Induction and transmission of *Bacillus thuringiensis* tolerance in the flour moth *Ephestia kuehniella*

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The use of *Bacillus thuringiensis* (Bt) endotoxins to control insect vectors of human diseases and agricultural pests is threatened by the possible evolution of resistance in major pest species. In addition to high levels of resistance produced by receptor insensitivity (5, 16, 17), several cases of tolerance to low to medium levels of toxin have been reported in laboratory colonies of lepidopteran species (3, 18). Because the molecular basis of some of these cases of tolerance to the toxin are not known, we explored alternative mechanisms. Here, we present evidence that tolerance to a Bt formulation in a laboratory colony of the flour moth *Ephestia kuehniella* can be induced by preexposure to a low concentration of the Bt formulation and that the tolerance correlates with an elevated immune response. The data also indicate that both immune induction and Bt tolerance can be transmitted to offspring by a maternal effect and that their magnitudes are determined by more than one gene.

coagulation | endotoxin tolerance | immune induction | maternal effect | melanization

Endotoxins from the spore-forming bacterium, *Bacillus thuringiensis* (Bt), are the most valuable biopesticides used currently in commercial agriculture, forest management, and mosquito control (1). However, despite the use of Bt endotoxins in transgenic crops covering >11.4 million hectares (2), the precise details of how endotoxins bind to gut cells to kill insects are poorly understood (3). This limitation impedes our understanding of potential mechanisms of insect resistance to Bt endotoxins other than the loss or modification of receptors (4, 5), altered proteolysis of protoxin and/or toxin (6, 7), and repair and/or replacement of damaged cells (8). Although few insect species have developed resistance in the field (3, 9), genetic resistance to Bt-endotoxins has been selected in several species in the laboratory (3, 10).

Here, we describe an investigation of a laboratory culture of the flour moth *Ephestia kuehniella* for a possible correlation between systemic immune induction and Bt tolerance. We present evidence that Bt tolerance can be induced by low concentrations of a commercial Bt formulation, which acts as an immune elicitor. An elevated immune status can be transmitted to the next generation by a maternal effect, and the magnitude of the immune response is determined by more than one gene.

Experimental Procedures

Bt Formulation. The toxin used was a commercial formulation of Bt endotoxins (Syngenta, North Ryde, NSW, Australia), consisting of Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, proteins, and spores.

Insects. The initial Bt-susceptible colony of *E. kuehniella* was a long-established laboratory colony that had been maintained without selection for >100 generations. Larvae and adults were reared, and bioassays were conducted at 25°C with a 14:10 h light/dark schedule. The larval diet was a 10:2:1 mixture of oat bran/wheat germ/brewer's yeast.

Selection. The selection process involved allowing successive generations of females to lay eggs onto standard diet into which the Bt formulation had been mixed. Five separate lines were maintained, and each was founded by five breeding pairs. Initially, each line was reared on a concentration of 50 ppm of the Bt formulation. For each generation, the concentration was increased: first to 100 ppm, then to 500, 1,000, and finally 2,000 ppm of the Bt formulation. The five lines were then pooled to form a single Bt-tolerant colony.

Melanization Assays. We chilled 8–10 larvae on ice for 5 min and then washed them first with ice-cold 70% ethanol solution and then with ice-cold PBS. Hemolymph was extracted by cutting off a foreleg and bleeding each larva directly into 1.5 ml of ice-cold PBS. The solution was centrifuged for 5 min at 5,000 rpm, and the cell-free supernatant was transferred to a cuvette. The absorbance was first measured at 280 nm to determine the relative protein concentration, and then the absorbance at 490 nm was recorded every 1 min for 90 min on a 100s spectrophotometer (Varian).

Tolerance Bioassay. Virgin females from the susceptible and tolerant colonies were mated with a male from their respective colonies and then maintained in a plastic bag (8 × 16 cm). After a female laid her eggs, the bag was cut into strips and the number of eggs on each strip was counted. The strips were then placed face-up on diet containing, variously, 0, 100, 500, 1,000, or 2,000 ppm of the Bt formulation. After 7 days, the plastic strips were removed carefully and the number of unhatched eggs remaining was recorded. Larvae were maintained for an additional 28 days, and the number of surviving larvae was counted. There were five replicates for each colony.

