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Cry1Ab protein levels in phytophagous insects feeding on transgenic corn: implications for secondary exposure risk assessment

Graham Head, Christopher R. Brown, Mark E. Groth & Jian J. Duan*
Monsanto Company, Ecological Technology Center, 800 North Lindbergh, St. Louis, MO 63141, USA
**Author for correspondence (E-mail: jian.j.duan@monsanto.com)*

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Abstract

Enzyme-linked immunosorbent assays (ELISA) and bioassays were used to estimate levels of Cry1Ab protein in four species of phytophagous insects after feeding on transgenic Bt-corn plants expressing Cry1Ab protein or artificial diets containing Cry1Ab protein. The level of Cry1Ab in insects feeding on sources containing the Cry1Ab protein was uniformly low but varied with insect species as well as food source. For the corn leaf aphid, *Rhopalosiphum maidis* (Fitch), feeding on diet solutions containing Cry1Ab protein, the level of the protein in the aphid was 250–500 times less than the original levels in the diet, whereas no Cry1Ab was detected by ELISA in aphids feeding on transgenic Bt-Corn plants. For the lepidopteran insects, *Ostrinia nubilalis* (Hübner), *Helicoverpa zea* (Boddie), and *Agrotis ipsilon* (Hufnagel), levels of Cry1Ab in larvae varied significantly with feeding treatment. When feeding for 24 h on artificial diets containing 20 and 100 ppm of Cry1Ab, the level of Cry1Ab in the larvae was about 57 and 142 times lower, respectively, than the original protein level in the diet for *O. nubilalis*, 20 and 34 times lower for *H. zea*, and 10 to 14 times lower for *A. ipsilon*. Diet incorporation bioassays with a susceptible insect (first instar *O. nubilalis*) showed significant Cry1Ab bioactivity present within whole body tissues of *R. maidis* and *O. nubilalis* that had fed on diet containing a minimum of 20 ppm or higher concentrations (100 or 200 ppm) of Cry1Ab, but no significant bioactivity within the tissues of these insects after feeding on transgenic Bt-corn plants. The relevance of these findings to secondary exposure risk assessment for transgenic Bt crops is discussed.

Introduction

Bacillus thuringiensis (Berliner) is a naturally occurring gram-positive bacterium commonly present in soil. Various strains of *B. thuringiensis* (Bt) are capable of producing crystal (Cry) proteins (δ-endotoxins) or inclusion bodies that have selective insecticidal effects against different groups of insects (see reviews in Schnepf et al., 1998). Microbial preparations containing Bt cry proteins (as well as cell bodies and spores) have been used as foliar sprays in agricultural and forest settings for several decades. Partly because of their selectivity and short half-life, Bt-based microbial insecticides are generally considered to have fewer adverse impacts on the environment than synthetic chemical insecticides.

In the last decade, modified and/or truncated genes of various subspecies of *B. thuringiensis* encoding insecticidal proteins have been genetically engineered into crop plants such as corn (= maize), cotton, potato, tobacco, and tomato for the purpose of pest insect control. By expressing the genes encoding insecticidal proteins, these transgenic crop plants are protected from damage by susceptible insect pests. Some transgenic crop varieties (e.g., corn, potato, and cotton) expressing Bt Cry proteins have become widely accepted by farmers as alternatives to chemical insecticides for control of economically important insect pests such

as lepidopteran and coleopteran pests (see review in Lewellyn et al., 1994; Persley, 1996; Federici, 1998).

Although the insecticidal activities of Bt protein toxins are not altered in the transgenic crops, the continuous expression of Bt Cry proteins in most plant structures throughout the growing season has raised several environmental concerns (see reviews in Williamson, 1992; Jepson et al., 1994). One such concern centers on the possible impact of this novel pest control technology on upper trophic level non-target organisms such as arthropod predators and parasitoids through crop plant-based food chains (e.g., Hilbeck et al., 1998a, b, 1999). Current testing guidelines from the United States Environment Protection Agency for registration of transgenic Bt crop plants expressing Cry protein require direct exposure (feeding) tests of the expressed insecticidal proteins with predators and parasitoids (EPA, 1989, 1994). Unlike herbivores, however, most arthropod predators and parasitoids rarely consume plant tissues. Thus, for the majority of predators and parasitoids, the degree of secondary exposure, if any, is primarily determined by 'prey' factors such as the intake and possible accumulation of the plant-produced Bt proteins by phytophagous prey. To date, little is known about the level of the Cry proteins in phytophagous insects following their ingestion of transgenic Bt crops or other food sources containing Cry proteins.

