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Expression of Cry3Bb1 in transgenic corn MON88017

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To evaluate the effects of transgenic expression of Coleopteran-specific *Bt* protein Cry3Bb1 on target and nontarget insects in fields with *Bt* crops, it is necessary to quantify the Cry3Bb1 contents in the plants. Here, we describe the optimization and validation of the quantitative detection of Cry3Bb1 by adapting the commercially available qualitative PathoScreen double antibody sandwich−enzyme-linked immunosorbent assay (DAS-ELISA) for quantitative measurements. The optimized method had an average accuracy of 84−109% and was used to quantify the Cry3Bb1 contents of different tissues of *Bt* corn MON88017 at four developmental stages during three years (2005−2007) in a field trial in Germany. The Cry3Bb1 contents were determined based on both dry weight and fresh weight. Cry3Bb1 expression was highest in young leaves (228.4 μg/g dw and 35.5 μg/g fw) and lowest in pollen (3.8 μg/g fw). In root tissues, the Cry3Bb1 content declined during the growing season from 130 to 40 μg/g dw. A significant decline of Cry3Bb1 contents was also observed during the growing season in other plant tissues. The Cry3Bb1 contents of different plant tissues strongly correlated to each other. On the basis of the total corn biomass produced on 1 hectare, it was estimated that up to 905 g of Cry3Bb1 is produced per hectare *Bt* corn MON88017.

KEYWORDS: Cry3Bb1; *Bacillus thuringiensis*; protein expression; ELISA; Western blot

INTRODUCTION

The western corn rootworm (WCR, *Diabrotica virgifera virgifera* LeConte) is one of the most serious insect pests of corn (*Zea mays* L.) in North America (*1*). It was introduced to Serbia in the early 1990s and spread rapidly to other European countries including Italy, Austria, Czech Republic, France, and Germany (*2*−*5*). The larvae of WCR feed on corn roots and cause yield losses by reducing water uptake and nutrient absorption. The damaged plants become susceptible to the entry of stalk and root-infecting microorganisms and finally tilt over (*6*). Recently, transgenic corn producing Cry3Bb1 protein from *Bacillus thuringiensis* (*Bt*) sp. *kumatomoensis* has been developed as a biotechnological alternative to the conventional application of chemical insecticides and crop rotation for WCR control. The first transgenic corn hybrid MON863 for the WCR control was registered in the United States in 2003 (*7*). A second corn line, named MON88017, was registered in the United States in 2005. It expresses a modified Cry3Bb1 protein differing at six amino acid positions from the Cry3Bb1 protein in *B. thuringiensis* sp. *kumatomoensis* and at one position from the Cry3Bb1 protein expressed in MON863. In MON88017, the Cry3Bb1 protein expression is driven by the enhanced 35S promoter (e35S) from cauliflower mosaic virus. The recombinant gene contains the rice actin intron, the 5′ leader sequence from wheat chlorophyll a/b-binding protein, and the 3′ nontranslated region from wheat. MON88017 further expresses a transgenic 5-enolpyruvylshikimate-3-phosphat synthase protein from *Agrobacterium* sp. strain CP4 (CP4 EPSPS) conferring tolerance to glyphosate (*8*).

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Detailed information about the level of Cry3Bb1 protein within different parts of MON88017 is one of the important parameters to evaluate the effect of Coleopteran-specific Cry3Bb1 protein on target and nontarget insects exposed to this crop. So far, expression data have only been collected from field experiments in the United States and in Argentina (*8*). It is known from transgenic corn expressing Cry1Ab protein that recombinant protein contents vary with plant variety, plant age, and environmental conditions (*9*, *10*). Thus, it is important to quantify the Cry contents in different geographic areas. In Germany, MON88017 was planted for the first time in a field trial from 2005 to 2007. Monitoring the tissue-specific expression of Cry3Bb1 is still necessary to provide basic data for the biosafety research in Europe.

For detecting Cry proteins, different immunological methods such as Western blot, immunohistochemical staining, enzyme-linked immunosorbant assay (ELISA), and lateral flow strips (LFD) have been developed. ELISA is an efficient analytical detection method that can be used for both qualitative and quantitative testing. It offers a simple, fast, and reliable protein determination, even at low concentrations (*11*, *12*). To date, several laboratories have applied the PathoScreen kit (Agdia Inc., Elkhart, IN) for the quantitative determination of Cry3Bb1 protein in root exudates, plant biomass, pollen, soil, and the guts or excretes of earthworms collected from glasshouse and in a field planted with *Bt* corn (*13*−*16*). The PathoScreen double antibody sandwiched (DAS)-ELISA has been developed for a qualitative Cry3Bb1 detection, and no studies have been reported to validate its quantitative application. In our study, we optimized the quantitative measurement of CryBb1 using the qualitative

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PathoScreen DAS-ELISA. Its ability to reliably quantify Cry3Bb1 was validated by determining the accuracy and the precision of the test. The optimized test was used to quantify the content of Cry3Bb1 protein of different plant tissues of MON88017 during the vegetation period over 3 years.

MATERIALS AND METHODS

Cultivation and Collection of Plant Samples. Event MON88017 and the near isogenic nongenetically modified variety DKC5143 were planted near Würzburg, Germany. The experimental field was designed as a randomized block in eight replicates for each *Bt* and non-*Bt* isoline (for details, see ref *18*). Plot dimensions were 31.5 m width by 40.5 m length. Each plot consisted of 42 rows with a distance of 75 cm between rows and 15 cm between plants. The cultivation of maize was carried out according to the local common agricultural practice.

