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Surveillance of Cry1Ab protein and *cry1Ab* DNA in liquid manure, soil and agricultural crops under Bt-maize cropping and slurry management of cows fed Bt-maize (MON810)

Helga Gruber

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Abbreviations

BBCH	**B**iologische **B**undesanstalt, **B**undessortenamt und **CH**emische Industrie
BH	field site **B**aumanns**h**of
Bt	***B**acillus **t**huringiensis*
DNA	**d**eoxyribo**n**ucleic **a**cid
DM	**D**ry **M**atter
CCα	decision limit
CCβ	detection capability
cry	**c**rystal
CTAB	**C**etyl**t**rimethyl**a**mmonium**b**romide
ELISA	**e**nzyme **l**inked **i**mmunosorbent**a**ssay
FAO	**F**ood and **A**griculture **O**rganisation of the United Nations
FI	field site **Fi**nsing
GIT	**g**astro**i**ntestinal **t**ract
GR	field site **Gr**ub
gm	**g**enetically **m**odified
LOD	**l**imit **o**f **d**etection
NH	field site **N**eu**h**of
NT	**n**on-**t**ransgenic
PCR	**p**olymerase **c**hain **r**eaction
PTMR	**p**artial **t**otal **m**ixed **r**ation
PU	field site **Pu**ch
SD	**s**tandard **d**eviation
T	**t**ransgenic

1 Abstract

Cultivation of genetically modified (gm) maize (Bt-maize; event MON810) leads to introduction of recombinant *cry1Ab* DNA and the insecticidal δ-endotoxin Cry1Ab into soil by way of root debris and harvest residues. Another agricultural pathway leading to the potential entry of the recombinant molecules is the application of liquid manure from cattle fed gm maize. To assess *cry1Ab* DNA and Cry1Ab protein entry by these pathways and their potential accumulation and persistence in soil a liquid manure field study and an experimental Bt maize cultivation field trial were accomplished. For the liquid manure study five cows each were fed a ration containing transgenic maize MON810 or near-isogenic maize components. Slurry produced was collected, stored and applied to grassland and maize field lots. Soil samples and the following grass and maize crop were collected from the slurry treated lots. To investigate the fate of Cry1Ab protein and *cry1Ab* DNA introduced into soil by Bt maize harvest residues soil was collected from four experimental field sites cultivated with MON810 and a near-isogenic non Bt-maize variety over nine growing seasons, the longest time of experimental Bt maize cultivation documented so far.

For analysis of the diverse agricultural sample matrices specific and sensitive methods for the detection of *cry1Ab* DNA and quantification of Cry1Ab protein were developed and validated. Qualitative endpoint PCR was applied for detection of a 206 bp *cry1Ab* gene fragment in feed, slurry and soil samples. Immunoactive Cry1Ab protein was quantified using an enzyme linked immunosorbent assay validated for each matrix according to the criteria specified in the European Commission Decision 2002/657/EC. Additionally, immunoblot analysis was performed to show the fragmentation pattern of immunoactive Cry1Ab protein in the agricultural samples.

The 206 bp *cry1Ab* DNA fragment was present in Bt maize MON810 plants and transgenic feed but not detectable in any of the slurry samples after feeding gm maize. Thus no indication for *cry1Ab* DNA application to agricultural soil by way of liquid manure was given. Quantification of Cry1Ab protein revealed a continuous degradation of the protein beginning with an average of 7.75 µg Cry1Ab g^{-1} harvested maize plant material, along 278 ng Cry1Ab g^{-1} feed sample down to 21 ng Cry1Ab g^{-1} liquid manure. The major fraction of Cry1Ab protein was degraded already by the processing of Bt maize plants to feed as feed contained only 2.6% of the recombinant protein detected in the plant. Calculated amounts around 500 mg Cry1Ab protein ha^{-1} could be applied to fields by common slurry management of farms feeding Bt-maize MON810. This adds up to less than 0.15% of Cry1Ab protein introduced directly by gm maize plant residues. None of the soil samples from lots treated with liquid manure from Bt-maize fed cows was positive for the presence of Cry1Ab protein at the assay detection limits, neither was the recombinant protein detected in grass crop or

isogenic maize plants grown on the slurry treated lots. Overall, the Cry1Ab protein revealed no extraordinary stability as it showed a higher rate of degradation in the assessed agricultural processes compared to total protein. The immunoblot analyses of maize MON810 plants, feed and liquid manure revealed a proceeding degradation of the recombinant 65 kDa Cry1Ab protein molecule into smaller immunoreactive fragments of 42, 34 and 17 kDa.

In the Bt maize cultivation field trial Cry1Ab protein was detected on one out of four field sites in concentrations of 2.91 ng and 2.57 ng Cry1Ab protein g^{-1} soil in samples collected six weeks after harvest of the eighth growing season. At no site Cry1Ab protein was detected in soil sampled in spring before the next farming season, although a calculated load of 100 ng Cry1Ab g^{-1} soil corresponding to 462 g Cry1Ab ha^{-1} could be introduced into soil by maize crop residues into the 30 cm top-soil layer. Thus no experimental evidence for accumulation or persistence of Cry1Ab protein in different soils under long-term Bt-maize cultivation was drawn from the field study.

1.1 Zusammenfassung

Beim Anbau von gentechnisch verändertem (gv) Mais (Bt-Mais, Event MON810) wird über Wurzelabrieb und Erntereste rekombinante DNA und insektizides δ-endotoxin Cry1Ab in den Boden eingebracht. Ein zweiter landwirtschaftlicher Eintragspfad für die rekombinanten Moleküle ergibt sich beim Ausbringen von Gülle von Rindern, die mit gv Mais gefüttert wurden. Ein Gülle-Feldstudie und der Versuchsanbau von Bt-Mais dienten dazu, den Eintrag von *cry1Ab* DNA und Cry1Ab Protein auf diesen Pfaden sowie deren mögliche Akkumulation und Persistenz im Boden zu untersuchen. Für die Gülle-Studie wurden je fünf Kühe mit einer Mais MON810-haltigen Futterration oder einer entsprechenden Ration mit isogenen Maiskomponenten versorgt. Die anfallende Gülle wurde gelagert und auf Dauergrünland und Maisflächen ausgebracht. Von den so behandelten Flächen wurden Bodenproben, der Grasschnitt und die Maispflanzen gesammelt. Um den Verbleib von Cry1Ab Protein und *cry1Ab* DNA aus Ernterückständen im Boden zu untersuchen, wurden Bodenproben von Mais-Versuchsflächen herangezogen. Auf diesen Flächen wurde Bt Mais MON810 und eine nah isogene Maissorte über neun Vegetationsperioden angebaut. Dies stellt den längsten Zeitraum dar, in dem Bt-Mais Daueranbau bisher wissenschaftlich dokumentiert wurde.

Für die Analyse der verschiedenen landwirtschaftlichen Probenmatrices wurden spezifische und sensitive Methoden zum Nachweis von *cry1Ab* DNA und zur Quantifizierung von Cry1Ab Protein entwickelt und validiert. Die Polymerasekettenreaktion (PCR) wurde zum qualitativen Nachweis eines 206 bp *cry1Ab* Genfragments in Futtermitteln, Gülle und Boden herangezogen. Zur Quantifizierung von immunaktivem Cry1Ab Protein diente ein enzyme-

linked immunosorbent assay (ELISA), der für jede Probenmatrix nach der EU-Kommissions Richtlinie 2002/657/EC validiert wurde. Zusätzlich wurde die Fragmentierung des immunaktiven Cry1Ab Proteins in den unterschiedlichen landwirtschaftlichen Proben im Immunoblot dargestellt.

Das 206 bp *cry1Ab* DNA Fragment war in Bt Mais MON810 Pflanzen und transgenen Futtermitteln vorhanden, während es in den Gülleproben aus Bt-Mais Fütterung nicht nachweisbar war. Es ergibt sich in dieser Studie somit kein Hinweis auf den Eintrag von *cry1Ab* DNA in landwirtschaftliche genutzte Böden über Gülle. Die Quantifizierung des Cry1Ab Toxins zeigt einen kontinuierlichen Abbau des Proteins mit durchschnittlichen Gehalten von 7.75 µg Cry1Ab g^{-1} Maiserntegut, 278 ng Cry1Ab g^{-1} Futtermittel und 21 ng Cry1Ab g^{-1} Gülle. Der Hauptanteil des Cry1Ab Proteins wurde durch die Verarbeitung der Bt Maispflanzen zu Futtermitteln abgebaut, in denen noch 2.6% des rekombinanten Cry1Ab Proteins aus Mais nachgewiesen wurde. Die errechneten 500 mg Cry1Ab Protein ha^{-1}, die von einem Bt-Mais fütternden Betrieb pro Güllegabe auf Ackerflächen ausgebracht werden, entsprechen 0.15% des Cry1Ab Proteins welches direkt durch Bt-Mais Ernterückstände in den Boden eingetragen werden kann. In keiner Bodenprobe der mit Gülle aus Bt-Mais Fütterung behandelten Flächen wurde Cry1Ab Protein detektiert. Entsprechend wurde das rekombinante Protein auch im Gras- oder isogenen Mais Aufwuchs nicht nachgewiesen. Insgesamt wies das Cry1Ab Protein keine außergewöhnliche Proteinstabilität auf, da es sich in den beobachteten Prozessen im Vergleich zum Gesamtprotein durch eine höhere Abbaurate auszeichnete. Die Immunoblotanalysen von Mais MON810 Pflanzen, Futtermitteln und Gülle zeigten einen fortschreitenden Abbau des rekombinanten 65 kDa Cry1Ab Proteinmoleküls in kleinere immunaktive Fragmente von 42, 34 und 17 kDa.

Im Bt Mais Feldversuch wurde Cry1Ab Protein auf einem der vier Versuchsstandorte in Konzentrationen von 2.91 ng und 2.57 ng Cry1Ab Protein g^{-1} Boden sechs Wochen nach der Ernte der achten Anbausaison bestimmt. Auf keinem Standort wurde im Frühjahr vor dem nächsten Maisanbau Cry1Ab Protein im Boden nachgewiesen, obwohl die errechneten, über Erntereste in den 30 cm tiefen Oberboden eingetragenen Cry1Ab Mengen bei 100 ng Cry1Ab g^{-1} Boden beziehungsweise 462 g Cry1Ab ha^{-1} liegen. Aus dem Langzeitfeldversuch ergibt sich somit kein Hinweis auf eine Akkumulation oder Persistenz des Cry1Ab Proteins in verschiedenen Böden unter Bt Mais Daueranbau.

