Heritability of Tolerance to the Cry1Ab Toxin of *Bacillus thuringiensis* in *Chilo suppressalis* (Lepidoptera: Crambidae)

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ABSTRACT Heritability of *Chilo suppressalis* (Walker) tolerance to the Cry1Ab toxin of *Bacillus thuringiensis* Berliner was estimated using a half-sibling design. Artificial diet with and without Cry1Ab was infested with progenies of 20 males, each mated with 2 females, and mortality was scored 5 d after infestation. The progeny of each female was reared and scored separately. Mean mortality of the 20 families on the Cry1Ab diet was 46.5%. The effects of both male parent and of female parent within male parent were significant. Heritability was estimated to be 0.52, suggesting that a high proportion of phenotypic variation was because of genetic differences. Mortality on the Cry1Ab diet was not correlated with mortality on control diet, indicating that differences among families in tolerance to Cry1Ab were not attributable to differences in general fitness. Our results indicate that "high dose" Bt rice plants may be particularly important for Cry1Ab resistance management in *C. suppressalis* populations.

KEY WORDS *Bacillus thuringiensis*, *Chilo suppressalis*, insecticide resistance, heritability, rice

Chilo suppressalis (WALKER), generally known as the Asiatic rice borer, is a major pest of rice in Europe and temperate Asia and is also widespread in tropical Asia. Many rice varieties with moderate resistance to stem borers have been released (Khush 1995), but high levels of resistance to lepidopterous stem borers and foliage feeders have not been identified in rice germplasm (Heinrichs et al. 1985). Consequently, there has been substantial interest in genetic engineering of rice with toxin genes from *Bacillus thuringiensis* Berliner to enhance resistance to lepidopterous pests, especially stem borers (Fujimoto et al. 1993, Wunn et al. 1996, Ghareyazie et al. 1997, Nayak et al. 1997, Cheng et al. 1998, Datta et al. 1998). As yet Bt rice varieties are not available to farmers, but field tests have begun in China (G. Ye, personal communication).

The long-term effectiveness of Bt rice will depend in part on the genetic potential for the evolution of resistance to Bt toxins in stem borer populations and the implementation of suitable resistance management strategies. Methodologies have been developed to help predict the rate of evolution of insect resistance to insecticides (Roush and McKenzie 1987, Firko and Hayes 1990). Most models assume that resistance is attributable to a mutation at a single locus. In contrast, quantitative genetic models make no assumption regarding the number of genes involved, and the expression of a trait is assumed to depend on environmental as well as genetic factors (Firko and Hayes 1990, Tabashnik 1992). As a tool for resistance risk assessment, quantitative genetics enables predictions to be made regarding the speed and magnitude of genetic change associated with resistance. Quantitative genetic techniques allow the heritability of a trait (i.e., the proportion of the total phenotypic variation as a result of the average effects of genes [Falconer and Mackay 1996]) to be measured. The heritability of a quantitative variable can be estimated using offspring-parent regression, threshold trait analysis (Firko and Hayes 1990, Omer et al. 1993), or sibling analysis (Bull et al. 1982, Tabashnik and Cushing 1989, Firko and Hayes 1991). In full-sibling analysis, 1 male is mated with 1 female and variation within and among families of offspring is compared (Firko and Hayes 1990). In half-sibling analysis, >1 female is mated with each male to produce both full and half-sibling families. Variation among males and among females within males are compared by nested analysis of variance (ANOVA).

In this study, we estimate the heritability of tolerance to the Cry1Ab toxin of *B. thuringiensis* in a population of *C. suppressalis.* We use the term "tolerance" in the sense of the response to a toxin by an individual, family, or population. Cry1Ab is the toxin that has been most often used in rice transformation for stem borer resistance (Fujimoto et al. 1993, Wunn et al. 1996, Ghareyazie et al. 1997, Nayak et al. 1997, Cheng et al. 1998, Datta et al. 1998).

Materials and Methods

Insects. Rice stubble (straw remaining rooted in the soil after harvest) was collected from 5 rice fields ≈10 km apart from each other, in Laguna Province, Philippines. The straw was dissected and *C. suppressalis* pupae were collected and sexed under a dissecting

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microscope. Male and female pupae were transferred to separate 500-ml plastic cups lined with moist filter paper.

Half-Sibling Mating. To produce half-sibling families, we established 100 cages, each consisting of a potted 45-d-old rice plant 'IR62' covered with a cylindrical Mylar cage. IR62 is a semidwarf *indica* rice variety typical of the rice varieties that predominate in tropical irrigated areas (Khush 1995). We have found it to be more suitable for rearing stem borers than comparable varieties. One male and 1 female were released into each cage. The moths were provided with a cotton swab dipped in 10% honey solution, and the honey was renewed every 2 d. Males were transferred to new cages with virgin females after the 1st females with which they were caged had laid eggs. Females were kept in the same cages for further oviposition. To produce approximately synchronous hatching times for the eggs laid by the 2 females mated to each male, the eggs laid by the 1st female were incubated at ≈25°C and those of the 2nd female at 28°C. Egg masses from each of the 2 females mated by the same male, for which eggs hatched on the same day, were used for the bioassays. We were able to establish such half sibling families from 20 males.

