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Biological activity of Cry1Ab toxin expressed by Bt maize following ingestion by herbivorous arthropods and exposure of the predator *Chrysoperla carnea*

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Abstract. A major concern regarding the deployment of insect resistant transgenic plants is their potential impact on non-target organisms, in particular on beneficial arthropods such as predators. To assess the risks that transgenic plants pose to predators, various experimental testing systems can be used. When using tritrophic studies, it is important to verify the actual exposure of the predator, i.e., the presence of biologically active toxin in the herbivorous arthropod (prey). We therefore investigated the uptake of Cry1Ab toxin by larvae of the green lacewing (*Chrysoperla carnea* (Stephens); Neuroptera: Chrysopidae) after consuming two Bt maize-fed herbivores (*Tetranychus urticae* Koch; Acarina: Tetranychidae and *Spodoptera littoralis* (Boisduval); Lepidoptera: Noctuidae) by means of an immunological test (ELISA) and the activity of the Cry1Ab toxin following ingestion by the herbivores. Moreover, we compared the activity of Cry1Ab toxin produced by Bt maize to that of purified toxin obtained from transformed *Escherichia coli*, which is recommended to be used in toxicity studies. The activity of the toxin was assessed by performing feeding bioassays with larvae of the European corn borer (*Ostrinia nubilalis* (Hübner); Lepidoptera: Crambidae), the target pest of Cry1Ab expressing maize. ELISA confirmed the ingestion of Bt toxin by *C. carnea* larvae when fed with either of the two prey species and feeding bioassays using the target pest showed that the biological activity of the Cry1Ab toxin is maintained after ingestion by both herbivore species. These findings are discussed in the context of previous risk assessment studies with *C. carnea*. The purified Cry1Ab protein was more toxic to *O. nubilalis* compared to the plant-derived Cry1Ab toxin when applied at equal concentrations according to ELISA measurements. Possible reasons for these findings are discussed.

Key words: biological activity, Chrysopidae, ELISA, Neuroptera, non-target effects, risk assessment, transgenic plants

Introduction

A major concern regarding the deployment of insect resistant transgenic plants is their potential impact on non-target arthropods, in particular on natural enemies that are important for natural pest regulation (Groot and Dicke, 2002; Dutton et al., 2003). To assess the risks of insect resistant transgenic plants, it has been suggested to follow a tiered testing system going from laboratory 'worst case' to field studies (Cowgill and Atkinson, 2003; Dutton et al., 2003; Poppy and Sutherland, 2004). 'Worst case' laboratory studies in which the insecticidal protein is offered directly to the test organisms at excessive concentrations are followed by extended laboratory studies in multitrophic testing systems, which include natural routes of exposure to the toxin as well as species interactions. When assessing non-target effects of insect resistant transgenic plants on natural enemies by performing such multitrophic feeding experiments it is important to verify that representatives of the lower trophic level (prey or host arthropod) contain the insecticidal protein in a biologically active form, given that an organism is only at risk if it is actually exposed to the hazard source. For Bt maize, it has been shown that different herbivorous arthropod species ingest variable amounts of Cry1Ab toxin (Head et al., 2001; Raps et al., 2001; Obrist et al., 2005). Using Enzyme-linked Immuno-Sorbent Assays (ELISA), Dutton et al. (2002) measured Cry1Ab concentrations in *Tetranychus urticae* Koch (Acarina: Tetranychidae) which were of the same order of magnitude as those found in Bt maize. In contrast, 1st instar *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) contained only one fourth of these concentrations after feeding on Bt maize.

To assess the risks of Bt maize on natural enemies, various studies have been performed at different tiers (for review see Dutton et al., 2003; O'Callaghan et al., 2005). In particular, *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), an important predator in many maize growing areas, has thoroughly been studied, since this predator was found to be negatively affected by Cry1Ab in a first tier ('worst case') study by Hilbeck et al. (1998a). The authors reported reduced survival of immature *C. carnea* when purified and trypsinized Cry1Ab toxin produced by *Escherichia coli* was mixed into an artificial diet at a concentration of 100 $\mu g\ ml^{-1}$. However, another study by Romeis et al. (2004) revealed that larvae of *C. carnea* were not affected when toxin from the same source was incorporated in a sucrose solution. In tritrophic studies, *C. carnea* was found to be negatively affected in both development and survival when fed lepidopteran

larvae (*S. littoralis* and *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae)) reared on Bt maize (Hilbeck et al. 1998b; Dutton et al., 2002). In contrast, Dutton et al. (2002) found no detrimental effects when the predator was offered Bt maize-fed *T. urticae*, a herbivore unaffected by the Cry1Ab toxin. This finding was surprising since the amount of toxin measured in *T. urticae* was four times higher than that measured in *S. littoralis*. Dutton et al. (2003) and Romeis et al. (2004) suggested that the negative effects observed on *C. carnea* were most likely not caused by susceptibility of the predator to the toxin but were due to the fact that the lepidopteran larvae were affected by the toxin. Such prey quality-mediated effects have been reported in other studies with insect resistant transgenic plants (Ashouri et al., 2001; Couty et al., 2001; Bernal et al., 2002; Bell et al., 2003).

