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CRY1AB TOXIN PRODUCTION OF *MON 810* TRANSGENIC MAIZE

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Abstract—Levels of Cry1Ab toxin were detected in genetically modified maize of genetic event *MON 810* against near isogenic maize as negative control by two commercial immunoassays. The immunoassays were characterized for their cross-reactivity (CR) between Cry1Ab protoxin and activated toxin, and were compared with each other for toxin detection in a reference plant sample. Cry1Ab toxin levels, corrected for active toxin content using the CR values obtained, were monitored in maize DK-440 BTY through the entire vegetation period. The toxin concentration was found to show a rapid rise in the leaves to 17.15 ± 1.66 μg/g by the end of the fifth week of cultivation, followed by a gradual decline to 9.61 ± 2.07 μg/g by the 16th week and a slight increase again to 13.51 ± 1.96 μg/g during the last 2 weeks due to partial desiccation. Similar but lesser fluctuation of toxin levels was seen in the roots between 5.32 ± 0.49 μg/g at the less differentiated V1 stage and 2.25 ± 0.30 μg/g during plant development. In contrast, Cry1Ab toxin levels appeared to be stably 1.36 ± 0.45, 4.98 ± 0.31, 0.47 ± 0.03, and 0.83 ± 0.15 μg/g in the stem, anther wall, pollen, and grain, respectively. Toxin concentrations produced at the VT-R4 phenological stages under actual cultivation conditions were compared with each other in three different years within an 8-year period. Environ. Toxicol. Chem. 2010;29:182–190. © 2009 SETAC

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INTRODUCTION

Event *MON 810* maize contains *cry1Ab* transgene, as known to be originated from *Bacillus thuringiensis* (Bt) and inherited in its several subspecies [1]. Within the agricultural utilization of genetically modified organisms (GMOs) transgenic tomato was the first model for Bt plants published by Monsanto scientists [2]. In this plant, a truncated *cry1* transgene of *B. thuringiensis* ssp. *kurstaki* HD-1 was used. *MON 810* maize varieties contain *cry1Ab* transgene also in a shortened form. The full length of *B. thuringiensis* ssp. *kurstaki cry1Ab* gene produces a 131 kDa Cry1 protoxin, while *MON 810 cry1Ab* transgene expresses a 91 kDa N-terminal fragment of it—so called preactivated toxin form, as documented in the official registration and reassessment documents of Bt plants of genetic event *MON 810*, listed in the database of AGBIOS (Merrickville, ON, Canada), the Canadian company dedicated to providing public policy, regulatory, and risk assessment expertise for products of biotechnology (http://agbios.com/docroot/decdocs/02-269-010.pdf; http://agbios.com/cstudies.php?book = FSA&ev = MON810&chapter = Expressed&lang). DiPel® (Valent BioSciences) as a biological insecticide against Lepidoptera larvae contains 80% Cry1A (a, b, and c) and 20% Cry2 (A and B) toxins produced by the different *cry* genes located on bacterial plasmids [3]. Cry protoxins of *B. thuringiensis* act as trypsin-activated toxins in sensitive insect larval groups. However, to become active and exert their insecticidal activity, protoxin molecules have to be processed in the insect midgut by trypsin-like and other proteases. Proteolytic activation usually leads to rapid removal of the highly conserved C-terminal protoxin tail. Various cysteine residues in this part are involved in intramolecular disulfide bridges that cross-link the different protoxin molecules in the unsolubilized crystal. At the N-terminal end, a few residues are cleaved off more slowly resulting in a final toxic fragment of molecular size between 60 and 70 kDa [4–7]. Smaller protoxins lacking the conserved C-terminal region are minimally trimmed at both ends upon proteolytic activation.

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, Cry1A toxin production of the cultivars to date has not been properly explored. In the Monsanto documents registered in the databases of AGBIOS (see above), the U.S. Environmental Protection Agency (http://www.agbios.com/cstudies.php?book = FSA&ev = MON810&chapter = Expressed&lang), and the U.S. Department of Agriculture (http://www.agbios.com/docroot/decdocs/05-242-021.pdf), mostly the same, nonpublished measurements are cited, referring to Cry1Ab toxin content of 7.93 to 10.61, 0.09, and 0.19 to 0.91 μg Cry1Ab/g fresh weight in leaf, pollen, and grain, respectively, in the U.S. documentation, and 7.59 to 15.06 and 0.35 to 0.69 μg Cry1Ab/g fresh weight in leaf and grain, respectively, in the European Union documentation. These studies also mention levels of Cry1Ab toxin expressed in the first-, second-, and third-leaf levels in pooled overseason samples from four-leaf to pollination stage, that is, 9.78, 8.43, and 4.91 μg Cry1Ab/g fresh weight, respectively. Nonetheless, this limited and partial information presented by Monsanto is hard to judge without suitable specifications on exact enzyme-linked immunosorbent assay (ELISA) methods of determination applied.

