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Effects of Activated Bt Transgene Products (Cry1Ab, Cry3Bb) on Immature Stages of the Ladybird *Adalia bipunctata* in Laboratory Ecotoxicity Testing

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Abstract Insect-active *Bacillus thuringiensis* (Bt) proteins are expressed by several transgenic crop plants to control certain pests, but nontarget organisms such as ladybirds also can be exposed to these proteins in the field. We developed an improved ecotoxicity testing protocol and conducted feeding trials in a laboratory setting to test for possible adverse effects of different concentrations of microbially produced trypsin-activated Cry1Ab and Cry3Bb toxins on the coccinellid *Adalia bipunctata*. Larval/pupal mortality, development time, and overall body mass accumulation were recorded. Even at the lowest concentration (5 μg/ml), *A. bipunctata* larvae fed with the lepidopteran-active Cry1Ab toxin exhibited significantly higher mortality than the control group. In experiments with the coleopteran-active Cry3Bb, only a concentration of 25 μg/ml resulted in a marginally significantly higher mortality compared to the control. Both experiments revealed a slight decline in mortality at the highest concentration of 50 μg/ml, though this was statistically significant only in the Cry1Ab treatment. No differences were detected for development time and body mass of newly emerged adults. Dilutions of the expression vector pBD10—used as a control to exclude effects of the toxin production method—at concentrations between 10 and 100 μg/ml revealed no significant effects on either of the studied parameters. This suggests that the increased mortality of larvae in the toxin feeding trials was caused directly by the activated Bt toxins and raises questions regarding their commonly postulated specificity and their mode of action in *A. bipunctata*. Implications of the reported results for ladybird populations and their biological pest control functions in transgenic crop ecosystems are discussed.

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Transgenic Bt plants expressing insect-active Cry toxins of the bacterium *Bacillus thuringiensis* (Bt) were developed to control important agricultural pests. Two major transgenic insect-active crops, Bt maize and Bt cotton, are grown on an increasing area in several countries worldwide (James 2007). It is commonly postulated that Bt plants and their expression products, activated Bt toxins, are specifically efficacious against a limited number of target insects, while nontarget organisms, including pollinators and predacious arthropods, are not affected (e.g., Shelton et al. 2002; O'Callaghan et al. 2005). Studies with target species have identified a rather complex mode of action, which involves toxin receptors in their midgut cells (de Maagd et al. 2001; Whalon and Wingerd 2003).

Predators can encounter transgene products like plant-expressed Bt toxins when feeding on plant material, for example, pollen, nectar, or leaf exudates, and, additionally, when preying on other organisms that have consumed transgenic plant tissue or toxin-loaded prey (Harwood et al. 2005; Zwahlen and Andow 2005). Further, prey organisms that have ingested transgene products might not only pass the unchanged transgene product from the plant to the

predator but also modify it by their own metabolism, potentially altering their biochemical activity and becoming more or less harmful to predators (Andow and Hilbeck 2004). Testing of predators for potential adverse effects of the expressed insect-active compounds in transgenic plants is, therefore, important considering their important biological control function as an ecosystem service. Several studies have investigated the effects of transgenic Bt plants on predaceous insects under laboratory conditions. In some cases, the tested species were significantly adversely affected (results that cannot be reliably predicted or explained yet), while in others no effects were observed (for more detail see reviews by Lövei and Arpaia 2005; O'Callaghan et al. 2005; Hilbeck and Schmidt 2006; Romeis et al. 2006).

Ladybirds represent abundant and highly valued biocontrol agents, particularly of aphids, mealy bugs, and mites (Obrycki and Kring 1998; Dixon 2000). Probably most, if not all, species also consume other insects and plant material, such as pollen (Lundgren et al. 2004, 2005). The two-spotted ladybird, *Adalia bipunctata* (L.), is an abundant polyphagous coccinellid species in many ecosystems in Europe (Hodek and Honěk 1996; Omkar and Pervez 2005). It has often been used for ecotoxicological studies with pesticides and was, therefore, selected for this study as a test species with regard to the risk assessment of transgenic crop plants in Europe.