Plants were harvested in 3 years (2005−2007) at four growth stages, which are specified according to the growth scale developed by the Biologische Bundesanstalt fuer Land- and Forstwirtschaft and Chemical Industry (BBCH) (*19*): BBCH19 (nine or more leaves unfolded), BBCH30 (beginning of stem elongation), BBCH63 (flowering, anthesis), and BBCH83 (ripening, early dough: kernel content soft, about 45% dry matter). In addition, silks and pollen were collected between BBCH61 and BBCH65 in 2006 and 2007 (BBCH61: male flowers, stamens in middle of tassel visible; female flowers, tip of ear emerging from leaf sheath; BBCH65: male flowers, upper and lower parts of tassel in flower; female flowers, stigmata fully emerged). Two *Bt* plants were randomly sampled in each plot. As nontransgenic control, two DKC5143 plants were collected from a single plot. At BBCH19 and BBCH30, the whole plants, and at BBCH63 and 83, only plant segments were collected. The plant material was transported at 4 °C to the laboratory, where different tissues (root, stalk, and upper leaf = second upper leaf, lower leaf = second lower leaf, anther, silk, pollen, and grain) of each plant were aliquoted. To remove soil particles, the roots were gently washed under running water and then dried with tissue papers. The leaf samples were cut from the middle of the second upper leaf and the second lower leaf, respectively. Pollen was collected from individual plants in a paper bag and transferred into a 2 mL Eppendorf reaction tube after separation from anther and tassel material. Plant tissues were weighed and split into two parts: One-half was stored at −80 °C until further analysis, and the other half was dried at 80 °C for dry weight determination.

To determine the plant biomass, five complete corn plants were randomly taken from five different plots at four growth stages (BBCH19, BBCH30, BBCH63, and BBCH83). The harvested plants were divided into root, stalk, leaf, and grain, cut into small pieces (5 mm), and mixed well. Fresh and dry weights of each proportion and the whole plant were determined. About 1−5 g of each tissue sample was subjected to the ELISA.

Preparation of Tissue Extracts. For protein extraction from roots, stalks, leaves, and anthers, tissue samples of 1−5 g samples were soaked in 5−10 volumes of phosphate-buffered saline with Tween 20 (PBST buffer) supplied with the ELISA kit and then homogenized at 24000 rev/min for 60 s with a T25 Ultra-Turrax homogenizator (Kinematica, Switzerland). Pollen, silks, and grains were ground using a mortar, pestle, and liquid nitrogen and were extracted in 10 volumes of PBST buffer. All plant extracts were kept on ice for 30 min, and 1 mL of the extract was transferred to 1.5 mL Eppendorf reaction vials, which were centrifuged at 5000*g* for 5 min. The supernatant was used for the quantification of Cry3Bb1. To determine the extraction efficiency, after the supernatant was collected, the pellet was ground in 1 mL of PBST buffer followed by vigorous vortexing. The suspension was kept on ice for 10 min and again centrifuged as described above. The supernatant was recovered again, and the extraction procedure was again repeated (in total four times). All supernatants were transferred into the wells of the PathoScreen microtiter plate, and the Cry3Bb1 toxin concentration was determined by ELISA.

ELISA Measurement of Cry3Bb1 in Plant Samples. The commercial PathoScreen kit (Agdia Inc.) is a DAS-ELISA and was developed to qualitatively detect Cry3Bb1 protein in corn seeds and leaves. Two different PathoScreen kit versions (methods A and B) were used during 2005 and 2007 (**Table 1**). The differences between method A and method B are (i) the type of the detecting antibody (polyclonal antibody for method A and monoclonal antibody for method B), (ii) the development time after adding the substrate, and (iii) the wavelength to read ELISA (450 nm for method A and 620 nm for method B). All other conditions were the same for both methods (**Table 1**). All samples were incubated with the capture antibody for 16 h at 4 °C in a humid box, instead of for 2 h at room temperature (22 °C) as recommended by the manufacturer. Both methods A and B were validated for the following parameters: calibration (range), sensitivity [limit of quantification (LOQ) and limit of detection (LOD)], specificity (cross-reaction), precision (intra- and interassay), accuracy (recovery), and ruggedness (*17*). Intra-assay precision was evaluated by determining the variation coefficient (CV) between replicates assayed at three different concentrations of Cry3Bb1 in spiked samples derived from independent assays performed on three or four different days for the methods B and A, respectively. Interassay precision indicated the variation occurring between separate assays and was assessed using the average precision for all concentrations. On the basis of the validation, the sensitivity of both methods was compared, and a correction factor to compare results from both methods was calculated.

Table 1. PathoScreen Methods of DAS-ELISA Used during 2005−2007[a]

condition altered	PathoScreen method A	PathoScreen method B
provided in	2005, 2006, before May 2007	after May 2007
capture antibody	polyclonal antibody	polyclonal antibody
detector antibody	polyclonal antibody	monoclonal antibody
Cry3Bb1 standard	lot C1205 and C1446	lot C1446
incubation temperature	4 °C	4 °C
incubation time	16 h	16 h
development time	15 min	20 min
stop reaction	3 M H_2SO_4	−[b]
ELISA reading[c]	450 nm	620 nm

[a] Method A, yellow color (450 nm) (color development was stopped by H_2SO_4); method B, blue color (620 nm) (color development without stop reaction). [b] −, not performed. [c] Measurement of color produced in the ELISA at different wavelengths.

The following samples were used to validate the ELISA measurement:

1. Negative control, plant extracts from near isogenic variety DKc5143.
2. Cry3Bb1 (Agdia) (Standard), lyophilized Cry3Bb1 toxin provided by Agdia; it was solubilized in 2 mL of PBST buffer, aliquoted, and stored at −20 °C.
3. Cry3Bb1 (NW), the full-length Cry3Bb1 protein was expressed in *E. coli* using the plasmid pMON 70855 (provided by Monsanto Co.) and purified at the DLR Rheinpfalz.
4. Trypsinized Cry3Bb1 (NW), Cry3Bb1 (NW) was treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated bovine pancreas trypsin (Sigma) with a ratio of 1/10 (w/w) for 30 min at 37 °C.
5. Spiked sample, Cry3Bb1 (NW) (0.66, 1.5, and 3.375 ng/mL) spiked in PBST buffer and in leaf extract from near isogenic variety DKc5143.
6. Plant samples, extracts from root, stalk, leaf, pollen, anther, silk, and grain of MON 88017.

