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ORIGINAL PAPER

Detection of Cry1Ab toxin in the leaves of *MON 810* transgenic maize

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Abstract The distribution of Cry1Ab toxin was detected in the leaves of genetically modified maize of genetic event *MON 810* by enzyme-linked immunosorbent assay. Cry1Ab toxin contents in the leaves at reproductive (milk, R3) phenological stage were measured to be between 3,878 and 11,148 ng Cry1Ab toxin/g fresh weight. Toxin content was significantly lesser (significant difference (SD)= 1,823 ng Cry1Ab toxin/g fresh leaf weight, $p<0.01$) in leaves at the lowest leaf level, than at higher leaf levels, probably due to partial leaf necrotisation. A substantial (up to 22%) plant-to-plant variation in Cry1Ab contents in leaves was observed. When studying toxin distribution within the cross and longitudinal sections of single leaves, lesser variability was detected diagonally, with approximately 20% higher toxin concentrations at or near the leaf vein. More significant variability (SD=2,220 ng Cry1Ab toxin/g fresh leaf weight, $p<0.01$) was seen lengthwise along the leaf, starting at 1,892 ng Cry1Ab toxin/g fresh weight at the sheath and rising to maximum concentration at the middle of the lamella. Cry1Ab toxin content may suffer significant (SD=2,230 ng Cry1Ab toxin/g fresh leaf weight, $p<0.01$) decreases in the leaf due to necrotisation. The results indicate that the longitudinal dimension of the leaf has more significance for sampling purposes than the diagonal position.

Keywords Genetically modified organism (GMO) · *MON 810* maize · Cry1Ab toxin · Enzyme-linked immunosorbent assay (ELISA)

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Introduction

European corn borer (*Ostrinia nubilalis*, Hübner) resistant first generation genetically modified (GM) plants express a foreign gene (*cry*) for protein derivatives of crystalline endotoxins (Cry toxins) produced by *Bacillus thurigiensis* (Bt) var. *kurstaki* (Berliner) [1, 2]. Such transgenic GM maize lines produce a truncated version of Cry1Ab proteins (genetic events *SYN-EV176-9, SYN-BT011-1*, *MON 810*). Since this Cry toxin is considered an insecticidal active ingredient of these plants, and since characteristics of the GM plants possibly influenced by environmental factors constitute an essential part in their environmental risk assessment [3–5], it is of crucial importance to possess proper methods for toxin monitoring. The most commonly used formats are lateral flow devices and 96-well microplate-based enzyme-linked immunosorbent assay (ELISA) [6], and various innovative analytical techniques have also been developed for quantitative or qualitative detection of Cry1Ab protein including microsphere-based immunoassays [7–9] or an immunomagnetic electrochemical sensor [10]. The use of ELISAs for Cry1Ab monitoring has been reported extensively [6, 7, 11–20].

Although *MON 810* cultivars (Agrigold, Asgrow, DeKalb, Garst, Golden Harvest, Monsanto, Pioneer, etc.) were registered in the USA and Europe as early as in 1996 and 1998, respectively, Cry1Ab toxin production of the cultivars to date has not been properly explored. The Monsanto documents [21–24] mostly refer to data (Table 1) from field trials in Europe and the U.S. from 1994 to 1996 as non-published studies. These sources mention Cry1Ab toxin content expressed in the first, second and third leaf levels (the lowest leaf level assigned as first level), 9,870, 8,430 and 4,910 ng Cry1Ab/g fresh weight, respectively. Nonetheless, this limited and partial information presented by

Monsanto is hard to judge without suitable specifications on cultivars and exact ELISA methods of determination applied. During the last decade, only a few papers were published about Cry1Ab toxin production of *MON 810* cultivars (Table 2), even though the entire phenomenon of variable toxin production is still unclear. *MON 810* cultivars produce Cry1Ab toxin in a tissue- and time-specific manner [25–27]. Soil quality, especially its nitrogen fertility also has a strong influence on Cry1Ab toxin expression [28], and multi-stack varieties have been reported to have expressed toxin content two fold higher than in single stack *MON 810* cultivars [29]. As reported Cry1Ab toxin content often scatter over wide ranges within a single survey, concerns regarding non uniform expression levels of the transgene and plant-to-plant variation in Cry1Ab contents were raised [27, 30]. Our aims in this work were to measure Cry1Ab toxin content in the leaves of *MON 810* DeKalb cultivar DK-440 BTY under experimental cultivation and to assess Cry1Ab distribution among leaf levels and within individual leaves of using the validated commercial Abraxis Bt-Cry1Ab/Ac ELISA method.