Laboratory ecotoxicity testing forms a core part of the environmental risk assessment (ERA) of Bt plants prior to their commercial release. Standard protocols adopted from testing chemical environmental stressors (like pesticides), as outlined in guidance documents issued by national regulatory agencies and international organizations (USEPA, OECD), are typically used as the scientific basis for testing transgenic insecticidal plants for regulatory purposes. (Mendelsohn et al. 2003; Andow and Hilbeck 2004). While this approach to risk assessment has attracted criticism with regard to the applied protocols stemming largely from the ecotoxicity testing schemes of chemicals (Andow and Hilbeck 2004; Andow et al. 2006; Lang et al. 2007), this type of testing currently still constitutes the main part of nontarget testing in transgenic plants. Therefore, we aimed at improving the existing protocols by using activated toxins, which is what transgenic plants are supposed to produce, and larvae of nontarget organisms instead of adults, as those are supposed to be more susceptible.

In this study, we investigated possible adverse effects of two activated Bt proteins (Cry1Ab, Cry3Bb), which are expressed, for instance, by several commercial transgenic Bt maize lines (*Zea mays*, e.g., MON810 or MON863), on the preimaginal development of *A. bipunctata*. Both toxins target different groups of pest species: Cry1Ab is most efficacious against lepidopteran caterpillars, while Cry3Bb is most active against certain herbivorous coleopteran larvae. As part of an integrated environmental risk assessment, these experiments are intended to provide initial information on possible hazards of these transgene products.

Materials and Methods

Test Dilutions

Stock solutions of all substances tested were provided by Plant Research International, Wageningen, The Netherlands. Bt protoxins (Cry1Ab and Cry3Bb) were cloned in *E. coli* using Cry protein expression vectors based on the plasmid pBD10 (containing either the *cry1Ab* gene or the *cry3Bb* gene), solubilized, and trypsin-activated as described by Bosch et al. (1994). To exclude any effects caused by this microbial production process, feeding trials were also performed with solutions from pBD10; pBD10 is the so-called "empty expression vector cassette" without the *cry* genes. Toxin stock solutions were diluted in deionized water to concentrations of 5, 25, and 50 μg/ml, respectively, and pBD10 stock solutions to concentrations of 10, 50, and 100 μg/ml. The wider range and higher maximum of concentrations employed for pBD10 were chosen to test for its effects at very high doses compared to the toxins. Dilutions were tested for the presence of toxins using ImmunoStrips (Agdia, Elkhart, IN, USA) and stored at a temperature of 4°C during the course of the experiment. As a control treatment for all experimental series, we applied a buffer (50 mM $NaHCO_3$, pH 10, 100 mM NaCl), which had also been used for the production of the stock solutions. At the beginning of each repetition, dilutions and control solutions were filled separately into brown glass vials (25 ml) with a spray-head and stored at a temperature of 4°C.

Insects

All individuals of *A. bipunctata* used in the experiments originated from synchronously laid egg masses purchased from Andermatt Biocontrol, Grossdietwil, Switzerland. Upon arrival, egg masses were immediately transferred into transparent plastic petri dishes (110 mm in diameter) with round filter paper on the bottom and incubated under controlled environmental conditions (25 ± 1.5°C temperature, 70% ± 5% relative humidity, and a photoperiod of 16:8 h [L:D]). No food was offered to the neonate larvae before the start of the experiment, but they were allowed to feed on each other or on unemerged *Adalia* eggs for < 24 h. Cannibalism appears to be important for successful larval development in many ladybird species (Agarwala and Dixon 1992).

Experimental Setup

Transparent plastic petri dishes were prepared as experimental containers for *A. bipunctata* larvae by cutting holes in the center of the lids to allow constant airflow and covering them with fine mesh to prevent the larvae from escaping.