2 Introduction

2.1 Genetically modified maize

The global area planted with biotech crops comprised 148 million hectares in 2010 with genetically modified (gm) maize accounting for a major proportion as 47 million hectares were cultivated with gm maize (*1*). One of the most common genetic modifications enables plants to synthesize a δ-endotoxin originating from *Bacillus thuringiensis* (Bt) var. kurstaki, which is directed against the larvae of the European corn borer (*Ostrinia nubilalis*, Hübner). This lepidopteran organism is considered to be one of the major pests worldwide in maize cultivation as it leads to the destruction of four percent of the yearly maize plants according to estimations of the Food and Agriculture Organization of the United Nations (FAO). The lepidopteran larvae start to feed on developing maize leaves and then bore into the stalk and excavate tunnels towards the maize root head. This leads to insufficient maintenance of water and nutrient balance and reduced mechanical stability of the maize plant. Heavy infestation can lead to the break down of a whole maize crop. Furthermore, infections by *Fusarium spp*. are facilitated in infested and weakened maize plants, leading to a toxic load with mycotoxines like Deoxynivalenol or Zearalenone, which may cause severe health problems in humans and animals (*2, 3*). Cropping of gm maize is an efficient strategy to combat the European corn borer that increasingly replaces commonly used chemical and biological measures of pest control. The first Bt maize cultivars have been registered and commercialized in 1996 in the United States, followed by their approval in other countries all over the world, including countries of the European Union in 1998 (*4*). Since the commercial release of Bt maize, public concern has been expressed about potential effects of Cry1Ab protein and *cry1Ab* DNA released into the environment. These concerns are currently met by studies considering the biosafety of this crop, which add up to the prescribed biosafety assessment performed in Europe by the EFSA before approving gm crops.

2.2 Cry proteins

Bt maize (MON810) is altered by insertion of a truncated version of the *cry1Ab* gene under control of the 35S promoter from the cauliflower mosaic virus (CaMV) into the plants genome. This *cry1Ab* gene encodes for a shortened insecticidal Cry1Ab protein of 65 kDa molecular size. The Cry1Ab protein belongs to a large family of crystal (Cry) proteins stored originally in crystalline inclusion bodies in endospore forming Bt strains. The Cry proteins are classified according to their amino acid sequence, size and structure (*5*). Each class exhibits

toxicity to a narrow range of target organism species e.g. the insect family of Lepidoptera for the Cry1Ab protein (*6, 7*). Cry proteins consist of three distinct structural domains responsible for their toxic mode of action (*8, 9*) (*Figure* 1). Once ingested the Cry proteins are solubilized and activated to their toxic form by gut proteases of susceptible insect larvae. Activated toxin binds to specific receptors localized in the midgut epithelial cells (*10, 11*), invades the cell membrane and forms cation-selective ion channels that lead to the disruption of the epithelial barrier and larval death by osmotic cell lysis (*12, 13*). Due to both the lack of Cry protein activation process in the mammalian acidic gut (*14*) and the absence of Cry protein receptors in the mammalian small intestine (*15, 16*), Cry toxins are considered harmless or non-toxic to humans and farm animals (*17, 18*).

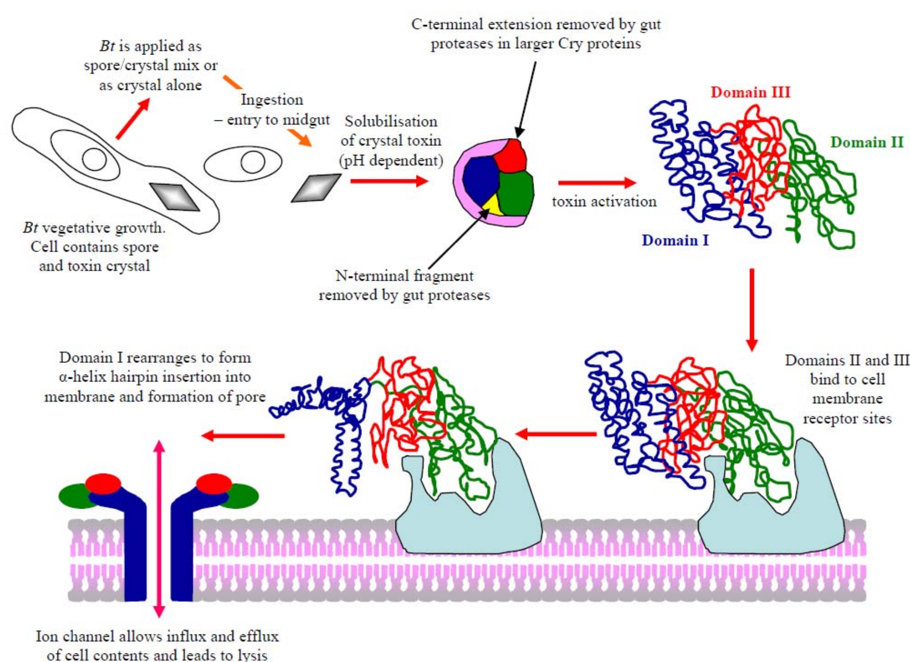

Figure 1: Toxicity mechanism of Cry proteins. The process of solubilisation, proteolysis and receptor binding of Cry protein domains II and III is followed by insertion and pore formation of domain I. Adapted from de Maagd et al (2001).

2.3 Fate of novel protein and DNA in the environment

Insecticidal proteins and novel DNA synthesized by Bt plants enter the soil by different pathways, e.g. in root exudates or root debris during the vegetative period (*19, 20*), after transgenic plants have been harvested and residual biomass is incorporated into soil (*21, 22*) and by some minor input from pollen (*23*).
A range of studies have investigated the entry and degradation of Cry proteins in field soils as well as the adsorption of the novel proteins on soil particles on a laboratory scale (*24-28*). In laboratory experiments, as a result of the interaction of the Bt toxins with soil constituents, the insecticidal proteins became partially resistant to microbial degradation (*20, 29, 30*). Several studies report that clay minerals and humic substances are the most important adsorbents in soils, because of their large specific surface area and high cation-exchange capacity (*26, 31*). However, the potential persistence of Bt toxins in field soils, as well as their microbial degradation, have to be regarded as a function of soil type, environmental conditions, source of the proteins (plant produced versus purified form), and the particular Cry protein studied (*32*). Up to now no accumulation of any Cry protein could be demonstrated from field soils under Bt maize cultivation for time periods of up to five years (*33-38*).
The potential effects of recombinant Cry proteins introduced into the agricultural environment were investigated in a wide range of studies on non-target organisms such as earthworms, nematodes and soil microbiota (*39*), beneficial insects (*40-43*) and the potential selection and enrichment of toxin-resistant target insects (*11, 44, 45*). However, to the present knowledge, no scientific evidence for Bt-maize posing a severe hazard to any of these non-target organisms was drawn from these studies (*4, 46*). Regarding the entry of gm plant DNA into the soil compartment, a potential horizontal gene transfer of transgenic plant DNA to soil borne bacteria is still discussed (*47*). So far, no evidence for such a putative transfer exists from field and microcosms investigations using marker genes from transgenic plants and indigenous soil and plant-associated bacteria (*48-50*). However, in laboratory studies bacterial incorporation of purified plant DNA fragments was enforced under artificial conditions and at very low frequencies (*49, 51, 52*).
It is common agricultural practice on dairy farms to collect the bovine slurry for liquid manuring of fields used for crop production. If gm maize is a component of the feed, insecticidal Cry1Ab protein and modified maize DNA may enter the agricultural soil by way of incompletely digested gm maize material in the liquid manure.
The fate of Cry1Ab protein and novel DNA during feed processing of Bt maize plants and during the passage through the cows gastrointestinal tract (GIT) has been addressed in a

range of studies so far. After harvest of maize plants, degradation of plant DNA and protein starts by autolytic processes and microbial activity (*53*). In the digestive tract DNA and protein fragments are further degraded by microbial enzymatic processes and gastric acid (*54-57*). In a long-term feeding study investigating dairy cows fed Bt maize MON810, fragments of *cry1Ab* DNA were detectable in the feed ration but not in feces of the animals (*58*). Concerning the novel protein, feed processing e.g. ensiling to maize silage leads to degradation of the recombinant protein (*59, 60*). After ingestion, microbial activity in the rumen leads to further degradation of the protein. There is evidence that a fraction of proteins passes through the rumen and is partly degraded in the small intestine. Thus, immunoactive Cry1Ab protein fragments can also be found in contents of the GIT and in the feces of cows and pigs fed gm feed (*55, 59, 61, 62*).

Considering these results in view of manuring practice on dairy farms, a potential entry of recombinant Cry1Ab protein, but not recombinant DNA, by way of liquid manure as a mixture of feces and urine has to be assumed into the agricultural environment, particularly the soil compartment. Accordingly, the fate of Cry1Ab protein introduced into soil and its unwanted transfer to crops cultivated on fields treated with liquid manure from cows provided with gm food has to be addressed. Furthermore, the quantitative degradation of Cry1Ab protein was not yet followed over the whole agricultural pathway from the Bt maize crop over liquid manure, soil and subsequent crops by a single validated detection method. The degradation products of Cry1Ab protein in this agricultural process have been assessed to a limited extent so far (*61*).

In order to trace even minute amounts of Cry1Ab protein or recombinant DNA in feed, liquid manure, different soils and various crops, highly specific and sensitive extraction and detection methods are needed. For Cry protein analysis immunological assay systems like enzyme linked immunoassays (ELISA) are most commonly used to date, whereas current recombinant DNA detection methods are based on polymerase chain reaction (PCR) systems. To ensure the collection of solid data these methods also need to be validated according to existing international guidelines for analytical procedures, like the guideline laid down in the European Commission Decision 2002/657/EC.

