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Immunological analysis of phloem sap of *Bacillus thuringiensis* corn and of the nontarget herbivore *Rhopalosiphum padi* (Homoptera: Aphididae) for the presence of Cry1Ab

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Abstract

Phloem sap of transgenic *Bacillus thuringiensis* (Bt) corn expressing a truncated form of the *B. thuringiensis* δ-endotoxin Cry1Ab, sap sucking aphids feeding on Bt corn and their honeydew were analysed for presence of Cry1Ab using ELISA. Phloem sap of Bt and non-Bt corn was collected using a newly developed technique with a microcapillary being directly inserted into the phloem tubes. Using this technique, no Cry1Ab was detected in the phloem sap. In contrast, measurable concentrations of Cry1Ab in the range of 1 ppb were detected when phloem sap of pooled leaf samples was extracted using EDTA buffer. This was probably because of Cry1Ab toxin released from damaged cells. When analysing apterous adults of *Rhopalosiphum padi* L. and their honeydew, no Cry1Ab could be detected. In contrast, Cry1Ab was clearly detected in both larvae of the leaf chewing herbivore *Spodoptera littoralis* (Boisduval) and their faeces, showing that Cry1Ab is detectable after ingestion and excretion by herbivores. These results suggest that *R. padi* ingests or contains no or only very low concentrations of Cry1Ab in the range of the detection limit. In consequence it is hypothesized that *R. padi* as an important prey for beneficial insects in corn is unlikely to cause any harm to its antagonists due to mediating Bt toxin.

Keywords: *Bacillus thuringiensis*, biosafety, phloem sap, *Rhopalosiphum padi*, *Spodoptera littoralis*, transgenic plants

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Introduction

Adoption rates of transgenic insect resistant crops expressing activated δ-endotoxins of the soil bacteria *Bacillus thuringiensis* (Bt) are among the highest for a new technology introduced into the agricultural industry. In 1998, Bt crops were grown on approximately 8 million hectares worldwide representing almost a doubling of the area planted from 1997 to 1998 (James 1999). This was due to increased acreage in the USA, Canada, Mexico and Australia and new introductions of insect resistant crops in China, Spain, France, South Africa and Argentina.

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Commercially available transgenic crops worldwide are cotton, corn, potato and tomato which are engineered to express truncated forms of single Bt proteins against chewing insect pests. Current Bt cotton cultivars produce the Cry1Ac protein targeted to control the tobacco budworm, most varieties of Bt corn express the Cry1Ab toxin to control the European corn borer, Bt potatoes produce the Cry3A protein to control the Colorado potato beetle (Federici 1999) and the Bt tomato 5345 express the Cry1Ac to control certain lepidopteran pests (EPA 1997). Bt proteins cause toxicity upon ingestion and the target organisms are killed when feeding on plant tissues containing the endotoxins.

Insecticides based on *B. thuringiensis* have been used in many regions of the world for more than 30 years (Federici

1999). Like synthetic chemical insecticides Bt formulations are sprayed on the surface of the plants where foliage feeding larvae of target and nontarget insect pests ingest them. Due to the external application, herbivores of other feeding guilds like sapsucking herbivores or cell content feeders had never ingested Bt and hence side-effects on these nontarget herbivores were not of much concern. However, the situation changed for transgenic Bt plants. In most of the corn and cotton varieties expression of Cry1A toxins is driven by the constitutive cauliflower mosaic virus (CaMV) 35S promoter (Sims & Berberich 1996; Jouanin *et al*. 1998). This promoter is active in all plant tissues indicating that Cry1A toxins are present in essentially all plant cells, albeit at varying concentrations. Consequently, cell sucking herbivores such as thrips and spider mites feeding on transgenic Bt plants may also ingest Bt toxin. Due to the specificity of Bt toxins cell sucking herbivores would not be expected to be lethally affected by Cry1A toxins but may represent potential mediators of the toxins to beneficial insects, which in turn might be affected.

An open question is whether toxins are also transported in the phloem sap. Corn usually is infested by grain aphids such as *Rhopalosiphum padi* L., which represent an important prey for beneficial insects. Because aphids are obligatory phloem sap feeders, the question whether Cry1Ab toxin is present in the phloem sap of transgenic plants and whether aphids ingest Bt toxin is of great ecological relevance. CaMV 35S-activity was detected in the epidermis, cortex, pith tissue, mesophyll cells, trichomes and in the xylem and phloem of transgenic tobacco plants (Shi *et al*. 1994). Therefore, Cry1Ab can also be expected to be present in phloem and phloem companion cells in Bt corn. However, expression of a protein in phloem cells of transgenic plants does not necessarily mean that the protein will be translocated into the phloem sap because translocation is mainly determined by the size and/or structure of the protein (Shi *et al*. 1994).