The discrepancy of the observed effects on *C. carnea* when feeding on *T. urticae* and *S. littoralis* may also be due to other factors. The toxin may, for example, be broken down by salivary enzymes during ingestion by a natural enemy before reaching the gut as suggested by Armer et al. (2000). In particular, predators using extraoral digestion such as heteropteran, neuropteran or coleopteran species (Cohen, 1995) may break down the toxin during ingestion. The actual exposure of a natural enemy when feeding on Bt-containing prey can be verified by measuring the toxin content in the natural enemy itself. Another possible explanation is that the toxin in certain herbivores may be deactivated or degraded by gut proteases into a non-toxic form, which is still measured with ELISA. In fact, different protein detection methods have already been reported to deliver inconsistent results. For example, Pang et al. (1999) showed that digested *B. thuringiensis* δ-endotoxin (Cry1Aa) appeared to be intact when analysed by native protein techniques. The protein however, exhibited no insecticidal activity and sequencing data revealed a cleavage site in domain II. Furthermore, effectiveness of Bt toxins can be influenced by secondary plant compounds (Sivamani et al., 1992; Appel and Schultz, 1994; Olsen and Daly, 2000) or ingested particles in the herbivore gut (Ben-Dov et al., 2003). All these factors could influence the actual exposure of natural enemies to bioactive Cry1Ab in herbivores.

The objective of this study was to investigate the actual exposure of a predator to Cry1Ab toxin, using the system Bt maize – *T. urticae*/*S. littoralis* – *C. carnea*. The uptake of Cry1Ab toxin by *C. carnea* when feeding on *T. urticae* and *S. littoralis* kept on Bt maize was measured using ELISA, and the biological activity of Cry1Ab toxin following ingestion by each of the two herbivore species was investigated by means of a feeding bioassay with the target lepidopteran

pest *O. nubilalis*. Moreover, the activity of Cry1Ab toxin expressed by Bt maize was compared to that of purified Cry1Ab toxin produced by transformed *E. coli*, which has often been used for first tier toxicological studies (MacIntosh et al., 1990; Hilbeck et al., 1998a; Mendelsohn et al., 2003; Romeis et al., 2004).

Material and methods

Plants

Transgenic Bt maize (Event Bt11, NT4640Bt; Syngenta, formerly Northrup King) (referred to as Bt^+) expressing a synthetic gene encoding a truncated version of the Cry1Ab protein derived from *Bacillus thuringiensis* subsp. *kurstaki* HD-1 and the corresponding non-transformed near isogenic line (NT4640) (Bt^-) as a control were used for insect rearing and experiments. In this transgenic maize variety the Cry1Ab expression is driven by the constitutive CaMV35S promoter. Plants were grown individually in plastic pots (3 l) in the greenhouse at 24 ± 4°C, 70 ± 10% RH and fertilised using 6% N:6% P_2O_5:26%K_2O at a concentration of 1 g l^{-1} with every watering. Seven- to nine-week-old plants were used for all experiments (6th–9th leaf stage). Plant material used for ELISA and feeding bioassays was collected from the 5th oldest leaf and frozen at −80°C.

Insects

Eggs of *O. nubilalis* were obtained from INRA (Institut National de la Recherche Agronomique, Le Magneraud, France). Egg masses were stored at 5°C for a maximum of 2 days and subsequently kept in a climatic chamber (25 ± 1°C, 70 ± 10% RH, 16:8 L:D) until egg hatch and initiation of the experiment.

Separate colonies of *T. urticae* were kept on either Bt^- or Bt^+ maize plants in the greenhouse, at environmental conditions of 24 ± 4°C, 70 ± 10% RH. Spider mites were collected in a tray kept underneath infested leaves by shaking the leaves using a stick. Thereafter, they were either offered to *C. carnea* or transferred into Eppendorf tubes and stored at −80°C for ELISA and for extraction solutions (described below).

Eggs of *S. littoralis* were provided by Syngenta (Stein, Switzerland) and kept at 25 ± 1°C, 70 ± 10% RH, 16:8 L:D. Upon emergence, larvae from 2–3 egg masses were transferred into a cellophane bag

(20.5 cm×40 cm) which was fixed on the 5th leaf of a whole maize plant (7–9 weeks old, 6th–9th leaf stage) and sealed with clips to prevent escape. After having fed for 24 h on Bt^- or Bt^+ plants, larvae were collected and either offered alive to *C. carnea* or stored as described above for *T. urticae*.