Induction Bioassay. We placed 50 adults from the susceptible colony on fresh diet for 12 h. After 18 days, 800 of the resulting susceptible larvae were randomly allotted into 40 replicates of 20 larvae each. Then, 16 replicates were transferred to diet containing 50 ppm of the Bt formulation (induction treatment) and 24 replicates to fresh diet (control treatment). After an additional 48 h, four replicates from each treatment condition were transferred to diet containing, variously: 100, 500, 1,000, or 2,000 ppm of the Bt formulation. An additional four replicates from each of the control treatments were transferred to fresh diet and diet containing 50 ppm of the Bt formulation. All larvae were then maintained for 14 days, and larval survivorship was recorded.

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Abbreviations: Bt, *Bacillus thuringiensis*; LC_{50}, median lethal concentration; S×T, susceptible female × tolerant male; T×S, tolerant female × susceptible male.

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Reciprocal Crosses. Crossing tolerant and susceptible individuals will produce different outcomes depending on the mode of transmission of the phenotypic trait. If tolerance is due to a dominant or semidominant trait, and is not sex-linked, then the susceptibility of the reciprocal crosses will be equal. Assuming that tolerance is based on a recessive trait, we postulate that tolerance is detected only if the gene is sex-linked. Because females are hemizygous in lepidopteran species, tolerance will be observed only in the female offspring from a susceptible female with a tolerant male (female offspring shown in bold): $(X^TY)_F \times (X^SX^S)_M$; F_1, $(X^TX^S)_M$; $(X^SY)_F$; and $X^SY_F \times (X^TX^T)_M$; F_1, $(X^SX^T)_M$;$(\mathbf{X^TY})_F$.

If tolerance is based on maternal transmission, all offspring of tolerant females (shown in bold) will be tolerant. Because similar outcomes are expected if transmission occurs by maternal inheritance (e.g., mitochondrial gene mutation) or maternal imprinting, these modes of inheritance are not excluded at this stage. $T(XY)_F \times S(XX)_M$; F_1, $T(\mathbf{XX})_M$;$T(\mathbf{XY})_F$; and $S(XY)_F \times T(XX)_M$; F1, $S(XX)_M$;$S(XY)_F$.

If tolerance is based on some combination of the crosses shown above, then intermediate outcomes will be observed.

Bioassays using neonate offspring of reciprocal crosses were performed by following the method described above for the tolerance bioassays, with virgin females from the susceptible and tolerant colonies mated with males from their reciprocal colony and then maintained in plastic bags until the female had laid her eggs. There were five replicates per cross.

To test whether the elevated immune response is transmitted by a maternal effect, two additional sets of melanization assays were performed. The first set used hemolymph from the offspring of both susceptible female × tolerant male (S×T) and tolerant female × susceptible male (T×S). In the second set, female offspring from the S×T cross were backcrossed with males from the tolerant colony. Third-instar F_2 (S×T)×T offspring were either fed diet containing a low (50 ppm) concentration of Bt formulation for 48 h or maintained on fresh food, and melanization assays were then performed.

Relationship Between Bt Tolerance and the Rate of the Melanization Reaction. Virgin females from the tolerant colony were mated with males from the susceptible colony and placed individually on fresh diet to produce individual cohorts of full siblings. After 28 days, a melanization assay and a 14-day bioassay with 25 larvae per replicate and five concentrations of the Bt formulation (0, 1,000, 2,000, 4,000, and 8,000 ppm) were performed for each cohort. There were nine replicates.

Statistical Analysis. Median lethal concentration (LC_{50}) values were estimated by probit analysis using POLO-PC software (LeOra Software, Berkeley, CA). Samples for which the 95% confidence intervals did not overlap were considered to be significantly different. Resistance ratio was expressed as the ratio of the LC_{50} of the relevant sample to that of the susceptible colony. All other analyses were performed by using the generalized linear model platform in JMP (Version 4.0.4, SAS Institute, Cary, NC), with continuous factors centered by their means (11).

Results

Relationship Between Bt Tolerance and Immune Response. Five groups of five breeding pairs of *E. kuehniella* were exposed to a low (50 ppm) concentration of the Bt formulation, which potentially can act as an immune elicitor (12). The Bt-formulation concentration was increased to 100, 500, 1,000, and 2,000 ppm in each subsequent generation. Insects developed tolerance in each of the treated groups, whereas the nonexposed colony remained susceptible (Table 1, Tolerant and Susceptible). Given that the founder colonies were small (10 individuals), we asked whether the observed increase in tolerance was associated with an elevated immune response rather than the selection of a preexisting Bt-resistance allele.

