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LARVAL DEVELOPMENT OF *SPODOPTERA EXIGUA* (LEPIDOPTERA: NOCTUIDAE) LARVAE ON ARTIFICIAL DIET AND COTTON LEAVES CONTAINING A *BACILLUS THURINGIENSIS* TOXIN: HERITABLE VARIATION TO TOLERATE CRY1AC

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ABSTRACT

Studies were conducted to determine if beet armyworms, *Spodoptera exigua* (Hübner), possess the genetic variation necessary to respond to selection for improved tolerance of the *Bacillus thuringiensis* Berliner (Bt) toxin Cry1Ac. *Spodoptera exigua* individuals that pupated earliest when fed the Cry1Ac diet (ca. the first 20% to pupate) produced offspring that developed significantly faster on the Cry1Ac diet than their parental-control strain. In addition, after two generations of selection, the selected population reached pupation 2 d faster than the Parental population. The selected group also developed significantly faster on transgenic-Bt cotton leaves (cv. NuCOTN 33B) than the Parental strain. Individuals selected to more rapidly develop on media containing Cry1Ac developed no more rapidly on artificial diet containing Cry2Aa than the parental, control colony of *S. exigua*.

Key Words: *Spodoptera exigua*, *Bacillus thuringiensis*, insecticide resistance, heritability, Cry1Ac

RESUMEN

Se llevaron a cabo estudios para determinar si el gusano trozador de la remolacha, *Spodoptera exigua* (Hübner), posee la variación genética necesaria para responder a la selección para la tolerancia mejorada de toxín Cry1Ac de *Bacillus thuringiensis* Berliner (Bt). Los individuos de *Spodoptera exigua* que se empuparon más temprano cuando fueron alimentados de una dieta de Cry1Ac (ca. los primeros 20% que se empuparon) produjieron descendientes que se desarollaron significativamente más rapidos con la dieta de Cry1Ac que el grupo control de la raza de su parientes. Además, después de dos generaciones de selección, la población seleccionada se empuparon 2 dias más rapida que la población pariente. El grupo seleccionado tambien se desarrolló significativamente más rápido en las hojas de algodón transgénicas-Bt (cv. NuCOTN 33B) que la raza Pariente. Los individuos seleccionados para desarrollarse más rápidos en un medio contiendo Cry1Ac no se desarrollaron más rápidamente en la dieta artificial con Cry2Aa que en la colonia control pariente de *S. exigua*.

Cotton varieties containing a gene from *Bacillus thuringiensis* (Berliner) (Bt) that expresses the insecticidal protein Cry1Ac have been commercially available since 1996. The Cry1Ac-expressing varieties of Bt cotton were developed primarily to control the tobacco budworm, *Heliothis virescens* F. and the pink bollworm, *Pectinophora gossypiella* (Saunders). The abilities of different species of insects feeding on cotton to tolerate Bt proteins will play an important role in determining how Bt cotton influences the population dynamics of these pests.

Spodoptera exigua (Hübner), a secondary pest of cotton, is more tolerant of Cry1Ac than the tobacco budworm (Gould & Tabashnik 1998). Although *S. exigua* can survive initial encounters with cotton tissue expressing Cry1Ac, its larval development is delayed. Delayed larval development not only may slow the buildup of population size in this species, but it may have the potential to increase its generation time. One important step is to determine if the genetic potential exists in *S. exigua* to become more tolerant of Cry1Ac, i.e., develop more rapidly on tissue containing Cry1Ac. The purpose of this research is to determine if there is genetic potential in this species to more rapidly finish larval development when feeding on food sources containing Cry1Ac. In addition, the performance of *S. exigua* on a second Cry protein is also investigated.

MATERIALS AND METHODS

Insect Colony and Diet

The *S. exigua* colony used during this study is maintained at the USDA-ARS, Southern Insect Management Research Unit, Stoneville, MS. Lar-

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vae were fed a standard artificial diet developed for Lepidoptera (Raulston & Lingren 1972). Cry1Ac diet was prepared by mixing a concentrated stock solution of MVPII powder into 300-500 ml batches of artificial diet to obtain a final concentration of 1.0 µg/ml Cry1Ac (approx.). A single neonate larva was placed into a 30-ml cup containing approx. 10 ml of Cry1Ac diet. This concentration (6.8 µg/g dry weight of diet) is comparable to reported amounts of Cry1Ac present in cotton tissue (6.7 µg/g dry weight of diet; Greenplate 1999) during the latter part of the growing season (116 d after plating). Larvae from all test groups were also placed on non-Cry1Ac diet as a control. Environmental conditions for these tests were 27 ± 1°C, 45-60% RH, and a photoperiod of 14:10 (L:D).