3 Aim of the Study

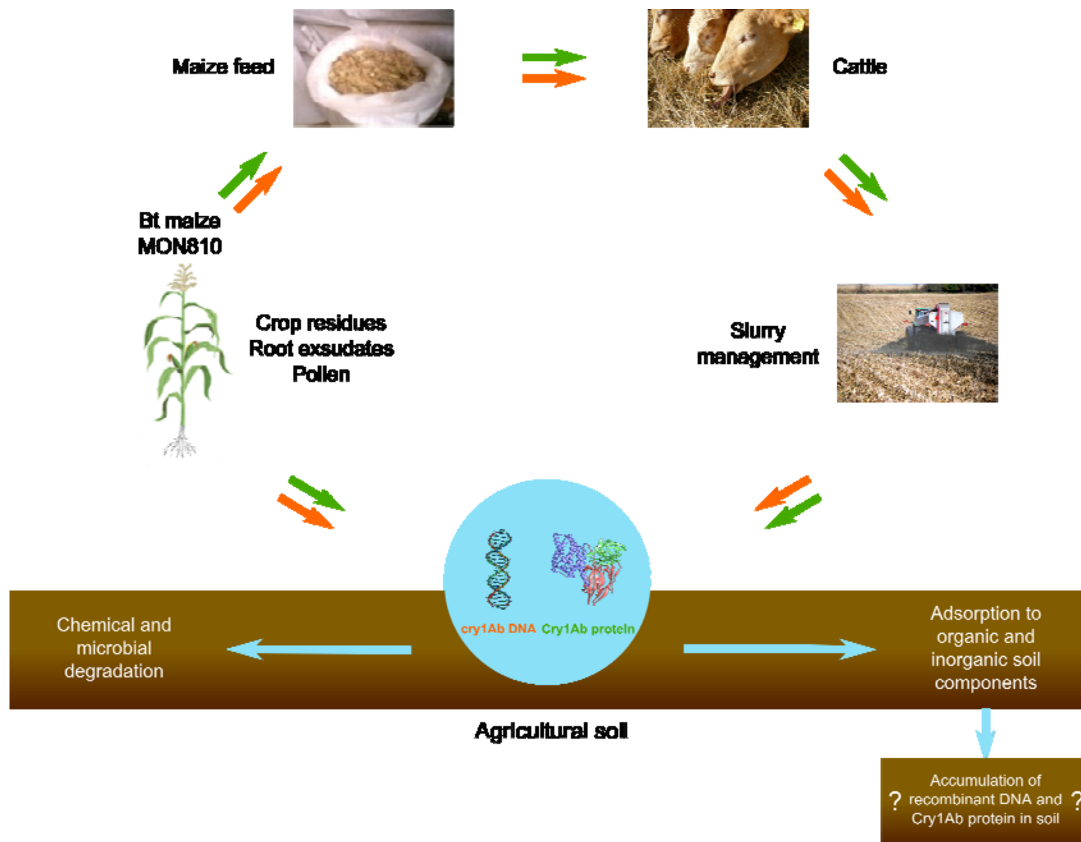

Figure 2: Schematic presentation of the aims of this study

The objectives of the present experimental field study were to investigate the entry of Cry1Ab protein and *cry1Ab* DNA into soil by two different pathways in agricultural procedures operating with Bt maize MON810 (Figure 2). In the direct pathway, the novel molecules are released to soil by Bt maize plant residues during the vegetation period and after harvest. The second indirect pathway enters the soil by liquid manure from cattle receiving Bt maize in their diet and will be addressed in this study for the first time. Major aspects of this work were the development and optimization of immunological assay systems to quantitatively determine Cry1Ab protein and the development of DNA isolation procedures to enable PCR analysis in the agricultural sample matrices of maize plants, maize feed, liquid manure, soil, as well as maize and grass crop and to validate them according to criteria laid down in international guidelines. Additionally, immunoblot analysis was applied to gather further information on the degradation of the insecticidal Cry1Ab protein molecule running through the investigated agricultural processes. Amounts of Cry1Ab protein turned over in liquid

manuring practice should be balanced and the amounts introduced into soil evaluated in comparison to the direct Cry1Ab protein entry by harvest residues on Bt-maize fields. Finally, analysis of soil obtained from fields after Bt maize long-term cultivation will meet the issue of a potential accumulation and persistence of the insecticidal protein or *cry1Ab* DNA in soil. Altogether, the study is aimed at providing data about the temporary availability of the recombinant Cry1Ab protein and *cry1Ab* DNA in the agrarian ecosystem, which is an important issue regarding the biosafety assessment of this gm crop.

4 Materials and Methods

4.1 Experimental maize field sites and maize cultivars

The four experimental Bt maize field sites Baumannshof, Puch, Neuhof and Grub/Finsing were located in Bavaria in the south of Germany. The main features of the field sites and their soil characteristics are given in Table. 1.

Table 1: Experimental sites and soil characteristics in the field trial investigating Bt maize long-term cultivation over a time period of nine growing seasons

field site	absolute altitude [m]	FAO soil type	pH $CaCl_2$	soil texture 0-30 cm [% Clay/ % Silt/ % Sand]	temperature mean [^oC]	average rainfall [mm/year]
Baumannshof	365	cambisol	4.8	4/15/81	7.8	636
Puch	550	rendzina	5.8	18/70/12	8.0	920
Grub/Finsing	525	luvisol	7.0	30/42/28	7.4	967
Neuhof	516	planosol, gleysol	6.8	21/6/18	7.6	764

Each experimental field contained four replicate plots cultivated with the Bt-maize variety Kuratus (event MON810) and four replicate plots planted with the near-isogenic-maize variety Gavott (both cultivars from KWS Saat AG, Einbeck, Germany). The size of the replicate plots was 750 m^2. A detailed view of the plot design is given in Figure 3.

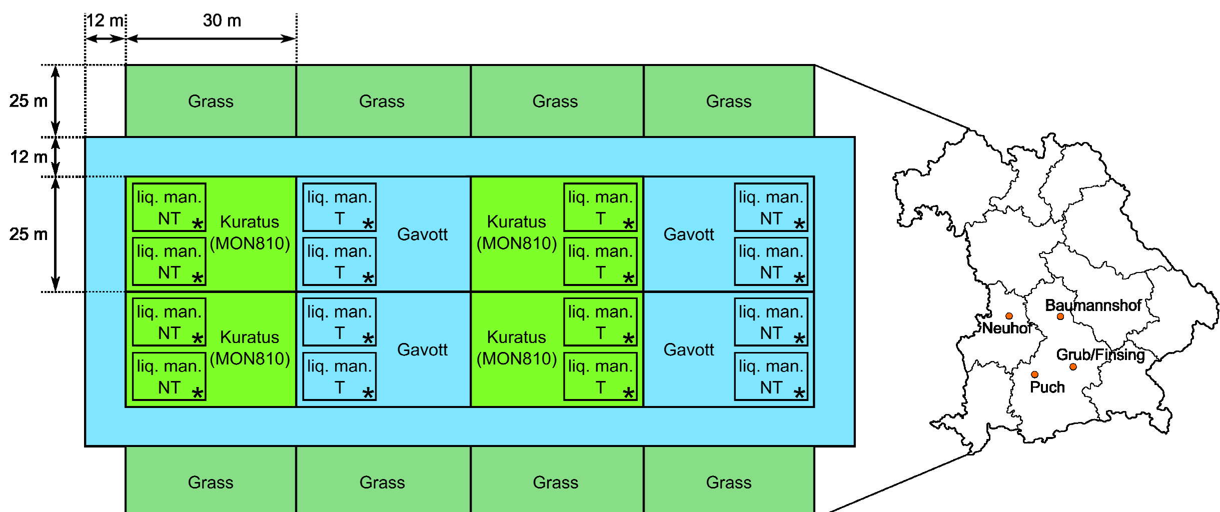

Figure 3: Experimental field design of Bt maize long term cultivation and application of liquid manure from Bt maize fed cattle to maize fields. Transgenic maize (light green) and a non-transgenic variety (blue) were grown on replicate plots of 750 m^2 size, surrounded by a 12 m framing strip of isogenic maize. Rectangles (∗) within the plots represent 6 m^2 small plots treated with liquid manure of transgenic (T) or non-transgenic (NT) origin on the sites Grub and Finsing.

Growing of gm (MON810) and non-gm maize cultivars was started in the year 2000 and continued without interruption until the end of the maize growing season 2008. The whole maize plants were harvested and used as grain maize according to good agricultural practice. Chopped plant residues, maize stubbles and root material remained on the field plots.

4.2 Feeding experiment and liquid manure field trial

For the liquid manure field trial ten dairy cows were selected out of a 25-month feeding study conducted on 36 lactating Bavarian Fleckvieh at the Bavarian State Research Center (Bayerische Landesanstalt für Landwirtschaft, Grub, Germany). A detailed description of the whole gm maize feeding experiment is given in Steinke et al. 2010 and Guertler et al. 2010. The daily diet contained a partial total mixed ration (PTMR) based on transgenic maize for the target group or non-transgenic maize for the control group as shown in Table 2. Non-transgenic and transgenic maize components with equivalent feed values assured a comparable feeding regime of the target and the control group (*63*).

Table 2: Feed composition of the partial total mixed ration (*58*) as given to cows in the liquid manure field trial

Component	Ratio (DM)
Maize silage[1]	41.9%
Maize cobs[1]	21.2%
Grass silage	11.0%
Straw	5.9%
Molasses	1.4%
Concentrates • Rapeseed meal • Maize kernels[1] • Mineral mixture • Urea	18.6% • 51.1% • 41.2% • 5.3% • 2.4%

[1]Ingredients contained 100% MON810 in gm ration or conventional maize in non-gm ration

For the liquid manure field trial, five cows each of either the target group fed on PTMR containing 63 % gm maize MON810 or the control group fed the conventional maize ration were kept in a stable equipped with special slurry sampling facilities for a time period of six days. The entire stall manure of each group was collected and pooled. Storage was carried out in four slurry tanks according to agricultural practice at ambient temperature for further experimentation (store 1 and 2: slurry control group; store 3 and 4: slurry experimental group).

The liquid manure was applied in the vegetation period of 2007 to grassland and maize plots located on two experimental field sites in Grub and Finsing (Southern Bavaria, Germany). Maize fields were under experimental maize cultivation before and during the liquid manure field trial for seven (Grub, as one of the long term cultivation sites) and one year (Finsing) whereas the grassland was under common permanent agricultural cultivation. Experimental Bt maize and non-Bt maize cultivation was performed on four replicate plots as stated above. Grassland was manured four times each time after cutting the grass, maize fields were treated twice (before and after germination of seeds) (*64*). Liquid manure of the Bt-maize fed group and the control group was applied to four replicate small plots of 6 m^2 on grassland (Figure 4) and within maize fields (Figure 3) according to the time schedule shown in Figure 5. On the maize fields, liquid manure of transgenic and non-transgenic origin was applied to Bt-maize- and non-Bt-maize-parcels, resulting in a total of 16 small plots of manure application. The manure was evenly applied with a watering-can in amounts of 2.5 L/m^2 on grassland and 4 L/m^2 on maize fields following a scenario of a maximum load allowed in good agricultural practice

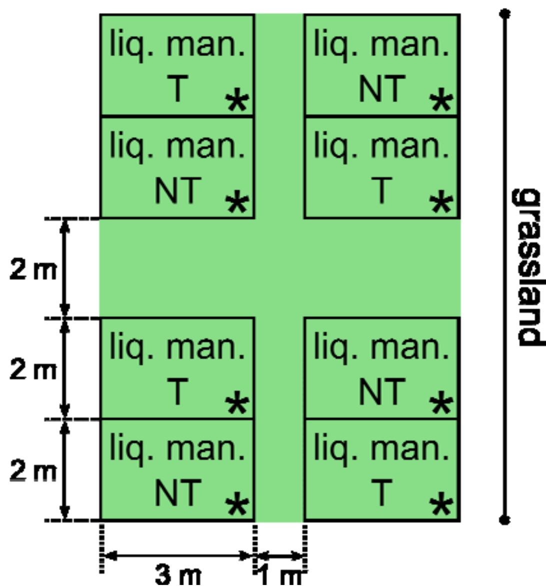

Figure 4: Application scheme of liquid manure on grassland. Liquid manure was obtained from cows fed either transgenic (T) or non-transgenic (NT) maize and applied to small plots of 6 m^2 size.