Eggs of *C. carnea* were collected from our permanent laboratory colony (Romeis et al., 2004) and kept separately in a climatic chamber (25 ± 1°C, 70 ± 10% RH, 16:8 L:D) until they hatched. Experiments were conducted with food-deprived 1st instars (12–24 h old).

ELISA

Quantification of Cry1Ab toxin was conducted with ELISA kits from EnviroLogix Inc. (Portland, Maine, USA). Cry1Ab standards at concentrations 0, 0.5, 2.5 and 5 ppb were used as calibrators. Spectrophotometric measurements were conducted with a microtiter plate reader (Dynatech MR 5000) at 450 nm and data were analysed using the software package Biolinx 2.0 (Dynatech Laboratories Inc.) and Dynex Revelation G 3.2 (Dynex Technologies).

Purified Cry1Ab toxin

Purified Cry1Ab toxin (M. Carey, Dept. Biochemistry, Case Western Reserve University, Cleveland, Ohio) was produced from Cry1Ab protoxin from *B. thuringiensis* subsp. *kurstaki* HD-1 that was expressed as a single gene product in *E. coli*. Inclusion bodies containing Cry1Ab protoxin were dissolved and trypsinized and the Cry1Ab toxin was isolated using high-performance liquid chromatography (HPLC) (Pusztai-Carey et al., 1994). All experiments were performed with protein from the same aliquot.

Bt uptake by 1st instar Chrysoperla carnea

In order to verify if Bt content in the predator correlates with the amount of ingested Bt-containing prey, individual weights of first instar *C. carnea* were measured on a microbalance (Mettler Toledo, MX5, division $d = 1$ μg; tolerance ± 2 μg, Switzerland). Larvae were subsequently placed individually in vials (25 mm Ø, 7 mm high) containing either adult *T. urticae* (sorted out under the binocular) or 1st instar *S. littoralis* which had been kept on Bt^- or Bt^+ (as described above) and allowed to feed *ad libidum* for 3.5 h. After feeding, larvae were weighed again and then frozen at −80°C. To obtain enough

material for quantifying Bt toxin in predators using ELISA, 70–80 whole ground *C. carnea* fed with *S. littoralis* larvae, and 30–40 *C. carnea* fed with *T. urticae*, were taken for one sample. For all Bt^+ treatments three samples were collected to obtain three independent replicates of the ELISA. One sample was taken for the Bt^- treatment to verify that there was no cross-reaction in the negative samples. Furthermore, three samples of the respective prey (approximately 8 mg of *S. littoralis* and 1 mg of *T. urticae*) were taken to determine the Bt toxin concentration. Bt^+ samples were extracted at the following concentrations (mg material per ml extraction buffer): 8 and 1 mg ml^{-1} for Bt^+-fed *S. littoralis* larvae and *T. urticae*, respectively; 16 and 2 mg ml^{-1} for *C. carnea* after consuming Bt^+-fed *S. littoralis* larvae or *T. urticae*, respectively. Each extraction solution was diluted (2× and 4×) for ELISA measurements. All control samples (Bt^-) were analysed undiluted.

Activity of Cry1Ab toxin in herbivores

In order to verify the biological activity of Cry1Ab toxin following ingestion by *T. urticae* and *S. littoralis*, we conducted two bioassays. For both bioassays, extraction solutions of Bt^+-fed *T. urticae* and *S. littoralis* larvae were incorporated into an artificial diet, which was subsequently offered to *O. nubilalis* larvae. Weight of *O. nubilalis* larvae was used as a parameter for the activity of the Cry1Ab toxin, because susceptibility of neonate larvae is reflected by a restricted increase of larval weight at sublethal toxin concentrations (Sims and Holden, 1996; Marcon et al., 1999; Head et al., 2001).

Extraction solutions for Ostrinia nubilalis *feeding bioassays*
Prior to the assays, seven extraction solutions were prepared, representing the following treatments: *S. littoralis* larvae (Bt^- and Bt^+), *T. urticae* (Bt^- and Bt^+), maize leaf (Bt^- and Bt^+) and purified Cry1Ab toxin. The solutions containing *T. urticae*, *S. littoralis* and maize leaves were made at equal proportions of 120 mg sample material per ml ELISA extraction buffer. For this purpose 1.2 g *S. littoralis* larvae, 0.8 g *T. urticae* and 0.8 g leaf material (both Bt^- and Bt^+ for each sample type) were macerated in 10, 6.67, and 6.67 ml extraction buffer, respectively. After centrifugation (10 min, 1200×*g*) the supernatant was stored at 5°C. The solution with purified Cry1Ab toxin was prepared by adding 100 μg to 1 ml extraction buffer and by diluting it to get a solution of 200 ng toxin ml^{-1}. In addition, a solution consisting of pure extraction buffer was prepared for a control

diet to determine whether the added arthropod or plant materials (Bt^-) had any impact on *O. nubilalis*.