Materials and methods

Seeds of *MON 810* maize cultivar DK-440 BTY and its near isogenic variety DK-440 were kindly provided by Monsanto Hungária Ltd. (Budapest, Hungary). Both *Bt* maize and near isogenic varieties were grown at the Ecological Experimental Station of the Plant Protection Institute, Hungarian Academy of Sciences (Julianna-major, Nagykovácsi, Hungary). Cry1Ab toxin was obtained from three sources, purchased from Abraxis Inc. LLC (Warminster, PA, USA), acquired from Sándor Szobszlay (Department of Environmental Protection and Safety, Szent István University, Gödöllő, Hungary), and received from Luke Masson (Biotechnology Research Institute, National Research Council of Canada, Montreal, Quebec, Canada) [31]. Chemicals were purchased from Sigma-Aldrich Ltd. (Budapest, Hungary) unless stated otherwise. Data were analysed using Statistica software by StatSoft Inc. (Tulsa, OK, USA). Standard curves for analytical determinations were calculated by simple linear regression in a narrow concentration range (up to 4 ng/mL) and sigmoid regression for a broader concentration range (up to 50 ng/mL) using the four-parameter logistic model [32]. Cry1Ab concentrations determined by linear regression are expressed as mean±standard deviation (sd) unless stated otherwise. Statistical uniformity of detected concentrations was determined in unpaired two-sample Student's *t*-tests. Significant differences (SD) among Cry1Ab concentrations were calculated using one-way analysis of variance (ANOVA) with the probability level (p) specified. Post-hoc analysis (Fisher's least significant difference (LSD test) was used to assign homogeneous groups (in which members are not statistically different from each other) at the probability level of the ANOVA tests in each experiment on Cry1Ab toxin content in leaves (cross sampling, longitudinal sampling and necrosis sampling measurements). Statistical grouping of the data in each experiment is assigned by capital letters in parentheses denoting separate homogeneous groups, but homogeneous groups in one experiment are not related to those in the other experiments.

Enzyme-linked immunosorbent assays

Cry1Ab toxin content in maize leaves was determined by commercial 96-well microplate format sandwich immunoassay, Abraxis Bt-Cry1Ab/Ac ELISA kit (#PN 51001, Warminster, PA, USA). For sample preparation, approximately 20 mg of ground maize samples were homogenised in 0.5 mL of manufacturer-provided extraction buffer, and were centrifuged at 12,000 rpm for 3 min. Samples were further diluted with phosphate-buffered saline (PBS) buffer as needed, at least 1:50. Immunoassays were carried out in 96-well ELISA microplates according to protocols provided by the manufacturers, in sequential steps, with a washing step with phosphate-buffered saline (PBS with 0.02% Tween 20) buffer (pH 7.4) in between each. Samples and calibrators were added to the plates in triplicates. Monoclonal Cry1Ab-specific mouse antibodies in PBS buffer were added, after the addition of the samples and calibrators, and the plate was incubated for 30 min at room temperature, washed, followed by the addition of an anti-mouse IgG-HRP enzyme conjugate, and the plate was incubated for a further 30 min at room temperature. Finally, hydrogen peroxide as a substrate and tetramethyl benzidine as a chromophore diluted in citrate buffer (pH 5.0) were added. Colour development was measured in the kinetic mode at a wavelength of 650 nm, the colour reaction was stopped after 20 min by adding 4 M sulphuric acid, and the colour intensity was read at 450 nm wavelength on an iEMS microtiter plate reader (Labsystems, Helsinki, Finland). Calibrators were put on every microplate at concentrations between 0.25 and 4 ng/mL, assays were used for determination of the analyte concentration in the linear range of determination by linear regression. The limit of detection (LOD) claimed by the manufacturer, defined as the concentration corresponding to an assay signal of 3 standard deviations above the background signal (negative corn leaf sample) interpolated from a Cry1Ab standard curve by linear regression is 0.125 ng/mL Cry1Ab in corn leaf extract [33]. Consequently, the lower limit of detection in the plant samples was 156 ng Cry1Ab toxin/g fresh weight.