In the present study, we use enzyme-linked immunosorbent assays (ELISA) and bioassays with a susceptible insect species to estimate levels of Bt protein in different groups (or species) of phytophagous insects after feeding on transgenic corn plants expressing the Cry1Ab protein or artificial diets containing different levels of purified Cry1Ab protein.

Materials and methods

Insect sources and rearing

The insects assayed in this study included one homopteran species [*Rhopalosiphum maidis* (Fitch), the corn leaf aphid (CLA)] and three lepidopteran species [*Ostrinia nubilalis* (Hübner), the European corn borer (ECB) , *Helicoverpa zea* (Boddie), the corn earworm (CEW), and *Agrotis ipsilon* (Hufnagel), the black cutworm (BCW)]. All of these species are pests of corn. Previous studies (MacIntosh et al., 1990) indicated that CLA is not susceptible to Bt Cry proteins including Cry1Ab, whereas the Cry1Ab susceptibility of ECB, CEW, and BCW ranked high, moderate, and low, respectively.

CLA used in this study originated from adults and nymphs collected from non-transgenic corn plants at the Monsanto Technical Center (Jerseyville, IL) on August 22, 1998 and were maintained on potted non-transgenic corn plants in a growth chamber (Percival Scientific Inc, Boone, IA) with controlled climatic conditions (27 °C, 50% r.h. and a photoperiod of L14:D8 h). ECB larvae used in this study originated from eggs supplied by Iowa State University, where an ECB colony is maintained on artificial diet. Larvae of CEW and BCW originated from eggs purchased from ECOGEN (Langhorne, PA), where they are mass-reared on artificial diet.

For feeding treatments, CLA (both adults and nymphs) were allowed to feed on one of five food sources: transgenic Bt corn plants (MON 810), non-transgenic corn plants (LH198), or artificial diets containing 0, 20 or 200 ppm of pure Cry1Ab protein. The transgenic Bt corn event contained a modified synthetic cry1Ab gene encoding a nearly full-length Cry1Ab protein, with an expression rate of 8–13 ppm in green leaves (fresh weight), whereas the non-transgenic corn plant was an isogenic line not genetically modified. Both transgenic and non-transgenic corn plants were grown in the growth chamber described previously. The artificial diets used for aphid-feeding treatments consisted of 20% (w/v) sucrose solutions with addition of 1% (w/v) yellow food coloring. The Cry1Ab protein used in the artificial diet treatments was produced at Monsanto (Chesterfield, MO) by Bt strain EG 2236 containing a single gene that encodes the full-length Cry1Ab protein. Inclusion bodies containing the full length Cry1Ab protein were isolated and purified according to procedures described in MacIntosh et al. (1990). For diet preparation, the purified full-length Cry1Ab protein was dissolved in deionized water and then added to the diet solution to produce concentrations of 20 and 200 ppm. The diet solution containing no Cry1Ab protein served as a negative (diet-only) control.

For host plant treatments, CLA (both adults and nymphs) were reared on potted transgenic or non-transgenic Bt corn plants placed in growth chambers. For artificial diet treatments, aphids were maintained in individual cells of a rearing plate (24 cell microculture plate, 4–5 aphids per cell) and provided with the diet solution through a paraffin membrane on a 'diet' plate. The diet plate was identical to the rearing plate; each cell of the diet plate was filled with 1 ml

of the diet solution and covered with a thin layer of paraffin film (i.e., the membrane). The diet plate then was placed on top of the rearing plate with the paraffin film side facing down so that aphids could access the diet by piercing the thin layer of paraffin film with their feeding stylets. A series of Western blots were carried out on samples of Bt-containing diet at the beginning and end of tests to verify the integrity of the Cry1Ab protein during the course of the experiment.