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During the past decade, only a few studies were published about Cry1Ab toxin production of *MON 810* cultivars, even though the entire phenomenon of variable toxin production is still unclear. Cry1Ab toxin content were reported in the leaves of cultivars Agrigold A6609Bt (0.76–2.30 μg/g [8]), Asgrow RX799Bt (0.77–2.39 μg/g [8]), Monsanto Novelis (0.44–11.07 and 0.32–11.07 μg/g [9,10]), Pioneer P31B13Bt (0.66–2.20 μg/g [8]), and Pioneer P33V08Bt (0.66–2.17 and 0.35–0.53 μg/g [8,11]). Parallel Cry1Ab levels in tissues other than leaves have been published for cultivar Monsanto Novelis, reporting toxin concentrations for root, stem, anther wall, and grain of 0.34 to 2.31, 0.35 to 2.06, 0.30 to 6.65, and 0.01 to 0.51 μg/g [9] and 0.27 to 4.17, 0.08 to 2.61, 0.30 to 6.65 and 0.01 to 0.51 μg/g [10], respectively. The data also reveal that *MON 810* cultivars produce Cry1Ab toxin in a tissue- and time-specific manner. Soil quality, especially its nitrogen fertility, also has strong influence on Cry1Ab toxin expression [11], and it has been noted that twofold higher toxin levels are expressed in multistack varieties than in single stack *MON 810* cultivars [12]. Moreover, toxin levels should be followed up on a regular basis in the characterization of the variety to show that it is distinct, uniform, and stable (DUS), and to assess how transgenes behave after decades of reproduction in a novel genomic context, whether Bt toxin expression has not undergone changes over time.

An additional technical problem has been that both Monsanto and independent studies on Cry1Ab toxin production in these cultivars used a commercial ELISA kit EnviroLogix Cry1Ab/Cry1Ac QuantiPlate (EnviroLogix) based on antibodies against protoxins [13]. This kit was unexpectedly withdrawn from the market in 2005, and was replaced with a qualitative ELISA method, EnviroLogix Cry1Ab/Cry1Ac QualiPlate. The analytical problem of why the Cry1Ab/Cry1Ac QuantiPlate has disappeared is still uncertain.

Because Bt crops mostly contain truncated transgenes, these plants (including *MON 810* maize) produce the preactivated toxin protein. As a result, it is improper in the analytical sense to directly apply ELISA systems devised against Cry1Ab/Cry1Ac protoxins to the measurement of plant toxin levels by using analytical standards of the protoxin protein. Two ways to overcome this problem is either to use plant-produced toxins as analytical standards, or to convert the protoxin standard into activated toxin before the analytical determination. An obstacle to the first approach is the lack of availability of plant-produced toxin proteins. The second approach, however, is readily available, as the enzymatic cleavage and activation of the protoxin into activated toxin has been studied in detail, and protocols for such enzymatic activation have been established [14–18].

The official registration and reassessment documents of Bt plants, including *MON 810*, submitted by the variety owners ([12,19]; http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad2/2-id_health.pdf) and listed in the AGBIOS database (see above) claim that enzymatic cleavage of Cry1Ab protoxin and of the plant produced truncated toxin result in the same 63-kDa protein. Moreover, it is also claimed that this active toxin and the truncated toxin produced by Bt plants or expressed in *Escherichia coli* were recognized with the same immunoreactivity by Cry1Ab-specific antibodies [19]. Monoclonal antibody lines generated against the activated toxin recognized the protoxin [9,20,21], and this affinity was not sequence- but conformation-dependent, as antibody binding to the protoxin was severely altered at elevated temperature [20]. The possible effect of protein conformation and other posttranslational factors in immunorecognition has also been emphasized [13,22]. Moreover, some (but not all) antibodies inhibited insect toxicity of the activated toxin, indicating antibody binding on the same protein epitopes that are responsible for toxin anchoring on their epithelial cell receptors in the insect midgut [20]. Polyclonal IgY antibodies against the activated toxin and their purified fraction monospecific to the active toxin could not be used for recognition the Cry1Ab protein produced by Bt maize (*MON 810* or *Bt 176*) neither in an indirect competitive, immobilized antigen-based ELISA, nor in a Western blot system [9].

The aims in the present study were to assess Cry1Ab toxin production of *MON 810* genetic event under experimental cultivation; to compare different Cry1Ab-specific ELISA kits for this purpose; and to test the detection efficacy of these kits for different Cry1Ab proteins (protoxin and activated toxin). Initial results [23] were obtained on EnviroLogix Cry1Ab/Cry1Ac QuantiPlate, and were revised with a similar commercial ELISA method, Abraxis Bt-Cry1Ab/Ac ELISA kit.

MATERIALS AND METHODS

MON 810 maize cultivar DK-440 BTY and its near isogenic variety DK-440 were kindly provided by Monsanto Hungaria in two batches. Cry1Ab toxin was obtained from two sources, purchased from Abraxis LLC and received from Luke Masson (Biotechnology Research Institute, National Research Council of Canada, Montreal, QC) [24]. Chemicals were purchased from Sigma-Aldrich, unless stated otherwise.