The plant extracts were diluted with PBST buffer (10 mM phosphate-buffered saline, 0.05% v/v Tween-20) and subjected to ELISA. Most samples collected from seasons 2005 and 2006 and a part of 2007 were quantified with method A. Samples collected at two BBCH63 and -83 in 2007 were measured with method B. All samples were measured in duplicate. Data were analyzed using the Microplate Manager 5.2.1 program (BioRad Laboratories Inc.). The optical density (OD) values of the negative control were subtracted from the OD values of test samples. The concentration of Cry3Bb1 was determined in plant samples in μg/g fresh weight (fw) and then recalculated into μg/g dry weight (dw). Estimates of the biomass of different tissues and their Cry3Bb1 contents were used to estimate the total Cry3Bb1 amount expressed in the field at different growth stages.

Western Blot Analysis. The specificity of the antibodies used in the ELISA was verified by Western blotting of sodium dodecyl sulfate (SDS) polyacrylamide gels (*20*). The gels were electro-blotted onto PVDF membrane (Hybond-P Transfermembran, Amersham Pharmacia). The

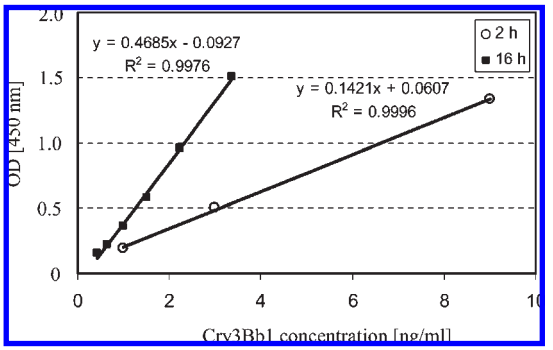

Figure 1. ELISA standard curves of Cry3Bb1 protein detection applying method A and using Cry3Bb1 (Agdia) as a standard at two different incubation conditions (22 °C for 2 h vs 4 °C for 16 h). Note that similar standard curves were obtained for method B (data not shown).

blots were incubated with rabbit anti-Cry3Bb1 antibodies (A30332, Agdia) at a dilution of 1/1000 in blocking buffer [PBST buffer and 1% bovine serum albumin, Fraktion V (Carl Roth)]. Goat antirabbit IgG linked to horseradish peroxidase (Sigma) diluted at a ratio of 1/7000 in blocking buffer was applied to detect binding of the primary antibody by using chemiluminescent substrates [1 mL of solution A (0.1 M Tris-HCl, pH 8.6, and 0.025% luminol), 0.5 μL of H_2O_2 (30%), and 100 μL of solution B (0.11% *para*-hydroxycoumarin acid in dimethyl sulfoxide)].

Statistical Analysis. Statistical analyses were performed using SAS software packages (SAS Institute, version 9.1.3, 2007). All data sets were tested for normality and homogeneity using Shapiro-Wilk and Levene tests. Data were Log-transformed for analysis of variance. The means of Cry3Bb1 contents were computed using PROC-MEAN. For pairwise comparison of differences between the means among developmental stages and between plant tissues, least-squares means (PROC MIXED, LSMEANS statement with option Adjust = Tukey) were performed. For testing the correlation of Cry3Bb1 contents in different tissues of the same plant, a Pearson correlation analysis using the CORR procedure in the SAS package was performed.

RESULTS

Optimization and Validation of PathoScreen for Quantitative Measurements of Cry3Bb1. To compare the signal produced in the ELISA, dilution series of Cry3Bb1 (Agdia) protein standard were generated and incubated with the capture antibody at two different conditions (22 °C for 2 h vs 4 °C for 16 h). Extension of the incubation time at low temperature increased the sensitivity of the test by a factor of 3, as indicated by the increase of the OD values after incubation for 16 h as compared to incubation for 2 h (**Figure 1**). A linear response of OD to protein concentrations was observed in a range of 0−2 OD units.

To estimate the extraction efficiency of Cry3Bb1 from different plant tissues, leaf, root, and stalk samples at BBCH19, pollen at BBCH63, and grain at BBCH83 were chosen, and four sequential extractions of each tissue were performed and measured. The extracted Cry3Bb1 protein concentration of the first extraction ranged from 5.6 to 43.0 μg/mL extract, whereas the second extraction yielded only 0.04−0.39 μg/mL, corresponding to 1−2% of the first extraction (**Figure 2**). Further subsequent extractions gave only trace amounts of less than 10 ng/mL, corresponding to 0.1% of the first extraction. As the extraction efficiency of the first extraction was 99% for leaves, stalk, and grain and 98% for roots and pollen, only one round of protein extraction was supplied in further tissue preparations.

The performance characteristics of PathoScreen methods A and B for a quantitative measurement of Cry3Bb1 are given in **Table 2**. Protein standard curves obtained on the same day and on different days showed a slight variability, with an average within-day

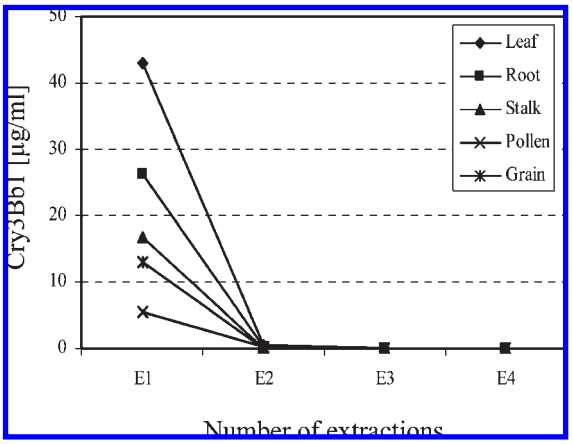

Figure 2. Extraction efficiency of four subsequent extractions (E1−E4) of Cry3Bb1 from leaf, root, stalk (BBCH19), pollen (BBCH63), and grain (BBCH83) of MON88017.