Table 1 Immunoreactive Cry1Ab toxin content in *MON 810* event maize in Monsanto studies

Location	Cry1Ab toxin content (ng Cry1Ab/g fresh weight)			Reference
	Leaf	Pollen	Grain	
USA	7,930–10,340	90	190–390	[23] referred by [21]
EU	7,590–9,390	–	420–690	[21]

Cultivation and sampling

Plants were cultivated with six border rows of near isogenic maize on plots of 400 m^2 area fenced off from other cultivation sites in Julianna-major. Phenological stages were designated by Ritchie et al. [34]. Leaf samples of DK-440 BTY and its near isogenic line, DK-440 were taken from different leaf levels at developmental stage R3. Samples were taken, as appropriate, either as leaf strips or leaf discs. For Cry1Ab toxin content determination at various leaf levels, leaf strips of 5 mm width approximately 80 mg in weight were cut from the middle section of each green leaf. In the case of sample position studies within individual leaves (Fig. 1), leaf discs of 5 mm in diameter were collected from the tenth leaf level (the lowest leaf level assigned as first level). For diagonal analysis, 5-mm-diameter leaf discs were cut from the middle part of leaves taken from the tenth leaf level of the maize plant: the centre disc contained the leaf vein; subsequent discs were obtained in 1-cm distance from each other towards leaf edge. For longitudinal analysis, 5-mm-diameter-wide leaf strips were cut at the middle between vein and perimeter of the tenth leaf lamella. Leaf tips were sampled by cutting the last 10-mm long portion of the leaf towards its end. Leaf tip samples were prepared with and without necrotic perimeter. Samples were immediately processed upon sampling for fresh and dry weight measurements, or were stored frozen (−40 °C) for toxin content determination by ELISA.

Internal reference laboratory samples were also prepared from corn tissues collected from the field, and were used for ELISA reliability determination. Corn leaves (approx. 1 kg) collected from six corn plants at the R1 phenological stage were chopped and lyophilised in batches in 1-litre volume round flasks. The dry material was further homogenised in a Retsch GM 200 cutting mill (Retsch GmbH, Haan, Germany), and was stored in aliquots at −60 °C. Another reference plant sample (#OR4) containing leaves, roots and stem of DK-440 BTY was prepared, and stored frozen in aliquots. This sample represented all plant matrices, in natural weight proportions in silage, was dried and ground together, and was used as an internal laboratory standard for analytical quality control.

Leaf surface area measurements

For measuring the necrotic proportion of the leaf tips, 27–29 mg samples of the tenth leaf levels were cut, similar to sampling for ELISA. Leaf surfaces were measured in quadruplicate leaf samples, in five repetitions each, by portable leaf area meter LI-3000 (LI-COR Biosciences Inc, Lincoln, NE, USA) complemented with LI-3050A transparent belt conveyer accessory. Area determinations allowed a resolution of 1 mm^2 (1×1 mm scanning area) with an accuracy of ±2% specified by the manufacturer. Measurements were carried out on leaf tips with and without necrotic perimeter, if detected.

Results and discussion

Analytical characterisation of Cry1Ab detection by ELISA

The commercial Bt-Cry1Ab/Cry1Ac ELISA system, used in this study, is validated and distributed by the manufacturer (Abraxis Inc., Warminster, PA, USA) for quantitative determination of Cry1Ab and Cry1Ac endotoxins in

Table 2 Immunoreactive Cry1Ab toxin content in *MON 810* event maize cultivars

Cultivar	Cry1Ab toxin content (ng Cry1Ab/g fresh weight)[a]					Reference
	Leaf	Root	Stem	Anther wall	Grain	
Agrigold A6609Bt	760–2300					[25]
Asgrow RX799Bt	770–2390					[25]
Monsanto Novelis	440–11070	340–2031	350–2060	300–6650	10–510	[26]
Monsanto Novelis	320–11070	270–4170	0.08–2610	300–6650	10–510	[27]
Pioneer P31B13Bt	660–2200					[25]
Pioneer P33V08Bt	350–530					[28]
Pioneer P33V08Bt	660–2170					[25]