Bioassay. One day before expected egg hatch, the egg masses from each female were sterilized separately. The egg masses were soaked in 70% ethanol for 30 s, transferred to 10% chlorine bleach for 5 min, washed 3 times with sterile distilled water, and air dried in a laminar flow hood. Egg masses from each female were placed in separate sterilized vials until hatching began. Two types of artificial diet were prepared (with and without Cry1Ab toxin) and distributed into scintillation vials (≈5 ml/vial). To make 1 liter of diet we added 115.4 g of modified southwestern corn borer diet (Bioserv, Frenchtown, NJ) (21.1 g wheat germ, 25.0 g casein, 25.0 g sucrose, 7.0 salt mixture, 0.2 g linseed oil, 0.1 g cholesterol, 37.0 g corn cob grits) to 7.4 g agar dissolved in water. Water was added to bring the volume to ≈1 liter and when the mixture cooled to below 65°C, 5 g choline chloride, 17.6 g corn starch, 7.4 g Vanderzant vitamin mix (Bioserve), and 4 ml formaldehyde (10%) were added. The Cry1Ab diet contained 0.6 μg/ml activated toxin, which we found to cause ≈50% mortality in preliminary experiments. The toxin was added to the diet immediately after adding the choline chloride, corn starch, and so on. Sixty larvae per female were transferred to scintillation vials containing Cry1Ab diet, and 30 larvae per female were transferred to vials containing control diet, with 10 larvae per vial. The vials were incubated in a growth chamber at 28°C and a photoperiod of 14:10 (L:D) h. Mortality was recorded 5 d after infestation.

The Cry1Ab toxin was a gift of Mycogen (San Diego, CA) and was provided in the form of toxin encapsulated in dead transgenic *Pseudomonas fluorescens* (Gelernter 1990). To prepare the toxin for incorporation into artificial diet, 100 mg of the formulated toxin was dissolved overnight at 37°C in 5 ml of 50 mM sodium bicarbonate buffer (pH 9.5) with 10 mM dithiothreitol and 1 mg/ml trypsin. Unsolubilized material was removed by centrifugation and the protein concentration of the supernatant was determined by the method of Bradford (1976).

Data Analysis. The arcsine-transformed larval mortality data were analyzed by nested ANOVA using PROC general linear model of the SAS package (SAS Institute 1998), to detect differences among males (families) and females within males (subfamilies). The heritability of tolerance to Cry1Ab by *C. suppressalis* was estimated using the formulas of Tabashnik and Cushing (1989) and Bull et al. (1982). In these calculations, mortality is considered as a threshold character with tolerance to the insecticide as the underlying continuous variable.

Results and Discussion

Mortality per family (i.e., all progeny of a single male) on the Cry1Ab diet ranged from 12.5 to 89.8% with a mean of 46.5% (Table 1). On the Cry1Ab diet, the effect of male parent on mortality was highly significant ($F = 8.32$; df = 19, 195; $P < 0.0001$), as was the effect of female parent within males ($F = 3.15$; df = 20, 195; $P < 0.0001$). On control diet, there was a significant effect of male parent on mortality ($F = 3.05$; df = 19, 78; $P = 0.003$) but not of female parent within male parent ($F = 0.48$; df = 20, 78; $P = 0.96$). Mean control mortality was ≤7% for all families except 1, in which mean control mortality was 20%. When the family with high control mortality was removed and the data were reanalyzed, the effect of male parent on control diet was not significant ($F = 1.22$; df = 18, 74; $P = 0.26$). We do not know the reason for the high control mortality in the one family. If this family also had the highest mortality on Cry1Ab diet, it would suggest that the family had lower general fitness compared with the rest of the families tested. However, the mean mortality of progeny of the same male parent on Cry1Ab diet (34%) was not high compared with other male parents (mean = 46.5%).

Because the effect of male parent on control mortality was not significant when we excluded the 1 family with the very high control mortality and the effect of female parent within male parents was not significant, fitness differences on the artificial diet that we used do not appear to be the reason for the differential mortality of male parent and of female parent within male parents on Cry1Ab diet. There was no correlation among families between mortality on Cry1Ab and control diet ($r = 0.02$, $P = 0.90$, $n = 20$ males), further suggesting that differential mortality of the families on Cry1Ab diet was not the result of general fitness differences but rather to their differential tolerance to Cry1Ab.