4.3 Sampling procedures

In the presented study samples of feed, liquid manure, soil, maize crop and grass crop were collected according to the time schedule given in Figure 5 and analysed for the presence of Cry1Ab protein and *cry1Ab* DNA.

Representative PTMR feed samples of the transgenic and the non-transgenic diet were collected weekly within the long term Bt maize feeding experiment. Feed samples collected within the period of liquid manure retrieval from the cows were selected and ground finely in liquid nitrogen using mortar and pestle.

Liquid manure samples were collected from slurry stores at the beginning of storage and at each date of manuring the experimental field lots (Figure 5). Each sample was pooled from six subsamples taken with a 35 mm diameter sampling tube after exhaustive homogenization of the slurry from the tank.

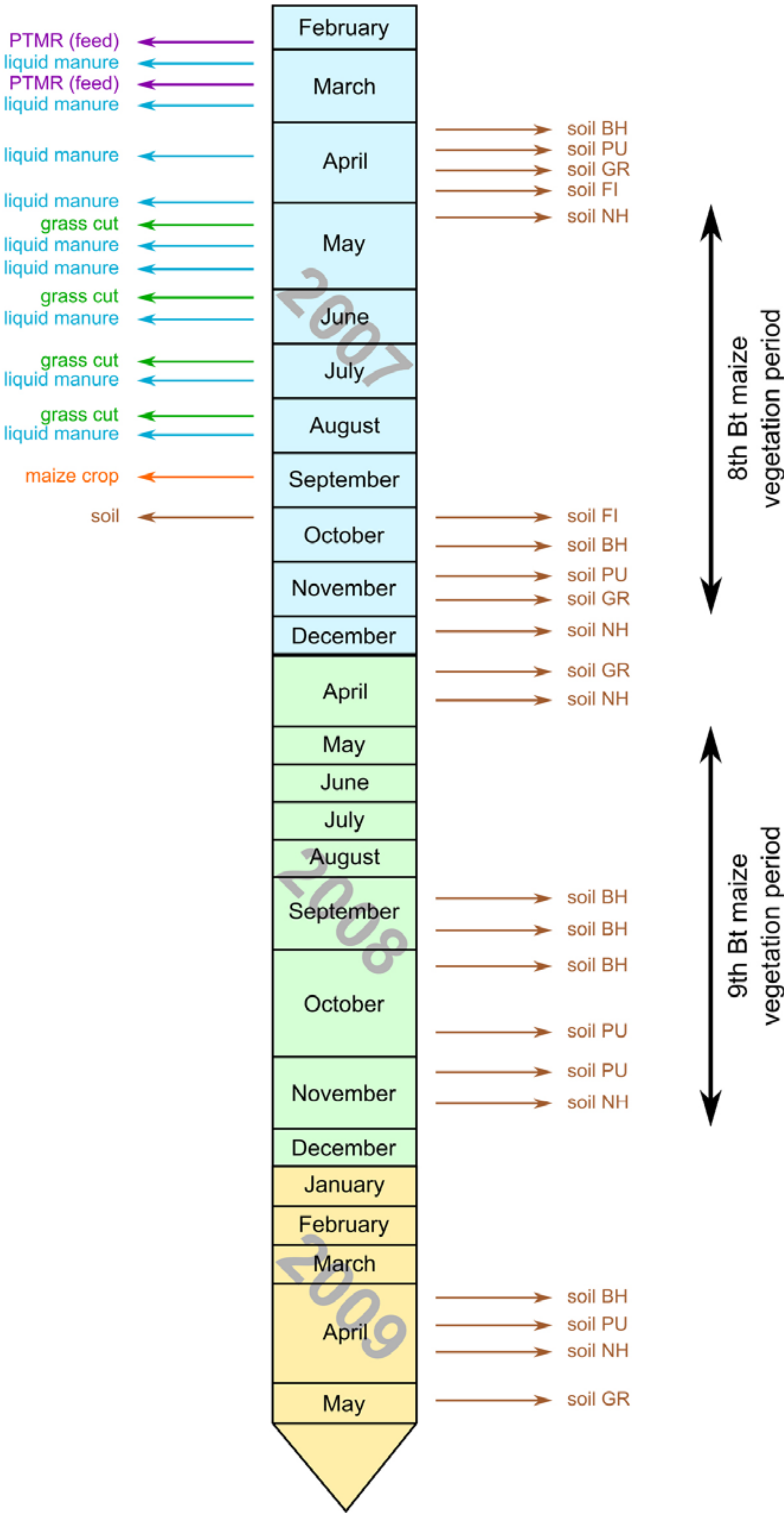

Figure 5: Sampling schedule of the experimental field trials investigating Cry1Ab protein and *cry1Ab* DNA entry into agricultural soil by way of harvest residues or liquid manure from Bt maize fed cattle.

Grass cut from each slurry treated grassland parcel was harvested at four cutting dates. The time period between liquid manure application and cropping was between 28 and 44 days. Grass from each parcel was mixed, one aliquot drawn from the core of the crop, chopped to small pieces and ground in liquid nitrogen.

Mature maize plants at growth stage of BBCH85 (ripening stage) (*65*) were harvested by cutting with a sickle from each experimental field parcel treated with liquid manure. Ten representative plants were selected and chaffed by a crop chopper to pieces of approximately 5 cm diameter size. Aliquots of the well mixed chaffed material were ground in liquid nitrogen.

For Western Blot analysis, one additional set of maize plants of the varieties Kuratus (transgenic) and Gavott (near-isogenic) were grown in the glasshouse at 22 °C with a weekly nitrogen phosphorus potassium (NPK) treatment. The leaves of four plants of each variety were collected in the growth stages of BBCH14 (5 leaves) and BBCH69 (10-11 leaves) omitting the uppermost and undermost leaves. Leaves were immediately reduced to small pieces using a scalpel, ground in liquid nitrogen and frozen at -20 °C.

Soil samples were taken from the liquid manure treated 6 m^2 maize and grassland lots at the end of the vegetation period in depth of 0-30 cm and 30-60 cm from maize field plots and 0-15 cm from grassland plots. In the years 2007, 2008 and 2009, soil samples from experimental maize fields under long term Bt maize cultivation were collected from 750 m^2 in depths of 0-30 cm and 30-60 cm. In each case, ten individual drilling cores of 4.5 cm diameter were mixed and combined to one soil sample per plot and depth. The soil samples were stored under cold-condition until further processing. In the laboratory, soil was homogenized and sieved at 2 mm mesh size. Aliquots of the fraction < 2 mm were stored at -20 °C until analysed for Cry1Ab protein or *cry1Ab* DNA.

4.4 Cry1Ab protein quantification

For the detection and quantification of Cry1Ab protein, an enzyme linked immunosorbent assay (ELISA), based on highly specific polyclonal antibodies raised in crossbred rabbits through immunization with 65 kDa active Cry1Ab toxin and labeled with biotin after affinity purification, was used (*66*). A schematic view of the quantitative sandwich-ELISA is given in Figure 6. The precision of the Cry1Ab-ELISA was assessed by determinig intra-assay and inter-assay coefficients of variation (CV) calculated from the analysis of Cry1Ab protein control samples at three concentrations of 0.3, 1.2 and 5.0 ng mL^{-1} in nine independent assays.

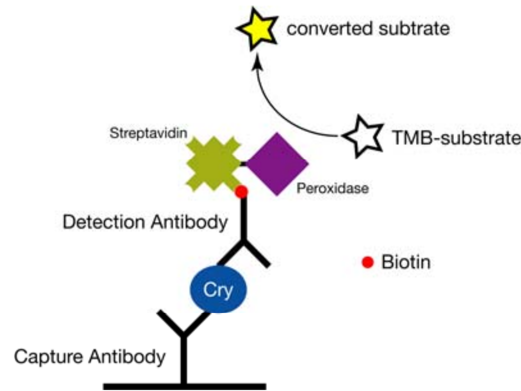

Figure 6: Assembly of the Sandwich Enzyme linked immunosorbent assay for sensitive and specific quantification of Cry1Ab protein

Cry1Ab protein extraction procedures for maize plant material, grass, liquid manure and soil were developed on the basis of a PBST extraction buffer system and are described in detail in (*64, 67*). For Cry1Ab protein analysis in PTMR, samples were extracted as previously described (*58*).

50 µL of each sample extract was applied as a concentrate or in adequate dilution in the assay and the Cry1Ab protein concentrations were determined in unknown samples by interpolation from a Cry1Ab protein calibration curve. HPLC purified trypsin activated Cry1Ab protein (65 kDa, generously provided by Dr. William J. Moar, Auburn University, USA) served as a calibrator for quantification of Cry1Ab protein. Results are presented as ng of Cry1Ab protein g^{-1} matrix wet weight or µg of Cry1Ab protein g^{-1} protein as determined by bicinchoninic acid (BCA) assay according to Smith et al. 1985.

For each sample matrix, the ELISA was validated according to the criteria specified in the recently adopted European Commission Decision 2002/657/EC for the performance and validation of screening and confirmatory analytical methods as outlined in previous publications (*66, 68*). The decision limits (CCα) and detection capabilities (CCβ) were calculated by the formulas

(1) CCα = (background noise level for Cry1Ab in blanks) × 3

Blank samples were fortified at the respective Cry1Ab concentration levels of CCα, and detection capabilities (CCβ) calculated from the equation

(2) CCβ = CCα + 1.64 × standard deviation of samples spiked at CCα.

According to the EU Commission Decision 2002/657/EC, false compliant rates (β-error: samples below CCα, when spiked at a concentration level of CCβ) of <5% are prescribed for screening assays. Similarly, <5% false compliant results (α-error: blanks exceeding the CCα value) are accepted.

The mean analytical recovery of Cry1Ab protein in plant material, PTMR, liquid manure and soil was determined separately for each matrix by spiking eight blank samples at three different concentration levels of Cry1Ab protein (5, 20 and 60 ng g^{-1} sample) and measuring Cry1Ab concentrations in the spiked samples by applying the Cry1Ab protein ELISA.