[a] Cry1Ab levels were measured with EnviroLogix Cry1Ab/Cry1Ac QuantiPlate Kit

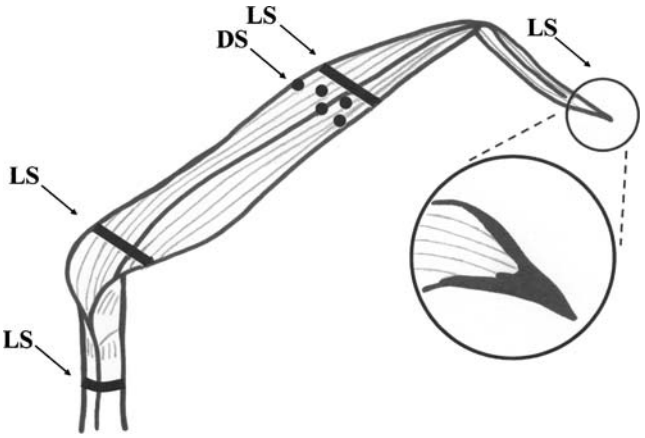

Fig. 1 Schematic representation of sampling positions for longitudinal and diagonal analysis of Cry1Ab toxin determination in individual maize leaves. Leaves were collected at dough (R4) phenological stage from the tenth leaf level (carrying female flowers). Sampling locations indicated as leaf strips for longitudinal sampling (*LS*) and leaf discs for diagonal sampling (*DS*)

microbial preparations and plants (cotton, corn). A limiting feature of the immunoassay in detecting the plant-expressed Cry1Ab protein is that it is based on antibodies directed against a 131-kDa molecular weight microbial protoxin, either purified from *B. thuringiensis* ferments or produced by transgenic microbes containing the *cry1Ab* gene encoding it. Moreover, the method uses the same protoxin as analytical standard [F. Rubio, Abraxis Inc., Warminster, PA, USA, personal communication]. In contrast, the *cry1Ab* transgene present in *MON 810* maize cultivars expresses a 91-kDa N-terminal fragment of it, so called preactivated toxin form [22]; consequently, ELISA results on plants are subject to correction for preactivated toxin.

Detection by standard curves using manufacturer-provided Cry1Ab standards at concentrations of 0, 0.25, 0.5, 1, 2 and 4 ng/mL (linear regression) and standards from three origins (Abraxis Inc., Szent István University, Hungary and Biotechnology Research Institute, Canada) at concentrations of 0.01 to 50 ng/mL (sigmoid regression) are shown in Fig. 2. The standard curves showed an upward sigmoid course typical for sandwich ELISA systems, and IC_{50} values appeared identical for the standard curves obtained with Cry1Ab toxin from three origins (12.28±0.53, 12.61±0.63 and 13.25±0.37 ng/mL for Cry1Ab from Abraxis Inc., Szent István University, Hungary and Biotechnology Research Institute, Canada, respectively). Moreover, a standard curve obtained with Cry1Ab from Abraxis Inc. in maize leaf extract also showed a course identical (IC_{50}=11.93±0.55 ng/mL for Cry1Ab) indicating no matrix effect under the experimental conditions. LODs determined from the sigmoid standard curves are 5 ng/mL for Cry1Ab in buffer and 6 ng/mL for Cry1Ab in leaf extract.

To achieve lower LODs, the commercial Bt-Cry1Ab/Cry1Ac ELISA system utilises linear regression to the standard curve in the concentration range of 0 to 4 ng/mL. Such forced shifting of the LOD downwards is driven clearly by commercial interests and not analytical considerations as the further the tested concentration is from the inflexion point of the sigmoid curve (IC_{50}), the more the accuracy of the data is deteriorating. The lower curvature part of the sigmoid curve is certainly less reliable for calibration than the middle, quasi-linear section, and should ELISA signals in the concentration range of the thus lowered detection limit fall significantly above background,