For the lepidopterans (ECB, CEW, and BCW), feeding treatments were the same as for CLA except that 100 ppm Cry1Ab was used to replace the 200 ppm diet treatment because of the lepidopterans' known susceptibility to Cry1Ab protein. For feeding treatments, newly-hatched larvae (first instars) were transferred to individual cells of a micro-plate (128 cells per plate, 4–5 larvae per cell) filled with artificial diet mixture (0.5 ml per cell) containing different Cry1Ab treatments or 5–10 pieces of fresh corn-leaf disks (each about 0.75 cm in diameter) cut from transgenic or nontransgenic Bt corn plants (at V4–V6 stages). The artificial diet used to rear the lepidopteran larvae was standard soybean-based multiple species lepidopteran diet (King & Hartley, 1992), purchased from Southland Products, Inc. (Lake Village, AR).

Insect sample collection. After a minimum of seven days on corn plants, or three days on artificial diets, all aphids (adults and nymphs) were collected into 1.5 ml centrifuge tubes using a small paintbrush. Aphids were not allowed to feed on artificial diet treatments for more than three days mainly because microbial infestation (molds) began to occur in older diet. For ELISA and bioassay, about 30 mg (fresh weight) aphid samples (consisting of 96–120 nymphs and adults from one rearing plate or 2–4 plants) were produced for each replicate; a total of five replicates of aphid samples were collected for each feeding treatment. For bioassay, aphid samples collected from each of the five replicates were pooled together to produce a minimum of 12 mg of lyophilized (dry weight) sample. Collected aphids were killed immediately by freezing, and stored in a freezer (-80 °C) for use in ELISAs and bioassays. Low aphid mortality ($< 5\%$) was observed in all the feeding treatments at the time of sample collection; dead aphids were excluded from the sample collection.

For the lepidopterans, all larvae were collected 24 h after being placed on food sources, and then stored under the same condition as for aphids for use in ELISAs and bioassays. For both ELISA and bioassay, the amount of lepidopteran larval tissues produced for each replicate was about the same (consisting of 300–400 larvae) as for aphid feeding treatments. For ELISA, five replicates of the insect samples were produced for each species in each feeding treatment. For bioassay, only lyophilized (dry weight) ECB larvae (pooled from all the five replicates) were collected. While little mortality was observed in BCW in any of the feeding treatments during the 24-h feeding period, about 10% of ECB and 5% of CEW died on artificial diets containing 100 ppm Cry1Ab. Less than 5% of ECB and CEW died on transgenic Bt corn during the 24 h feeding period. All dead insects were excluded from the sample collection.

ELISA. Prior to the assay, frozen insect samples (of each replicate) were thawed, weighed into a 1.5 ml centrifuge tube, and mixed with a phosphate-buffered saline solution with 0.07% (v/v) Tween 20 ($1\times$ PBST) at a insect:buffer ratio of 1:10. Immediately following addition of $1\times$ PBST, the insect samples were homogenized for approximately 10 s at 3000 rpm with a Wheaton overhead stirrer. The homogenized insect-buffer solution was then stored at -20 °C until the time of assay (about one week later). At that time, the samples were thawed and centrifuged at 12 000 rpm for 5 min. Following centrifugation, the extract supernatants were analyzed by quantitative Cry1Ab polyclonal ELISA.

A 96-well microplate (Nunc MaxiSorp, Roskilde, Denmark) was coated with a polyclonal rabbit anti-Cry1Ab antibody. A seven-point standard curve of pure trypsinized Cry1Ab protein [produced and purified according to procedures of MacIntosh et al. (1990)] was added to the plate, along with appropriate blanks and controls. The test and control samples were diluted further in the ELISA plate (25 μl/well), and allowed to incubate, along with the standards and controls, with an alkaline phophatase conjugated polyclonal second antibody. Following an overnight incubation at 4 °C, the unbound proteins were washed off and the ELISA plate was developed with p-nitrophenyl phosphate/ethanolamine substrate. The absorbances were measured at 405–655 nm with a BioRad 3550 microplate reader (BioRad, Hercules, CA). Concentrations of Cry1Ab were determined in each sample by extrapolating an optical density reading against the standard curve of pure Cry1Ab protein. The appropriate dilution factors were calculated and Cry1Ab expression results were converted to the full-length