For two generations, individuals that completed larval development more rapidly when feeding on Cry1Ac diet were selected based on the number of days required to reach pupation (≤16 d; Fig. 1, "Selected" colony). In addition to testing the Selected colony on Cry1Ac diet, its performance was also assessed on non-Cry1Ac diet. The Parental strain was always tested on both types of diet at the same time as the selected colony.

First and Second Generations

During the first generation, the number of days to pupation for each tested individual from the Parental colony was recorded. Parental-colony larvae were tested on both Cry1Ac (N = 510) and non-Cry1Ac diets (N = 60). The pupae resulting from individuals tested on Cry1Ac diet were placed into three groups: (1) the "Selected" group was composed of individuals that had pupated by 14-16 d on Cry1Ac diet; (2) an "Intermediate" group was composed of individuals that pupated by 17-20 d on Cry1Ac diet; and (3) a "Slow" group was composed of individuals that pupated after 21 d of exposure to Cry1Ac diet. Adults from each group were mated *inter se* to produce their second-generation progenies. The larval developments (days to pupation) of these offspring were compared among the above groups and also to the Parental colony on both Cry1Ac (N > 200 per group) and non-Cry1Ac diets (N = 30 per group). In addition, larvae from the two extreme groups ("Selected" and "Slow") and the Parental strain were weighed at 7 d. The weights were used as another measure of larval development on Cry1Ac and non-Cry1Ac diets.

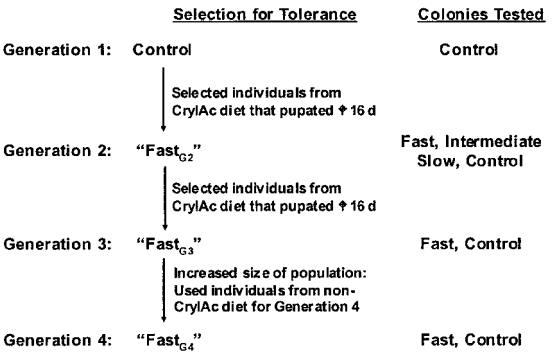

Fig. 1. Mating design for the selection and tests of *S. exigua* individuals and colonies more tolerant of Cry1Ac.

Differences between groups for the number of days to pupation were analyzed via non-parametric statistics. Comparisons between two groups (first generation) were made using Wilcoxon rank-sum tests (SAS 1995). Comparisons of >2 groups (second generation) were made with Kruskal-Wallis ANOVA (SAS 1995). When Kruskal-Wallis results indicated significant differences, means were separated via pairwise-comparisons among the groups using the Wilcoxon rank-sum tests. Bonferroni adjustments were made in the significance levels for follow-up tests to control the overall Type I error at $P = 0.05$ (n = 4 groups, therefore significance was set at $P < 0.008$).

Third Generation

Third-generation individuals from the Selected colony were tested on Cry1Ac and non-Cry1Ac diets and compared with the unselected, Parental colony. As with the previous generations, the number of days to pupation was recorded for each individual. Comparisons between the Selected and Parental groups were made via Wilcoxon rank sum tests (SAS 1995).

Fourth Generation

Most of the third-generation larvae from the Selected group were fed non-Cry1Ac diet in order to not put the colony through another bottleneck and also to increase the size of the colony for the fourth generation of testing on plant tissue. As a consequence, parents of the fourth-generation larvae from the Selected group underwent no selection for improved tolerance of Cry1Ac. During the fourth generation, larvae from the Parental colony and Selected colony were assayed on transgenic-Bt cotton leaves (cv. NuCOTN 33B) and non-Bt cotton leaves (cv. DP5415). Growth of larvae on both types of cotton leaves was evaluated via 10-d weights.