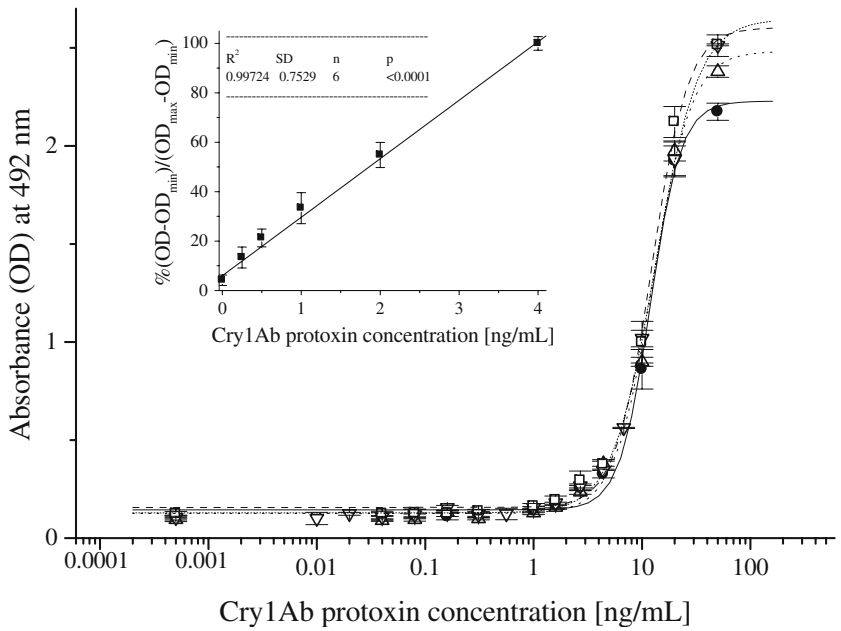

Fig. 2 Standard curves for Cry1Ab toxin in the Abraxis Bt-Cry1Ab/Cry1Ac ELISA system based using sigmoid and linear regression. Sigmoid regression on standard curves in PBS spiked with Cry1Ab from Abraxis (□, *dashed line*), Szent István University, Hungary (⋀, *dotted line*), Biotechnology Research Institute, Canada (▽, *short dotted line*), and in leaf extract spiked with Cry1Ab from Abraxis (●, *solid line*). Linear regression on standard curves in PBS spiked with Cry1Ab from Abraxis is shown in the insert

their validity for calibration may become questionable. Nonetheless, the ELISA system provides stable calibration lines in the concentration range of 0.25 to 4 ng/mL. The insert in Fig. 2 displays the average of normalised linear regression lines determined in 37 individual determinations with a regression coefficient (R^2) of 0.997 (regression coefficients in individual determinations ranged between 0.964 and 0.9995) and an LOD of 0.125 ng/mL as claimed by the manufacturer. Normalisation itself does not affect LOD, but allows comparison among individual standard lines of different optical density signals. It has also to be noted that sigmoid regression curves were obtained with pure Cry1Ab protein dissolved in PBS, while linear regression lines were obtained with manufacturer standard solutions in a buffer of undisclosed exact composition. The solution contained a stabiliser protein for Cry1Ab [F. Rubio, Abraxis Inc., Warminster, PA, USA, personal communication], but it is proprietary information whether the manufacturer applied solubiliser substances.

Accuracy of the toxin concentration detected by the ELISA method was evaluated using standard addition method [35]. An internal reference lyophilised corn leaf sample, collected in the R1 phenological stage, was subjected to ELISA analysis. Cry1Ab toxin content was measured in the leaf extract diluted 1:500 by PBS, and was found to contain 1.25±0.03 ng/mL Cry1Ab toxin corresponding to 15.61±0.41 μg/g Cry1Ab/g lyophilised leaf tissue (with the 19.2%±1.1% measured dry weight content of the lyophilised corn leaf sample, this concentration represents 3.00±0.08 μg/g Cry1Ab/g raw leaf tissue.) The diluted extract (250 μl) was spiked at three levels by the addition of various volumes (100, 200, 250 μl) of a standard solution (4 ng/mL Cry1Ab), the spiked solutions being supplemented in all cases to 500 μl, and Cry1Ab concentrations were detected by ELISA. The ELISA signal plotted against the concentration of the added standard provided excellent linear correlation (r^2=0.9982), and on the basis of the four spiked concentrations (0, 0.8, 1.6 and 2 ng/mL, respectively) the initial concentration in the diluted extract was found to be 1.29±0.04 ng/mL Cry1Ab toxin corresponding to 16.20±0.47 μg/g Cry1Ab/g lyophilised leaf tissue (3.12±0.09 μg/g Cry1Ab/g raw leaf tissue). As revealed in Student's t test (t=1.56, p<0.01), no significant difference was found between the Cry1Ab toxin concentration determined by the calibration curve (1.25± 0.03 ng/mL) and the standard addition method (1.29± 0.04 ng/mL), the 3.9% difference in the determined Cry1Ab concentration indicates no substantial matrix interaction at the dilution (1:500) applied.