As a food source for the ladybird larvae, which could easily be prepared with the test substances, we used sterilized eggs of the flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), purchased from Andermatt Biocontrol, Grossdietwil, Switzerland. *E. kuehniella* eggs were considered a high-quality food for coccinellids, including *A. bipunctata* larvae compared to aphids (Lanzoni et al. 2004; De Clerq et al. 2005). In each replicate, about 30–50 mg of *Ephestia* eggs (depending on the age of the ladybird larva in order to provide food on an ad libitum basis) was applied with a paintbrush to a small square of plain cardboard (ca. 33 × 33 mm). The eggs were sprayed with the respective treatment dilution and the cardboard was placed on a round filter paper on the bottom of the petri dish. A single ladybird larva (ca. 24 h) was placed into each prepared petri dish using a moist soft paintbrush. To prevent larvae from escaping, all petri dishes were sealed with strips of parafilm along the margin.

The experiments were performed with 30 individual replicates of each concentration of Cry1Ab, Cry3Bb, or pBD10 and independently repeated four times. In each repetition, ladybird larvae from different shipments were used. The same environmental conditions (25 ± 1.5°C temperature, 70% ± 5% relative humidity, and a photoperiod of 16:8 h [L:D]) were applied throughout. Every second day throughout the larval development, mortality and developmental stage of all larvae were recorded and cardboards with sprayed *Ephestia* eggs were renewed. Fourth-instar larvae were checked for pupation every day. After pupation, cardboards were removed and the pupae were checked for emergence of adults every day. Newly emerged adults were weighed on a precision balance (Mettler Toledo AG 104; readability, 0.1 mg) after emergence as soon as their elytra had thoroughly hardened.

Statistical Data Analysis

Each of the three experiments (with Cry1Ab, Cry3Bb, and pBD10, respectively) was analyzed separately for each recorded parameter: total mortality, total preimaginal development time (defined as time between hatching of the first larval stage from egg until emergence of the adult from pupa), and body mass of young adults after emergence (representing preimaginal accumulation in body mass).

Total mortality for each experiment was calculated as the percentage (rate) of all larvae which did not develop to the adult stage. Data sets on mortality in each of the four independent repetitions ($N = 4$) were analyzed using a contingency table analysis following a categorical model. In this model, independence between paired observations is tested with a likelihood ratio test. If this test detected significant independence within a data set, pairwise chi-square tests were used to evaluate differences between the single protein concentrations.

For preimaginal development time and body mass, we calculated the arithmetic means from the individual measurements of each protein concentration within each of the four independent repetitions to produce the data set for further analysis. Untransformed data of all data sets were then tested for normal distribution using a Shapiro-Wilk *W*-test (goodness of fit). If, according to the result of this test, a normal distribution applied to a data set, overall differences between the different concentrations were analyzed with a one-way analysis of variance (ANOVA). If, however, no normal distribution could be detected in a data set, we used the nonparametric Kruskal-Wallis *H*-test to analyze differences in the overall data set. Following the results of the Shapiro-Wilk *W*-tests, a Kruskal-Wallis *H*-test was applied only to analyze differences in the data set for total preimaginal development time of the experiment with pBD10, while all other analyses were conducted with an ANOVA. In all tests, the establishment of significance was based on a probability level of $p < 0.05$. All statistical calculations were conducted with JMP software, version 5.0 (SAS Institute Inc. 2002).

Results

Total Preimaginal Mortality Rates

In the contingency table analysis of our feeding experiment with trypsin-activated Cry1Ab toxin, the likelihood ratio test indicated a highly significant independence between paired observations in the data set of mean preimaginal mortality rates ($\chi^2 = 31.681$; df = 3; $p < 0.0001$). Mortality rates were significantly different between the control treatment (16.7%; mean of the four independent repetitions) and all three concentrations of the toxin. We recorded an 11% higher preimaginal mortality compared to the control ($\chi^2 = 4.126$; df = 1; $p = 0.0422$) when larvae were fed with a concentration of 5 µg/ml. Mortality increased by more than 30% compared to the control when larvae were fed with a concentration of 25 µg/ml ($\chi^2 = 29.665$; df = 1; $p < 0.0001$) and by a little less than 20% compared to the control ($\chi^2 = 11.591$; df = 1; $p = 0.0007$) when larvae were fed with a concentration of 50 µg/ml throughout their entire development (Table 1). Further, compared to the treatments with 5 µg/ml, mortality