4.5 Cry1Ab protein fragmentation analysis by immunoblot

The fragmentation of Cry1Ab protein from maize MON810 was recorded by immunoblot analyses of protein extracts from maize plants, PTMR samples and liquid manure samples. Amounts of approximately 5 µg (maize plants), 50 µg (PTMR) or 220 µg (liquid manure) total protein were applied to 12 % reducing SDS-polyacrylamide gels and proteins were resolved by gel electrophoresis at 120 V for 150 min, followed by blotting of the proteins onto nitrocellulose membranes (Protran AB 85, Whatman, Dassel, Germany). Immunodetection with polyclonal Cry1Ab protein specific antibodies (rabbit 0.1 µg mL^{-1}) and visualization of immunoactive Cry1Ab fragments was performed according to the procedures described earlier (*61*). HPLC purified trypsin activated Cry1Ab protein (65 kDa) served as a positive control for the presence of Cry1Ab protein in the respective samples.

4.6 cry1Ab DNA analysis

4.6.1 DNA extraction procedures

For DNA extraction from PTMR, liquid manure and soil, a Cetyltrimethylammoniumbromide (CTAB)-based extraction protocol (Swiss Food Manual, 2004) was optimized and used as described in detail elsewhere (*58*). DNA concentrations of all samples were determined by measuring the optical density (OD) at 260 nm and the ratio 260/280nm was used for DNA integrity determination (Nanodrop 1000, Thermo Scientific). The recovery rate of each extraction procedure was determined by spiking samples with predefined copy numbers of *cry1Ab* DNA, followed by re-extraction and qualitative analysis of the DNA.

4.6.2 Polymerase Chain Reaction

Qualitative end-point PCR was performed using 100 ng genomic DNA to test for the presence of a 206 bp fragment of *cry1Ab* (Accession No. AY326434) in PTMR and liquid manure samples. As a positive DNA extraction control, a 365 bp fragment of ribosomal *18S* RNA was amplified. Additionally, a 173 bp fragment of chloroplast specific multi copy gene *rubisco* (Accession No. X86563) and a 202 bp fragment of the maize specific single copy *opaque-2* gene (Accession No. X15544) were amplified as a positive control. Primer sequences are given in Table 3. Cycling conditions for amplification of the *opaque-2* gene fragment are given in Gruber et al. 2011c. The cycling conditions for amplification of the other three gene fragments are summarized in (*69*). Isolated DNA from gm maize grain

(MON810) served as a positive control for *cry1Ab*, *opaque-2* and *rubisco*. The PCR products were separated by gelelectrophoresis and visualized by staining with ethidiumbromide.

Table 3: Primer sequences as used for the DNA analysis and fragment size of the amplicons

target gene	fragment size (bp)	primer sequences	reference
cry1Ab	206	For: 5′-CCT GGA GAA CTT CGA CGG TA-3′ Rev: 5′-TCG TGC CGT AGA GAG GAA AG-3′	(*69*)
cry1Ab	280	For: 5’-CAA GTG TGC CCA CCA CAG C -3’ Rev: 5’-GCA AGC AAA TTC GGA AAT GAA -3’	*this study*
rubisco	173	For: 5′-AGC TAA TCG TGT GGC TTT AGA AGC C-3′ Rev: 5′-TGG TAT CCA TCG CTT TGA AAC CA-3′	(*56*)
opaque-2	202	For: 5’-CTG GAG AAA GAG TGC CCT TG -3’ Rev: 5’-TGG CTA ACC GGT TTA CGT TC-3’	(*70*)
18S	365	For: 5′-AAG TCT TTG GGT TCC GGG-3′ Rev: 5′-GGA CAT CTA AGG GCA TCA CA-3′	(*69*)

4.7 Statistics and data analysis

Final data are presented as ng of Cry1Ab protein g^{-1} wet weight sample and as µg Cry1Ab protein per g total protein. Data are presented as mean ± standard deviation of replicate samples analysed. Student’s t-test was used to compare the means of Cry1Ab concentrations in maize plants, PTMR, liquid manure and soil of transgenic and non-transgenic origin, considering a P-value below 0.05 as significant.

According to the validation criteria laid down by the European Commission Decision 2002/657/EC Cry1Ab protein values below the decision limit of CCα indicated the absence of the novel protein in the respective sample with an α-error of 5%.

In the qualitative end-point PCR analysis the limit of detection (LOD) is presented as the lowest detectable number of gene copies g^{-1} sample after gene amplification in spiked samples.

5 Results and Discussion

5.1 Assay validation

At the beginning of this study, an ELISA, validated according to the guidelines of the European Commission Decision 2002/657/EC for Cry1Ab protein detection in sample material of animal origin was available (*58, 66*), but not validated for the agricultural matrices investigated in the present study. Similarly, sensitive analytical methods for detection and quantification of novel DNA in animal samples were developed before (*58, 71*) but not assessed for the analytical matrices of liquid manure and soil. Hence, the development of extraction and quantification methods for Cry1Ab protein and the development of detection methods for *cry1Ab* DNA in the new matrices was a major task of this study. Based on the sensitive and specific ELISA system, Cry1Ab protein quantification methods in the matrices of maize plant material, grass crop, feed, liquid manure and soil were developed and optimized. The Cry1Ab protein assay was validated for each matrix by determining the decision limit CCα, the detection capability CCβ of the ELISA and analytical recovery rates. A qualitative endpoint PCR assay (*58*) was adopted for the analysis of the recombinant *cry1Ab* DNA in liquid manure and soil from one representative experimental field site. For *cry1Ab* DNA detection LODs were determined. Results of the validation procedure are summarized in Table 4.

5.1.1 Cry1Ab protein ELISA analytical assay performance

The analytical range of the assay was 0.04 to 20 ng Cry1Ab mL^{-1} extraction buffer, which corresponds to 0.4 ng to 200 ng Cry1Ab protein g^{-1} matrix and allowed for determination of Cry1Ab down to the analytical limit of 0.4 ng Cry1Ab protein g^{-1} matrix(*68*).. A good assay precision was documented by a mean intra-assay CV of 5.9 % and a mean inter-assay CV of 14.6 % (n=9; three Cry1Ab protein concentrations) (*64*).

5.1.2 Analytical recovery of Cry1Ab protein

The immunoassay operated well for the investigated matrices with mean analytical recoveries in the range from 68% to 99%. The recoveries differed in the investigated matrices as summarized in Table 4.

Table 4: Decision limits (CCα), detection capabilities (CCβ) and analytical recoveries of the Cry1Ab protein ELISA and limits of detection (LOD) for qualitative endpoint PCR analysis of *cry1Ab* DNA in different sample matrices

Sample material	CCα [ng g^{-1} wet weight]	CCβ [ng g^{-1} wet weight]	Mean Cry1Ab protein recovery[a] [%]	LOD *cry1Ab* gene fragment [copies g^{-1} wet weight]
Feed (PTMR)	4.00	6.00	78.9 ± 13.2	37 [b] (206 bp)
Liquid manure	1.20	1.41	71.2 ± 5.1	10^3 (206 bp)
Soil	2.00	3.06	68.1 ± 9.9	3.3 x 10^5 [c] (280 bp)
Plant material	1.38	2.00	98.8 ± 7.2	n.d.

[a] Mean recovery ± S.D. after spiking with three concentrations of Cry1Ab protein (eight replicates per spike concentration)
[b] (*58*)
[c] soil representing the experimental field site Puch

A broad variance of analytical recoveries of Cry1Ab protein was observed in representative soil samples of experimental Bt-maize fields ranging from 49.1 % to 88.9 % (*67*). The percentage of Cry1Ab protein, recovered from the different soils when fortified with known amounts of Cry1Ab protein, was strongly correlated (R=0.912) to the clay content of the soils assessed as shown in Figure 7. High clay contents were associated with low recovery rates, whereas, lower clay contents resulted in high recovery of Cry1Ab protein from soil. It has been reported before that physical and chemical soil properties exert a strong effect on the adsorption of protein molecules to the surface active particles in soil. In the present study clay minerals proved to be the major compounds responsible for the Cry1Ab adsorption in the investigated soils. Similar findings were reported earlier concerning the binding of novel protein to the soil matrix (*26, 72-74*).

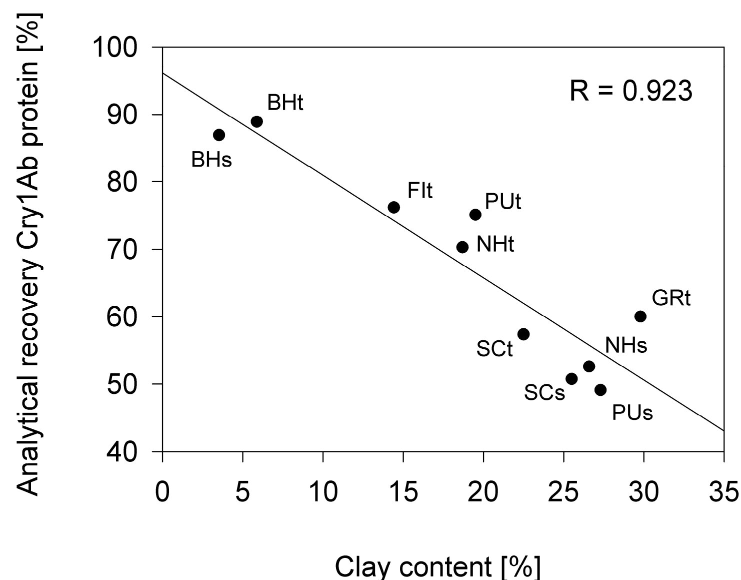

Figure 7: Correlation analysis of analytical recovery of Cry1Ab protein determined in spiking experiments and clay contents of experimental field site soils. Dots represent mean values for each field site (PU: Puch; BH: Baumannshof; NH: Neuhof; GR: Grub; SC: Schwarzenau; FI: Finsing) and soil depth (t: topsoil 0-30 cm; s: subsoil 30-60 cm) calculated from eight replicate samples analysed.

5.1.3 *cry1Ab* DNA detection by PCR

For analysis of feed PTMR and liquid manure samples, a sensitive and well-established conventional PCR method for detection of a small *cry1Ab* gene fragment of 206 bp was adopted. Its suitability for the investigated slurry matrix was proven by determination of the LOD for the 206 bp *cry1Ab* gene fragment, which is in line with its LOD reported earlier for bovine feces (*58, 70*). An LOD of 3.3×10^5 gene copies g^{-1} soil was determined for the detection of the 206 bp *cry1Ab* DNA fragment in soil samples from the Puch field site. It is well known that PCR analysis of complex environmental samples like soil may be hampered by poor DNA recovery due to degradation of the DNA by nucleases and irreversible nonspecific binding of the target DNA to soil particles. Furthermore, the activity of the polymerase may be inhibited by organic and anorganic compounds like humic acids, fulvic acids or metals (*75-77*).