Enzyme-linked immunosorbent assays

Cry1Ab toxin content in maize plants was determined by commercial 96-well microplate format sandwich ELISAs, EnviroLogix Cry1Ab/Cry1Ac QuantiPlate® (AP 003) and Abraxis Bt-Cry1Ab/Ac ELISA kit (PN 51001). For sample preparation, approximately 20 mg of ground maize samples were homogenized in 0.5 ml of manufacturer-provided extraction buffer, and were centrifuged at 12,000 rpm for 3 min. Samples were further diluted as needed, but at least by 1:10. 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 reagent addition. Samples and standards (calibrators) were added to the plates in triplicates. In the case of the EnviroLogix Cry1Ab/Cry1Ac QuantiPlate, Cry1Ab-specific antibodies conjugated to horseradish peroxidase as a reporter enzyme, diluted in PBS (with 0.05% Tween 20; pH 7.4) were added after 15 min of incubation, and the plate was further incubated for 1 h at room temperature. Finally, the plate was washed, and hydrogen peroxide as a substrate and tetramethyl benzidine as a chromophore diluted in citrate buffer (pH 5.0) were added. Color development was measured in the kinetic mode at a wavelength of 620 nm, the color reaction was stopped after 20 min by adding 4 M sulfuric acid, and the color intensity was read at 450-nm wavelength on an iEMS microtiter plate reader (Labsystems, Helsinki, Finland). Tests with the Abraxis ELISA

kit were carried out similarly, but with one additional step: polyclonal Cry1Ab-specific rabbit antibodies in PBS buffer were added after the addition of the samples and standards, the plate was incubated for 30 min at room temperature, washed, followed by the addition of a goat anti-rabbit IgG-horseradish peroxidase enzyme conjugate, and the plate was further incubated for 30 min at room temperature. Standards were put on every microplate at concentrations ranging 0.5 to 5 ng/ml for EnviroLogix Cry1Ab/Cry1Ac QuantiPlate and 0.2 to 4 ng/ml for Abraxis Bt-Cry1Ab/Ac ELISA kit (representing the linear range of the sigmoid standard curves), analyte concentrations were determined by linear regression. The limit of detection (LOD) in the extracts was 0.14 ng/ml and 0.125 ng/ml in the EnviroLogix and Abraxis ELISA systems, respectively.

To assess analytical accuracy and precision of the ELISA systems, Shewhart analytical control charts [25] were plotted on concentration values determined in the ELISA systems for standard samples at medium concentration levels, that is, 2.5 ng/ml and 1 ng/ml for EnviroLogix Cry1Ab/Cry1Ac QuantiPlate and Abraxis Bt-Cry1Ab/Ac ELISA kit, respectively. Cross-validated concentration values for such controls were determined in each performed analysis by linear regression from the corresponding analytical standard line, and were plotted sequentially as shown in Figure 1 for the two immunoassays. The charts depict variability of the actual concentration values detected for the standard samples selected, and consider their closeness to target specifications (nominal standard concentrations) over time. Performance characteristics of the analytical measurements are considered well controlled if no consistent trends emerge among values detected and if values do not fall outside the prespecified limits, upper and lower control limits (UCL, LCL, respectively) and upper and lower warning limits (UWL, LWL, respectively) derived from mean analyte concentration values.