Table 1 Mean mortality rates (%) of individual preimaginal stages of *Adalia bipunctata* in laboratory feeding experiments conducted with different concentrations of Cry1Ab, Cry3Bb, and pBD10

Experiment	Sprayed substance	Concentration	Mortality (%)							
			n	Larval stage					Pupa	Total*
				1st	2nd	3rd	4th	Total		
a	Cry1Ab	0 µg/ml (ctrl)	120	14.2	1.0	1.0	0.0	15.8	1.0	16.7^{c}
		5 µg/ml	120	24.2	3.3	0.0	0.0	26.7	1.1	27.5^{b}
		25 µg/ml	120	44.2	3.0	1.5	3.1	48.3	1.6	49.2^{a}
		50 µg/ml	120	31.7	4.9	1.3	0.0	35.8	0.0	35.8^{b}
b	Cry3Bb	0 µg/ml (ctrl)	120	20.8	1.1	0.0	0.0	21.7	0.0	21.7^{b}
		5 µg/ml	120	15.8	5.0	2.1	0.0	21.7	0.0	21.7^{b}
		25 µg/ml	120	24.2	5.5	4.7	1.2	32.5	1.2	33.3^{a}
		50 µg/ml	120	25.8	3.4	2.3	0.0	30.0	1.2	30.8^{ab}
c	pBD10	0 µg/ml (ctrl)	120	7.5	0.9	0.9	1.9	10.8	3.7	14.2^{a}
		10 µg/ml	120	10.0	1.9	1.9	2.0	15.0	0.9	15.8^{a}
		50 µg/ml	120	9.2	0.9	3.7	0.0	13.3	2.0	15.0^{a}
		100 µg/ml	120	9.2	0.0	1.9	0.0	10.8	3.7	14.2^{a}

Note: ctrl, control. *n*, number of tested individuals. * Values with different superscript letters are significantly different at a level of $p < 0.05$ within the same experiment

rates were significantly higher in the treatments with 25 µg/ml ($\chi^2 = 12.040$; df = 1; $p = 0.0005$) but not in the treatments with 50 µg/ml ($\chi^2 = 1.930$; df = 1; $p = 0.1648$). Preimaginal mortality was significantly different for Cry1Ab concentrations of 25 versus 50 µg/ml ($\chi^2 = 4.379$; df = 1; $p = 0.0364$).

For the feeding experiment with Cry3Bb, the likelihood ratio test revealed a nearly significant result ($\chi^2 = 6.830$; df = 3; $p = 0.0775$). Pairwise chi-square tests were performed to avoid ignoring marginally significant differences between one of the tested concentrations and the control. Within the data set, the total mean mortality rates of *A. bipunctata* preimaginal stages were significantly different between the treatment with a toxin concentration of 25 µg/ml and the control (33.3%; $\chi^2 = 4.120$; df = 1; $p = 0.0424$). The control and a concentration of 5 µg/ml revealed an identical value for this parameter (21.7% mortality). At a concentration of 50 µg/ml, mortality was not significantly different (30.8%; $\chi^2 = 2.615$; df = 1; $p = 0.1059$) compared to the control (Table 1). Also, mortality at a toxin concentration of 50 µg/ml was not significantly different from that at toxin concentrations of 5 µg/ml ($\chi^2 = 2.615$; df = 1; $p = 0.1059$) and of 25 µg/ml ($\chi^2 = 0.172$; df = 1; $p = 0.6782$).

No independence of paired observations could be detected in the experiment with pBD10 ($\chi^2 = 0.181$; df = 3; $p = 0.9806$). Here, total mean mortality rates were not significantly different between the control treatment (14.2%) and either one of the three different concentrations (10 µg/ml, 15.8%; 50 µg/ml, 15.0%; 100 µg/ml, 14.2%; Table 1).