Table 1. LC_{50} values for neonate offspring of tolerant, susceptible, and genetic crosses of T×S and S×T adults

Cross	LC_{50} (ppm)	95% C.I.	Slope	RR
Susceptible	231	203–262	2.62	—
S×T	476	372–580	2.39	2
T×S	1,200	1,038–1,372	2.22	5.2
Tolerant	1,816	1,489–2,227	2.02	7.9

Differences in control mortality (mean 3.51%) were not significant ($F = 0.185$, $P = 0.905$). C.I., confidence interval; RR, resistance ratio.

To investigate this hypothesis, we first measured melanization reactions of susceptible and tolerant *E. kuehniella* larvae. Compared with susceptible larvae, cell-free hemolymph (Fig. 1) from tolerant larvae showed significantly increased melanization reactions ($F = 397.1$, $P < 0.0001$), a hallmark of elevated immune responses in insects.

Effects of Pretreatment with a Low Concentration of Bt Formulation. To examine a possible correlation between the immune-induction process and Bt tolerance in the absence of genetic selection, we exposed susceptible *E. kuehniella* larvae to a low concentration of the Bt formulation and subsequently exposed the same larvae to high concentrations of the toxin. Pretreated larvae had a significantly higher LC_{50} value (Table 2) than larvae that were not pretreated. In addition, cell-free hemolymph from susceptible *E. kuehniella* larvae exposed to a low concentration of the Bt formulation for 48 h displayed significantly elevated melanization reaction rates ($F = 4{,}685.7$, $P < 0.0001$) compared with untreated larvae (mean ± SD absorbance at 490 nm after 90 min: induced susceptible, 294 ± 19.8; noninduced susceptible, 15 ± 2.8). This finding suggests that a transient induction of some component(s) of the immune system may be the basis for Bt tolerance in these larvae.

Transmission of Bt Tolerance and Immune Status by a Maternal Effect. Because tolerant insects were cultured on food containing the Bt formulation from egg-hatch onward, if some component(s) of the immune response is the basis for Bt tolerance in these larvae, then neonates must be either immune-induced immediately after emergence or already in an elevated immune state at the time of emergence. The latter process would involve a transmission from parent to offspring, most likely by a maternal effect.

To investigate a possible maternal effect, we performed two crosses, one in which females from the tolerant colony were individually crossed with susceptible males (T×S) and a recip-

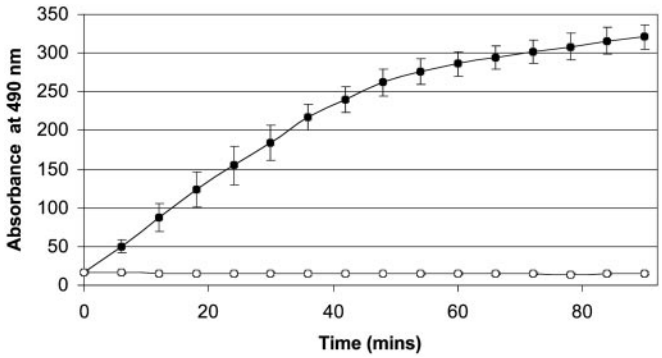

Fig. 1. Melanization assay of cell-free hemolymph from tolerant and susceptible larvae. Bars represent SD. ●, hemolymph from tolerant larvae; ○, hemolymph from susceptible larvae.