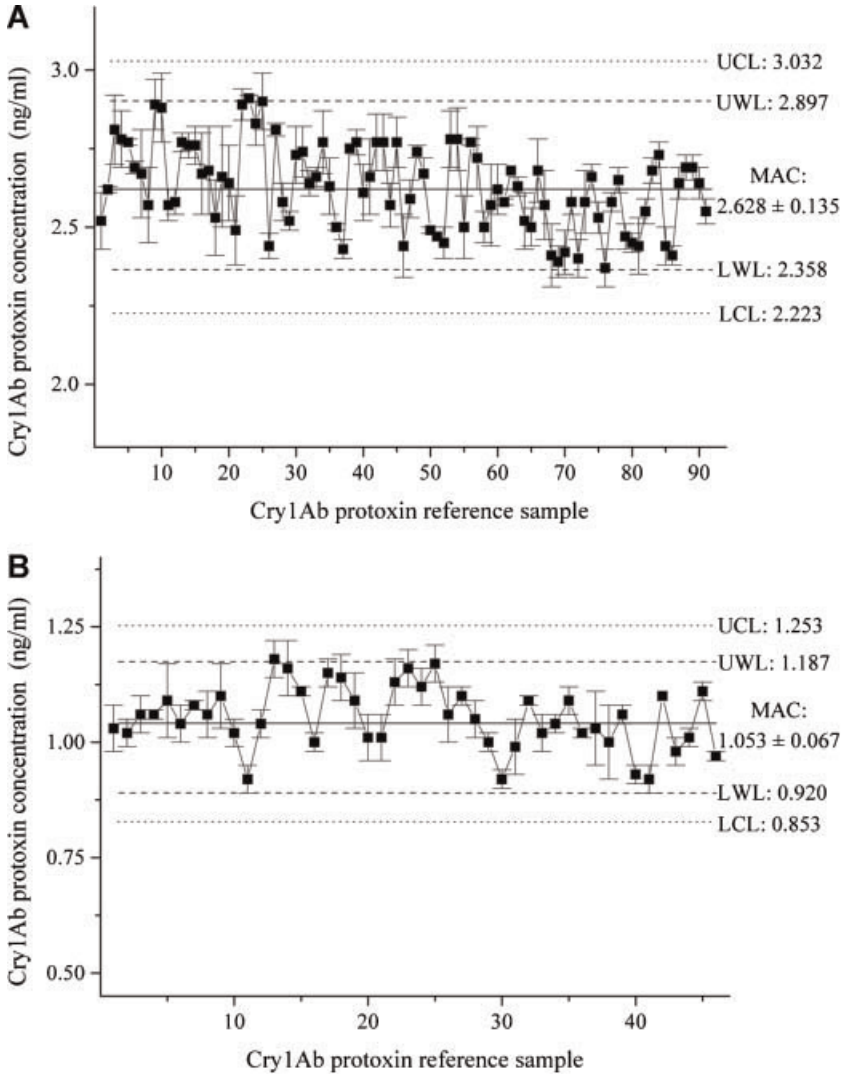

Fig. 1. Analytical control chart of determination of Cry1Ab protoxin content by the EnviroLogix Cry1Ab/Cry1Ac QuantiPlate system at concentration level 2.5 ng/ml (**A**) and Abraxis Bt-Cry1Ab/Ac ELISA kit at concentration level 1 ng/ml (**B**). Performance characteristics, i.e., upper control limit (UCL), lower control limit (LCL), upper warning limit (UWL), lower warning limit (LWL) were derived from mean analyte concentration (MAC). Control and warning limits were calculated from the average of 91 determinations for EnviroLogix Cry1Ab/Cry1Ac QuantiPlate providing control limits UCL 3.03 ng/ml and LCL 2.22 ng/ml, and warning limits UWL 2.90 ng/ml and LWL 2.36 ng/ml. Control limits were calculated from the average of 46 determinations for Abraxis Bt-Cry1Ab/Ac enzyme-linked immunosorbent assay providing control limits UCL 1.25 ng/ml and LCL 0.85 ng/ml, and warning limits UWL 1.19 ng/ml and LWL 0.92 ng/ml.

Enzymatic activation of Cry1Ab protoxin

To obtain the active Cry1Ab toxin, the microbial Cry1Ab protoxin was cleaved by trypsin according to literature method [15]. Enzyme treatments were carried out in 1-ml vials using 50 mM carbonate buffer (pH 10.5) containing 10 mM dithiotreitol (DTT) as a diluent. In each treatment, 5 μl of protoxin solution in the buffer at concentrations ranging from 0.18 to 7.5 μg/ml was added to 45 μl of trypsin (EC 3.4.21.4) solutions at concentrations ranging from 1.8 to 416.6 ng/ml at various toxin/trypsin ratios ranging between 0.45 and 55, and the mixture was incubated for 2 h at 37°C. To stop enzymatic activity, 5.6 μl of phenylmethanesulfonyl fluoride (PMSF) at a concentration of 10 mM was added, the mixture was vortexed at 540 rpm for 10 s. Aliquots of these vials, upon appropriate dilution (1:1000) in PBST buffer, were transferred onto 96-well ELISA microplates, and the binding affinity was detected by performing the ELISA protocol. Inactivation of the enzyme not only allowed exact control of the duration of the enzymatic reaction, but also excluded the possibility that antibodies could be degraded in the ELISA process. As negative controls, aliquots of protoxin similarly incubated without or with inactivated trypsin were also subjected to ELISA. Trypsin was inactivated by heat, keeping the enzyme aliquot at 90°C for 2 min and by preincubation with PMSF at 37°C for 30 min.

Cross-reactivities

The comparison of the ELISA signals obtained on protoxin solutions at set concentrations to those obtained by the same ELISA system on the trypsinized active toxin solutions provides immediate information on the cross-reactivity (CR) of the given ELISA system between Cry1Ab toxin and protoxin. Accordingly, CR between the activated Cry1Ab toxin and Cry1Ab protoxin was defined as the percentage ratio of the concentration values detected by ELISA for the two toxin forms at the applied concentrations.

Cultivation and sampling

Both Bt and near isogenic varieties were grown at the Ecological Experimental Station of the Plant Protection Institute, Hungarian Academy of Sciences (Julianna-major, Nagykovácsi, Hungary) in the 2001, 2005, and 2008 growing seasons. *Bacillus thuringiensis* plants were cultivated on plots of 200 to 400 m^2 area with three to six border rows of near isogenic maize and fenced off from other cultivation sites. Cry1Ab toxin production in *MON 810* maize cultivars was followed for four months upon planting. Plant samples of DK-440 BTY and its near isogenic line, DK-440 were taken nine times in approximately two-week intervals from various plant parts at least in four repetitions. Four entire plants were collected and dissected at each sampling time, and organs including, when appropriate, leaves, stem, root, anther, pollen, and grain were separated. Plant samples were immediately processed upon sampling for fresh and dry weight measurements and for toxin content determination by ELISA, or were stored frozen (−40°C). Entire leaves were subjected to sample preparation in the V1 phenostage, while leaf samples from the V3 phenostage on were taken as one to three stripes of 3- to 5-mm width and approximately 80-mg weight each, cut from the middle section of each green leaf. Composite samples were prepared from at least nine individual leaf stripes in equal amounts from each leaf of the plant, so that all green leaf levels were represented equally. Unused leaf stripes were stored frozen until possible parallel determinations. Therefore, the majority of the determinations were done at least in quadruplicates, ranging up to eight replications in certain cases. Phenological stages are designated by Ritchie et al. [26].

A reference laboratory sample (OR4) was prepared from collected plant materials for validation purposes. The sample containing leaves, roots and stem of DK-440 BTY, to represent all plant matrices, in natural weight proportions in silage, dried and ground together was prepared, and stored frozen in aliquots. This sample was used as internal laboratory standard for analytical quality control.

RESULTS AND DISCUSSION

ELISA with Cry1Ab protoxin and activated toxin

A common feature and disadvantage of the available commercial ELISA systems for the detection of Cry1Ab/Cry1Ac toxins is that these immunoassays are based on antibodies directed against microbial protoxin forms, either purified from *B. thuringiensis* ferments or produced by transgenic microbes containing the *cry1Ab/cry1Ac* gene encoding these protoxins. Moreover, these methods use the same protoxin as analytical standard; therefore, these immunoassays are validated to detect protoxin molecules in a quantitative manner (D. Grothaus, Monsanto, St. Louis, MO, USA, personal communication; F. Rubio, Abraxis, Warminster, PA, USA, personal communication).