For quality assurance, an internal reference sample (#OR4) was used. Cry1Ab toxin concentration measured in this sample in two different years (2006 and 2009) provided 3,432±1,337 and 3,522±284 ng/g concentrations, respectively. Student's t test revealed no statistical difference between the two determinations (t=0.15, p<0.01) indicating reliable detection of Cry1Ab toxin during a several-year period.

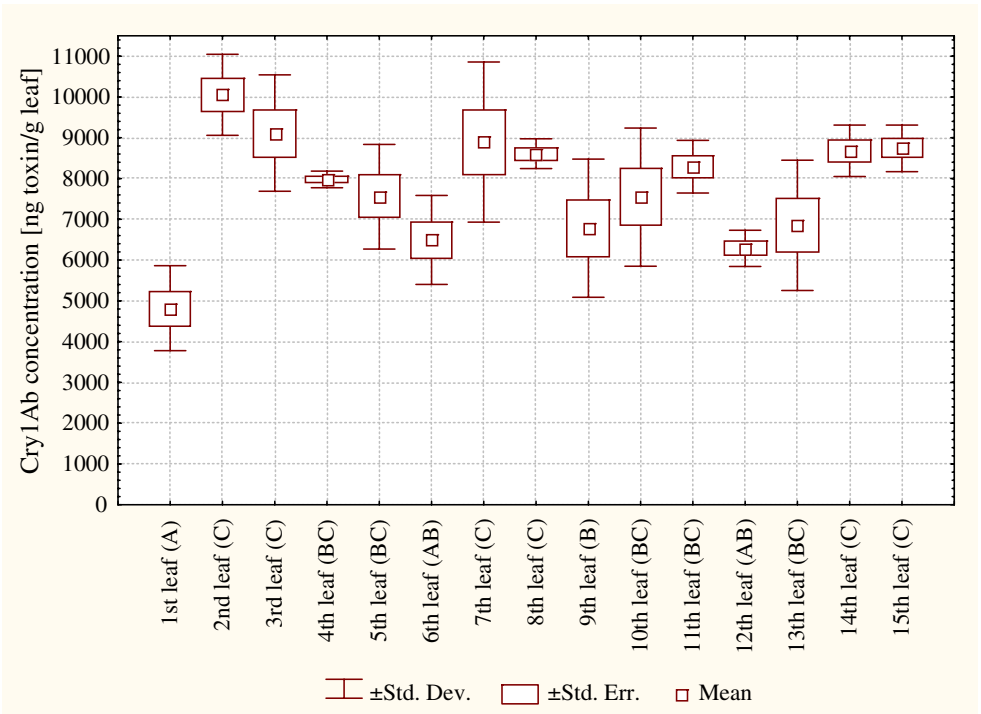

Fig. 3 Cry1Ab toxin content of *MON 810* maize (DK-440 BTY) leaves originated from different leaf levels at milk (R3) phenological stage. Lowest leaf level corresponds to the visible first leaf. Different capital letters indicate significant differences (SD=1,823 ng Cry1Abtoxin/g fresh leaf weight, p<0.01)

Table 3 Cry1Ab toxin content of tenth leaf level

Plant organ leaf (10^{th} leaf level)		Cry1Ab toxin content [a] average ± standard deviation (ng Cry1Ab/g fresh weight)
middle cross sampling [b]	vein	9885 ± 877 (B)
	near vein	10552 ± 1065 (B)
	2 cm from vein	8509 ± 351 (A)
	3 cm from vein	8571 ± 480 (A)
	edge	8194 ± 562 (A)
longitudinal sampling [c]	sheath	1892 ± 223 (C)
	lamella base	5535 ± 1073 (E)
	lamella middle	8924 ± 1507 (F)
	lamella tip	4579 ± 1864 (D)
	green lamella tip [d]	7749 ± 1598 (EF)
Stem		1071 ± 101
necrosis sampling [e]	unnecrotised (green) lamella	9023 ± 1697 (I)
	partially necrotised (yellow) lamella	6089 ± 1229 (H)
	necrotised (brown) lamella	2550 ± 153 (G)

[a] Cry1Ab toxin levels were determined in the milk (R3) phenological stage for diagonal and longitudinal sampling and in the dough (R4) phenological stage for necrosis sampling. Plant samples were analysed by Abraxis Bt-Cry1Ab/Ac ELISA kit at least in quadruplicates

[b] Different capital letters in parentheses (A, B) indicate significant differences (SD=1,155 ng Cry1Ab toxin/g fresh leaf weight, $p<0.01$) within cross sampling measurements. Homogeneous groups A and B are not related to those assigned by letters C–I