precision of 3.6 (method A) and 3.3% (method B) and a between-day precision of 7.1 (method A) and 5.2% (method B). The LOD and LOQ of method B were 3- and 4-fold lower than those of method A, indicating that the sensitivity of the test increased by the replacement of the polyclonal anti-Cry3Bb1 antibody (method A) by a monoclonal antibody (method B) as the detection antibody. Western blot analyses showed that the provided capture antibody recognized the full-length Cry3Bb1 protein (77 kDa) and its degradation products of 70, 55, and 46 kDa in grain extracts stored for 1−2 years at −80 °C (**Figure 3**). No immune-reactive band was observed in the extracts from the near isogenic sample, indicating that the antibody is specific only against Cry3Bb1. Intra-assay variability showed a low CV of 3.9 and 3.4% for methods A and B, respectively. The variations between separate assays performed on different days were 17.7 and 9.8% for methods A and B, respectively, and thus 4.5- and 3-fold higher than intra-assay variation for the two methods. Method B produced a 1.3-fold higher recovery of Cry3Bb1 protein than method A (**Table 2**). The ruggedness of method A was 4.9% when aliquots of the same extract were independently measured on two different days, while it was 10.5% when freshly extracted samples were measured on different days (**Table 2**).

Cry3Bb1 Contents in Different Tissues of MON88017 during the Growth Stages. Cry3Bb1 contents were determined for 880 plant tissue samples by using the validated PathoScreen methods A and B. The Cry3Bb1 contents measured in the different years 2005, 2006, and 2007 are provided as Supporting Information, Tables S1 and S2. The expression levels were significantly different among three years ($F = 24.3$, df = 828, and $P < 0.0001$). To get an overall seasonal expression pattern of Cry3Bb1 in MON88017 and determine whether there was a correlation of Cry3Bb1 protein expressed in different tissues in each plant, statistical analysis was performed on data pooled over 3 years (**Tables 3** and **4**).

Differences among Tissue Samples. The mean Cry3Bb1 contents differed significantly between most plant tissues at each developmental stage (BBCH19: $F = 145.6$, df = 38, and $P < 0.0001$; BBCH30: $F = 14.9$, df = 53, and $P < 0.0001$; BBCH63: $F = 70.1$, df = 83, and $P < 0.0001$; and BBCH83: $F = 72.7$, df = 68, and $P < 0.0001$) (**Table 3**). No statistical differences were found between stalk and root [df = 53 and adjustment (adj) $P = 0.135$ and df = 68 and adj $P = 0.839$] and between stalk and lower leaf (df = 53 and adj $P = 0.337$ and df = 68 and adj $P = 0.213$) at both BBCH30 and BBCH83. In addition, the Cry3Bb1 contents of the lower leaves and the upper leaves did not differ at BBCH30

Table 2. Summary of Optimization and Validation Experiments for Cry3Bb1 Detection Using PathoScreen ELISA Method[a]

performance characteristics	method A	method B
calibration	$n = 4$	$n = 4$
standard curve working range (ng/mL)	0.44−3.38	0.44−3.38
within day precision (% CV)	3.6	3.3
between day precision (% CV)	7.1	5.2
sensitivity		
LOD (ng/g fw)	0.41	0.14
LOQ (ng/g fw)	0.73	0.18
specificity		
recognize Cry3Bb1 (Western blot, **Figure 3**)	full-length (77 kDa) and degraded products (70, 55, and 46 kDa)	
precision (repeatability RSDr)	$n = 3$	$n = 4$
intra-assay (%)	3.9	3.4
inter-assay (%)	17.7	9.8
accuracy		
% average recovery	84.1	109.2
(% min − max recovery)	(62−107)	(85−141)
% CV	18.4	13.4
ruggedness		
% CV of an aliquot from frozen extract on two different days ($n = 4$)	4.9	ND
% CV of the fresh extracts on two different days ($n = 11$)	10.5	ND

[a] n = number of replicates, CV = coefficient of variation, RSDr = % repeatability, and ND = not determined.

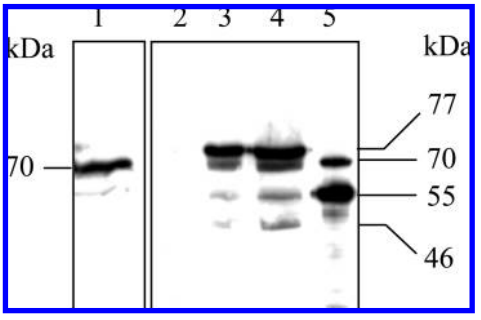

Figure 3. Western blot analysis of Cry3Bb1 protein expressed in *E. coli* and in transgenic MON88017. The membrane was incubated with rabbit anti-Cry3Bb1 antibodies (A30332, Agdia). Lane 1, Cry3Bb1-Agdia; lane 2, negative control (extract from near isogenic variety DKc5143); lanes 3 and 4, plant produced Cry3Bb1 protein from grain extracts (sample collected in 2006 and 2005, stored at −80 °C for 1 and 2 years, respectively); and lane 5, trypsinized Cry3Bb1 (NW).

(df = 53 and adj $P = 0.061$) and at BBCH63 (df = 83 and adj $P = 0.762$). The highest Cry3Bb1 contents were found in young tissues, for example, the upper leaves (100−228 μg/g dw, corresponding to 24.7−35.5 μg/g fw) or in the silk (110 μg/g dw, corresponding to 11.7 μg/g fw at BBCH63) (Supporting Information, Table S2). The lowest Cry3Bb1 contents were observed in pollen (3.8 μg/g fresh weight) and in kernels (27 μg/g dw, corresponding to 9.4 μg/g fw). The maximum−minimum variability of the Cry3Bb1 contents of most plant tissues was 2−9-fold over 3 years. Only the lower leaves showed a higher variation at BBCH83 with 0.7−133 μg/g dw, which was caused by partial senescence of the lower leaves during the very dry summer 2006 (Supporting Information, Tables S1 and S2).

Differences among Growth Stages. Comparison among four growth stages revealed that the highest Cry3Bb1 contents were found in all tissue samples when plants were young (at BBCH19). With increasing plant age, the mean Cry3Bb1 contents significantly declined ($P < 0.0001$), except for the lower leaf at BBCH30 and BBCH63 (df = 38, $t = 0.89$, and $P = 0.394$) (**Table 3**). The highest decline of Cry3Bb1 protein was found in the stalk from BBCH21 to BBCH83, which was 5-fold (df = 53, $t = 26.1$, and $P < 0.0001$) (**Table 3**).