The estimated heritability for mortality for tolerance to Cry1Ab was calculated to be 0.52, meaning that 52% of the variation in tolerance was the result of genetic differences. Two families had mortality on the Cry1Ab diet considerably higher (89.8 and 82.1%) than the range observed for the rest of the families (12.5–64.6%). We therefore examined what propor-

Table 1. Percentage mortality of *C. suppressalis* larvae used in calculation of heritability of Cry1Ab tolerance with a half-sibling design

Male	Female	Diet (treatment)		Male	Female	Diet (treatment)	
		Cry1Ab	Control			Cry1Ab	Control
1	1	90	0	11	1	40.0	0
	2	89.6	3.3		2	35.0	0
	Mean	89.8	1.7		Mean	37.5	0
2	1	79.2	3.3	12	1	50.0	6.7
	2	85.0	0		2	23.3	0
	Mean	82.1	1.7		Mean	36.7	3.3
3	1	73.3	0	13	1	25.6	0
	2	55.8	0		2	46.7	0
	Mean	64.6	0		Mean	36.1	0
4	1	40.0	6.7	14	1	33.3	0
	2	86.7	6.7		2	38.3	0
	Mean	63.3	6.7		Mean	35.8	0
5	1	43.3	0	15	1	18.0	13.3
	2	81.7	0		2	50.0	26.7
	Mean	62.5	0		Mean	34.0	20
6	1	75.0	0	16	1	50.0	6.7
	2	43.3	0		2	15.0	3.3
	Mean	59.2	0		Mean	32.5	5
7	1	58.3	0	17	1	31.7	3.3
	2	56.7	0		2	25.0	0
	Mean	57.5	0		Mean	28.3	1.7
8	1	60.0	0	18	1	36.7	0
	2	45.0	0		2	18.3	0
	Mean	52.5	0		Mean	27.5	0
9	1	26.7	0	19	1	28.3	0
	2	71.7	0		2	18.3	0
	Mean	49.2	0		Mean	20.8	0
10	1	31.7	0	20	1	8.3	0
	2	63.3	0		2	16.7	6.7
	Mean	47.5	0		Mean	12.5	3.3

tion of the estimated heritability was attributable to these 2 families. When we removed the family with the highest mortality from our calculations, the effect of female parent within male parent was significant and the heritability estimate decreased to 0.29. When the 2 families with the highest mortality were removed, the effect of female parent within male parent was still significant and the heritability estimate decreased to 0.14. However, in both cases the effect of male parent was not significant ($P > 0.05$).

This study demonstrated heritable variation in *C. suppressalis* tolerance to a Bt toxin, and thus the potential for evolution of resistance to Bt rice in this species. It is not surprising that we detected heritable variation for tolerance to Cry1Ab, but the heritability estimate obtained from this small sample of individuals was relatively high. A review of heritability estimates of insecticide resistance in insect pests reported a range of 0.05–0.85 with a mean of 0.29 (Omer et al. 1993). In most of these studies, a higher heritability estimate was observed for conventional synthetic insecticides (mean = 0.42) than the estimates for *B. thuringiensis* spore/crystal mixtures and purified toxins (mean = 0.13). Tabashnik (1994) reported that realized heritability estimates for *B. thuringiensis* ranged from 0.22 to 0.61 with a mean of 0.33 in 11 experiments with *Plodia interpunctella* (Hübner) compared with a mean of 0.12, 0.04, and 0.13 in *Leptinotarsa decemlineata* (Say), *Aedes aegypti* (L.), and 6 lepidopterous species (other than *P. interpunctella*), respectively. However, realized heritability estimates are known to underestimate heritability values (Falconer and Mackay 1996).

Estimates of heritability and related parameters are specific to the populations and environments concerned, and thus predictions based on heritability estimates should be made with caution (Keiding 1986, Firko and Hayes 1990, Tabashnik 1992). If populations of *C. suppressalis* also show high heritability of tolerance to Cry1Ab in transgenic rice under field conditions, then resistance could evolve quickly under some scenarios. An example would be widespread planting of a *cry1Ab*-transformed cultivar that failed to cause high mortality of larvae heterozygous at resistance-associated loci (Tabashnik 1994) (i.e., a "low dose" cultivar). Our interest in quantitative variation for Cry1Ab tolerance in rice stem borers was stimulated in part by concern over Bt rice lines with low or declining titers of toxin. For example, Alinia et al. (2000) studied a rice line with a *cry1Ab* gene under control of the PEP carboxylase promoter. They found that insect resistance declined substantially at early reproductive stage in plants that received no nitrogen fertilizer, and also at flowering stage even in well-fertilized plants.

"High dose" plants serve to decrease the heritability of resistance by causing high mortality of larvae heterozygous at resistance-associated loci (Tabashnik 1994, Gould 1998). Our results indicate that it may be particularly important to develop high dose Bt rice plants in areas where *C. suppressalis* is an important pest. Modeling studies suggest that high dose plants

can provide effective resistance management when used in combination with refuges of non-Bt plants (Tabashnik 1994, Gould 1998). Studies are in progress at the International Rice Research Institute to evaluate the potential effectiveness of various spatial scales of refuges for resistance management in Bt rice (Cohen et al. 1997).

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