Both commercial kits, EnviroLogix Cry1Ab/Cry1Ac QuantiPlate and Abraxis Bt-Cry1Ab/Ac ELISA kit were applied to determine toxin concentration prior and after enzymatic activation of the Cry1Ab protoxin. Results of the ELISA signal comparison are shown in Table 1. Using EnviroLogix Cry1Ab/Cry1Ac QuantiPlate and Cry1Ab protoxin (Biotechnology Research Institute, National Research Council of Canada, Montreal, QC) enzymatically activated toxin provided detected concentration levels in the range of 290–325 ng/ml for the initial protoxin concentration of 750 ng/ml. In the case of the Abraxis Bt-Cry1Ab/Ac ELISA kit and Cry1Ab protoxin (Abraxis), the corresponding levels were 150–165 ng/ml detected activated toxin level for initial protoxin concentration of 275 ng/ml. As negative controls, protoxin solutions were treated with heat deactivated and PMSF preinhibited trypsin as well to illustrate the lack of nonspecific effects on the immunoassay signal. In these negative controls, 95.4 to 99.7% of the initial toxin concentration was detected.

Enzyme-linked immunosorbent assay signals in all cases decreased in result of the enzymatic treatment relative to protoxin concentrations determined in the untreated protoxin solution. This is perspicuous as Cry1Ab-specific antibodies raised against bacterial protoxin are being used in both ELISA systems, and these antibodies are expected to show lower affinity to the truncated toxin protein of lesser or altered antigenic epitopes. As seen from Table 1, toxin/trypsin ratios in these experiments ranged between 2 and 55 (w/w) corresponding to molar ratios of 0.36 and 10, respectively. Such high molar proportions (even excess) of trypsin relative to its substrate (the protoxin in this case) compared to physiological

Table 1. Cry1Ab protoxin and activated toxin content during enzymatic cleavage of the bacterial protoxin and activated toxin/protoxin cross-reactivities, measured with EnviroLogix Cry1Ab/Cry1Ac QuantiPlate and Abraxis Bt-Cry1Ab/Ac enzyme-linked immunosorbent assay (ELISA) kit

Concentration				Concentration	CR[a]
	Cry1Ab protoxin				
Trypsin	Nominal	Detected	Trypsin/toxin ratio (w/w)	Cry1Ab activated toxin detected (ng/ml)	Cry1Ab activated toxin/protoxin (%)
	(ng/ml)				
375	750	748.3 ± 86.9 (A)[b]	0.50	292.2 ± 70.6 (B)[b]	39.1 ± 9.4
75			0.10	323.7 ± 47.2 (B)[b]	43.3 ± 6.3
125	275	277.1 ± 10.5 (C) [c]	0.45	150.5 ± 8.0 (D)[c]	54.3 ± 3.1
25			0.09	153.8 ± 8.3 (D)[c]	55.5 ± 3.0
5			0.02	164.3 ± 14.1 (D)[c]	59.3 ± 4.8

[a] Cross-reactivity (CR) between the activated Cry1Ab toxin and Cry1Ab protoxin was calculated as the percentage ratio of the ELISA signals by the two toxin forms at the applied concentrations.

[b] EnviroLogix Cry1Ab/Cry1Ac QuantiPlate (EnviroLogix) and Cry1Ab (Biotechnology Research Institute, National Research Council of Canada) were used, the nominal Cry1Ab toxin level was 750 ng/ml. Different capital letters in parentheses after each detected concentration range indicate significant differences at 1% significance level (212.4 ng/ml).

[c] Abraxis Bt-Cry1Ab/Ac ELISA kit and Cry1Ab (Abraxis) were used; the nominal Cry1Ab toxin level was 275 ng/ml. Different capital letters in parentheses after each detected concentration range indicate $SD_{1\%}$ (28.8 ng/ml).

conditions are chosen deliberately, so that complete amide cleavage occurs, and that is why no dependence of the protoxin cleavage on trypsin concentration is observed in this trypsin/toxin proportion range. Similar results were obtained when protoxin was applied at initial concentrations in the range of 18 to 36 ng/ml, and trypsin/toxin ratios varied between 0.05 and 2.2 (w/w), that is, 0.28 and 12.1 (mol/mol) (data not shown). The observed decrease in the detection of toxin concentration when activated toxin was measured instead of protoxin of the same concentration is due to lower binding of the antibodies to this shortened protein than to their original immunogen, Cry1Ab protoxin, and these experiments revealed that average CR were 0.412 and 0.564 in the EnviroLogix Cry1Ab/Cry1Ac QuantiPlate and Abraxis Bt-Cry1Ab/Ac ELISA kit, respectively. While these immunoassays are suitable, from the immunoanalytical aspect, for measuring protoxin content, e.g., in microbial samples, toxin concentrations detected by them in plant samples containing the preactivated toxin have to be corrected by these CR values.