To clarify whether Cry1Ab toxin is translocated into the phloem sap of Bt corn and whether aphids pick it up, we analysed phloem sap of Bt and non-Bt corn and adult apterous *R. padi* reared on Bt and non-Bt corn for presence of Bt toxin using an immunological assay (ELISA). Because sapsucking insects of the suborder Homoptera digest little to no proteins in the gut (Stoger *et al*. 1999) most ingested protein should be excreted. Detection of Cry1Ab in honeydew produced by *R. padi* feeding on Bt corn would, therefore, provide direct evidence for the presence of Cry1Ab in phloem sap. Therefore, honeydew produced by *R. padi* was analysed as well.

The collection of phloem sap from cut leaves with ethylenediaminetetraacetate (EDTA)-buffer is a long known and widely used method (King & Zeewaart 1974). However, by cutting leaves, it is inevitable that cells will be damaged and their cell contents contribute to the overall amount of compounds isolated as 'phloem sap' compounds. To investigate the suitability of EDTA-exudation of phloem sap for the biosafety testing of transgenic insecticidal plants, we compared this method with a newly developed technique (Brandt *et al*. 1999). This method allows the collection of pure contents of specific plant cells, including phloem tubes, using microcapillaries.

Foliar-feeding insects definitely ingest Bt toxins when feeding on leaf tissue. Larvae of foliar-feeding species may, therefore, be used in comparison with aphids to see whether Cry1Ab can be detected in herbivores and faeces once it is ingested and excreted. For this purpose, we analysed larvae of the Egyptian cotton leafworm *Spodoptera littoralis* (Boisduval) and their excretions for presence of Cry1Ab when they had fed on Bt corn and non-Bt corn.

The objectives of this study were: (i) to analyse phloem sap of Bt and non-Bt corn for presence of Bt toxin; (ii) to compare two different phloem sap collection methods; and (iii) to analyse two different herbivores feeding on Bt corn for presence of Cry1Ab in the insects and their excretions.

Materials and methods

Plants

Three different corn hybrids were used in the analyses. One hybrid (N4640Bt; Novartis, formerly Northrup King) was genetically modified with a truncated, synthetic version of a gene from *Bacillus thuringiensis* var. *kurstaki* HD-1 coding for the Cry1Ab insecticidal δ-endotoxin. The expression is driven by the constitutive CaMV 35S promoter. The other hybrid used was the corresponding untransformed hybrid variety (N4640). For the remainder of this manuscript, these two hybrids will be referred to as (Bt +) and (Bt –) corn, respectively. In order to compare different Bt corn hybrids, phloem sap collection using the microcapillary technique was also conducted with another transgenic hybrid variety, the commercially available Event 176 from Novartis Seeds. Event 176 contains two tissue specific promoters: the maize phosphoenolpyruvat-carboxylase (PEPC) promoter, which is expressed in green tissues, and a maize pollen specific promoter (Koziel *et al*. 1993).

(Bt +) and (Bt –) plants were potted in pairs in plastic containers (15 cm in diameter) in composted garden soil and cultivated in glasshouse cabins at 25–30 °C and 70–80% relative humidity (rH). All plants were fertilized once a week with 30 mL Loncin (nitrogen : potassium : phosphate = 16:16:16%). Analyses were conducted when plants were 4–5 weeks old (5–6 leaf stage) using the youngest fully grown leaf. Plants used for microcapillary-based phloem sampling were grown at the Max-Planck Institute for Molecular Plant Physiology in Golm, Germany, in growth chambers under controlled conditions of 20 °C, 60% rH and a photoperiod of 16:8 light : dark (L:D).

Insect species

Rhopalosiphum padi was taken from the permanent laboratory colonies of the Swiss Federal Research Station for Agroecology and Agriculture, Zurich, Switzerland. Aphids were reared on (Bt +) and (Bt –) corn in climate chambers at constant 20 °C, 65% rH and a photoperiod of 16:8 (L:D) hours.

Egg masses of *Spodoptera littoralis* were kindly provided by Novartis Crop Protection, Basel, Switzerland where they were reared as a laboratory colony on an artificial diet. Until they were used, they were kept on a well defined standard meridic diet designed for mass rearing of the European corn borer *Ostrinia nubilalis* Hübner at 20–25 °C (12:12) temperature, 65% rH and a photoperiod of 16:8 (L:D) hours.

Enzyme-linked immunosorbent assay (ELISA)

A Cry1Ab/Cry1Ac Plate Kit of EnviroLogix Inc. Portland, Maine (Catalogue No. AP003) was used. The Kit represents a 'sandwich' ELISA with a detection range between 1 and 12 ppb Standards in different concentrations (0, 1, 4 and 6 ppb Cry1Ab toxin) were provided as calibrators. The spectrophotometric measurements were conducted with a microtiter plate reader (Dynatech MR 5000) at 450 nm. Data were analysed using the software packages Biolinx 2.0 (Dynatech Laboratories Inc.) and Dynex Revelation G 3.2 (Dynex Technologies).