An ELISA was performed with all the extraction solutions as described above. Each Bt^+ solution was diluted (3×, 6×, 12×, 24× and 48× for *S. littoralis*, 25×, 50×, 100×, 200× and 400× for *T. urticae* and leaf material, 10×, 20×, 40×, 80×, 160× for purified toxin) in order to get a range of measurable concentrations. The control solutions (Bt^- arthropod and leaf material) were measured undiluted.

1st bioassay

The purpose of the 1st bioassay was to determine whether the Cry1Ab toxin contained in *S. littoralis* larvae and *T. urticae* is biologically active when compared to Cry1Ab in Bt^+ maize and to a control (Bt^- samples). Therefore, equal amounts of each sample material (i.e., extraction solutions containing *S. littoralis*, *T. urticae*, maize leaf each Bt^- and Bt^+, respectively) were incorporated in meridic diet and offered to neonate *O. nubilalis* larvae.

A standard quantity of 2.5 ml from each extraction solution (Bt^- and Bt^+ from *S. littoralis*, *T. urticae*, maize leaf, respectively) was mixed each with 2.5 ml extraction buffer. This quantity was chosen because preliminary experiments have shown that the expected toxin quantities contained in these solutions caused a measurable impact on weight increase of *O. nubilalis* larvae. Each of the prepared 5 ml solutions was incorporated into 45 g of liquid agar based Ivaldi–Sender diet (Bathon et al., 1991). To avoid degrading the Bt toxin, the temperature of the liquid diet was kept below 60 °C. The diets were distributed in Petri dishes (9 cm×1.6 cm) and covered with tissue paper. After cooling, the solidified diets were cut into pieces of approximately 0.5 g and distributed into cells of bioassay trays (C-D International, Pittman, NJ). Individual 1–12 h old *O. nubilalis* larvae were introduced to each of the cells, which were subsequently sealed with a vented acetate cover (C-D International, Pittman, NJ). Forty *O. nubilalis* larvae were tested per treatment. After 7 days of incubation in the climatic chamber (25 ± 1°C, 70 ± 10% RH, 16:8 L:D), the weight of each *O. nubilalis* was recorded. Since mortality was low (<5%) and did not differ among treatments (Chi-square test; $\chi^2_3 = 0.006$, $p = 1.0$), data of dead larvae, or larvae that escaped during the experiment were not included in the analyses.

All statistical analyses were computed in STATISTICA (version 6, Statsoft Inc., Tulsa, USA). After verifying that there were no statistical differences among *O. nubilalis* weights of all control groups (pure extraction buffer, Bt^--fed *S. littoralis*, Bt^--fed

T. urticae, and Bt^- leaves) (ANOVA, $F_{3,154}=1.7$, $p=0.17$) these datasets were pooled for further analyses. Since variances of Bt^+ treatments were not homogeneous, comparison of mean weights of *O. nubilalis* feeding on the different Bt^+ diets and the control were analysed using Kruskal–Wallis ANOVA. *Post hoc* comparisons of mean ranks were made using Mann–Whitney *U*-test with Bonferroni correction. Six pairwise comparisons of all treatments lead to an adjusted $\alpha=0.008$.

In addition, a dose-response curve was drawn for the decreasing *O. nubilalis* weights as a function of increasing Cry1Ab toxin concentrations in the different Bt^+ diets. The concentrations in the diets were derived from the ELISA measurements of the extraction solutions. The model was determined with purified Cry1Ab toxin prior to the assay using non-linear regression analyses and was described with the function $Y=(a+bX^c)^{-1}$.

2nd bioassay

The aim of the second bioassay was to determine whether Cry1Ab toxin in Bt^+ maize-fed *S. littoralis*, *T. urticae* and in Bt^+ maize have equal degrees of biological activity. Therefore, the Cry1Ab toxin concentration was kept constant in all diets by adapting the quantities of extraction solutions to be added to the *O. nubilalis* diets. These quantities were calculated based on the ELISA measurements of the extraction solutions. In addition, the activity of plant-derived Cry1Ab toxin was compared to that of purified toxin using the same method.