Total Preimaginal Development Time and Body Mass of Newly Emerged Adults

No statistically significant differences in the total preimaginal development times of *A. bipunctata* (between hatching of the first larval stage from egg and emergence of adult beetles; ANOVA, $F = 0.587$, df = 3, $p = 0.6350$) were documented between the control treatment and any of the three concentrations of the activated Bt toxin Cry1Ab (Table 2). Values ranged between 15.7 and 16.6 days. Similarly, there were also no statistically significant differences in the total preimaginal development times ($F = 2.249$; df = 3; $p = 0.1350$) between the control and any of the three different concentrations of Cry3Bb toxin. Values varied from 15.8 ± 0.1 days in the control to 16.2 ± 0.2 days in the treatment with a concentration of 25 µg/ml. Also, no statistically significant differences (Kruskal-Wallis *H*-test: $H = 0.975$; df = 3; $p = 0.8073$) were documented in the data sets for individuals fed with different concentrations of pBD10.

Body mass of newly emerged adults was not significantly different (ANOVA: $F = 0.877$; df = 3; $p = 0.4802$; Table 2). Mean body mass of adults was not significantly different between the control treatment and the three Cry3Bb toxin concentrations applied to their larval food (ANOVA: $F = 1.226$; df = 3; $p = 0.3429$). Also, no statistically significant differences were observed between the three concentrations of pBD10 and the respective control treatment (ANOVA: $F = 0.102$; df = 3; $p = 0.9574$).

Table 2 Mean developmental times (days, with standard error) of the individual preimaginal stages and mean body mass of newly emerged adults (mg, with standard error) of *Adalia bipunctata* in laboratory feeding experiments conducted with different concentrations of Cry1Ab, Cry3Bb, and pBD10

Experiment	Sprayed substance	Concentration	Development time (days)							Body mass (mg)	
			n	Larval stage					Pupa	Total*	*n*	Adult*
				1st	2nd	3rd	4th	Total				
a	Cry1Ab	0 μg/ml (ctrl)	100	2.8 ± 0.3	2.2 ± 0.0	2.4 ± 0.2	2.7 ± 0.2	10.1 ± 0.5	6.4 ± 0.1	16.6 ± 0.6[a]	100	10.5 ± 0.5[a]
		5 μg/ml	87	2.8 ± 0.3	2.2 ± 0.1	2.4 ± 0.2	2.6 ± 0.1	10.0 ± 0.5	6.5 ± 0.1	16.5 ± 0.5[a]	87	10.4 ± 0.6[a]
		25 μg/ml	61	2.8 ± 0.2	2.1 ± 0.2	2.1 ± 0.0	2.8 ± 0.3	9.7 ± 0.3	6.0 ± 0.3	15.7 ± 0.6[a]	61	10.4 ± 0.7[a]
		50 μg/ml	77	2.8 ± 0.3	2.2 ± 0.2	2.2 ± 0.1	2.6 ± 0.2	9.9 ± 0.2	6.3 ± 0.1	16.2 ± 0.3[a]	77	11.9 ± 1.2[a]
b	Cry3Bb	0 μg/ml (ctrl)	94	2.6 ± 0.3	2.2 ± 0.2	2.2 ± 023	2.9 ± 0.2	10.0 ± 0.2	5.8 ± 0.2	15.8 ± 0.1[a]	94	10.0 ± 0.3[a]
		5 μg/ml	94	2.8 ± 0.3	2.1 ± 0.1	2.3 ± 0.1	3.1 ± 0.2	10.1 ± 0.2	5.9 ± 0.2	16.1 ± 0.2[a]	94	9.5 ± 0.5[a]
		25 μg/ml	80	2.7 ± 0.2	2.3 ± 0.2	2.4 ± 0.2	3.1 ± 0.2	10.4 ± 0.2	5.8 ± 0.3	16.2 ± 0.2[a]	80	9.1 ± 0.3[a]
		50 μg/ml	83	2.8 ± 0.3	2.2 ± 0.1	2.3 ± 0.1	3.2 ± 0.2	10.5 ± 0.2	5.8 ± 0.2	16.3 ± 0.1[a]	83	9.1 ± 0.4[a]
c	pBD10	0 μg/ml (ctrl)	103	2.3 ± 0.1	1.9 ± 0.0	2.2 ± 0.1	2.9 ± 0.4	9.2 ± 0.6	5.6 ± 0.1	14.8 ± 0.5[a]	103	10.2 ± 0.5[a]
		10 μg/ml	101	2.4 ± 0.2	1.8 ± 0.1	2.1 ± 0.1	2.9 ± 0.4	9.2 ± 0.7	5.8 ± 0.1	15.1 ± 0.6[a]	101	9.8 ± 0.6[a]
		50 μg/ml	101	2.4 ± 0.2	1.8 ± 0.0	2.0 ± 0.0	2.9 ± 0.4	9.2 ± 0.6	5.9 ± 0.1	15.0 ± 0.6[a]	101	10.1 ± 0.4[a]
		100 μg/ml	103	2.4 ± 0.2	1.8 ± 0.1	2.0 ± 0.1	3.0 ± 0.5	9.3 ± 0.7	5.8 ± 0.1	15.1 ± 0.6[a]	103	10.0 ± 0.9[a]