Correlation between Tissues. A correlation analysis of the Cry3Bb1 contents of different tissues of the same plant was performed to test whether the variability among different tissues was independent. A significant correlation among the Cry3Bb1 contents of roots, stalk, and leaves was observed (**Table 4**). No correlation was observed with other tissues, such as anthers, silk, and grains (data not shown).

Estimation of Cry3Bb1 Amount Per Hectare. From plant samples taken during the seasons 2005−2007, the total biomass per hectare was estimated. During the growing season, the roots and above-ground corn biomasses increased steadily and were highest at BBCH83 (root, 10200 kg/ha, and above-ground biomass, 76700 kg/ha). The estimated Cry3Bb1 amounts produced on 1 hectare increased by a factor of about 4−4.5 from BBCH19 to BBCH83 and were about 85 and 820 g/ha in roots and above-ground biomass at BBCH83, respectively (**Figure 4**).

DISCUSSION

To provide confidence in the estimation of Cry3Bb1 contents in transgenic corn, quantification methods need to be validated. Our single-laboratory validation allowed us to characterize the performance characteristics of the commercially available PathoScreen kit for a quantitative detection of Cry3Bb1 in *Bt* corn MON88017 and to compare the Cry3Bb1 contents of different corn tissues during three subsequent years. An important parameter of a quantitative measurement of *Bt* proteins in transgenic corn is its extraction efficiency, which depends on tissue type, target protein, sample processing protocol, extraction buffer and protocol, time, and buffer−sample ratios (*12*). The

Table 3. Mean of Cry3Bb1 Contents of Different Tissues of MON88017 Collected during Four Developmental Stages (BBCH19, BBCH30, BBCH63, and BBCH83) in Three Growing Seasons 2005−2007[a]

		Cry3Bb1 (μg/g dry weight) (mean of data from 3 years 2005−2007)			
		developmental stage			
tissues		BBCH19	BBCH30	BBCH63	BBCH83
root	mean (SE)	129.7 (4.1) dA	99.0 (7.2) cA	65.8 (3.7) bB	40.3 (2.0) aC
	range	76.8−175.3	36.6−289.3	33.1−149.8	15.8−74.5
	n	48	48	48	48
stalk	mean (SE)	184.0 (8.7) dB	113.9 (6.5) cAB	47.3 (1.9) bA	37.4 (3.1) aBC
	range	70.1−320.8	31.3−206.3	14.0−74.6	12.2−107.2
	n	48	48	48	48
lower leaf	mean (SE)		126.8 (8.0) bBC	117.0 (8.4) bC	33.51 (4.3) aAB
	range		57.0−298.4	31.7−242.3	0.74−133.1
	n		48	48	48
upper leaf	mean (SE)	228.4 (11.0) dC	151.7 (8.6) cC	125.5 (4.1) bC	100.2 (3.5) aD
	range	116.4−391.6	22.8−304.8	63.6−196.8	53.0−162.6
	n	48	48	48	48
anther	mean (SE)			65.8 (2.1) B	
	range			37.5−108.1	
	n			48	
pollen (μg/g fresh weight)	mean (SE)			3.81 (0.2)	
	range			2.3−5.9	
	n			32	
silk	mean (SE)			110.2 (6.5) C	
	range			25.9−205.0	
	n			32	
grain	mean (SE)				27.1 (0.6) A
	range				7.2−59.4
	n				48

[a] SE = standard error, and *n* = number of samples. The range gives the minimum and the maximum value during the survey of 3 years. Means within a row followed by the same lowercase letter are not significantly different. Means within a column followed by the same capital letter are not significantly different ($P > 0.05$, PROC MIXED, LSMEANS, with option Adjust = Tukey, SAS Institute 2007).

Table 4. Correlation of Cry3Bb1 Contents in Different Tissues of the Same MON88017 Plant during Four Developmental Stages with the Reference Tissue Sample as Root (Mean of 3 Years) [a]

tissues	stalk	lower leaf	upper leaf
root	0.89 (64)	0.69 (48)	0.79 (64)
stalk		0.59 (48)	0.89 (64)
lower leaf			0.65 (48)

[a] Pearson correlation coefficients were calculated using SAS Procedure CORR; SAS Institute, 2007. Pearson correlation ($p < 0.0001$); numbers in brackets give the number of observations.

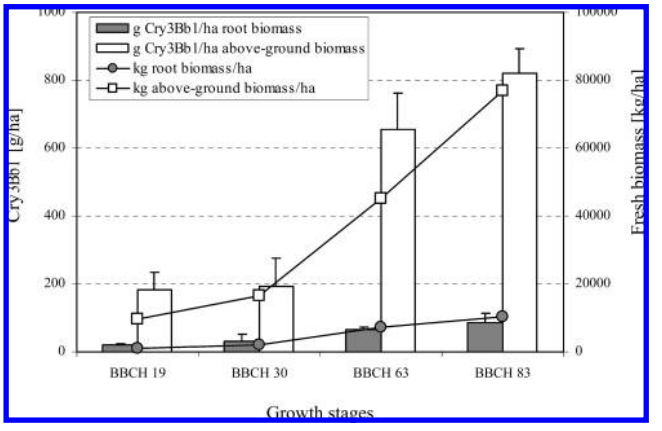

Figure 4. Estimated mean of Cry3Bb1 protein production of MON88017 per hectare during two growing seasons 2006 and 2007. The amounts of Cry3Bb1 produced per ha were calculated on the basis of Cry3Bb1 contents measured in root and above-ground plant tissues of single plants ($n = 8$), and their biomasses were determined at four growth stages and an estimate of 87200 plants per ha (~37000 plants/acre).

efficiency of Cry3Bb1 extraction was 98−99% for different tissues, dispelling the need of a second extraction.