Solutions of identical Cry1Ab concentration were prepared by mixing the appropriate quantity of each extract (4.22 ml of *S. littoralis*, 0.67 ml of *T. urticae* and 1.0 ml of leaf solution each Bt^- and Bt^+, and 4.71 ml of the solution containing purified Cry1Ab) with extraction buffer to yield a total of 5 ml of extraction solution of a toxin concentration of 350 ng ml^{-1}. This solution was added to 45 g of meridic diet as described above. This resulted in a concentration of 7 ng g^{-1} diet. This concentration was chosen because it caused easily detectable effects on weights of *O. nubilalis* larvae in preliminary assays. The following steps in this experiment were performed as described for the 1st feeding bioassay.

After verifying that there were no statistical differences among *O. nubilalis* weights of all control groups (buffer, Bt^--fed *S. littoralis*, Bt^--fed *T. urticae*, and Bt^- leaves) (ANOVA; $F_{3,139}=0.83$, $p=0.48$) these datasets were pooled for further analyses. Mortality did not differ among treatments (Chi-square test; $\chi^2_4=0.006$, $p=1.0$) and data

of larvae that escaped or died during the experiment were not taken into account for the analyses. Comparisons of means were made as described above, but with one additional treatment (purified Cry1Ab toxin), leading to 10 pairwise comparisons with a Bonferroni-adjusted $\alpha = 0.005$.

Results

Bt uptake by 1st instar Chrysoperla carnea

Mean weights (±SE) of *C. carnea* larvae increased from 59.7 ± 0.5 to 113.1 ± 2.0 µg (n = 124) after feeding on *T. urticae* and from 60.7 ± 0.4 to 115.5 ± 1.5 µg (n = 250) after feeding on *S. littoralis* larvae for 3.5 h. This corresponds to a weight increase of a factor of approximately 1.9. ELISA measurements revealed that the toxin content in Bt^+-fed *T. urticae* was approximately 10 times higher than that in Bt^+-fed *S. littoralis*. Accordingly, the Bt toxin concentration in *C. carnea* was about 10 times higher when fed with Bt^+-fed *T. urticae* than when fed with Bt^+-fed *S. littoralis* (Figure 1). No Cry1Ab toxin was detected in *C. carnea* larvae fed control herbivores (Bt^--fed *S. littoralis* and Bt^--fed *T. urticae*).

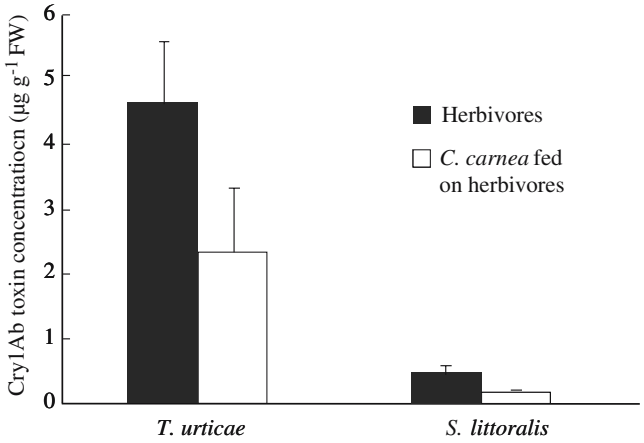

Figure 1. Mean (+SE) Cry1Ab toxin concentrations (µg g^{-1} fresh weight) in Bt^+ maize-fed herbivores (*Tetranychus urticae* and *Spodoptera littoralis*) and in 1st instar *Chrysoperla carnea* after having fed on each herbivore (n = 3 samples each consisting of 40–80 individuals).

Activity of Cry1Ab toxin in herbivores

ELISA of extraction solutions

Highest Bt toxin concentrations were measured in the extraction solution containing Bt^+-fed *T. urticae* (525.4 ng ml^{-1}), followed by the Bt^+ leaf extraction solution (350.1 ng ml^{-1}) and the Bt^+-fed *S. littoralis* extraction solution (82.9 ng ml^{-1}). A concentration of 74.4 ng ml^{-1} was measured in the solution containing purified Cry1Ab toxin. This corresponds to approximately 37% of the value that would have been expected from the amount added to the solution (200 ng ml^{-1}). Traces of Cry1Ab toxin (below the limit of quantification ranging from 0.5 to 1 ng ml^{-1}) were measured in the control extraction solutions. Trace amounts in Bt^--fed spider mites have been detected earlier (Dutton et al., 2002), and could be attributed to cross-reaction with other proteins resulting from the high sample/buffer ratio at which the undiluted controls were analysed.

1st bioassay

The levels of Cry1Ab toxin in the diets offered to *O. nubilalis* were calculated from the ELISA measurements of the extraction solutions. The resulting concentrations were 4.1, 26.3 and 17.5 ng g^{-1} diet for the Bt^+-fed *S. littoralis*, Bt^+-fed *T. urticae*, and Bt^+ leaf, respectively.