Note: ctrl, control. *n*, number of tested individuals for each parameter. * Values with the same superscript letter are not significantly different at a level of $p < 0.05$ for a parameter within the same experiment

Discussion

Based on the common knowledge of the mode of action and the efficacy of the two tested Bt toxins to certain groups of target species, Lepidoptera or herbivorous Coleoptera larvae, it would not be expected that a predacious coleopteran nontarget species exhibits susceptibility to the lepidopteran-active protein Cry1Ab. However, the increased preimaginal mortality rates in our laboratory feeding trials suggest a direct toxic effect of Cry1Ab and, to a much lesser extent, of the coleopteran-active Cry3Bb in their trypsin-activated form to the predator *A. bipunctata* under the experimental conditions applied. A further unexpected finding is the slight decline in preimaginal mortality at the highest concentrations (50 µg/ml) of both toxins compared to the medium concentrations used in our experiment (25 µg/ml). This observation suggests the lack of a typical dose-response relationship in the studied system.

The highest mortality occurred during the early larval stages (Table 1). Affected individuals died quickly, without long times of sickness, and surviving individuals did not show longer development times or lower body mass values than controls. While this suggests that sublethal effects do not exist with regard to these parameters, effects on other parameters (e.g., fecundity, longevity) cannot be excluded and should be investigated in additional studies. Although we observed no indication of a feeding deterrent behavior, this aspect should be given attention, as reduced feeding and decreased food utilization could also impose a higher mortality, which has been reported from herbivores (Deml et al. 1999; Hussein et al. 2005). On the other hand, toxin concentrations should be quantified throughout the course of feeding experiments to ensure that degradation of toxins is not responsible for the lack of mortality in later larval stages.

To explain our observations, we consider that additional studies on the mode of action in nontarget species are necessary, including histological and molecular aspects (Hilbeck and Schmidt 2006). As even in target pests the mode of action of Bt toxins is not fully understood and new aspects are still being discovered (Crickmore 2005), the sum of unexplained effects observed here might indicate a different mode of action of the Bt toxin in predacious arthropods, and other nontarget organisms as well. In a recent paper, interactions of Bt toxins and the community of midgut bacteria were suggested to play a key role in Bt toxicity (Broderick et al. 2006). Such indications should be followed scientifically also in the light of other recent studies reporting unexpected effects (Rosi-Marshall et al. 2007; Bøhn et al 2008). Further, genetically determined differences in the sensitivity of nontarget test organisms cannot be ruled out to explain our findings. Such differences might lead to a lethal response in one genotype and the lack of a recognizable response in the other. Systematic studies with different genotypes of predators should be conducted to test this hypothesis.

Initial experiments, like the ones conducted in this study, provide important data on toxicological responses of nontarget organisms to Bt toxins. Nonetheless, they cannot simply be extrapolated to describe the potential ecological impacts of Cry proteins in the field, but have to be complemented with experiments under ecologically more realistic conditions (Lang et al. 2007).