The plate-to-plate variation for each point of the standard curves of Cry3Bb1 (Agdia) was low (CV 7.1% for method A and 5.2% for method B), which indicates good reproducibility (**Table 2**). However, the standard curves' working range was relatively narrow (8-fold), and time-consuming investigations for determining the optimal dilution of various plant tissue samples are necessary to fit the narrow working range. On the other hand, it allowed detecting even very low Cry3Bb1 concentrations, for example, those in pollen. The LOD and LOQ of method A were 3−4 times higher than those of method B and were in good agreement with the lower limit of the working range of the standard curve. As shown by Western blotting, the antibody recognized both the full-length Cry3Bb1 protein (77 kDa) and its degradation products of Cry3Bb1 (70, 55, and 46 kDa), which accounted for less than 10% of total Cry3Bb1 (**Figure 3**). The 55 kDa degradation product was similar in size to the trypsinized Cry3Bb1 (NW), which bioactivity has been demonstrated in bioassays with first instar larvae of the Colorado potato beetle *Leptinotarsa decemlineata* (data not shown). Hence, it is assumed that at least this degradation product is still biologically active. Some Cry3Bb1 protein degradation may have occurred in the plant or in the collected samples during storage or sample preparation. To minimize this degradation, all plant samples were analyzed as soon as possible after harvest, and all extraction steps were performed on ice or at 4 °C. There is no information about the Cry3Bb1 (Agdia). The size of the Cry3Bb1 (Agdia) standard included in the Western blot analysis was identical to the 70 kDa band of the trypsinized Cry3Bb1 sample (**Figure 3**). Although there was no further information provided by Agdia,

we assume that this form is more stable than the full-length Cry3Bb1 protein (77 kDa) and was applied as protein standard.

Whereas the within-assay repeatability RSDr was only 3.9 and 3.4%, the interassay precision was 17.7 and 9.8% RSDr and thus considerably higher. Comparable results were also obtained from a collaborative study of 40 laboratories, where the variation of the ELISA method to quantify Cry1Ab in MON810 ranged from 6.5 to 18.8% in intra-assay and from 13.8 to 23.5% in interassay (*11*). The Cry3Bb1 recovery of nontransgenic tissue samples spiked with a bacterial expressed Cry3Bb1 protein ranged from 62 to 107% (method A) and 85 to 141% (method B) of the spiked concentration and is near the ideal accuracy range (80 to 120%) of an immunoassay (*21*). Also, the coefficient of variation for measured recoveries at each fortification level is in the acceptable range of less than 20% (*17*). The change of the detection antibody from a polyclonal antibody in method A to a monoclonal antibody in method B could be one of the reasons for the observed over-recovery and increased sensitivity of method B. An average recovery rate of 60% for method A was obtained in a round robin study of six laboratories from Germany and Switzerland, although two out of the six laboratories involved did not use an extended incubation time (*22*). A similar accuracy (73−122%) of an ELISA for quantification of Cry9C protein was obtained for the samples spiked with corn flour Cry9C, whereas only a 27−113% recovery was obtained, when bacterial expressed Cry9C protein was spiked (*23*).

In general, the validation parameters of method B were superior to method A, presumably due to the change of a polyclonal (method A) to a monoclonal detection antibody (method B). Both methods were used to determine the Cry3Bb1 contents in various tissues over 3 years. Because of the change from method A to method B in the course of our investigations and to the different sensitivities of both methods, a correction factor of 0.77 (84.1/109.2, see **Table 2**) was applied to all measurements obtained with method B (BBCH63 and BBCH83 in 2007), to make them comparable with measurements obtained using method A (all other measurements in 2005−2007). Thus, the differences in Cry3Bb1 contents obtained in different years should not derive from different quantification methods. However, a systematic bias in the measurements depending on the methodology has to be noted.

Cry3Bb1 protein could be determined in all analyzed tissue types. This was expected as the Cry3Bb1 expression in MON88017 is driven by the constitutive e35S promoter (*8*). Young plants expressed more Cry3Bb1 protein than old plants; highest Cry3Bb1 contents were found at BBCH19 with 228 μg/g dw for leaves, 184 μg/g dw for stalks, and 130 μg/g dw for roots, whereas the mean Cry3Bb1 contents of silk were 110 μg/g dw at BBCH63, kernel (27 μg/g dw at BBCH83), and pollen (3.7 μg/g fw at BBCH63), respectively. Except for the grain (27 μg/g dw), these Cry3Bb1 contents were 2.5−6.6-fold lower than those reported from studies in the United States in 2002 and Argentina in 2003 and 2004 (*8*). Differences in Cry3Bb1 contents were also reported in other studies. For example, the concentration of Cry3Bb1 protein in DKC46-23, a hybrid of event MON863, was estimated to be about 1.6 ± 0.2 μg/g of dry biomass (mixture of roots, stems, and leaves) (*13*). The Cry3Bb1 contents of whole MON863 plants were only measured based on fresh weight and ranged between 38 and 54 μg/g fw at 35 days postplanting (*24*). The discrepancies in the Cry3Bb1 measurements could be caused by differences between field- and laboratory-grown plants and by reporting Cry protein values based on tissue fresh weight (*13*). Also the source, age of the plant samples, methods of extraction and analysis, antibodies-based reagents used for quantifying the *Bt* protein, and the protein standards may influence the results of measurements (*25*, *13*).

Comparison of Cry3Bb1 contents among different growth stages revealed a significant 2−5-fold decrease in roots, stalks, and leaves from BBCH19 to BBCH83 on both a dry weight and fresh weight basis. A similar decrease of Cry3Bb1 protein during the growing season had been observed for MON863, although it did not negatively affect the protection of the root system from larval feeding (*7*). While Cry1Ab protein was found with highest amount at anthesis of Novartis *Bt* corn, Event 176 (*9*). Cry1Ab protein was also found to increase gradually from the seedling to maturing stage of different *Bt* rice lines (*26*, *27*). These variations in the Cry expression during the growing season clearly indicate the necessity to examine the Cry contents on transgenic plants during the season, if solid information on their expression profile is needed. Although our study was limited to only one location, it is most likely that Cry3B1 contents may differ when *Bt* corn is grown in different environments as it was observed for Cry1Ab in MON810 (*10*). In general, the variation in Cry3Bb1 levels among individual plants was lower than 10-fold for most plant tissues. It was consistent with the observation in *Bt* corn Event 176, which expressed Cry1Ab protein specific for Lepidopteran species (*9*). The only exception was the Cry3Bb1 contents of lower leaves at BBCH83 which showed an up to 180-fold variation. This was mainly due to the extremely dry weather during summer 2006, causing a withering of the lower leaves and thus strongly diminished total protein contents.