To test for differences in larval growth between the Selected and Parental groups on leaves of Bt and non-Bt cotton, a single leaf from the upper canopy of cotton plants was placed into a circular leaf cup. Five larvae were placed into each cup. To help control for environmental heterogeneity in the performance of the cotton, tests were

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blocked by the location where the cotton was grown. NuCOTN 33B and DP5415 were planted in four sets of paired plots. For each block, leaves were collected from the same set of paired plots and replaced every 2 d. Ten cups per insect group per cotton variety were randomized on a tray for each block. Two blocks were set up with the first egg clutch from both colonies, while the remaining two blocks were set up the next day with larvae from the second egg clutch. As a consequence, egg clutch and block are not independent of each other. In our ANOVA, block effects and cups nested within (block × cotton variety) treatments were considered random sources of error. Insect group was considered a fixed source of variation. Proc Mixed was used to analyze the log-transformed weights and estimated the denominator degrees of freedom based on Satterthwaite's approximation (Littell et al. 1996).

Performance on Cry1Ac and Cry2Aa

The duration of larval development for larvae from the Selected and Parental colonies was compared on non-Bt, Cry1Ac, and Cry2Aa diets. The duration of larval development was measured as the number of days required to finish larval development (days to pupation). The purpose was to determine if the performance of colonies when feeding on Cry1Ac and Cry2Aa was correlated.

Cry1Ac diet was prepared as described above. Cry2Aa was supplied by B. Moar, Auburn University. A concentration of 10 µg per ml Cry2Aa was chosen for investigating larval development. In other work, the expression of Cry2Ab, a similar protein to Cry2Aa, in BollgardII cotton was approx. 10× greater than the expression of Cry1Ac. The Cry1Ac concentration was increased 10-fold to determine our concentration of Cry2Aa used in this experiment.

RESULTS

Generations 1 and 2

The development of *S. exigua* larvae was significantly different on Cry1Ac compared with non-Cry1Ac diet (Wilcoxon test $P < 0.0001$). Days to pupation for the Parental colony during the first generation ranged from 15-28 d when larvae were reared on 1.0 µg/ml Cry1Ac diet. All individuals had pupated by 14 d on the non-Cry1Ac diet.

Individuals from the Selected group produced second-generation offspring that pupated significantly earlier on the Cry1Ac diet than individuals from the Parental, Intermediate, and Slow groups (Fig. 2A; K-W ANOVA H = 149.94; df = 3; P < 0.0001). All groups differed significantly in their development on the toxic diet (P < 0.0001 for all comparisons). Individuals from the Selected group pupated ca. 1 d earlier than the larvae from the Parental colony. On non-Cry1Ac diet there were also significant differences among groups in their days to pupation (H = 79.93; df = 3; P < 0.0001). Greater than 90% of the larvae from the Selected, Intermediate, and Parental groups had pupated by 14 d. The only group to significantly differ from the others was the Slow group. Individuals from the Slow group took significantly longer to pupate (mean ± SE = 13.90 ± 0.10, 14.00 ± 0.08, 16.14 ± 0.08, and 14.77 ± 0.10 d, for Selected, Intermediate, Slow, and Parental, respectively).

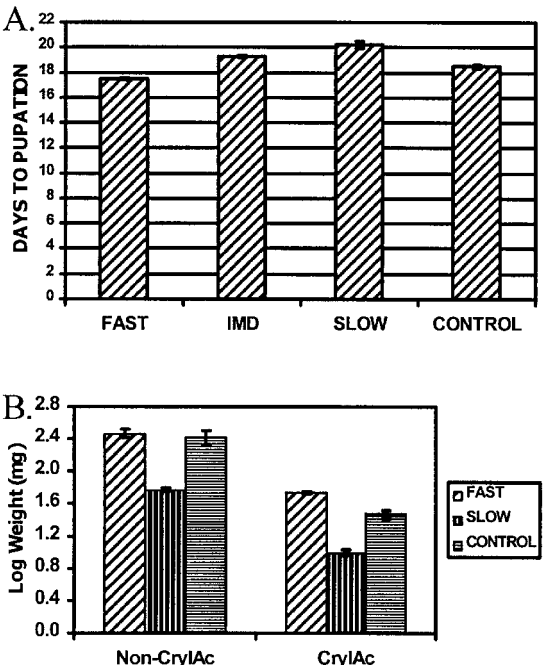

Fig. 2. Second-generation tests of *S. exigua* groups. (A) Days to pupation for mating groups feeding on Cry1Ac diet and (B) average (±SE, mg) log weight of mating groups on non-Cry1Ac and Cry1Ac diets. Bars with the same letter do not differ significantly as determined by Wilcoxon tests (A) and analysis of least-squared means (B).