Mean weights of *O. nubilalis* larvae feeding for 7 days on diets containing an equal quantity of either Bt^+-fed *S. littoralis*, Bt^+-fed *T. urticae*, Bt^+ leaf material or control extraction solutions differed significantly among treatments (Kruskal–Wallis ANOVA, $H_{3,269} = 139.9$; $p < 0.0001$) (Figure 2). Pairwise comparisons of means showed that weights of control insects were significantly higher compared to those of any of the Bt^+ treatments and revealed a clear difference between weights of *O. nubilalis* kept on Bt^+-fed *S. littoralis* and Bt^+-fed *T. urticae* diet (Mann–Whitney *U*-test for all comparisons $p < 0.0001$). No statistically significant difference was found between weights of *O. nubilalis* kept on Bt^+-fed *T. urticae* and Bt^+ leaf diet when using the adjusted $\alpha = 0.008$ ($U = 492$, $p = 0.037$). When *O. nubilalis* weight is plotted against the Cry1Ab concentrations in the diets calculated from ELISA measurements of extraction solutions, a strong dose-response curve is obtained ($Y = (0.135 + 0.006X^{1.255})^{-1}$, $R^2 = 0.56$, $F_{3,266} = 887.7$, $p < 0.0001$) (Figure 2).

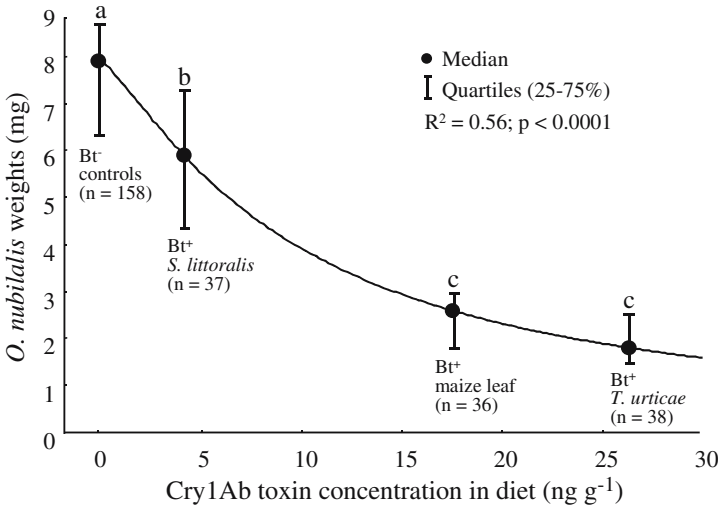

Figure 2. Median of weights of *Ostrinia nubilalis* larvae (mg ± quartiles) after feeding 7 days on diets containing different quantities of Cry1Ab due to the addition of equal quantities of Bt^+-fed *Spodoptera littoralis*, Bt^+-fed *Tetranychus urticae*, Bt^+ maize leave, or control (Bt^-) extraction solutions. Different letters above the bars represent differences among treatments (Mann–Whitney *U*-test with Bonferroni-adjusted $\alpha = 0.008$). In addition, a dose-response curve representing *O. nubilalis* weights as a function of the Cry1Ab toxin concentrations in the diets is shown. The model is described by the function $Y = (0.135 + 0.006X^{1.255})^{-1}$.

2nd bioassay

Mean weights of *O. nubilalis* larvae feeding for 7 days on diets containing equal toxin concentration (7 ng g^{-1}) originating from Bt^+-fed *S. littoralis*, Bt^+-fed *T. urticae*, Bt^+ leaf, purified Bt toxin and control extraction solutions were different among treatments (Kruskal–Wallis ANOVA, $H_{4,\ 290} = 94.0$; $p < 0.0001$) (Figure 3). Pairwise comparisons showed that none of the treatments containing either Bt^+ plant or Bt^+-fed arthropod extraction solutions differed from each other when using the adjusted $\alpha = 0.005$ (Mann–Whitney *U*-test for *S. littoralis* vs. *T. urticae*: $U = 498.5$, $p = 0.03$; *S. littoralis* vs. leaf: $U = 523$, $p = 0.04$; *T. urticae* vs. leaf: $U = 634$, $p = 0.72$). However, all of these treatments were statistically different from the control (*S. littoralis*: $U = 1930$, $p = 0.003$; *T. urticae*: $U = 1252.5$, $p < 0.0001$; leaf: $U = 1370$, $p < 0.0001$). Purified Cry1Ab toxin was shown to cause a significantly stronger effect on weights of *O. nubilalis* larvae compared to the plant-derived Bt^+ treatments, although toxin concentrations in the diets were equal according to ELISA (all $p < 0.0001$).