Phloem sap analyses

Phloem sap from (Bt +) and (Bt –) corn was collected by using two different methods. The microcapillary technique was recently developed at the Max-Planck Institute for Plant Physiology Golm, Germany (Brandt *et al*. 1999). Additionally, the widely used exudation from cut leaves using EDTA (King & Zeewaart 1974) was applied. Phloem sap collection and analysis by both methods were repeated twice.

Microcapillary technique. Sieve tube sap was collected according to the microsampling procedure for fluorescent companion cells described in Brandt *et al*. (1999) with the following modifications. For identification, sieve elements were loaded overnight with the fluorescent dye carboxyfluorescin diacetate (Sigma, Deisenhofen, Germany) according to Grignon *et al*. (1989). The next day one leaf, still attached to an intact plant, was fixed on the stage of a fluorescence microscope and a glass microcapillary with a tip diameter of approximately 1 μm was inserted from the abaxial side into a labelled vein. The glass microcapillary was slowly moved through the tissue until fluorescent sap entered the tip. After 10–30 min, when approximately 3 μL of sap was collected, the tip was withdrawn and the sample was immediately released into a reaction vial containing 10 μL extraction buffer provided by the ELISA Kit. Additionally, the sap from at least three leaves (approximately 10 μL) was combined to increase the amount of phloem sap (pooled samples). After collection of phloem samples parts of the leaf used were cut and immediately frozen in liquid nitrogen for storage. Later, these leaf tissue samples were analysed to verify the expression of Cry1Ab in the test plants (see below). All samples were stored at –20 °C until use. Three plants per hybrid (N4640Bt, N4640, Event 176) were used for phloem sap collection.

Exudation from cut leaves using EDTA. Leaves were cut from eight plants for N4640Bt and N4640 and immediately recut under EDTA-buffer (20 mM Ethylendiaminetetraacetate-Na2-salt-dihydrat, pH 7) to prevent air contact with the phloem. Afterwards, leaves were individually placed upright in plastic containers (4 cm length × 2 cm width × 7 cm height) filled with 4 mL of EDTA-buffer with approximately 0.5 cm of the basal part of the leaves immersed in the solution. Additionally, from four of the plants, pooled samples of three leaves immersing in EDTA-buffer were prepared the same way. Containers were placed in plastic boxes (22 cm length × 10 cm width × 8 cm height) and the lid replaced with wet filter paper to maintain high humidity. They were placed on a shaker for 3 h at room temperature for exudation. After the exudation period, the samples were frozen at –80 °C, freeze dried and resolved in 3 mL extraction buffer. The samples were stored at –20 °C until use.

Plant expression of Cry1Ab was verified by analysing leaf material. Leaf pieces of (Bt +) and (Bt –) plants were weighed, homogenized with 5 mL extraction buffer using extraction bags and a hand model homogenizer (Bioreba AG) and the plant sap diluted 1:50 with extraction buffer.

Extraction procedures for both sampling techniques are summarized in Table 1.

Analyses of R. padi *and honeydew*

For one sample, one hundred apterous *R. padi* each from single (Bt +) and (Bt –) corn plants were collected, weighed, homogenized in 1 mL extraction buffer and analysed. Aphid analyses were repeated three times (trial A, B and C), sample size in the trials ranged from three to five per treatment (for details see Table 2).

For honeydew collection, approximately 60 apterous *R. padi* were caged (three cages of 20 aphids each) on each of four (Bt +) and four (Bt –) plants in the first trial and on each of eight (Bt +) and eight (Bt –) corn plants in the second trial, respectively. The cages consisted of petri dishes (diameter approximately 3 cm) with foam rubber at the edges to prevent the plant tissue from being damaged.

Table 1 Results of the ELISA test of leaf tissue analyses and of phloem sap samples of control plants (N4640) (Bt +) plants (N4640Bt) and Event 176 plants. The number of replicates and mean (SD) of the amount of material used, the amount of extraction buffer used and the dilution, and the concentrations of Cry1Ab in the buffer solution (ppb) and in the tissue analysed (µg Cry1Ab/fresh weight) is given