Comparison of the Cry1Ab ELISA kits

The ELISA system developed by EnviroLogix (Cry1Ab/Cry1Ac QuantiPlate) has been a widely used immunoassay in Bt plant analysis both in monitoring Cry1Ab protein expressed in GM crops and for surveillance of transgenic protein in produce or animal tissues [6,13,27–34], until its sudden removal from the market in 2005. The similar immunoassay developed and commercialized by Abraxis (Bt-Cry1Ab/Ac ELISA kit) appeared to be a suitable replacement: it is also currently used for quantitative determination of Cry1Ab and Cry1Ac toxins [35], and the two ELISA systems are similar in their construction (both are sandwich ELISAs, use antibodies against bacterial protoxin with protoxin standards). Yet there also appear to be distinctive differences between the two immunoassays: the Abraxis kit uses polyclonal rabbit antiserum and anti-rabbit IgG-peroxidase conjugate, while the EnviroLogix Cry1Ab/Cry1Ac QuantiPlate applies antiserum-peroxidase conjugate (therefore, the assay sequence is two steps shorter than that of the former), and significant variation was seen in their CRs to the activated toxin (see above). Therefore, the two immunoassays had to be validated against each other. For this purpose, a reference laboratory sample (OR4) was used: toxin concentration measured by sample OR4 was evaluated by EnviroLogix Cry1Ab/Cry1Ac QuantiPlate in 2002 to contain 4.64 ± 0.36 μg toxin/g dry weight-based on the protoxin standard curve (raw concentration), which represented 11.26 ± 0.88 μg activated toxin/g dry weight content with the CR value of the immunoassay considered. Measuring the same sample by Abraxis Bt-Cry1Ab/Ac ELISA in 2006 and 2009 provided 1.94 ± 0.75 μg/g and 1.99 ± 0.16 μg/g raw toxin concentration, representing 3.43 ± 1.34 μg/g and 3.52 ± 0.28 μg/g activated toxin concentration, respectively. This indicates that EnviroLogix Cry1Ab/Cry1Ac QuantiPlate provided 2.36 times higher values in raw toxin concentration than the Abraxis Bt-Cry1Ab/Ac ELISA kit, corresponding to a 3.24 times difference in the calculated activated toxin content (Table 2). Results in the Abraxis kit underestimating the toxin content relative to the EnviroLogix ELISA system were similar to frozen leaf samples as well (data not shown). This indicates that the two kits measure toxin concentrations in the same range, but their direct comparability to each other is questionable, as also indicated in the literature [32].

To test analytical characteristics and applicability of the two immunoassays, Shewhart analytical control Charts [25] were plotted (Fig. 1) on concentration values determined at two absorbance wavelengths in the ELISA systems (2.5 ng/ml for the EnviroLogix Cry1Ab/Cry1Ac QuantiPlate and 1 ng/ml for the Abraxis Bt-Cry1Ab/Ac ELISA kit). Mean analyte concentration values determined for the controls applied were 2.63 ± 0.14 ng/ml and 1.05 ± 0.07 ng/ml for EnviroLogix Cry1Ab/Cry1Ac QuantiPlate and Abraxis Bt-Cry1Ab/Ac ELISA kit, respectively. Corresponding assay precision values were found to be 5.14% for EnviroLogix Cry1Ab/Cry1Ac QuantiPlate and 6.36% for the Abraxis Bt-Cry1Ab/Ac ELISA kit, appearing sufficient for analytical determination. Determination accuracy was also found proper for both immunoassays, statistically not differing from zero (4.39% ± 4.82% in the case of the EnviroLogix Cry1Ab/Cry1Ac QuantiPlate and

Table 2. Cry1Ab toxin content of a dried maize reference sample (OR4) detected by two enzyme-linked immunosorbent assay (ELISA) methods

	Toxin concentration in sample OR4	
	as detected by ELISA	corrected to activated toxin
ELISA method applied[a]	(μg toxin/g dry wt)	
EnviroLogix Cry1Ab/Cry1Ac QuantiPlate, 2002	4.64 ± 0.36 (A)[b]	11.26 ± 0.88 (B)[b]
Abraxis Bt-Cry1Ab/Ac ELISA kit, 2007	1.94 ± 0.75 (C)[c]	3.43 ± 1.34 (D)[c]
Abraxis Bt-Cry1Ab/Ac ELISA kit, 2009	1.99 ± 0.16 (C)[c]	3.52 ± 0.28 (D)[c]

[a] EnviroLogix Cry1Ab/Cry1Ac QuantiPlate (EnviroLogix) and Abraxis Bt-Cry1Ab/Ac ELISA kit were used.
[b] Different capital letters in parentheses indicate significant differences at 1% significance level (0.50 μg/g).
[c] Different capital letters in parentheses indicate significant differences at 1% significance level (0.99 μg/g).

5.31% ± 6.67% in the case of the Abraxis Bt-Cry1Ab/Ac ELISA kit). From the control chart, it is also apparent that accuracy improved with time, as higher values near upper warning limit were more frequent at the beginning of the analytical scheme commenced.

Cry1Ab toxin levels in maize DK-440 BTY

Monitored Cry1Ab toxin levels in *MON 810* maize cultivar DK-440 BTY, detected in the first year of cultivation, showed both among sample and temporal fluctuation: relative errors among multiple determinations of individual samples ranged between 0.2% and 7.6%, while those among samples of the same kind taken in parallel (at the same time) from different plants showed a tremendously greater variation between 9.7 and 57.8%, with similar variabilities within leaf, root, stem, or grain samples. This indicates quantitative determination of acceptable accuracy by the ELISA method, yet significant inherent biological variability among samples of individual plants at the same developmental stage.