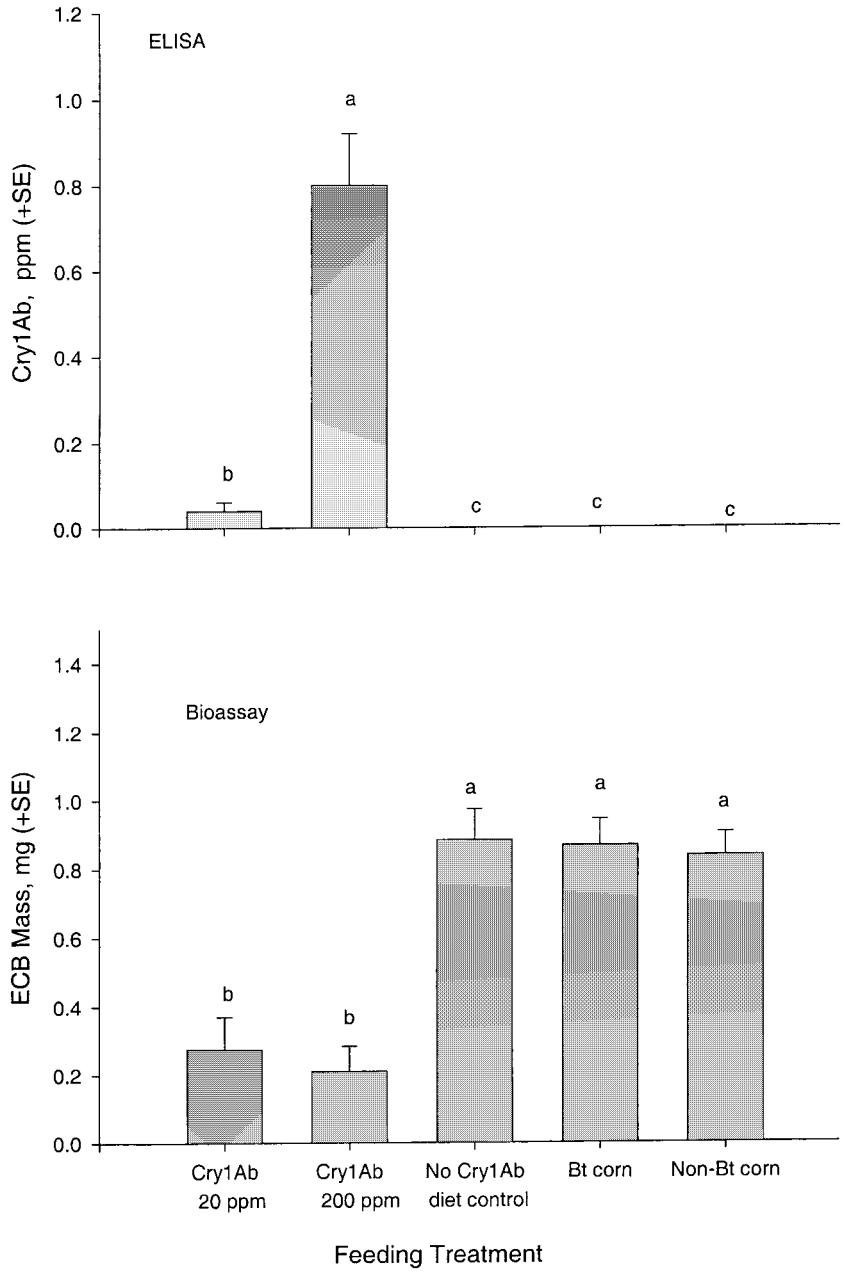

Figure 1. Level of Cry1Ab (ppm) in fresh (non-lyophilized) tissues of corn aphids feeding on different food sources (as determined by ELISA), and biological activity of the lyophilized (dry weight) corn aphid tissues (as determined by diet incorporation bioassay using first instar ECB). Bars followed by the same letters within each graph indicate no significant difference among treatments (LSD tests, $P < 0.05$).

protein expression rate and reported on a part per million, fresh weight basis.

Bioassay. The purpose of the bioassay was to determine if the level of Cry1Ab determined by ELISA was an accurate measure of the amount of biologically active Cry1Ab protein to which an insect feeding on these herbivores would be exposed. The amount of biologically active Cry1Ab in CLA and ECB larvae feeding on different food sources was assayed against first instar ECB larvae, which are particularly susceptible to Cry1Ab.