Tissue analysed	*n*	Amount material	Buffer/dilution	ppb	µg Cry1Ab/g fresh weight
Microcapillary technique					
leaf tissue control	3	206.4 (17.6) mg	5 mL/1 : 50	14.4 (2.2)	0
leaf tissue (Bt +) plants	3	236.3 (3.3) mg	5 mL/1 : 50	216.5 (5.1)	4.6 (0.1)
leaf tissue Event 176	3	331.5 (62.0) mg	5 mL/1 : 50	289.5 (58.2)	4.4 (0.2)
phloem sap (single leaf samples) of control plants	5	~3 µL	300 µL	< 1	—
phloem sap (single leaf samples) of (Bt +) plants	5	~3 µL	300 µL	< 1	—
phloem sap (single leaf samples) of Event 176	5	~3 µL	300 µL	< 1	—
phloem sap (pooled leaf samples) of control plants	3	~10 µL	300 µL	< 1	—
phloem sap (pooled leaf samples) of (Bt +) plants	3	~10 µL	300 µL	< 1	—
phloem sap (pooled leaf samples) of Event 176 plants	3	~10 µL	300 µL	< 1	—
EDTA technique					
leaf tissue control plants	8	438.6 (39.6) mg	5 mL/1 : 50	< 1	0
leaf tissue Bt plants	8	428.8 (62.7) mg	5 mL/1 : 50	7.1 (0.7)	4.2 (0.5)
phloem sap (single leaf samples) of control plants in EDTA buffer	8	4 mL EDTA	3 mL	< 1	—
phloem sap (single leaf samples) of (Bt +) plants in EDTA buffer	8	4 mL EDTA	3 mL	< 1	—
phloem sap (pooled leaf samples) of control plants in EDTA buffer	4	4 mL EDTA	3 mL	< 1	—
phloem sap (pooled leaf samples) of (Bt +) plants in EDTA buffer	4	4 mL EDTA	3 mL	1.1 (0.1)	—

Detection limit: ppb = 1.

Table 2 ELISA of *Rhopalosiphum padi* and *Spodoptera littoralis*, their host plants (leaf tissue) and their excretions (honeydew and faeces, respectively) when fed on control (N4640) and (Bt +) plants (N4640Bt). Given is the number of replicates (*n*), mean (SD) of the amount material used, the amount of extraction buffer used and the dilution applied, the concentration of Cry1Ab in the buffer solution (ppb) and the calculated concentration of Cry1Ab in the respective tissues

Tissue analysed	*n*	Amount material	Buffer/dilution	ppb	µg Cry1Ab/g fresh weight
***R. padi* — trial A**					
leaf tissue Bt plants	4	256.0 (21.0) mg	5 mL/1 : 50	81.52 (52.93)	1.55 (0.97)
aphids (control)	4	31.29 (3.14) mg	1 mL	< 1	< 0
aphids (Bt)	4	36.56 (2.87) mg	1 mL	< 1	< detection limit
***R. padi* — trial B**					
leaf tissue control plants	4	531.47 (131.98) mg	5 mL/1 : 50	3.35 (1.56)	0
leaf tissue Bt plants	5	432.06 (47.91) mg	5 mL/1 : 50	294.67 (28.08)	3.44 (0.40)
aphids (control)	5	24.56 (3.33) mg	1 mL	< 1	0
aphids (Bt)	5	24.43 (2.20) mg	1 mL	0.88 (0.36)	0.03 (0.01)
***R. padi* — trial C**					
leaf tissue Bt plants	2	440.7 (90.0) mg	5 mL/1 : 100	359.55 (59.71)	4.83 (2.35)
aphids (control)	4	21.16 (1.46) mg	1 mL	< 1	0
aphids (Bt)	3	16.94 (0.95) mg	1 mL	< 1	< detection limit
honeydew — trial B					
leaf tissue control plants	7	210.21 (79.82) mg	5 mL/1 : 50	2.98 (1.28)	0
leaf tissue Bt plants	8	227.33 (30.15) mg	5 mL/1 : 50	478.61 (70.86)	10.53 (0.95)
honeydew (control)	8	32.93 (55.49) mg	3 mL	1.40 (1.57)	0.34 (0.59)
honeydew (Bt)	8	23.51 (9.24) mg	3 mL	0.61 (0.63)	0
S. littoralis					
leaf tissue control plants	2	429.41 (5.21) mg	5 mL/1 : 50	4.82 (0.38)	0
leaf tissue Bt plants	4	449.20 (80.65) mg	5 mL/1 : 50	243.12 (53.13)	2.73 (0.54)
larvae (control)	2	64.47 (3.55) mg	3 mL	< 1	0
larvae (Bt)	4	56.82 (5.07) mg	3 mL	5.98 (0.88)	0.32 (0.05)
faeces (control)	2	6.02 (0.88) mg	2 mL/1 : 50	0.15 (0.00)	0
faeces (Bt)	4	4.41 (1.18) mg	2 mL 1 : 50	116.03 (43.39)	2.98 (0.64)

Detection limit: ppb = 1.

They were lined with a preweighed sheet of plastic foil on which the honeydew was deposited. After 48 h, plastic sheets were reweighed and the honeydew produced on one plant dissolved in 3 mL extraction buffer. Samples were stored at –20 °C until analysis.