Cry1Ab toxin was not detected in any sample taken from the near isogenic maize line (negative control). Considering the LOD of the EnviroLogix Cry1Ab/Cry1Ac QuantiPlate and Abraxis Bt-Cry1Ab/Ac ELISA kit (0.14 ng/ml and 0.125 ng/ml, respectively), corresponding CR values and actual sample preparation parameters, it means that the near isogenic maize line does not contain Cry1Ab toxin above 85 or 55 ng/g fresh weight concentrations.

A clear trend of Cry1Ab toxin production has been seen in DK-440 BTY during plant development. Most intense toxin expression started at the germination stage in an onset starting from 0.83 ± 0.15 μg/g in the seed to 8.06 ± 1.43 μg/g at germination stage. Intensive toxin production continually increased in the leaves during the first month, and reached a maximum of 17.15 ± 1.66 μg/g by the five-leaf stage (V5), gradually dropping to near the initial level (9.61 ± 2.07 μg/g) by the R4 phenophase, followed by an increase again to 13.51 ± 1.96 μg/g during the last two weeks (R5), most likely due to partial desiccation of the leaf material at the end of the growing season (Fig. 2). A similar fluctuation to a significantly lesser extent was seen in Cry1Ab toxin levels in the roots from 5.32 ± 0.49 μg/g at the less differentiated germination stage, and set in a steady level of 2.25 ± 0.30 μg/g by the R5 phenophase (Fig. 2). Cry1Ab toxin levels appeared similarly stable at 1.36 ± 0.45 μg/g in the stem by the R5 phase (no significant differences among data), while detected toxin concentrations in the anther wall and pollen were 4.98 ± 0.31 and 0.47 ± 0.03 μg/g, respectively. Thus, toxin levels showed the following pattern: leaf > anther wall > root > stem > grain > pollen.

These toxin levels measured are in agreement with the official *MON 810* documentation (see above) and with certain literature data [9,10], where toxin levels were detected in the same range, although with a strongly fluctuating trend. Concerns have also been raised regarding unpredictability and erratic expression values of Bt toxins in plants [36]. Other studies reported considerably lower Cry1Ab concentrations in Bt maize hybrids of the *MON 810* event, e.g., 350 to 534 ng/g in the leaves [11] and 190 to 390 ng/g [37], 160 ng/g [38], or 8 to 509 ng/g [9,10] in the seed/kernel. Literature data indicates that Cry1Ab toxin content appears to be proportional with chlorophyll content within a single leaf [8] with highest level (600 ± 50 and 590 ± 70 ng Cry1Ab toxin/g fresh wt) at the green leaf tip, and lowest in the white-yellow leaf base (180 ± 10 and 220 ± 70 ng Cry1Ab toxin/g fresh wt) indicating that tissues with low chlorophyll content correlate with decreased Cry1Ab toxin levels. 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 [9,10]. Studying four Chinese domestic Cry1A-producing transgenic maize lines, Liu and Wang measured elevated Cry1A toxin levels in the green (photosynthesizing) parts of the plants. Toxin levels were found in various fresh tissues in the following order leaf > husk leaf > pith > tassel > ear pith > pollen > silk [39], and displayed a continuous increase during plant development among different developmental stages. It must be noted, however, that all these values reported in the scientific literature rely on protoxin-based immunoassays; therefore, they should be subject to correction due to lower CR of the plant expressed Cry1Ab toxin with the antibodies generated against the microbial protoxin.

Cry1Ab toxin production at VT-R4 phenological stages

As seen from the temporal toxin distribution data (Fig. 2), toxin levels do not change significantly in the plant in the reproductive stages, between the silking (R1, emergence of the pistil) and dough (R4, intensive starch accumulation in kernel) stages. Therefore, a comparison of toxin levels at these mature stages in different years provides information on year-to-year variations of toxin production of the plant. *MON 810* maize was sampled at subsequent years at similarly developed stages, and