[c] Different capital letters in parentheses (C–F) indicate significant differences (SD=2,220 ng Cry1Ab toxin/g fresh leaf weight, $p<0.01$) within longitudinal sampling measurements. Homogeneous groups C–F are not related to those assigned by letters A, B and G–I

[d] Lamella tip with necrotised edges removed

[e] Different capital letters in parentheses (G–I) indicate significant differences (SD=2,230 ng Cry1Ab toxin/ g fresh leaf weight, $p<0.01$) within necrosis sampling measurements. Homogeneous groups G–I are not related to those assigned by letters A–F

Cry1Ab distribution among leaf levels

Average Cry1Ab toxin content in the leaves of *MON 810* maize cultivar DK-440 BTY in the milk (R3) phenological stage, determined by Abraxis Inc Bt-Cry1Ab/Ac ELISA kit, ranged between 4,821 and 10,054 ng toxin/g fresh leaf weight (Fig. 3). In contrast, Cry1Ab toxin was not detected in any samples taken from the near isogenic maize line (negative control). Considering the claimed LOD of the Abraxis Bt-Cry1Ab/Ac ELISA kit (0.125 ng/mL) and actual sample preparation parameters, this means that the near isogenic maize line does not contain Cry1Ab toxin above 156 ng Cry1Ab/g fresh weight concentration. Cry1Ab toxin content in *MON 810* maize cultivar DK-440 BTY showed both within plant and plant-to-plant variations. Relative standard errors within single sample determinations in triplicates ranged between 0.6 and 6.2%, indicating the utility of the Abraxis Bt-Cry1Ab/Ac ELISA kit, as has also been reported in the literature [36]. Nonetheless, relative sample-to-sample fluctuation of toxin content within the same plant was found to be as high as 19–21%, and those between samples from different plants ranged up to 22%. Statistical analysis (ANOVA+t test) revealed that Cry1Ab toxin content in the lowest leaf level is significantly different in toxin content from all other leaf levels (SD=1,823 ng Cry1Abtoxin/g fresh leaf weight, $p<0.01$). On the basis of such groping, Cry1Ab toxin content was determined to be 4,821±1,042 ng Cry1Ab toxin/g at the lowest leaf level and 7,990±1,530 ng Cry1Ab toxin/g fresh weight at all higher leaf levels (ranging between 5,090 and 11,050 ng Cry1Ab toxin/g fresh weight). Cry1Ab toxin contents in the latter 14 leaf levels were grouped statistically into two separable groups ($p<0.01$). A possible explanation may be a difference in microclimatic conditions: adequate rates of nitrogen fertility during early growth are known to be essential for Cry toxins (including Cry1Ab) production by the plant, and nitrogen abundance may cause several-fold increase in Cry1Ab content [28], therefore differences in reachable nitrogen levels due to varying precipitation may cause such variability if certain actual leaf formation periods fell to dry climatic intervals. The data therefore, indicate quantitative determination of acceptable accuracy

by the ELISA method, yet significant biological variability among samples of individual plants at the same developmental stage.

These toxin concentrations measured are in agreement with the official *MON 810* documentation [21] and with certain literature data [26, 27], where toxin content was detected in the same range, with a strong fluctuating trend, and with statistical differences between top and bottom leaves [27]. Other studies reported lower Cry1Ab concentrations in corn borer resistant maize hybrids of the *MON 810* event, e.g., 350-534 ng/g in the leaves [28]. Nonetheless, on the average 20-fold differences can be seen in the toxin content even of the same *MON 810* maize variety (Novelis) depending on cultivation year or location [26, 27].

Cry1Ab distribution within single leaves

Aiming at a better understanding of the background of the high variability among Cry1Ab toxin content determinations in various leaf samples, the distribution of the toxin protein within single leaves was also studied. Toxin content in various segments of the leaf from the tenth leaf level carrying female flowers was determined both diagonally and lengthwise. Detected Cry1Ab toxin concentrations are listed in Table 3. When subjecting leaf discs sampled diagonally from the middle section of the tenth leaf to ELISA determination, average Cry1Ab toxin content fell into the range of 8,200–10,550 ng Cry1Ab toxin/g fresh leaf weight. Statistical analysis of the cross section data revealed that toxin concentrations were grouped into two significantly different groups (termed groups A and B in Table 3, SD=1,155 ng Cry1Ab toxin/g fresh leaf weight, $p<0.01$): Cry1Ab content at and near the leaf vein (group B in Table 3, 9,900–10,550 ng Cry1Ab toxin/g fresh weight,) appeared some 20% higher than towards leaf edge (group A in Table 3, 8,200–8,600 ng Cry1Ab toxin/g fresh weight). Even though toxin concentrations are somewhat lower than in the cross middle of the leaves, the lamella section appear to be better for sampling purposes for Cry1Ab toxin analysis due to better tissue homogeneity.