Table 2. Effect of preexposure to a low dose of toxin on the LC_{50} of susceptible *E. kuehniella*

Treatment	LC_{50} (ppm)	95% C.I.	Slope	RR
Control	1,940	1,252–4,128	0.94	—
Induction	32,610	8,404 to 1×10^6	0.52	16.8

Second instar larvae were exposed to 550 ppm of the toxin formulation. Control insects were kept on fresh food without toxin. After 2 days, a 14-day bioassay was performed. C.I., confidence interval; RR, resistance ratio.

rocal cross, in which susceptible females were crossed with tolerant males (S×T). When the melanization reaction rates of cell-free hemolymph from the offspring of the two crosses were analyzed, the offspring of the individual S×T crosses showed no signs of melanization, similar to susceptible larvae. In contrast, the cohorts of T×S larvae showed significantly elevated ($F = 82.7, P < 0.0001$), although variable, melanization reaction rates (Fig. 2). This finding suggests that the elevated immune status is transmitted to offspring by a maternal effect. Further, when individual cohorts of 28-day-old T×S larvae were analyzed for tolerance to the Bt formulation, there was a significant positive relationship ($r^2 = 0.966, F = 168.2, P < 0.0001$) between the degree of Bt tolerance and the rate of the melanization reaction (Fig. 2).

Differences in tolerance to the Bt formulation in offspring from the two crosses were investigated by using neonates. T×S neonates had a significantly higher LC_{50} value (Table 1) compared with S×T neonates.

Genetic Disposition for Bt Tolerance and Immune Induction. Comparison of the survival of susceptible and S×T neonates revealed that the LC_{50} of the S×T neonates was significantly higher (Table 1), indicating a genetic contribution to the variation in tolerance to the Bt formulation in addition to the maternal effect. We further examined the genetic contribution by using a backcross, in which any genetic disposition for immune induction was transmitted exclusively by the male line.

Female offspring from the S×T cross were backcrossed with males from the tolerant colony, and offspring were analyzed for melanization reactions. Noninduced larvae from the backcross (S×T)×T showed no significant melanization, similar to susceptible larvae. However, when induced with a low concentration of the Bt formulation, larvae from the (S×T)×T cross showed significantly stronger melanization reactions ($F = 680.1, P < 0.0001$) [mean ± SD absorbance at 490 nm after 90 min: induced (S×T)×T, 409 ± 27.6; noninduced (S×T)×T, 29 ± 4.2], which were also significantly higher ($F = 31.4, P < 0.0001$) than the melanization reactions of induced susceptible larvae [mean ± SD absorbance at 490 nm after 90 min: induced susceptible, 294 ± 19.8, induced (S×T)×T, 409 ± 27.6].

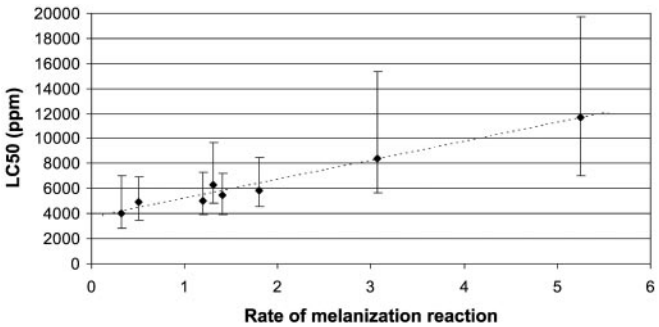

Fig. 2. Relationship between the rate of the melanization reaction [as the slope of the plot of absorbance vs. time (arbitrary units)] of cell-free hemolymph and the LC_{50} values of individual cohorts of 28-day-old T×S larvae. For one replicate, a melanization rate of 7.92 was recorded, but an LC_{50} value could not be calculated because mortality in the 8,000 ppm condition was <10%. Note that the rates of the melanization reactions ranged from 0.32 to 7.92. Dashed line represents fit in linear regression ($r^2 = 0.966$). Bars represent 95% confidence intervals.

Discussion

By exposing larvae from a laboratory culture of *E. kuehniella* to an increasing concentration of a complex formula of endotoxins and spores from *B. thuringiensis*, a tolerant colony, which survived on levels of the Bt formulation that was lethal to the starting colony, emerged within a few generations.

Many observations suggest that the basis of the tolerance may be an elevated immune status. (*i*) The tolerant colony displayed an elevated immune response (Fig. 1) compared with the susceptible colony. (*ii*) Induction of the immune response by a low concentration of the Bt formulation was correlated with a subsequent increase in tolerance to the Bt formulation (Table 2). Because susceptible larvae were immune-induced and exposed to the Bt formulation in the same generation, this experiment excludes the selection of a preexisting resistance allele as a cause of the development of tolerance. (*iii*) The level of the immune response in the T×S larvae, as measured by the rate of the melanization reactions, was variable among single-pair offspring but directly correlated with the extent of Bt tolerance (Fig. 2).