Expression of Cry1Ab in the plants used for both aphid and honeydew analyses was verified by analysing leaf material of the plants used as described above. To account for high toxin concentration in the plants dilution of plant sap in the third trial of the aphids analyses was 1:100 instead of 1:50 used in all other analyses. Extraction procedures are described in Table 1.

Analyses of S. littoralis *— larvae and faeces*

Forty larvae of *S. littoralis* were raised on standard meridic diet until they reached the L3/L4 — instar. They were then fed leaves of (Bt +) or (Bt –) corn for 24 h, respectively, with 10 larvae feeding on leaves of one plant. After 24 h, pooled samples of 4–5 larvae from one plant were homogenized in 3 mL of extraction buffer using a homogenizer. The faeces of all larvae feeding on one plant was collected and homogenized in 2 mL extraction buffer. For analyses, the samples were diluted 1:50 with extraction buffer (for details of extraction procedure see Table 2). Expression of Cry1Ab in the food plants was verified by analysing leaf material (see above). All samples were stored at –20 °C until use. Analyses of larvae and faeces of *S. littoralis* were repeated twice.

Results

Phloem sap analyses

The repetitions of phloem sap analysis of each of the two sampling techniques provided the same results. Therefore, only the results for the first analyses are shown here. Using the microcapillary technique, no Cry1Ab could be detected in the phloem sap of either (Bt +) or Event 176 plants. The extinction values were comparable to those of the control plants and the pure extraction buffer (Fig. 1, left). Also, no Cry1Ab was detected in the pooled samples. Again, extinction values were in the range of the control plants and the pure buffer (Fig. 1, left). Testing of leaf material revealed high expression in all three Bt(+) and Event 176 plants used (Table 1). Applying the EDTA-exudation technique, Cry1Ab could not be detected in the phloem sap of single leaf samples of (Bt +) plants either (Fig. 1, right). Extinction values again were comparable to those of the control plants and the pure buffer. However, the pooled samples revealed measurable optical density (OD) for Cry1Ab in the phloem sap of (Bt +) plants. Leaf material of Bt corn gave high OD values confirming expression of Cry1Ab in the plants. Table 1 gives the extraction procedures and the concentration of Cry1Ab in the tissues analysed.

Analyses of Rhopalosiphum padi *and honeydew*

The three trials of *R. padi* analysis gave inconsistent results. While in the first and the third trial no Cry1Ab could be detected in the aphids (Fig. 2A,C), the second trial gave some positive OD values for apterous *R. padi* at the detection limit (1 ppb) (Fig. 2B). In all trials, expression of Cry1Ab in the food plants was confirmed. Concentrations and extraction procedures for all three trials are provided in Table 2.

In honeydew, no Cry1Ab could be detected in the first trial (Fig. 3A). However, in the second trial the extinction value of the (Bt –) but not of the (Bt +) treatment revealed positive OD (Fig. 3B) at the detection limit. For concentrations and extraction procedures see Table 2.

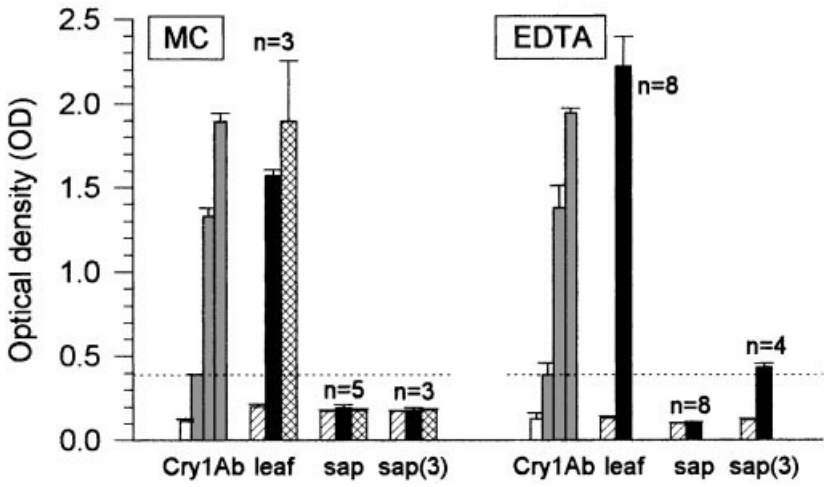

Fig. 1 Detection of Cry1Ab in green leaf tissue (leaf) and phloem sap (sap) of different varieties of Bt corn (N4640Bt, Event176) and control plants (N4640) using ELISA. Phloem sap was collected by microcapillary technique (MC, left) and by phloem exudation using EDTA-buffer (right). Bars show the extinction measured in optical density (OD) at 450 nm wavelength. Mean ± SD are given, numbers above bars indicate sample size. White bar = pure extraction buffer (negative control), grey bars = standard [purified Cry1Ab in extraction buffer at a concentration of (from left to right) 1, 4 and 6 ppb], striped bars = N4640, black bars = N4640Bt, crossed bars = Event 176, sap(3) = pooled samples of three leaves. The dashed line shows the detection limit (1 ppb).