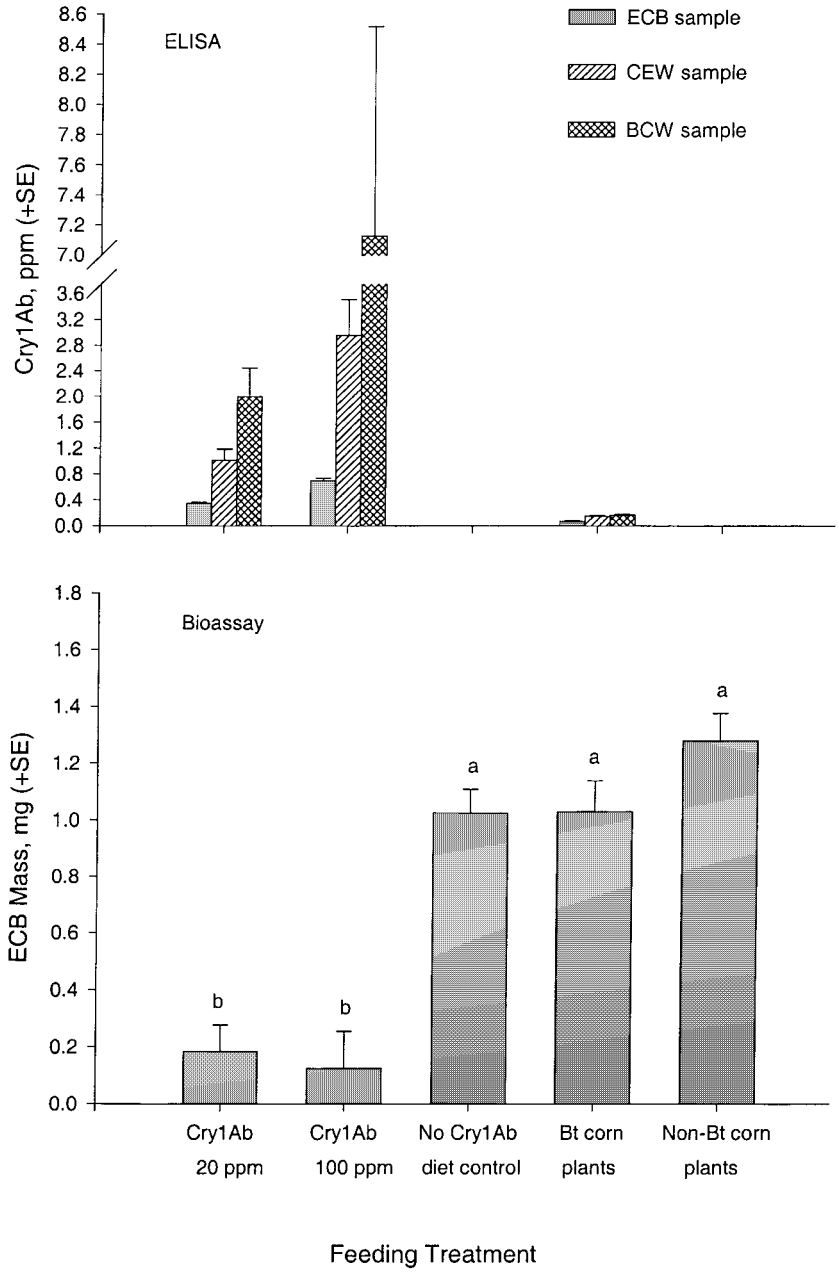

Figure 2. Level of Cry1Ab (ppm) in fresh (non-lyophilized) tissues of the larvae of European corn borer (ECB), corn earworm (CEW), and black cutworm (BCW) feeding on different food sources (as determined by ELISA), and biological activity of the lyophilized (dry weight) tissues of these European corn borer larvae (as determined by bioassay using first instar ECB). Multiple comparisons of means of Cry1Ab levels were not performed (for ELISA results) because of a significant interaction between insect species and feeding treatment. Bars followed by the same letters (for the bioassay results) indicate no significant difference among treatments (LSD tests, $P < 0.05$).