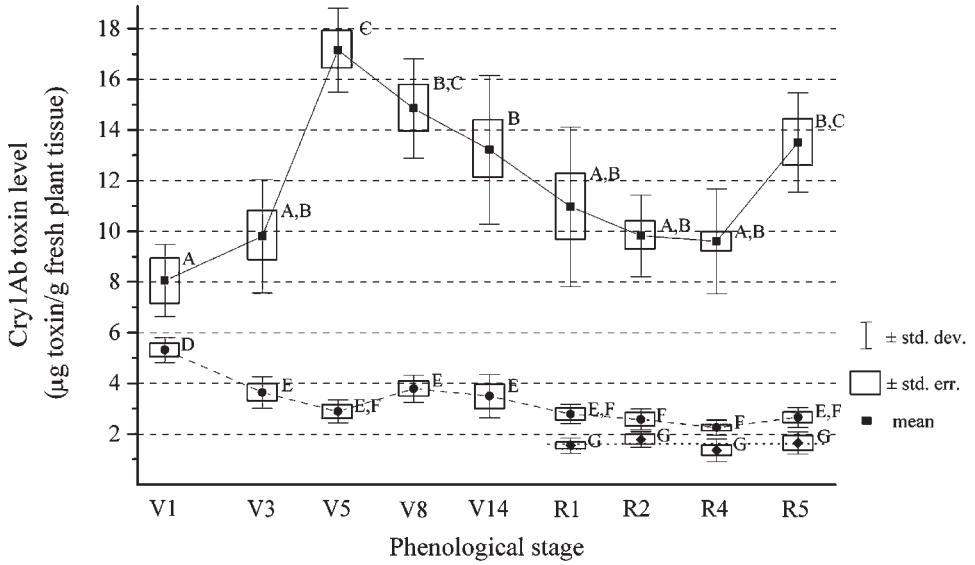

Fig. 2. Fluctuation of Cry1Ab toxin levels in the leaf (■), root (●), and stem (◆) during the development of DK-440 BTY (*MON 810*) maize plants detected with the EnviroLogix QuantiPlate enzyme-linked immunosorbent assay kit and calculated for activated toxin levels by the detected cross-reactivity. Plant samples analyzed at least in quadruplicates. Cry1Ab toxin were determined for first leaf (V1), third leaf (V3), fifth leaf (V5), eighth leaf (V8), fourteenth leaf (V14), silking (R1), blister (R2), dough (R4), and dent (R5) phenological stages. Different capital letters at each time point represent significant differences between samples of the same tissue at significant differences at 1% significance level of 3.68 and 0.92 μg Cry1Ab/g fresh tissue for leaf (A–C) and root (D–F), respectively. The Cry1Ab toxin content did not show statistically significant differences ($F_{calc} = 1.37$) among various stem samples (G). Error bars indicate standard deviation (std. dev.), rectangles indicate standard errors (std. err.).

toxin levels detected in various plant parts were compared with each other (Table 3). Cry1Ab content was uniformly the highest in the leaves and lowest in the grain and pollen. The toxin content was significantly lower in the root in 2005 than in other years, while the highest toxin production in this organ was the highest in 2008. Toxin content in the stem did not show statistically significant differences among the years studied ($F_{calc} = 2.99$). The largest relative variation was seen in the toxin level in the anther/tassel, which was related to the difference in maturity status of the plant sampled: anther was collected in a mature state (see high Cry1Ab toxin content of anther wall) at the stage of pollination in 2001, while samples from the immature tassel were collected at the early tasseling stage in 2008. The toxin content of pollen sampled in 2005 was below the LOD of the immunoassay using the present sample preparation method, and could be detected only by a modified sample preparation protocol for pollen allowing lower LOD (data not shown). Altogether a similar trend was seen in all three

Table 3. Cry1Ab toxin production of MON 810 maize at phenological stages between the tasseling vegetative stage (VT) and the dough reproductive stage (R4)

Plant organ[a]	Cry1Ab toxin content[b] (μg toxin/g fresh wt) 2001[c]	2005[d]	2008[e]
Leaf	9.83 ± 1.61 (A)	7.99 ± 1.34 (A,B)	6.12 ± 2.15 (B)
Root	2.58 ± 0.41 (C)	1.53 ± 0.43 (D)	3.82 ± 0.44 (E)
Stem	1.79 ± 0.31	1.07 ± 0.10	1.61 ± 0.60
Anther wall or tassel[f]	4.98 ± 0.31	NM[g]	1.31 ± 0.37
Pollen[f]	0.47 ± 0.03	below limit of detection	NM

[a] Cry1Ab toxin levels were determined in the tasseling–dough (VT-R4) phenological stages. Plant samples analyzed by enzyme-linked immunosorbent assay (ELISA) at least in quadruplicates.

[b] Different capital letters in parentheses after each detected concentration range indicate significant differences between samples of the same plant organ collected in different years at significant differences at 5% significance level (2.81 μg/g) for leaf (A, B) and at 1% significance level (1.05 μg/g) for root (C–E). The Cry1Ab toxin content did not show statistically significant differences ($F_{calc} = 2.99$) among stem samples in different years.

[c] Cry1Ab toxin levels detected in the plant leaf, root, and stem at developmental stage R4 and in pollen and anther at developmental stage R1 by EnviroLogix Cry1Ab/Cry1Ac QuantiPlate ELISA. Activated toxin/protoxin cross-reactivity value of 0.412 considered.

[d] Cry1Ab toxin levels in the plant leaf, root and stem at developmental stage R3 and in pollen at developmental stage R1 by Abraxis Bt-Cry1Ab/Ac ELISA kit. Activated toxin/protoxin cross-reactivity value of 0.564 considered.

[e] Cry1Ab toxin levels in the plant organs detected at tasseling stage by Abraxis Bt-Cry1Ab/Ac ELISA kit. Activated toxin/protoxin cross-reactivity value of 0.564 considered.

[f] Anther wall and pollen were sampled after pollination in 2001. Tassel was sampled before pollination (tasseling stage) in 2008, as the crop did not reach the pollination stage.

[kg] NM = not measured.

years sampled: the toxin was produced at the highest concentration in the leaves, where photosynthesis is the most intense.

In conclusion, Cry1Ab production of *MON 810* maize has been characterized both within a single vegetation period and among different growing seasons. For this purpose, two commercially available ELISA systems were evaluated for their CR to the activated form of the toxin, and the thus obtained CR values were used for correction of the raw ELISA results. It has to be mentioned that *MON 810* maize (and Bt maize, in general) produces more Cry1Ab toxin than seen in the literature, as none of the literature data are corrected for activated toxin content, but were obtained with protoxin standard. Therefore, we urge that ELISA systems be developed and made widely available that are based on antibodies raised against plant produced toxins, or specific even to enzymatically cleaved, activated form of Cry1Ab.

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