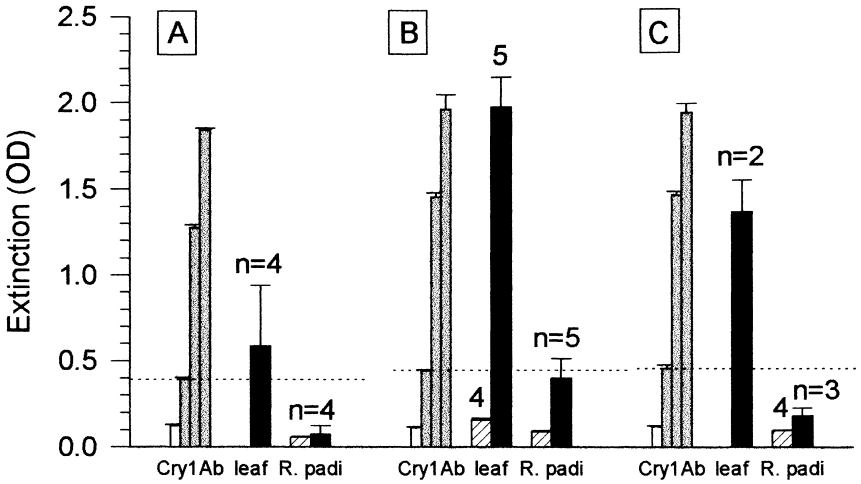

Fig. 2 Detection of Cry1Ab in green leaf tissue (leaf) of Bt corn (N4640Bt) and control corn (N4640) and in 100 apterous *Rhopalosiphum padi* sucking on these varieties in three trials (A, B, C). Bars show the extinction measured in optical density (OD) at 450 nm wavelength. Mean ± SD are given, numbers above bars indicate sample size. White bars = pure extraction buffer (negative control), grey bars = standard [purified Cry1Ab in extraction buffer at a concentration of (from left to right) 1, 4 and 6 ppb], striped bars = N4640, black bars = N4640Bt. The dashed lines show the detection limit (1 ppb).

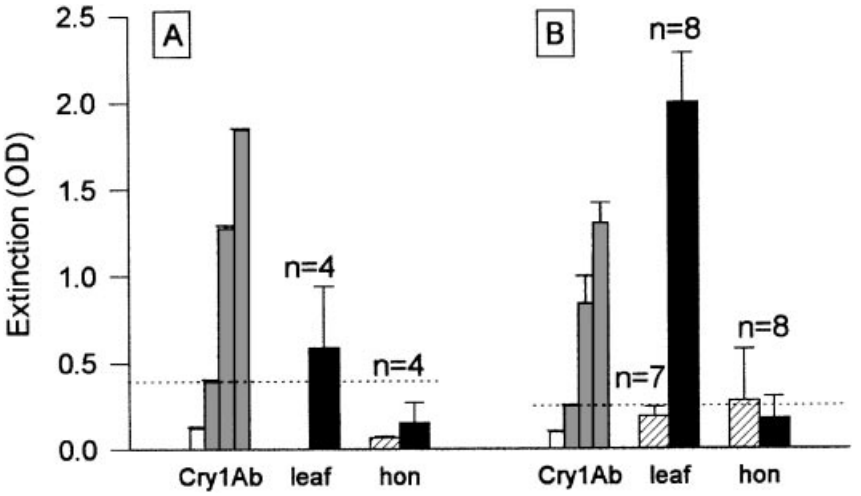

Fig. 3 Detection of Cry1Ab in green leaf tissue (leaf) of Bt corn (N4640Bt) and control corn (N4640) and in honeydew (hon) of *Rhopalosiphum padi* sucking on the plants in two trials (A, B). Bars show the extinction measured in optical density (OD) at 450 nm wavelength. Mean ± SD are given, numbers above bars indicate sample size. White bars = pure extraction buffer (negative control), grey bars = standard [purified Cry1Ab in extraction buffer at a concentration of (from left to right) 1, 4 and 6 ppb], striped bars = N4640, black bars = N4640Bt. The dashed lines show the detection limit (1 ppb).