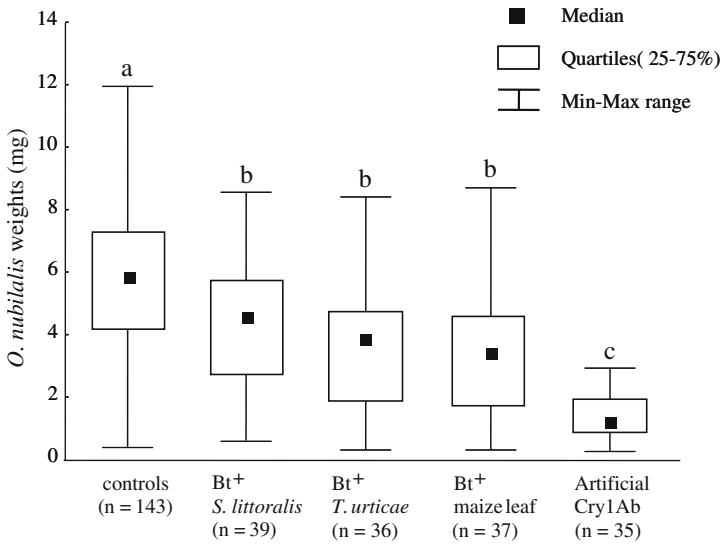

Figure 3. Median of weights of *Ostrinia nubilalis* larvae (mg ± quartiles) after feeding 7 days on diets containing Cry1Ab at a concentration of 7 ng g^{-1} caused by the addition of different quantities of Bt^+-fed *Spodoptera littoralis*, Bt^+-fed *Tetranychus urticae*, Bt^+ maize leaf, or artificial Cry1Ab toxin extraction solutions compared to a control. Different letters represent differences among treatments (Mann–Whitney *U*-test with Bonferroni-adjusted $\alpha = 0.005$).

Discussion

Risk assessment of insect-resistant transgenic plants with respect to natural enemies should include investigations of their exposure as well as their susceptibility to the transgene product. The exposure of species of higher trophic levels occurs following ingestion of food (e.g., herbivores) containing the active, insecticidal protein. Our results confirm that the predator *C. carnea* ingests the Cry1Ab toxin, when feeding on Bt maize-reared *T. urticae* and *S. littoralis* larvae. In addition, we have shown that the Cry1Ab toxin uptake by *C. carnea* varied according to the amount of toxin measured in the herbivores entailing a 10 times higher exposure when feeding exclusively on Bt^+-fed *T. urticae* than when feeding on Bt^+-fed *S. littoralis*. Cry1Ab toxin concentrations measured in *C. carnea* (approximately half of the concentration measured in the respective prey) corresponded well to the concentrations we would expect according to their food uptake (weight gain of a factor of 1.9 in a 3.5 h period). This indicates that *C. carnea* takes up the Cry1Ab toxin equally, not depending on the herbivore species or the toxin concentration in the herbivore. From

our results it appears that the toxin is not modified when ingested by the predator and we assume that exposure of *C. carnea* will correlate with Cry1Ab toxin concentration in any other herbivore it may consume.

Our feeding bioassays have shown that the biological activity of Cry1Ab toxin ingested by both *T. urticae* and *S. littoralis* was maintained resulting in measurable effects on weights of *O. nubilalis* larvae, which consumed diet incorporating extracts from the respective prey. The growth inhibition of susceptible *O. nubilalis* larvae as a function of the toxin concentrations (measured with ELISA), corresponded well to the model which was established to describe the dose-response curve of Cry1Ab toxin ($Y=(a+bX^c)^{-1}$). Moreover, all diets containing toxin originating from maize plants (Bt^+-fed *T. urticae*, Bt^+-fed *S. littoralis*, Bt^+ leaf) caused similar effects on weights of *O. nubilalis* larvae when applied at equal concentrations (based on ELISA measurements). These results show that the activity of Cry1Ab toxin in both Bt^+-fed *T. urticae* and Bt^+-fed *S. littoralis* remains the same as in Bt^+ maize, and that the toxin is not deactivated in the herbivore gut. The findings that Bt toxin ingested by herbivores remains active stands in agreement with previous studies by Head et al. (2001), who tested the activity of purified Cry1Ab toxin following ingestion by *O. nubilalis* and aphids.