Altogether, the analytical performance of the Cry1Ab protein assay in the investigated sample material fulfilled the EC validation guideline criteria and good analytical recoveries of the novel protein were achieved. Thus, the assay is well-suited for documenting Cry1Ab protein concentration levels when investigating the fate of Cry1Ab protein from gm maize introduced by agricultural processes into the soil compartment. The qualitative detection methods for c*ry1Ab* DNA established in the study revealed a high sensitivity and were well-adapted for PCR analysis of feed and liquid manure samples and soil samples of the field site Puch. After further optimization of extraction methods to gather DNA extracts of adequate purity and DNA yield in the other soil types, they could be applied for PCR analysis of soil samples from all experimental field sites.

5.2 Cry1Ab protein contents

By applying the validated highly sensitive and specific Cry1Ab protein ELISA, both the intact recombinant Cry1Ab protein molecule and immunoreactive Cry1Ab protein fragments are quantified as a whole. In the following results section the term Cry1Ab protein covers the entirety of immunoreactive Cry1Ab protein and its immunoreactive fragments.

5.2.1 Feed

Cry1Ab assay validation revealed a CCα of 4.00 ng g^{-1} PTMR (wet weight) and a CCβ of 6.00 ng g^{-1} PTMR (wet weight) (Table 4).

No Cry1Ab protein was detected in the non-transgenic PTMR feed samples of the control group, as the reported value of 2.6 ng Cry1Ab protein g^{-1} wet sample is clearly below the decision limit CCα determined for PTMR. In the transgenic PTMR, Cry1Ab protein was measured in amounts of 278 ± 29 ng Cry1Ab protein g^{-1} wet feed. This corresponds well with

amounts of 245.5 ng Cry1Ab protein g^{-1} feed sample reported by Paul et al. 2010 for samples analysed in course of the associated Bt-maize feeding study. A mean daily intake of 6 mg Cry1Ab protein per cow by the whole PTMR feed ration containing kernels cobs and silage was assumed by Guertler 2009.

5.2.2 Liquid manure

A CCα of 1.20 ng g^{-1} slurry (wet weight) and a CCβ of 1.41 ng g^{-1} slurry (wet weight) was determined in the validation procedure (Table 4).

22 samples were collected from the slurry stores within 29 weeks of storage and analysed for the presence of Cry1Ab protein. No liquid manure sample of the cows fed a non-transgenic ration was positive for the presence of Cry1Ab protein at the decision limit CCα. In the stores filled with slurry from the transgenic ration-fed cows Cry1Ab protein was detected in all samples (Figure 8). This finding goes in line with previous work reporting the presence of Cry1Ab protein in feces of Bt-maize fed cows (*58, 62*). In liquid manure, as a mixture of urine and feces, the Cry1Ab protein detection can be attributed to poorly digested transgenic maize plant feed material in the feces. Apparently, the Cry1Ab protein seems to be protected from degradation by proteolytic enzymes in the gastrointestinaltract (GIT) when enclosed in the heavy digestible parts of the maize plant (*78*). Regarding the effect of liquid manure storage on Cry1Ab protein quantities in samples collected at the time point of filling, the stores showed initial concentrations of 23.5 ± 0.9 and 18.2 ± 1.0 ng Cry1Ab protein g^{-1} slurry in store 3 and 4, respectively. During further storage, Cry1Ab concentrations in samples taken from the tanks continuously decreased to mean Cry1Ab concentrations of 51% of the initial values within 25 weeks of storing liquid manure (*64*).

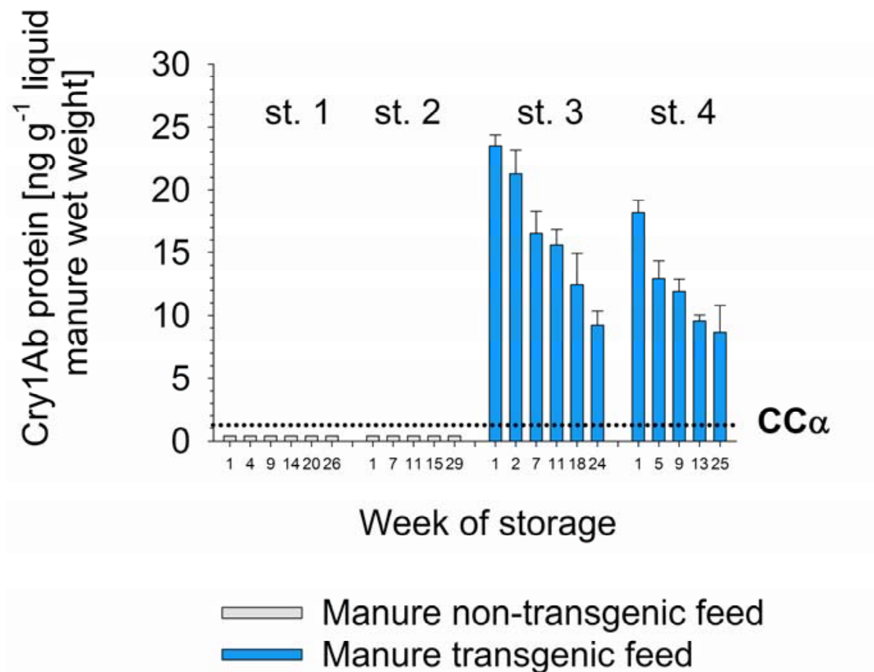

Figure 8: Detection and quantification of Cry1Ab protein in liquid manure samples derived from cows fed partial total mixed ration containing non-transgenic maize (store 1, store 2) or transgenic maize MON810 (store 3, store 4). Samples were analysed with proceeding time of storage in the slurry stores. The data are presented as mean ± SD values (n=4).

5.2.3 Soil-liquid manure field trial

For analysis of soil samples, a validated Cry1Ab protein ELISA with a CCα of 2.00 ng g^{-1} soil (wet weight) and a CCβ of 3.06 ng g^{-1} soil (wet weight) was applied (Table 4).

At the experimental field sites Grub and Finsing 32 soil samples in total were collected from maize field and grassland lots treated with liquid manure obtained from cows receiving non-transgenic or transgenic feed rations. Results of the analysis of soil samples for the presence of Cry1Ab protein are summarized in Table 5. In soil collected from maize lots and under grassland management at the field site Grub, all samples were below CCα. For soil samples collected at the Finsing field site, values exceeding CCα of 2.0 ng Cry1Ab protein g^{-1} soil were observed on the lots treated with slurry from non-transgenic and from transgenic origin. This can be due to a cross reaction of the polyclonal antibody used in the ELISA with organic substances in this soil horizon, which is rich in fine grass roots and organic material. Natural organic matter and, in particular, humic substances were identified as commonly interfering substances when environmental samples were analysed by ELISA before (*79-81*). Beyond the finding that there is no significant difference in soil samples of Finsing undergoing each slurry treatment, further methodological development is necessary to reduce unspecific

background absorbance in the ELISA and to give final evidence for the presence or absence of Cry1Ab protein in this special soil matrix rich in organic substances.

Table 5: Cry1Ab protein analysis in soil samples collected in the liquid manure field trial. Mean values and standard deviation of soil samples drilled from four replicate plots are shown. Values of 0.40 ng Cry1Ab protein g $^{-1}$ soil indicate measurements at or below the analytical detection limit.

Field site	Cultivation	Origin of liquid manure - maize feed	Cry1Ab protein [ng g^{-1} wet weight]
Grub	Bt-maize	non-transgenic	0.40 ± 0.00
	Bt-maize	transgenic	0.40 ± 0.00
	maize non-transgenic	non-transgenic	0.40 ± 0.00
	maize non-transgenic	transgenic	0.40 ± 0.00
Finsing	Bt-maize	non-transgenic	0.45 ± 0.09
	Bt-maize	transgenic	0.50 ± 0.14
	maize non-transgenic	non-transgenic	0.40 ± 0.00
	maize non-transgenic	transgenic	0.40 ± 0.01
Grub	grassland	non-transgenic	1.56 ± 0.32
	grassland	transgenic	1.68 ± 0.21
Finsing	grassland	non-transgenic	2.45 ± 1.95
	grassland	transgenic	3.42 ± 1.62

5.2.4 Maize and grass crop - liquid manure field trial

Cry1Ab assay validation revealed a CCα of 1.38 ng g^{-1} plant material and a CCβ of 2.00 ng g^{-1} plant material (wet weight) (Table 4).

Maize plants

Maize samples from the non-Bt maize variety Gavott were below the decision limit CCα, whereas samples from the MON810 Bt maize variety Kuratus revealed mean Cry1Ab protein concentrations ranging from 6.7 to 8.4 µg Cry1Ab protein g^{-1} chaffed plant material (*64*) (Figure 9). This is consistent with values of 5.5 – 6.4 µg Cry1Ab protein g^{-1} fresh weight for leaves of maize MON810 plants reported before (*82*). Consistent average Cry1Ab protein expression levels of 4.65 µg Cry1Ab protein g^{-1} plant material were stated by (*32*). Neither the near-isogenic maize plants nor the transgenic maize plants showed statistically significant differences in Cry1Ab protein concentrations depending on the treatment with liquid manure from cows fed non-transgenic or transgenic ration. This implies that no residual traces of the transgenic protein from undigested Bt maize plant material in liquid manure were dispersed to and persisted during the growth of the maize plants. These results are important with regard to concerns about the uptake of the Cry1Ab protein toxin by crops subsequently grown in soils in which Bt maize has been cultivated. In a study meeting these

concerns, Saxena and Stotzky 2002 demonstrated, that the toxin released to soil in root exudates of *Bt* maize, from the degradation of the biomass of Bt maize, or as purified toxin, was not taken up from soil or from hydroponic culture by non-Bt maize plants (*83*).