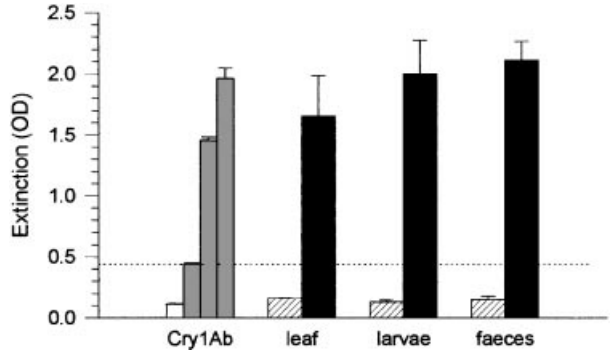

Fig. 4 Detection of Cry1Ab in green leaf tissue of Bt corn (N4640Bt) and control corn (N4640) (leaf) and in larvae and faeces of *Spodoptera littoralis* after a 24-h feeding period on these varieties. Bars show the extinction measured in optical density (OD) at 450 nm wavelength. Mean ± SD are given, sample size was 2 for the control treatment and 4 for the Bt treatment. White bars = pure extraction buffer (negative control), grey bars = standard [purified Cry1Ab in extraction buffer at a concentration of (from left to right) 1, 4 and 6 ppb], striped bars = N4640, black bars = N4640Bt. The dashed line shows the detection limit (1 ppb).

Analyses of Spodoptera littoralis *larvae and faeces*

In contrast to aphids and honeydew, Cry1Ab could be detected in the larvae of *S. littoralis* and in their faeces when fed transgenic (Bt +) corn (Fig. 4). The data presented are the results of the second trial confirming the results of the first trial. The OD value of the (Bt +) treatment for the leaves, the larvae and the faeces are in the range of 4–6 ppb Concentrations and extraction procedures are summarized in Table 2. In the faeces concentration of Cry1Ab was approximately tenfold higher than in the larvae (Table 2).

Discussion

When pure phloem sap was collected via microcapillaries, no Cry1Ab could be detected in the phloem sap. Even pooling samples to increase the amount of sap revealed no evidence for Cry1Ab presence. On the other hand, increasing the number of leaves for phloem sap exudation with EDTA-buffer led to positive extinction values and

measurable concentrations of Cry1Ab in the range of the detection limit. However, because cut leaf pieces were used for sap collection, it is likely that toxin originating from damaged cells was measured rather than traces of toxin transported in the phloem sap. Thus, phloem extraction by EDTA exudation seems to be inadequate for proving the presence or absence of transgenic insecticidal proteins in the phloem sap as it can easily lead to the wrong conclusions because of likely contamination with cell contents. In consequence, the microcapillary sampling technique proved to be more reliable and should be the preferred method for phloem sap collection in the future.

In transgenic tobacco plants expressing the snowdrop lectin (GNA) under the control of the CaMV 35S promoter, GNA was detected in phloem, xylem cells, parenchyma cells, mesophyll cells, cortex and pith tissue (Shi *et al*. 1994). GNA- or Soybean Trypsin inhibitor (SKTI)-expression driven by CaMV 35S enhance resistance of transgenic potato or rice plants against the peach-potato aphid *Myzus persicae* (Gatehouse *et al*. 1996), the glasshouse potato aphid *Aulacorthum solani* (Down *et al*. 1996) or the brown plant hopper *Nilaparvata lugens* (Lee *et al*. 1999), indicating that these toxins are most likely translocated from the phloem cells into the phloem sap. Additionally, using another constitutive promoter, the maize ubiquitin 1 promoter, expression of GNA leads to enhanced resistance of wheat and rice plants to the grain aphid *Sitobion avenae* (Stoger *et al*. 1999) and the brown plant hopper (Rao *et al*. 1998), again suggesting that these proteins are translocated into the phloem sap. Shi *et al*. (1994) provide direct evidence for the translocation of the GNA into the phloem sap when they detected the lectin in the honeydew produced by aphids feeding on tobacco plants that express GNA under the control of the phloem specific rice sucrose synthase promoter (Rss1).

With the CaMV 35S promoter controlling Cry1Ab expression in corn, it is expected that Cry1Ab is expressed in the phloem cells and phloem companion cells of Bt corn. However, our results provide no evidence that Cry1Ab is translocated into the phloem sap. One reason for the absence of the toxin in the sap may be its molecular size. GNA and SKTI are relatively small molecules with molecular weights of 12.5 kDa (Powell *et al*. 1998) and 21 kDa (Lee *et al*. 1999), respectively, whereas the expressed Cry1Ab has a molecular weight of approximately 65–69 kDa. Additionally, Cry1Ab signal sequences necessary for the transport into the phloem sap may be lacking (Shi *et al*. 1994) or the toxin may be bound to structural components of the sieve tubes (Shi *et al*. 1994).

No or only very low concentrations of Cry1Ab could be detected in adult *Rhopalosiphum padi* feeding on Bt corn. The extinction value in the range of the detection limit in one trial may indicate that under certain circumstances aphids do pick up some toxin. However, before final conclusions can be drawn, this result should be verified by more ELISA tests and/or additional Western blot analyses to reassure that it is actually the Cry1Ab which was measured. There is the possibility that aphids ingest little amounts of toxin during probing for host plant suitability. While searching for vascular tissues the stylets of most aphid species follow an extracellular path interrupted by brief intracellular punctures (Tjallingii 1988). These punctures have been shown to correlate significantly with the probability of nonpersistent virus acquisition (Powell *et al*. 1995; Collar *et al*. 1997) and it is hypothesized that during these cell punctures aphids ingest tiny volumes of intracellular plant sap directed to discriminate hosts from nonhosts (Powell *et al*. 1995; Collar & Fereres 1998). Also, the duration of cell sap ingestion is dependent on the physiological state of the aphids (Collar & Fereres 1998), which may explain why in only one out of three trials positive OD values were obtained.