Prior to bioassay, all CLA (adults and nymphs) and ECB larvae collected from different feeding treatments were lyophilized. Previous ELISAs with lyophilized CLA and ECB samples (unpublished data) indicated that lyophilization generally enhanced levels of Cry1Ab protein in the insect samples (dry weight) 7–10 times over that in the unlyophilized insect samples (fresh weight). Thus, it was expected that the use of lyophilized (instead of fresh) insect samples would enhance chances of detection of Cry1Ab activity with a bioassay.

The bioassay procedure was similar to that previously described by Sims & Holden (1996). Lyophilized aphid or ECB samples were thoroughly slurried with water in a 15-ml centrifuge tube on a Vortex Mixer, and then mixed at a ratio of 10 mg (dry weight tissue) per ml agar-based liquid diet. The insect tissue-diet mixture for each treatment was added to cells of the bioassay tray (C-D International, Pitman, NJ) at a rate of 0.5 ml per cell using a repeater pipette. After the diet mixture cooled and solidified, one first instar ECB (12–24 h old) was introduced into each of the cells of the bioassay tray. The bioassay tray was sealed with a vented transparent acetate cover, and placed in a growth chamber at 27 °C and 30–40% r.h. for 7 days. Following the 7-day incubation, the mass of ECB larvae in each feeding treatment was determined and compared with a standard curve of larval masses reared on diets containing various concentrations of Cry1Ab. A total of 14–34 first instar ECB were assayed with CLA or ECB tissues from each treatment.

Statistical analysis. Analysis of variance (ANOVA) was used to analyze for differences in levels of Cry1Ab determined by ELISA in insect tissues from different treatments. The mass of ECB exposed to CLA and ECB tissues from different feeding treatments were analyzed using the same ANOVA procedures. All statistical analyses was performed with JMP Statistical Discovery Software (SAS Institute, 1995).

Results

Level of Cry1Ab in aphids. While ELISA detected no Cry1Ab in CLA samples that fed on transgenic Bt corn plants, mean levels (± SE) of 0.04 (± 0.02) and 0.80 (± 0.12) ppm of Cry1Ab were detected, respectively, in samples of aphids that fed on artificial diets containing 20 and 200 ppm Cry1Ab (Figure 1–ELISA). No Cry1Ab was detected in aphid samples from diet-only controls or non-transgenic corn plants.

Results from bioassays (Figure 1–Bioassay) indicated that, in contrast to the diet-only control, the mean mass of ECB larvae was reduced significantly when assayed with lyophilized (dry) tissues of aphids that had fed on 20 or 200 ppm Cry1Ab-treated diets ($F = 10.23$, $df = 4, 100$, $P < 0.001$). However, there was no significant difference in the mean mass of ECB between 20 and 200 ppm Cry1Ab diet treatments, partly because only five of the ECB larvae survived on the diet containing lyophilized tissues of aphids that had fed on the 200 ppm Cry1Ab diet. While no mortality of ECB larvae occurred in the 20 ppm Cry1Ab diet treatment, about 58% of the ECB larvae died on the diet containing lyophilized tissues of aphids that had previously fed on 200 ppm Cry1Ab-treated diet. This indicates that levels of biologically active Cry1Ab in the lyophilized tissues of aphids feeding on 200 ppm Cry1Ab diet probably are higher than those of aphids feeding on diets containing lower concentrations (e.g., 20 ppm) of Cry1Ab. No significant differences were detected in mean masses of ECB larvae when assayed with lyophilized samples of aphids feeding on transgenic Bt corn plants, non-transgenic Bt corn plants, and diet-only control; nor did mortality of ECB larvae occur in any of these treatments. This indicated that Cry1Ab was not present at a biologically active level in aphids feeding on transgenic Bt-corn plants.

Level of Cry1Ab in lepidopteran larvae. Results from ELISAs (Figure 2–ELISA) indicated that the larvae of all three lepidopteran species contained Cry1Ab after consumption of transgenic Bt corn or Cry1Ab-treated artificial diets. No Cry1Ab was detected in any of the larvae feeding on diet-only controls and non-transgenic corn plants. The levels of Cry1Ab in the larvae varied significantly among species ($F = 20.70$, $df = 2, 60$, $P < 0.001$) as well as among different feeding treatments ($F = 43.35$, $df = 4, 60$, $P < 0.001$). There was also a significant interaction in level of Cry1Ab between insect species and feeding treatments ($F = 12.0$, $df = 8, 60$, $P < 0.001$). The level of Cry1Ab in ECB larvae was lower than that in CEW or BCW across all treatments. For transgenic Bt-corn treatments, the level of Cry1Ab in ECB larvae was about two times lower than in CEW or BCW larvae. For the 20 ppm Cry1Ab diet treatments, the level of Cry1Ab in ECB larvae was about three times lower than in CEW and six times lower than in BCW. For the

100 ppm Cry1Ab diet treatment, the level of Cry1Ab in ECB larvae was about four4 times lower than in CEW and 10 times lower than in BCW.