The 7-d log weights of larvae supported the days to pupation results during the second generation. There were significant differences among the log weights (mg) of the second-generation colonies on non-Cry1Ac diet (F = 158.43; df = 2, 55; P < 0.0001) and Cry1Ac diet (F = 155.15; df = 2, 203; P < 0.0001). On both diets the Slow group was significantly smaller than the Parental and Selected group (Fig. 2B). However, the Selected group grew significantly faster on the Cry1Ac diet than the Parental group, but there was no difference in growth on the non-Cry1Ac diet (Fig. 2B). The ratios of the average log weight on Cry1Ac relative to non-Cry1Ac diet were 0.706, 0.607, and 0.560 for the Selected, Parental, and Slow groups, respectively. In addition, the percentage differences

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for the log weight of groups on Cry1Ac relative to non-Cry1Ac were -29.43, -39.30, and -44.02% for the Selected, Parental, and Slow groups, respectively.

Generation 3

The improved growth of the Selected group continued into the third generation. During the third generation, the Selected group pupated significantly earlier than the Parental group when larvae were fed Cry1Ac diet ($P < 0.0001$; 16.24 ± 0.14 vs. 18.75 ± 0.15 d for the Selected and Parental groups, respectively). Most individuals (>90%) from both groups pupated by 14 d on the non-Cry1Ac diet ($P > 0.6$).

The mean days to pupation significantly differed among generations ($F = 46.65$; df = 2, 445; $P < 0.0001$). All pair-wise comparisons of least-squared means for generations differed from each other (all Bonferroni-adjusted *P*'s < 0.0001). The percentage of the total individuals feeding on Cry1Ac diet that pupated ≤15 d increased with each generation of selection (2.6, 6.2, and 33.3% for generation 1, 2, and 3, respectively). The mean number of days to pupation also decreased after each episode of selection (Fig. 3).

Generation 4

During the fourth generation, the Selected colony was compared with the Parental colony in 10-d tests involving leaves of NuCOTN 33B (Bt cotton) and DP5415 (non-Bt cotton). Two-way ANOVA found significant effects of cotton variety ($F = 84.90$; df = 1, 77.7; $P < 0.0001$), BAW colony ($F = 17.06$; df = 1, 647; $P < 0.0001$) and variety × colony ($F = 10.21$; df = 1, 647; $P = 0.0015$) on larval weights. Tests of least-squared means (Slice option of lsmeans statement, Proc Mixed) found no significant and significant differences between larval weights of Selected and Parental colonies when they were feeding on non-Bt leaves (DP5415 $F = 0.45$; df = 1, 646; $P = 0.5046$) and Bt leaves (NuCOTN 33B $F = 26.30$; df = 1, 648; $P < 0.0001$), respectively. Larvae from the Selected strain were significantly larger than individuals from the Parental strain after 10 d of feeding on Bt cotton leaves.

During the leaf tests, comparisons between the Selected and Parental groups were also made on artificial diets. There were no differences between the two groups after 11 d of feeding on non-Cry1Ac diet ($P = 0.099$, with slightly better growth in Parental group). However, larvae from the Selected group were significantly larger than larvae from the Parental group after feeding for 11 d on Cry1Ac diet (average weights = 151.3 and 75.3 mg, for Selected and Parental, respectively; $F = 37.00$; df = 1, 10.3; $P < 0.0001$).

Cry1Ac and Cry2Aa Tests

Larval development was influenced by larval diet ($F = 157.68$; df = 1,331; $P < 0.0001$; Fig. 5) and the interaction between larval diet and *S. exigua* colony ($F = 4.37$; df = 2,331; $P = 0.0134$). Larvae from the selected colony pupated significantly earlier than larvae from the Parental colony when exposed to Cry1Ac diet ($F = 9.55$; df = 1,331; $P = 0.0022$; Fig. 5). However, there were no significant differences in the number of days to reach pupation between the Parental and selected colonies when larvae fed on non-Bt diet or Cry2Aa diets ($P > 0.6$; Fig. 5).