The absence of measurable concentrations of Cry1Ab in the honeydew produced by aphids sucking on (Bt +) corn provides additional support that even if toxin is taken up, it is only in very small amounts. The positive OD of the honeydew of the (Bt –) treatment in the second trial may be due to a cross reaction between some components in the honeydew and the Cry1Ab-specific antibodies (P. Gugerli, personal communication). Further evaluation of the different compounds in honeydew and their potential to cross react are needed before conclusions can be drawn.

The data presented in this paper suggest that *R. padi* on (Bt +) corn represent no hazardous, Bt-containing prey to beneficial insects. This finding is supported by glasshouse feeding trials in which *Chrysoperla carnea* was fed with *R. padi* sucking on (Bt –) and (Bt +) corn plants, respectively. The leaves were not excised to reassure Bt expression and potential phloem transportation of the toxin in the leaves. No negative impact on mortality, larval weight or developmental time of *C. carnea* was found (A. Raps, unpublished data). Moreover, paired choice tests demonstrated that *C. carnea* larvae did not distinguish between *R. padi* fed (Bt –) or (Bt +) corn, while they showed a significant preference for *S. littoralis* larvae fed nontransgenic corn over those fed transgenic Bt corn (Meier & Hilbeck, 2001). This again provides evidence that even if there is Cry1Ab in aphids, this concentration is insufficient to cause harmful effects. Using another variety of Bt corn, Event 176, Lozzia *et al*. (1998) also did not find negative effects on biological parameters of both *R. padi* and *C. carnea* when conducting tritrophic feeding studies. Finally, in another experiment where convergent lady beetles (*Hippodamia convergens*) were fed aphids (*Myzus persicae*) sucking on potatoes expressing Cry3A endotoxin, no negative effects on survival, development or reproduction of the beetles were found (Dogan *et al*. 1996). However, in both studies cut leaves were used for feeding the aphids and it is not clear what impact the cutting of the

leaves has on the presence of Bt toxins in plant tissues and on potential phloem transportation.

The ELISA test used in this study provided quantitative results of Cry1Ab in leaf tissues of the two Bt corn varieties used and in the foliar feeding herbivore *Spodoptera littoralis*. The expression level of Cry1Ab in Event 176 was comparable to the upper range concentration of field grown Event 176 plants detected by Fearing *et al*. (1997). Further, the detection of Cry1Ab in *S. littoralis* which definitely ingests the toxin and in its faeces showed that Cry1Ab can still be detected after ingestion and excretion. The concentration of Cry1Ab in the faeces is approximately tenfold higher than in the larval body and comparable to the concentration of the fresh leaf tissue fed, suggesting that the food and hence Cry1Ab passes the gut quickly and is poorly digested. The high amount of toxin present in faeces of nontarget herbivores may become ecologically relevant as host faeces can be important in host location by parasitoids (Lewis & Jones 1971). Thus, changes in the volatile profile of faeces caused by 'poisoning' may affect the ability of host location by female parasitoids (Schuler *et al*. 1999).

In summary, our results indicate that aphids on Bt crops seem to represent a 'safe food' for beneficial insects. Even if the positive result in one of the trials was due to low concentrations of Cry1Ab, the concentration of the toxin in aphids would be more than hundred-fold lower than in *S. littoralis*. However, before drawing general conclusions about the safety of Bt crop species for aphid predators and parasitoids, more investigations on susceptibility of aphids antagonists to the Cry1A toxins and on phloem transportation of Bt toxins are recommended to be sure that there are no differences depending on the different plant species, promoters or toxins involved.

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The research in this paper is part of a larger study focused on nontarget effects of genetically modified crops expressing *Bacillus thuringiensis* δ-endotoxins on the general predator *Chrysoperla carnea*. A. Raps is a biologist specialized in multitrophic interactions between microorganisms, plants and herbivorous insects. Julia Kehr is a plant physiologist specialized in translocation processes in plants. P. Gugerli is a virologist and plant pathologist with special experience in immunological detection of viruses and specific proteins. W.J. Moar is an Associate Professor in Entomology investigating the toxicity of Bt Cry toxins and resistance development of herbivores. F. Bigler is an agroecologist and entomologist with special experience in biocontrol of insect pests and ecotoxicology of arthropods. A. Hilbeck is agroecologist specialized in plant and insect ecology and biocontrol.