Results from the bioassays (Figure 2–Bioassay) indicated that lyophilized tissues of ECB larvae feeding on 20 and 100 ppm Cry1Ab-treated artificial diet resulted in significant reductions in the mean mass of subsequently- assayed ECB larvae ($F = 27.05$, $df = 4$, 116, $P < 0.001$). This result verified that the Cry1Ab present in ECB larvae was still biologically active. However, the level of Cry1Ab accumulated in ECB larvae feeding on transgenic Bt corn plants for 24 h did not result in a significant reduction in the mean mass of assayed ECB larvae. While about 20% of the ECB larvae died on the diet containing lyophilized tissues of ECB larvae that had fed on 100 Cry1Ab-treated diet, little mortality (0–5%) occurred in any other (diet or host plant) treatment. This result indicated that levels of biologically active Cry1Ab was probably higher in the lyophilized tissues of ECB feeding on 100 ppm Cry1Ab diet than in those of ECB feeding on diet treated with 20 ppm Cry1Ab or transgenic Bt plant tissues containing 8-13 ppm of protein (Sanders et al., 1999).

Discussion

Results from this study indicate that the level of Cry1Ab in phytophagous insects feeding on sources containing the Cry1Ab protein is uniformly low but varies with insect species as well as food source. For CLA feeding on diet solutions containing Cry1Ab, the level of Cry1Ab in the aphid body was calculated to be 250–500 times less than the original Cry1Ab levels in the diet solution, whereas no Cry1Ab was detected by ELISA in aphids feeding on transgenic Bt-corn plants. Previous studies established that the transgenic Bt corn (MON 810) used in this study had 8–13 ppm of Cry1Ab in green tissues (Sanders et al., 1999). The non-detectable level of Cry1Ab in CLA thus might have resulted from one or both of two mechanisms: (a) Cry1Ab in transgenic corn plants may not be present in the phloem and therefore phloem-feeding insects are not likely to take in Cry1Ab , and (b) CLA may break down Cry1Ab after ingestion. Future studies on the distribution and level of Cry protein in the phloem of transgenic Bt plants are needed to determine which of the mechanisms plays a role in limiting the level of Cry1Ab protein in phloem-feeding insects.

Regardless, results from the ELISA and bioassay indicated that the levels of Cry1Ab in CLA through feeding on transgenic-Bt corn were below the detection limit of both assay methods; thus the biological risk from Cry1Ab- 'fed' aphids would be minimal if not zero. These results agree with previous laboratory and field observations that transgenic Bt corn plants (such as MON 810) expressing Cry1Ab do not have any adverse impact on field populations of aphidophagous predators such as lacewings and ladybird beetles (e.g., Orr & Landis, 1997; Pilcher, 1997; Lozzia et al., 1998; Manachini et al., 1999). It is likely that the secondary risk to predators and parasitoids posed by other phytophagous insects that feed in a similar way to aphids, particularly phloem feeders, also is negligible.

For the lepidopteran insects, levels of Cry1Ab in the larvae varied significantly with feeding treatment and insect species. When feeding for 24 h on artificial diet containing 20 and 100 ppm of Cry1Ab, respectively, the level of Cry1Ab accumulated in the surviving larvae was about 57 and 142 times lower than the original level of Cry1Ab in the diet for ECB, 20 and 34 times lower for CEW, and 10 to 14 times lower for BCW. Similar patterns were found for the transgenic Bt corn plant treatment. Based on an approximate expression rate of 10 ppm of Cry1Ab for the transgenic Bt corn used in this study, the level of Cry1Ab in the larvae would be 143 times less than the original level of Cry1Ab in the transgenic Bt-corn plants for ECB, 67 times less for CEW, and 59 times less for BCW.