The total of root and above-ground Cry3Bb1 production of MON88017 was estimated to 203 g/ha for BBCH19 and 905 g/ha for BBCH93, based on an estimate of 87200 plants/ha (~37000 plants/acre). Thus, at the ripening stage, the Cry3Bb1 production is considerably higher than Cry1 protein production in other Bt corn varieties, which produce about 4−10 g Cry1Ab/ha (Event 176, 25000 plants/acre), 465 g Cry1Ab/ha (MON810, 60000 plants/acre), and about 51 g Cry1F/ha (Cry1F corn, 60000 plants/acre) (*9*, *28*). The high expression level of Cry3Bb1 is necessary to target the WCR larvae, which are generally not very sensitive to the Cry3Bb1 toxin. The LC_{50} values in laboratory bioassays were 4−12 ng Cry1Ab/cm^2 for larvae of European corn borer as compared to 2.01−13.04 μg Cry3Bb1/cm^2 for laboratory populations and of 0.74−9.20 μg Cry3Bb1/cm^2 for field populations of WCR (*29*, *30*). In field experiments, Cry3Bb1 corn MON863 effectively protected corn plants from feeding damage by WCR (*7*). However, the observed Cry3Bb1 contents may be only sufficient to affect first instar WCR larvae. Feeding experiments on corn MON88017 grown in greenhouse and showing Cry3Bb1 contents similar to plants in our field experiment did not affect the mortality of second instars or adults of WCR but only delayed larval development (*31*). Hence, the low susceptibility of WCR to Cry3Bb1 may be only in part balanced by the overall Cry3Bb1 expression level. Although the Cry3Bb1 contents in *Bt* corn appeared to be sufficient for WCR control, no nontarget effects, such as change in individual number, weight, and the surviving rate, had been observed in field studies when a Cry3Bb1 corn hybrid was grown over three consecutive years (*14*, *15*).

Our study provided the detailed expression pattern of Cry3Bb1 in MON88017 in a field trial performed in Germany. The 3 years of measurements revealed a strong correlation of Cry3Bb1 contents in different plant tissues and confirmed the decline of Cry3Bb1 contents over the growing season as observed in experiments in the United States (*8*). However, our Western blot analyses provided evidence that the antibodies used in the PathoScreen method also recognize degradation products of Cry3Bb1 to a certain extent. As it is not known whether all of these degradation products are biologically active, it cannot be

excluded that the Cry3B1 measurements slightly overestimate the contents of active Cry3Bb1 in these plants. On the other hand, the amount of degradation products (< 10% of total Cry3Bb1) appears to be within the error range of quantification. Hence, it is not expected to significantly influence the measurements. Considering the validated test parameters, it can be concluded that the adopted PathoScreen method allows a rapid, sensitive, and reliable determination of the concentration of Cry3Bb1 in corn plant tissues.

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Supporting Information Available: Tables of Cry3Bb1 contents (μg/g dry weight) of different tissues of MON88017 collected during four developmental stages (BBCH19, BBCH30, BBCH63, and BBCH83) in three individual growing seasons 2005−2007. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Whitworth, R. J.; Wilde, G. E.; Shufran, R. A.; Milliken, G. A. Comparison of adult corn rootworm (Coleoptera: Chrysomelidae) sampling methods. *J. Econ. Entomol.* **2002**, *95*, 96–105.

(2) Kuhlmann, U.; van der Burgt, W. A. C. M. Possibilities for biological control of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, in central Europe. *Biocontrol News Inf.* **1998**, *19*, 59N–68N.

(3) Miller, N.; Estoup, A.; Toepfer, S.; Bourguet, D.; Lapchin, L.; Derridj, S.; Kim, K. S.; Renyaud, P.; Furlan, L.; Guillemaud, T. Multiple transatlantic introduction of the Western Corn Rootworm. *Science* **2005**, *310*, 992.

(4) Wutke, A.; Hummel, H. E. Ulrichs, Ch. Der Westliche Maiswurzelbohrer *Diabrotica virgifera virgifera* LeConte (Col.: Chrysomelidae) auf dem Weg nach Deutschland. *Gesunde Pflanzen* **2005**, *57*, 73–80.

(5) Baufeld, P.; Unger, J.-G. Der Westlicher Maiswurzelbohrer (*Diabrotica virgifera virgifera*) − Aktuelle Situation und Hintergründe. *Mitt. Julius Kühn-Inst.* **2008**, *417*, 233–234.

(6) Levine, E.; Oloumi-Sadeghi, H. Management of diabroticite rootworms in corn. *Annu. Rev. Entomol.* **1991**, *36*, 229–255.

(7) Vaughn, T.; Cavato, T.; Brar, G.; Coombe, T.; DeGooyer, T.; Ford, S.; Groth, M.; Howe, A.; Johnson, S.; Kolacz, K.; Pilcher, C.; Purcell, J.; Romano, C.; English, L.; Pershing, J. A method of controlling corn rootworm feeding using a *Bacillus thuringiensis* protein expressed in transgenic maize. *Crop Sci.* **2005**, *45*, 931–938.

(8) Sidhu, R. S.; Brown, S. Petition for the determination of nonregulated status for MON 88017 corn. *Monsanto Company 04-CR-108U*, **2004**; 277 pp.

(9) Fearing, P. L.; Brown, D.; Vlachos, D.; Meghji, M.; Privalle, L. Quantitative analysis of Cry1A(b) expression in *Bt* maize plants, tissues, and silage and stability of expression over successive generations. *Mol. Breed.* **1997**, *3*, 169–197.

