et al. 1999) and 520-fold resistance to Cry1Ac in the KS-SC strain from Kansas (Li et al. 2005a). Factors contributing to the differences in reported resistance levels among strains may...
include genetic differences among the strains as well as differences among experiments in the toxic materials tested, bioassay procedures, and susceptibility among the unselected strains used for calculating resistance ratios. Side-by-side comparisons among strains with identical methods could clarify the role of genetic versus environmental influences (González-Cabrera et al. 2001).

The results reported here show similar levels and inheritance of resistance to Cry1Ab toxin in the Europe-R and RSTT-R strains. Although the resistance ratio was somewhat higher for the Europe-R strain, no significant difference occurred between resistant strains in LC_{50} of Cry1Ab (Table 1). When the RSTT-R strain was created by pooling individuals from Nebraska-R (F_{10}) and Europe-R (F_{20}), the Europe-R strain was much more resistant than Nebraska-R (Siqueira et al. 2004b). Because Europe-R may be a primary source of the resistance alleles in RSTT-R, similarity between Europe-R and RSTT-R is not unexpected.

Fig. 3. Observed versus expected mortality at each of five concentrations tested of B. thuringiensis TRC Cry1Ab for O. nubilalis larvae from the backcross F_{1} (Europe-R × Nebraska-S) × Nebraska-S.

Fig. 4. Observed versus expected mortality at each of five concentrations tested of B. thuringiensis TRC Cry1Ab for O. nubilalis larvae from the backcross F_{1} (RSTT-R × Europe-S) × Europe-S.
Analysis of the F1 progeny from reciprocal crosses between the resistant and susceptible strains indicates that resistance to Cry1Ab was inherited as an autosomal trait in both the RSTT-R and Europe-R strains. These results are consistent with nearly all previous results with Bt resistance (Ferré and Van Rie 2002), including resistance to Dipel in the KS-SC strain of *O. nubilalis* (Huang et al. 1999).

For both resistant strains, D was close to zero, indicating that the LC50 values of F1 hybrid progeny were intermediate between the LC50 values of resistant and susceptible parent strains. h depended on the concentration of the toxin, with resistance more dominant as concentration decreased. Increased dominance of Bt resistance at low toxin concentrations has been reported in several other species (Liu and Tabashnik 1997, Sayyed et al. 2000, Liu et al. 2001, Tabashnik et al. 2004). The results with *O. nubilalis* tested here, none of the four Bt-resistant strains of other lepidopteran species previously analyzed for fit to models with one, two, or more loci showed better fit as the number of loci increased. Diamondback moth resistance to Dipel in the NO-QA strain from Hawaii (Tabashnik et al. 1992) and to Javelin (a formulation similar to Dipel) in the Loxa A strain from Florida (Tang et al. 1997) fit best to a one-locus model. Subsequent molecular work with the NO-QA strain mapped genetic control of its Cry1A resistance to a single chromosomal location (Heckel et al. 1999). With pink bollworm resistance to Cry1Ac in the AZP-R strain from Arizona, the best fit occurred with one- or two-locus models (Tabashnik et al. 2002), and molecular work identified a cadherin locus tightly linked with high levels of resistance. Diamondback moth resistance to Cry1C in the Cry1C-Sel strain from South Carolina fit best to a one-locus model (Zhao et al. 2005b), and subsequent molecular mapping with a related strain (SCI) revealed contributions from two linkage groups (Baxter et al. 2005).

Evidence from several independent research groups suggests that major genes conferring high levels of resistance to Cry1Ab are rare in *O. nubilalis*. In April 2006

### Table 3. Indirect tests for deviation between observed and expected mortality (df = 1) for monogenic and additive polygenic models