Reciprocal crosses of tolerant and susceptible insects revealed a transmission of the immune induction (Fig. 2) and tolerance (Table 1) from one generation to the next by a maternal effect. The most straightforward explanation for these observations is the incorporation of an immune elicitor into the oocyte by an immune-induced female. The elicitor can interact with embryonic tissues to induce the immune system of the neonate. By the time that the neonate starts feeding, the insect is already induced, thus increasing the chances of surviving the Bt formulation.

Bioassays showed that the S×T neonates were significantly more tolerant than the susceptible neonates, indicating a genetic contribution to the variation in tolerance in addition to the maternal effect. Further, the fact that the level of the immune response and Bt tolerance in the T×S larvae occurred across a range of values suggests that the observed variation in their magnitudes is determined by more than one gene.

Together, the results of this study suggest a model of Bt tolerance based on an elevated immune status. The immune response can be induced by low doses of the Bt formulation or by internal elicitors, which can be transmitted from the mother to her offspring. The magnitude of the maximum possible immune response is determined by more than one gene.

The results from the backcross, in which female offspring from the S×T cross were crossed with males from the tolerant colony, are consistent with this model. No immune induction was observed in the offspring from this backcross, confirming that the elevated immune status in the tolerant colony is based on a transient immune induction, which is initiated in each generation by a maternal effect. However, when offspring from the backcross were immune-induced by a low concentration of the Bt formulation, the observed melanization reaction was significantly greater than that detected in immune-induced susceptible larvae, indicating that the disposition to respond to an elicitor was determined genetically by alleles that were different in the tolerant colony, compared with the susceptible colony.

Attempts to use other elicitors, such as lipopolysaccharide or zymosan, to induce the immune system of *E. kuehniella* were not successful. Given that larvae feed on substrate, which is contaminated frequently by bacteria or fungi, it is possible that some insects, including *E. kuehniella*, *Galleria mellonella*, and *Drosophila melanogaster*, require direct elicitor contact with the hemolymph to be responsive. In this context, it is possible that

low concentrations of the Bt formulation may damage gut epithelium enough to allow elicitors to reach the hemolymph but not enough to be fatal. Additional experiments using elicitors, such as baculoviruses that can cross the gut epithelium, may shed light on the induction process.

The proposed immune-related tolerance mechanism is different from previously described Bt-resistance mechanisms (3) in which the observed reductions in Bt toxicity are explained almost exclusively in terms of alterations to receptor properties on the gut epithelium (4, 5). However, our results in *E. kuehniella* are consistent with recent observations of malaria-refractory *Anopheles gambiae* colonies, which have elevated oxidative activities compared with susceptible insects (13, 14).

Until the molecular mechanism of the Bt tolerance is identified, the immune-based model will remain speculative. One possible mechanism is that soluble immune components are secreted into the gut lumen in immune-induced larvae and interact with the mature toxin, causing its inactivation by a coagulation or melanization reaction. Alternatively, lectin-like immune molecules that mimic the toxin may engage the receptor in internalization reactions without causing osmofragility.

Likewise, the nature and molecular mechanism of transmission of the elicitor remains to be elucidated. It is known that the determination of dorsal–ventral polarity in the *Drosophila* embryo is mediated by a group of genes that are functionally involved in immune-defense reactions of the adult insect (15). Our observations, indicating that immune-induction processes in the embryo are caused by a maternal effect, raises the question of how the developmental and immune functions are separated in space and/or time. One possibility is that regulatory components involved in dorsal–ventral polarity are attached to the chorion and released into the perivitelline egg space, whereas immune elicitors may be stored in the yolk interacting with primordial mesodermal tissues after the dorsal–ventral axis has been established.

The finding that exposure to a low concentration of the Bt formulation resulted in subsequent increased Bt tolerance may also explain the observed decline in susceptibility of larvae with age. Whereas age-dependant Bt toxicity is difficult to understand in the context of resistance mechanisms based solely on receptor inactivation, it fits well with an induced tolerance. Because *Ephestia* larvae reduce food intake after an initial encounter with toxin-contaminated food, older larvae may be able to survive nonfeeding periods long enough for the induction of tolerance to occur. In contrast, younger larvae or neonates with less fat reserves may be unable to survive for sufficient time for induction to provide protection.

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