(10) Nguyen, H. T.; Jehle, J. A. Quantitative analysis of the seasonal and tissues specific expression of Cry1Ab in transgenic maize MON 810. *J. Plant Dis. Prot.* **2007**, *114*, 82–87.

(11) Stave, J. W. Protein immunoassay methods for detection of biotech crops: application, limitations, and practical considerations. *J. AOAC Int.* **2002**, *85*, 780–786.

(12) Grothaus, G. D.; Bandla, M.; Currier, T.; Giroux, R.; Jenkins, G. R.; Lipp, M.; Shan, G.; Stave, J. W.; Pantella, V. Immunoassay as an analytical tool in agricultural biotechnology. *J. AOAC Int.* **2006**, *89*, 913–928.

(13) Icoz, I.; Stotzky, G. Cry3Bb1 protein from *Bacillus thuringiensis* in root exudates and biomass of transgenic corn does not persist in soil. *Transgenic Res.* **2007**, *17*, 609–620.

(14) Ahmad, A.; Wilde, G. E.; Yan Zhu, K. Detectability of Coleopteran-specific Cry3Bb1 protein in soil and its effect on nontarget surface and below-ground arthropods. *Environ. Entomol.* **2005**, *34*, 385–394.

(15) Ahmad, A.; Wilde, G. E.; Yan Zhu, K. Evaluation of effects of Coleopteran-specific Cry3Bb1 protein on earthworms exposed to soil containing corn roots or biomass. *Environ. Entomol.* **2006**, *35*, 976–985.

(16) Mullin, C. A.; Saunders, M. C.; Leslie, T. W.; Biddinger, D. J.; Fleischer, S. J. Toxic and behavioural effects to *Carabidae* of seed treatments used on Cry3Bb1- and Cry1Ab/c-protected corn. *Environ. Entomol.* **2005**, *34*, 1626–1636.

(17) Lipton, C. R.; Dautlick, J. S.; Grothaus, G. D.; Hunst, P. L.; Magin, K. M.; Mihaliak, C. A.; Rubio, F. M.; Stave, J. W. Guidelines for the validation and use of immunoassays for determination of introduced proteins in biotechnology enhanced crops and derived food ingredients. *Food Agric. Immunol.* **2000**, *12*, 153–164.

(18) Rauschen, S.; Schultheis, E.; Pagel-Wieder, S.; Schuphan, I.; Eber, S. Impact of *Bt* -corn MON88017 in comparison to three conventional lines on *Trigonotylus caelestialium* (Kirkaldy) (Heteroptera: Miridae) field densities. *Transgenic Res.* **2009**, *18*, 203–214.

(19) Meier, U. *Growth Stages of Monos- and Dicotyledonous Plants*, 2nd ed.; Federal Biological Research Centre for Agriculture and Forestry: Braunschweig, Germany, 2001.

(20) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **1970**, *227*, 680–685.

(21) Stave, J. W. Detection of new or modified proteins in novel foods derived from GMO—Future needs. *Food Control* **1999**, *10*, 367–374.

(22) Nguyen, H. T.; Hunfeld, H.; Meissle, M.; Miethling-Graff, R.; Pagel-Wieder, S.; Rauschen, S.; Zurbruegg, C.; Eber, S.; Gessler, F.; Romeis, J.; Tebbe, C. C.; Nentwig, W.; Jehle, J. A. Round robin quantitation of Cry3Bb1 using the qualitative PathoScreen ELISA. *WRPS/IOBC Bull.* **2008**, *33*, 59–66.

(23) Trucksess, M. W. Determination of Cry9C protein in corn-based foods by enzyme-linked immunosorbent assay: Interlaboratory study. *J. AOAC Int.* **2001**, *84*, 1891–1901.

(24) Ward, D. P. Petition for determination of nonregulated status for the regulated article: Corn Rootworm protected corn event MON 863. *Monsanto Company #01CR-050U*, **2001**; 106 pp.

(25) Mendelsohn, M.; Kough, J.; Vaituzis, Z.; Matthews, K. Are *Bt* crops safe? *Nat. Biotechnol.* **2003**, *21*, 1003–1009.

(26) Wünn, J.; Klöti, A.; Burkhardt, P. K.; Biswas, G. C. G.; Launis, K.; Iglesias, V. A.; Potrykus, I. Transgenic Indica rice breeding line IR58 expressing a sythetic *cryIA*(*b*) gene from *Bacillus thuringiensis* provides effective insect pest control. *Bio/Technol.* **1996**, *14*, 171–176.

(27) Wu, G.; Cui, H.; Ye, G.; Xia, Y.; Sardana, R.; Cheng, X.; Altosaar, I.; Shu, Q. Inheritance and expression of the cry1Ab gene in *Bt* (*Bacillus thuringiensis*) transgenic rice. *Theor. Appl. Genet.* **2002**, *104*, 727–734.

(28) Clark, B. W.; Phillips, T. A.; Coats, J. R. Environmental fate and effects of *Bacillus thuringiensis* (*Bt*) proteins from transgenic crops: a review. *J. Agric. Food Chem.* **2005**, *53*, 4643–4653.

(29) Siegfried, B. D.; Zoerb, A. C.; Spencer, T. Development of European corn borer larvae on event 176 Bt corn: influence on survival and fitness. *Entomol. Exp. Appl.* **2001**, *100*, 15–20.

(30) Siegfried, B. D.; Vaugh, T. T.; Spencer, T. Baseline susceptibility of western corn rootworm (Coleoptera: Chrysomelidae) to Cry3Bb1 *Bacillus thuringiensis* toxin. *J. Econ. Entomol.* **2005**, *98*, 1320–1324.

(31) Meissle, M.; Pilz, C.; Romeis, J. Susceptibility of *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) to the entomopathogenic fungus *Metarhizium anisopliae* when feeding on *Bacillus thuringiensis* Cry3Bb1-expressing maize. *Appl. Environ. Microbiol.* **2009**, *75*, 3937–3943.

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