Discussion

Spodoptera exigua had sufficient genetic variation to respond to selection for improved tolerance of Cry1Ac. After two generations of minimal selection, the Selected group pupated approximately 2 d earlier on Cry1Ac diet than the Parental strain. The better growth of the Selected group on the leaves of Bt cotton supports that the Selected colony had become more tolerant of Cry1Ac

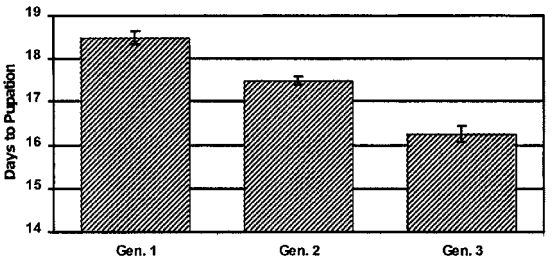

Fig. 3. Mean days to pupation (±SE) during after each generation of selection

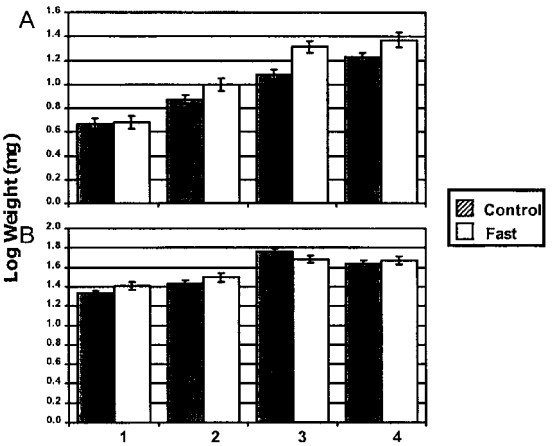

Fig. 4. Mean log weight (±SE, mg) of the Selected and Parental groups when tested on Bt cotton leaves (A) and non-Bt cotton leaves (B) during generation 4.

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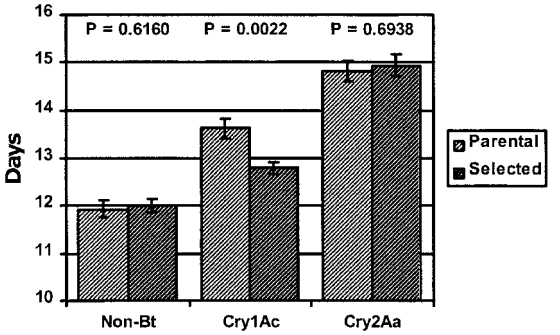

Fig. 5. Mean days to pupation (±SE) when the Selected and Parental colonies are exposed to artificial diets containing the no Bt proteins, Cry1Ac and Cry2Aa.

relative to the parental colony. In addition, the rapid response to selection suggests that there is little recessive gene action for development when larvae are exposed to tissue expressing Cry1Ac.

The growth of larvae on conventional cotton tissue was significantly more rapid than the growth of selected larvae on Cry1Ac-expressing tissue. There is a potential for assortative mating of adults based on the relative growth rates of larvae while feeding on expressing and non-expressing cotton tissue. The degree of assortative mating will be dependent on the overlap of larval development on the two types of tissue. As the population becomes more tolerant of the Cry1Ac tissue, there should be greater overlap in the development of larvae feeding on non-Cry1Ac tissue. Although there is little recessive gene action, one consequence of the greater overlap in larval development and its reduction in positive assortative mating will be to slow the evolution of improved growth when larvae are feeding on Cry1Ac-expressing tissue.

Unlike conventional insecticides used to control late-season populations of *S. exigua*, the continuous expression of Cry1Ac in cotton leaves may create unwanted selection for improved tolerance in field populations that are below economically-damaging densities. However, the lack of a significant relationship between larval development on Cry1Ac and Cry2Aa will delay the response to selection for improved growth on tissue expressing these two toxins or Cry proteins related to these proteins. Stacked varieties may therefore play an important role in the evolution of ecological characters that will influence population development and generation time.

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