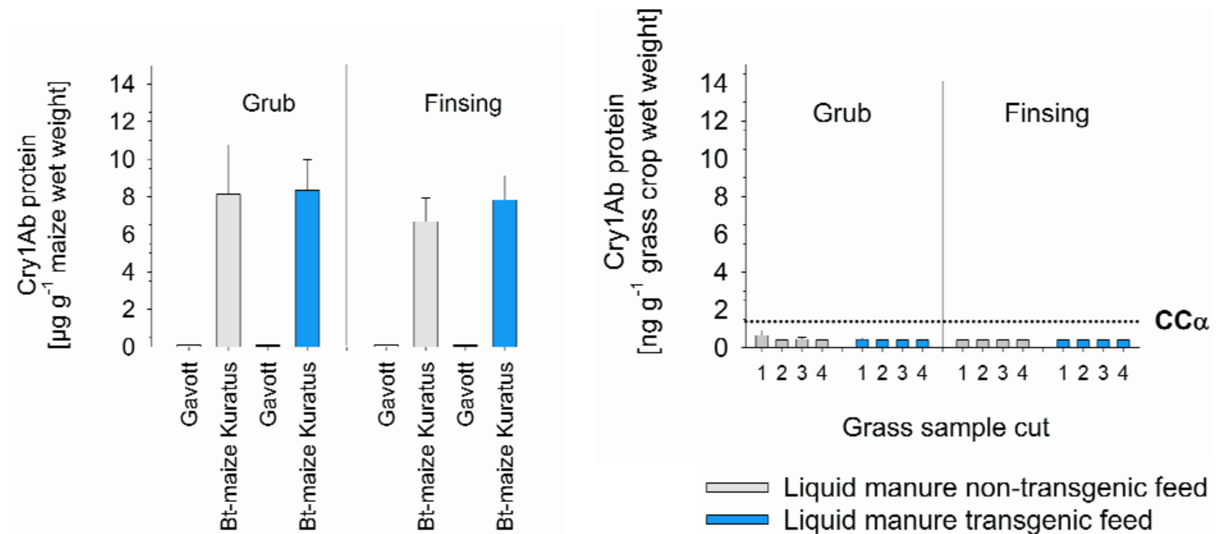

Figure 9: Cry1Ab protein determination in maize and grass crop harvested from field lots treated with liquid manure from cows fed partial total mixed ration containing non-transgenic maize (grey bars) or transgenic maize MON810 (blue bars). Data are presented as mean ± S.D. (n=4).

Grass

Grass crop was sampled at four cutting dates keeping a minimum time interval of 28 days to the preceding manuring as commonly practiced in grassland management. All grass samples analysed were clearly below CCα of 1.38 ng Cry1Ab g^{-1} plant material (Figure 9). Thus, no Cry1Ab protein was detected in any of the grass samples collected from grassland lots treated with slurry from cows fed Bt maize or from the control group fed non-transgenic feed. According to these results, grass crop, harvested from grassland under slurry management of a cattle farm feeding Bt-maize MON810, can be regarded as similar to grass produced under management omitting GM feed concerning the unwanted contamination of grass with the Cry1Ab toxin.

5.2.5 Soil under long-term Bt maize cultivation

Altogether 21 soil samplings were conducted on the four experimental field sites Baumannshof, Grub, Neuhof and Puch that were in the eights and ninth year of permanent Bt-maize cultivation during the study. Out of the reported field studies with gm maize, this is the longest duration Bt-maize was cultivated in consecutive growing seasons for an experimental analysis of Cry1Ab protein contents in soil (*34, 37, 67*). Environmental factors

were considered in the presented study by including different field sites into the experiment to assess the potential accumulation of transgenic Bt-protein under various soil types and climate regimes.

Soil samples were either collected in spring before sowing or in autumn after maize residues had been incorporated into soil after harvest. For analysis of soil samples, the validated Cry1Ab ELISA with a CCα of 2.00 ng g^{-1} soil and a CCβ of 3.06 ng g^{-1} soil was applied. The results of the quantitative Cry1Ab protein analysis of all soil samples are depicted in Figure 10.

No immunoactive Cry1Ab protein was detected in any of the soil samples taken from the control plots cultivated with non-transgenic maize. From the plots cropped with Bt maize, no transgenic protein was determined in soil samples from the field sites Baumannshof and Grub. Similarly, all soil samples from Puch field plots showed Cry1Ab values below CCα. However, in 2008, soil collected in Puch from transgenic plots directly after harvest showed mean Cry1Ab protein values of 1.17 ± 0.30 ng Cry1Ab protein g^{-1} soil (0-30 cm), which is below CCα but significantly elevated ($P< 0.05$) compared to the soil samples from non-Bt maize plots. In soil from Bt-maize plots of the experimental field site Neuhof collected in December 2007, six weeks after harvest, Cry1Ab protein was detected in quantities of 2.91 ± 1.54 ng Cry1Ab protein g^{-1} top soil and 2.57 ± 1.59 ng Cry1Ab protein g^{-1} lower soil. In the whole study, these autumn soil samples from Neuhof were the only samples revealing immunoreactive Cry1Ab concentrations higher than CCα (2.00 ng Cry1Ab protein g^{-1} soil). The presence of the recombinant protein in soil even six weeks after harvest could be attributed to soil water-logging on this field site so that Bt maize harvest residues and root material were kept in a poorly aerated environment at low temperatures. The decomposition rate of plant material will be greatly reduced under such field conditions as efficient aerobic microbial degradation of organic material is inhibited (*84*). As a consequence, persistence of the recombinant Cry1Ab protein for a few months after harvest is possible under exceptional and extreme environmental circumstances that lead to temporary protection of plant material from degradation.

Finally, in all soil samples taken in spring 2007, 2008 and 2009, Cry1Ab protein concentrations were close to and below the analytical detection limit of 0.4 ng Cry1Ab protein g^{-1} soil. Thus, no Cry1Ab protein was detected in soil on any of the four investigated experimental field sites in spring after up to nine consecutive Bt-maize growing seasons. These results are consistent with a range of studies giving report about a fast degradation of Cry1Ab protein (*33-35, 37, 38*) from transgenic plants in field soils. Also the Cry3Bb1 protein from Bt maize (event MON88017) did not accumulate in soil as outlined in a three years field trial (*36*).

The Cry1Ab quantities determined in the Neuhof 2007 bulk soil samples under cultivation of Bt-maize MON810 in the presented study are comparable with the data reported in earlier studies for the soil fraction < 2mm. Hopkins and Gregorich 2003 found trace amounts < 0.1 ng of Cry1Ab protein g^{-1} soil. Maximum concentrations of 3 ng Cry1Ab protein g^{-1} soil were reported by Baumgarte and Tebbe 2005 in soil samples collected during the vegetation period.

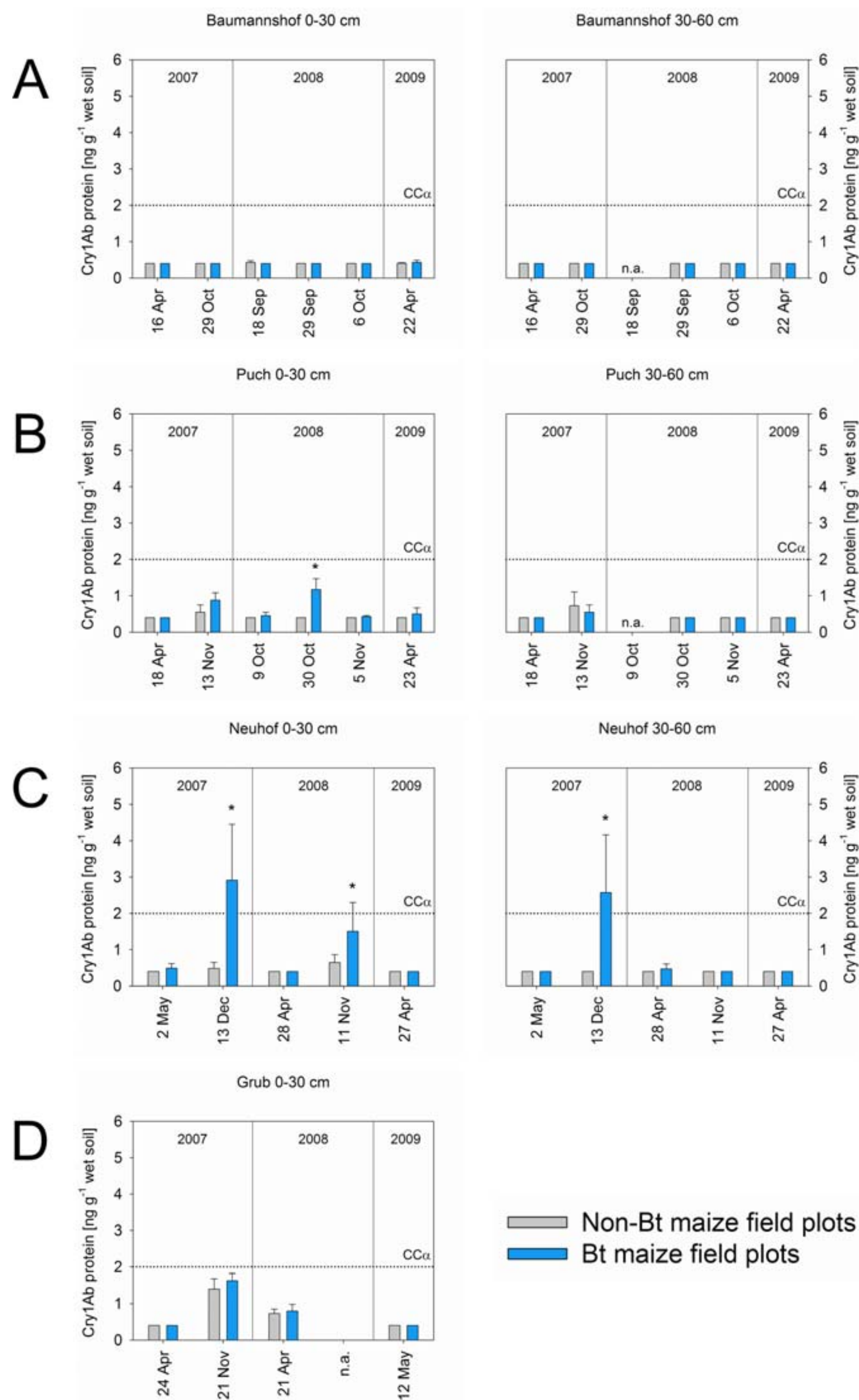

Figure 10: Cry1Ab protein contents in bulk soil samples collected in the depths of 0-30 cm and 30-60 cm on the experimental field sites Baumannshof (A), Puch (B), Neuhof (C) and Grub (D) cultivated with Bt maize MON810 (blue bars) and a near-isogenic non-Bt maize variety (grey bars) in the eighth (2007) and ninth (2008) growing seasons of consecutive Bt-maize cultivation. Data from 2009 represent Cry1Ab protein contents determined in soil samples collected terminally in spring after nine years long-term cultivation of Bt-maize MON810. The dotted line marks the decision limit CCα at 2.0 ng Cry1Ab protein g^{-1} wet soil. *indicate differences ($P < 0.05$) in Cry1Ab protein quantities in soil of Bt-maize and non-Bt maize field plots. n.a.: not analysed.