<table>
<thead>
<tr>
<th>Genotype model</th>
<th>1 locus</th>
<th>2 loci</th>
<th>5 loci</th>
<th>10 loci</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>χ²</td>
<td>P</td>
<td>χ²</td>
<td>P</td>
</tr>
<tr>
<td>Europe-R × Nebraska-S</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>1.52</td>
<td>0.218</td>
<td>0.32</td>
<td>0.572</td>
</tr>
<tr>
<td>3.7</td>
<td>13.10</td>
<td>&lt;0.001</td>
<td>7.96</td>
<td>0.005</td>
</tr>
<tr>
<td>11.1</td>
<td>2.47</td>
<td>0.116</td>
<td>0.99</td>
<td>0.320</td>
</tr>
<tr>
<td>33.3</td>
<td>5.20</td>
<td>0.023</td>
<td>2.66</td>
<td>0.103</td>
</tr>
<tr>
<td>100</td>
<td>10.23</td>
<td>0.001</td>
<td>5.61</td>
<td>0.018</td>
</tr>
<tr>
<td>300</td>
<td>3.36</td>
<td>0.067</td>
<td>1.93</td>
<td>0.165</td>
</tr>
<tr>
<td>900</td>
<td>0.42</td>
<td>0.517</td>
<td>0.22</td>
<td>0.639</td>
</tr>
<tr>
<td>Avg. mortality (%)</td>
<td>58</td>
<td>58</td>
<td>58</td>
<td>58</td>
</tr>
<tr>
<td>Mean difference (%)</td>
<td>9.5</td>
<td>6.1</td>
<td>4.6</td>
<td>3.6</td>
</tr>
<tr>
<td>RSTT-R × Europe-S</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.74</td>
<td>10.35</td>
<td>0.001</td>
<td>5.11</td>
<td>0.024</td>
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<tr>
<td>2.2</td>
<td>12.59</td>
<td>&lt;0.001</td>
<td>3.94</td>
<td>0.047</td>
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<tr>
<td>6.7</td>
<td>5.25</td>
<td>0.022</td>
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<td>0.099</td>
</tr>
<tr>
<td>20</td>
<td>8.89</td>
<td>0.003</td>
<td>3.29</td>
<td>0.070</td>
</tr>
<tr>
<td>60</td>
<td>14.15</td>
<td>&lt;0.001</td>
<td>5.46</td>
<td>0.019</td>
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<tr>
<td>180</td>
<td>6.74</td>
<td>0.009</td>
<td>3.33</td>
<td>0.068</td>
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<tr>
<td>540</td>
<td>1.31</td>
<td>0.252</td>
<td>0.65</td>
<td>0.420</td>
</tr>
<tr>
<td>Avg. mortality (%)</td>
<td>59</td>
<td>59</td>
<td>59</td>
<td>59</td>
</tr>
<tr>
<td>Mean difference (%)</td>
<td>16.5</td>
<td>9.1</td>
<td>6.3</td>
<td>3.4</td>
</tr>
</tbody>
</table>

* Probability values indicating significant differences between the observed and expected mortality (P < 0.05).
F₂ screens of 1,418 isofemale lines (5,672 alleles) from field populations in the United States and France, no alleles for resistance to Bt corn that produces Cry1Ab were detected (Andow et al. 1998, 2000; Bourguet et al. 2003). Although laboratory selection of O. nubilalis strains has yielded several strains with moderate levels of resistance (Huang et al. 1997, Bolin et al. 1999), we are not aware of any that are reported to complete development on Cry1Ab-producing corn (Huang et al. 2002). The resistance levels seen here for Cry1Ab are higher than those reported for other Bt-resistant strains of O. nubilalis, yet the results imply that the resistance is caused by more than one locus and that a major resistance allele was absent in the founding populations.

Although the strains studied here exhibit increased survival on Cry1Ab expressing leaf tissue, surviving larvae are extremely stunted and are unlikely to complete development (B.D.S., unpublished). The hypothesis that the resistance alleles occurring in laboratory-selected strains such as Europe-R and RSTT-R are relevant in the field can be tested only after resistance evolves in the field. So far, after >9 yr of large-scale use of Bt corn, field-evolved resistance to Bt toxins in O. nubilalis has not been documented. We hypothesize that a low initial frequency of major genes for resistance to Cry1Ab has been a major factor helping to delay resistance to Bt corn in this pest.

Acknowledgments

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