The differences in Cry1Ab levels among the three lepidopteran species were correlated with differences in the insects' susceptibility to Cry1Ab protein and diet quality. MacIntosh et al. (1990) established that the LC_{50} value of Cry1Ab protein for ECB was about nine times lower than for CEW, and 24 times lower than for BCW. For ECB, the feeding treatments containing Cry1Ab are likely to have resulted in lower food intake, and thus relatively low Cry1Ab intake. Nevertheless, diet incorporation bioassays indicated that lyophilized tissues of ECB larvae feeding on 20 and 100 ppm Cry1Ab-treated diets had significant bioactivity against first instar ECB. Although the bioassay did not detect any significant bio-activity with lyophilized tissues of ECB larvae feeding on transgenic Bt-corn plants (possibly because of the low level of Cry1Ab accumulation), higher levels of Cry1Ab in CEW and BCW (Figure 2–ELISA) would be expected to have significant, though very

limited, biological activity against susceptible insect species. Fortunately, current knowledge of the mode of action and insecticidal activity of Cry1Ab protein indicates little toxicity against other groups of insects (other than lepidopteran larvae) (e.g., MacIntosh et al., 1990). At the concentrations of Cry1Ab observed here, only activity against lepidopterous insects has been recorded. The extremely low levels of Cry1Ab protein in the lepidopteran larvae feeding on transgenic Bt corn plants or artificial diet containing high doses (100 ppm) of Cry1Ab are unlikely to have any toxic effect on relatively non-susceptible insect predators such as lacewings.

In a laboratory study with the predaceous lacewing, *Chrysoperla carnea* (Stephens), Hilbeck et al. (1998a) showed that, when reared on artificial diet containing 100 ppm Cry1Ab, significantly higher mortality (57%) of the lacewing larvae was observed than for larvae reared on control diet containing no Cry1Ab (30%). In subsequent laboratory studies, Hilbeck et al. (1998b, 1999) further showed that, when reared on larvae of *O. nubilalis* and *Spodoptera littoralis* (Boisduval) which had fed on transgenic Bt corn expressing Cry1Ab protein or artificial diet containing various concentrations (25–200 ppm) of Cry1Ab, significantly higher mortality (46–78%) of the lacewing larvae was observed than in the comparable negative (non-Bt protein) controls (26–37%). Based on the findings from the study reported here, however, the lethal effects of Bt Cry protein 'contaminated' prey reported in Hilbeck et al. (1998b, 1999) were likely to have resulted either from the unrealistically high concentrations of the Cry1Ab protein in the artificial diet or from the inferior quality of the prey intoxicated by feeding on insecticidal Bt proteins. In a recent study with snowdrop lectin (GNA) incorporated into artificial diet, Down et al. (2000) also suggested that the observed adverse effect of 'GNA-contaminated' aphids [*Myzus persicae* (Sulz)] on ladybird larvae (*Adalia bipunctata* L.) might be due to the suboptimal food quality of the aphids, instead of direct toxicity from the protein (GNA) itself.

Currently, the non-target organism risk assessment for transgenic crop plants expressing Bt Cry proteins in the US is governed by the rule or guidelines proposed by EPA in 1994 (EPA, 1994). These guidelines require prolonged direct exposure (continuously-feeding) tests of the concerned plant pesticides (e.g., Bt proteins) with representative predators and parasitoids. The doses of the plant pesticides for continuous feeding tests with non-target organisms (predators and parasitoids) are generally set 10–100 times higher than the maximum expression rate of the Bt protein in the transgenic plants. Results from the current study demonstrate that the levels of Cry1Ab protein accumulated in both target and non-target phytophagous insects are manyfold less than the original levels of Cry1Ab in both treated artificial diets and transgenic Bt corn plants. Based on these findings, two conclusions may be drawn: first, direct feeding on plant tissues probably represents the only significant route of exposure for non-target species, and second, the safety margin (10–100 times the maximum expression rate of Bt protein in the transgenic plants) set by the EPA (1989, 1994) for evaluation of the (secondary) exposure hazard to predators and parasitoids in the continuous feeding tests should have been high enough (perhaps unrealistically high) to ensure detection of toxicological hazard of the plant-produced insecticidal protein to upper trophic level organisms.

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