For longitudinal analysis, leaf strips were obtained evenly across the length of the leaf from the sheath to the leaf tip. Considerably lower toxin concentrations (1,892±223 ng Cry1Ab toxin/g fresh leaf weight) were detected at the sheath, while the highest toxin content (8,924±1,507 ng Cry1Ab toxin/g fresh leaf weight) was measured in the middle section of the leaf, with a decreasing trend towards the leaf tip. Statistical analysis grouped toxin content in the sheath, lamella base, lamella middle and tip into four separate groups (termed groups C to F in Table 3, SD=2,220 ng Cry1Ab toxin/g fresh leaf weight, $p<0.01$). To decipher whether the strongly decreasing trend in toxin concentration at the leaf tip is due to natural necrosis, toxin concentrations were also measured in leaf tip samples with and without their necrotised tissues at the leaf edges. Toxin contents detected in the leaf tip statistically significantly differed from each other when measured in the intact leaf tip (4,579±1,864 ng Cry1Ab toxin/g fresh leaf weight, statistical group D in Table 3) and with the necrotised tissues removed (7,749±1,598 ng Cry1Ab toxin/g fresh leaf weight, statistical groups E and F in Table 3), i.e. toxin concentration in the green part of the leaf tip fell into the same statistical groups as the lamella base and middle. The apparent increase in Cry1Ab toxin content (59.1±24.1%) due to the removal of the necrotised edges seemed to correlate with the necrosis ratio of the leaf tips (39.9±4.3%) assessed by scanning optical leaf surface area measurements detecting surfaces of the healthy and necrotised areas at the leaf tips where such necrotisation occurred (Fig. 1). Leaf tip necrotisation, therefore, is a plausible cause of the lower Cry1Ab toxin concentrations. To test this hypothesis, corn leaves were similarly collected at a later, dough (R4) phenological stage, when substantial necrotisation occurred on some leaves, and Cry1Ab toxin content was determined in living (green), partially necrotised (yellow) and necrotised (brown) leaves. The toxin content of leaves of these three different necrotisation rates were grouped into three separate statistical groups (termed groups G to I in Table 3, SD=2,230 ng Cry1Ab toxin/g fresh leaf weight, $p<0.01$). Thus, significantly decreased Cry1Ab concentrations were found in the yellow and brown leaf tissues (6,089±1,229 and 2,550±153 ng Cry1Ab toxin/g fresh leaf weight, respectively) compared to the healthy leaf (9,023±1,697 ng Cry1Ab toxin/g fresh leaf weight). Whether this decrease in Cry1Ab concentration is due to decomposition of the toxin during necrosis or to its active transport along with other plant proteins from the necrotising tissue [37] is an open question.

The data indicate a substantial variation in detectable Cry1Ab toxin content lengthwise along the leaf. Lower toxin concentrations at the sheath are closer to the toxin content detected in the stem (1,071±101 ng Cry1Ab toxin/g fresh weight), gradually increasing towards the lamella middle, and later remaining stable unless leaf tissue necrotisation causes further decrease. Cry1Ab toxin content apparently proportional with chlorophyll content is in agreement with literature data [25], when toxin concentrations within a single leaf were found highest (600±50 and 590±70 ng Cry1Ab toxin/g fresh weight) at the green leaf tip, and lowest in the yellow leaf base (180±10 and 220±70 ng Cry1Ab toxin/g fresh weight) indicating decreasing Cry1Ab toxin concentrations with decreasing chlorophyll content. Our results indicate that the location of leaf samples taken from a single maize plant and within a

single leaf affects the detectable Cry1Ab protein concentration in the sample. The longitudinal dimension of the leaf has more significance for sampling purposes than the diagonal position; therefore, the diagonal lamella middle in the middle section of the leaf is most advisable for taking leaf samples.

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