The fact that *C. carnea* is exposed to higher concentrations of biologically active Cry1Ab toxin when feeding on Bt^+-fed *T. urticae* than when feeding on Bt^+-fed *S. littoralis*, indicates that other mechanisms are responsible for the previously reported negative effects on *C. carnea* when offered Bt^+-fed lepidopteran larvae (Hilbeck et al., 1998b; Dutton et al., 2002). The most probable explanation is that *C. carnea* is affected by feeding on a food source of reduced quality rather than by the toxin itself. This assumption is supported by the studies by Romeis et al. (2004) who could not detect direct toxic effects on *C. carnea* larvae, when these were fed with sucrose solution containing purified Cry1Ab at 10,000-fold higher concentrations than those found in *S. littoralis* larvae. Chrysopids are known to have prolonged development when feeding on low quality food (Prinicpi and Canard, 1984). *Spodoptera littoralis*, as a lepidopteran species, was shown to be adversely affected by the Cry1Ab toxin when feeding on Bt maize (Dutton et al., 2002; 2005). Given that *S. littoralis* is a poor quality prey even without having been exposed to the toxin (Dutton et al., 2002) such prey-quality mediated effects on *C. carnea* are likely to occur when feeding on sublethally affected (sick) *S. littoralis*. An alternative hypothesis, to explain that effects on *C. carnea* were only

detected when fed with Bt-containing *S. littoralis* larvae is that the Cry1Ab toxin in the mid-gut of this herbivore may be present in a more active form than in Bt maize or Bt-fed *T. urticae*. The Cry1Ab protein in maize is expressed in a truncated form compared to the protoxin expressed by *B. thuringiensis* (Koziel et al., 1993). However, this does not necessarily mean that the plant-derived toxin is solubilised and in a biologically active form (Federici, 2002). Solubilisation of the Cry1Ab is mainly caused by the lepidopteran-specific alkaline pH of the gut juices whereas truncation is performed by various mid-gut proteases (Choma et al., 1990; Gill et al., 1992). Since *S. littoralis* is susceptible to the Cry1Ab (Dutton et al., 2002; 2005), it can be assumed that activation processes (solubilisation and/or cleavage) occur in its mid-gut. The fully activated toxin in the *S. littoralis* gut may cause adverse effects on *C. carnea* whereas the original, less processed protein in the mid-gut of *T. urticae* would not elicit such effects. However, to our knowledge, no evidence supporting this hypothesis has been provided to date and recent studies revealing that the toxin does not bind in *C. carnea* mid-gut make this hypothesis even less plausible (Rodrigo et al., 2004; R. de Maagd, personal communication, 2004).

Our results show that the purified Cry1Ab toxin produced by *E. coli* is more toxic to *O. nubilalis* larvae than the plant-originated Cry1Ab toxin when applied at equal concentrations according to the ELISA measurements. This difference could either be based on aberrant ELISA measurement leading to variable toxin concentrations in the diets (i.e., the activity of the purified toxin was underestimated by the ELISA), or could be caused by differing activities of Cry1Ab at the same concentration (i.e., the biological activity of the purified toxin exceeds that of the plant-derived toxin). The ELISA constantly detected only about one third of the *E. coli*-produced toxin that was actually added to the solution. This was also found in previous analyses at various dilutions (unpublished results) and may lead to the conclusion, that the ELISA underestimates the toxicity of the purified toxin. Although the source of the discrepancy in our ELISA data could not be determined, speculations explaining differences in the properties of plant-derived and *E. coli*-produced toxin can be made, for example by considering the method of production and/or state of activity. Differences may exist in the stability of the Cry1Ab toxins of different origin which could influence their degradation. Indications for this assumption are given by Palm et al. (1994) who reported that plant-derived toxin degraded within a shorter period than purified Cry1Ab toxin obtained from transformed *E. coli* in the soil. Finally,

the discrepancy could also be attributed to the presence of plant or arthropod material in the diet which may reduce the susceptibility of the lepidopteran larvae (Ben-Dov et al., 2003) or to toxin–plant compound interactions influencing the toxicity of the Cry1Ab protein (Olsen and Daly, 2000).

In our studies, we confirm that the Cry1Ab toxin remains biologically active when ingested by two herbivore species and that the toxin is more concentrated in *T. urticae* than in *S. littoralis* larvae. *Chrysoperla carnea* was therefore exposed to higher concentrations of Cry1Ab toxin when consuming Bt^+ maize-fed *T. urticae* than when consuming Bt^+ maize-fed *S. littoralis* larvae. The results of the present study provide further evidence that *C. carnea* is not susceptible to Cry1Ab, because no effects were found on the predator when fed with spider mites containing the toxin (Dutton et al., 2002). Thus, previously reported negative impacts on *C. carnea* following consumption of Bt^+ maize-fed *S. littoralis* resulted from a reduction in the prey quality, because the lepidopteran larvae themselves were affected by the Bt toxin.

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