To assess the ecological relevance of such a study and the risk that a given nontarget species encounters in the field, it is important to consider whether this species can be exposed to harmful transgene products in similar concentrations. *A. bipunctata* is a polyphagous ladybird which consumes a range of different aphid species, but their suitability as prey differs from species to species. The use of coccids and diaspids as alternative prey was also reported as well as the consumption of pollen in the absence of aphids (Omkar and Pervez 2005). Under laboratory conditions, aphids feeding on Cry1Ab-expressing maize (event Bt11) were shown not to incorporate the Bt toxin (Table 3), because it is, apparently, not transported in the phloem of these plants (Raps et al. 2001; Dutton et al. 2002). Exposure of ladybirds to Bt toxins in Cry1Ab maize fields is, therefore, rather unlikely as long as they feed solely on aphids. For other plants, especially dicotyledonous crops, it is not known whether phloem sap does contain

Table 3 Amount of Bt toxin in potential food sources of ladybirds from transgenic maize crop ecosystems

Food source	Transgenic expression product	Event	Toxin level	Reference
Pollen	Cry1Ab	Bt176	1.1–2.3 µg/g fw	AGBIOS (2008)
		MON810	7.9–10.3 µg/g fw	AGBIOS (2008)
	Cry3Bb	MON863	10–81 µg/g fw	AGBIOS (2008)
			34.7–101.0 µg/g fw	Duan et al. (2002)
		MON88017	25 µg/g dw	Canadian Food Inspection Agency (2006)
Rhopalosiphum padi (aphid)	Cry1Ab	Bt11	<0.001 µg/g fw	Dutton et al. (2005)

Note: fw, fresh weight; dw, dry weight

Bt toxins, but Cry proteins were found in low-concentration (4–6 ng/g fresh weight) in cotton aphids, *Aphis gossypii* Glover, and can be transferred into aphid-based food chains including ladybirds (Zhang et al. 2004, 2006). Contents of Cry3 toxins in aphids have not been studied to date.

Ladybird larvae and adults also consume plant material in the form of pollen, often to overcome food shortages (Lundgren et al. 2005). By feeding on pollen of transgenic Bt plants, larvae are indeed exposed to transgene products expressed by the plants. Amounts of Bt toxins in pollen can vary considerably between different transgenic maize lines and also within the same line, with values exceeding the toxin concentrations tested in this study, e.g., in Cry3Bb-expressing MON863 (Table 3). Duan et al. (2002) and Lundgren and Wiedenmann (2002), however, found no significant adverse effects of pollen from MON863 maize fed to larvae of the ladybird *Coleomegilla maculata* DeGeer, which could be in agreement with our finding that *A. bipunctata* larvae exhibited a lower susceptibility to Cry3Bb toxin than to Cry1Ab.

Consumption of alternative insect prey, which have ingested transgene products from plants, might also expose ladybirds to Bt toxins. Different species of herbivores and predators in Cry1Ab maize fields were found to incorporate the toxin at concentrations up to 2.59 μg/g, and also adult ladybirds of North American species in Cry1Ab maize fields were shown to contain low amounts of Cry1Ab, ranging from 0.2 to 1.3 μg/g (Harwood et al. 2005, 2007). Also, no published data exist on Cry3 toxin concentrations in arthropods. As the food choice of predators can change considerably from year to year in the same crop (Schmidt 2006), the uptake of Bt toxins from different food sources might also be different over time. To date, few studies have been published regarding the possible accumulation of Bt toxins in food webs. While some literature implies that Bt toxins do not accumulate in food webs (Harwood et al. 2005), Obrist et al. (2006) reported fluctuating concentrations in spider mites, *Tetranychus urticae* Koch (Acarina: Tetranychidae), that occasionally were three times higher than in maize leaves they had fed on. Zhang et al. (2006) found Bt concentrations in a ladybird to be higher than in its aphid prey. Thus, it cannot be excluded that ladybird larvae can be exposed to potentially hazardous concentrations of Cry1Ab or Cry3Bb, particularly when transgenic plants are cultivated extensively and for longer periods of time.

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