5.2.6 Toxicological relevance of Cry1Ab protein detected in soil

The ELISA applied enables for quantification of Cry1Ab and its immunoreactive degradation products (*61*) but gives no evidence for the remaining biological activity of the Cry1Ab protein molecules. The presence of bound toxin and its insecticidal activity can only be determined using sensitive organism species in biotoxicity assays at present.

Appropriate test organisms used in earlier studies are the target organism *Ostrinia nubilalis*, other lepidoteran species or susceptible soil dwelling invertebrates. The Cry1Ab protein susceptibility of the target organism *O. nubilalis* was described by a LC_{50} ranging from 0.10 to 0.34 µg of Cry1Ab per gram of diet in a study performed by Saeglitz et al. 2006 (*85*). Accordingly, *O. nubilalis* larvae fed on biogas reactor effluent containing Cry1Ab protein concentrations of 2.67 ng g^{-1} and below - the concentration range of the Neuhof-autumn 2007 soil in the present study - showed no increased mortality compared to a group receiving control effluent without Cry1Ab (*60*). Both free and humic acid bound Cry1Ab protein was insecticidal to larvae of the lepidopteran tobacco hornworm *Manduca sexta* with LC_{50} values between 215 - 304 ng 100 µL $^{-1}$ (*86*). Effects on the predator organism *Chrysoperla carnea* were determined after feeding Cry1Ab toxin in concentrations of 25, 50, 100 µg g^{-1} diet to the prey organism (*41*). A study on feeding solutions of recombinant Cry1Ab protein to the soil dwelling nematode *Caenorhabditis elegans* revealed verifiable effects on growth and reproduction at minimum concentrations of 118 and 41 mg Cry1Ab protein L^{-1} nutrient solution, respectively (*87*). This corresponds to Bt-toxin concentrations about 10,000 times higher than the concentrations measured in the experimental field soils of the present study. In the cited nematode study the test organism *C. elegans* identified deleterious effects of rhizosphere and bulk soil samples from fields with Bt maize (MON810) containing 0.51 and 1.31 ng Cry1Ab g^{-1} soil dry weight. However, though the observed effects correlated with concentrations of the Cry1Ab protein in soil they could not be explained by a direct toxicity of the Cry1Ab protein. These results of the nematode study point out that beneath quantification of Cry1Ab protein in soil samples additional assessment of Bt toxin using appropriate biotoxicity assays should gain in importance regarding further studies. Relating the Cry1Ab susceptibilities of test organisms to the concentrations of Cry1Ab protein determined in the field soils of the present study no toxicological and ecological effects on soil dwelling invertebrates should be expected. These assumptions are in line with the current knowledge of the effects of Cry proteins on soil dwelling organisms as summarized by (*88*). There, for the majority of soil born organisms no toxic effects were reported. Studies on the effects on soil nematodes show different findings, though with most of the results revealing no effects.

5.3 Cry1Ab protein degradation pattern in immunoblots

Cry1Ab protein quantification by the ELISA technique allowed for an easy and rapid analysis of the agricultural samples in the present study. The elaborate and time consuming Western Blot technique offers a more detailed and qualitative analysis of Cry1Ab protein as degradation products of the transgenic protein are separately visualized. In the present study, immunoblot analysis of MON810 maize plants, feed and liquid manure revealed a continuous degradation of the recombinant 65 kDa Cry1Ab protein molecule into smaller immunoactive fragments of approximately 42, 34 and 17 kDa (*64*). The time dependent fragmentation of Cry1Ab protein due to feed processing, storage and enzymatic proteolysis in the ruminal GIT has been described before (*61*) and fragments of similar size have been detected by other Cry1Ab specific antibody preparations (*56, 59, 89*). In the agricultural samples assessed in the present study, fragmentation of the Cry1Ab protein becomes apparent already in the early developmental BBCH14 and BBCH69 stages of the transgenic maize plants suggesting a continuous synthesis and breakdown of the recombinant Cry1Ab protein by endogenous plant proteases (Figure 11A). In transgenic feed, the same fragmentation pattern as in Bt maize plants was recorded with the full sized recombinant 65 kDa Cry1Ab protein and fragments of 42 kDa, 34 kDa and 17 kDa (Figure 11B). In liquid manure obtained from cows fed transgenic maize only the full sized 65 kDa Cry1Ab protein and a 34 kDa fragment was detected, whereas the 42 kDa and the 17 kDa fragment detected in transgenic plant and feed samples was not recorded. Successive degradation of the full sized recombinant Cry1Ab protein and the immunoactive 34 kDa fragment during slurry storage is documented by the immunoblot shown in Figure 11C. In an earlier study, the 34 kDa fragment was reported to be the most prominent immunoactive fragment in digesta and feces (*61*). These consistent findings could be a sign of a higher proteolytic stability of this 34 kDa degradation product.

Altogether, the fragmentation pattern of the Cry1Ab protein in addition to Cry1Ab protein quantity is an important criterion in the discussion on toxic effects of Cry1Ab protein introduced into the environment by agricultural processes. To the present knowledge, the toxic pathway of the recombinant Cry1Ab toxin is closely related to the presence of all functional domains of the whole trypsin resistant 65 kDa protein (*9, 90-92*). However, the toxic activity of Cry1Ab fragments smaller than 65 kDa has not been studied so far. With regard to the documented proteolytic stability of the 34 kDa Cry1Ab protein fragment, the biological activity of this fragment would be an interesting issue to be adressed in further studies.

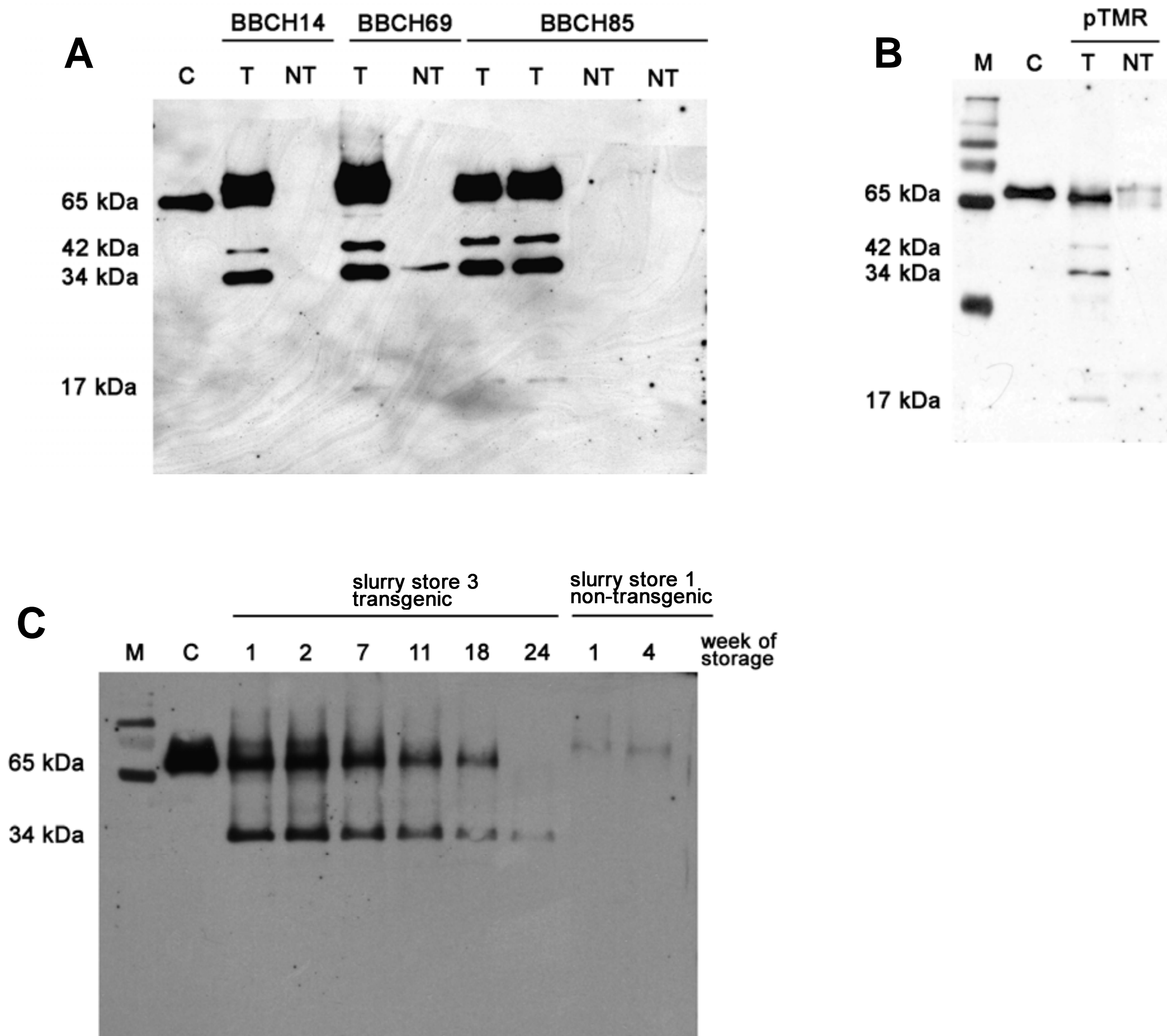

Fig 11: Western blot analysis showing immunoreactive full sized Cry1Ab protein and immunactive fragments in [A] transgenic (T) and non-transgenic (NT) maize plants in successive developmental stages of BBCH14, BBCH69 (plant leaves) and BBCH85 (chaffed maize crop), [B] total protein extracts (60 µg) of transgenic (T) and non-transgenic (NT) PTMR feed and [C] in liquid manure from Bt maize MON810 fed cows (store 3) and the control group (store 1))with proceeding time (weeks) of storage. In any blot, 500 pg of trypsin treated and HPLC purified Cry1Ab protein was used as a positive control (C), Prestained Protein Marker (M) served as a protein fragment size standard.

5.4 Quantitative Cry1Ab protein turnover

This is the first study that quantified Cry1Ab protein in respect of the fate of the recombinant protein introduced into agricultural soil entirely from Bt maize crop over feed and liquid manure into soil and investigated the following crop. Data were collected by an ELISA system validated for the various agricultural samples (Bt maize plants, feed, slurry and soil) and allow for balancing of Cry1Ab contents over the whole pathway. The quantitative degradation of the recombinant Cry1Ab protein and its immunoreactive fragments is summarized in Table 5.

Table 5: Quantification of Cry1Ab protein degradation by Bt maize crop processing and biodegradation in agricultural processes. Cry1Ab concentrations are given in mean values ± standard deviation. The quantitative decrease of the recombinant protein by each degradation step is shown by the percentages of Cry1Ab protein recovered in a sample in relation to Cry1Ab quantities in the primary Bt maize plant material or in relation to the previous degradation stage (red values).

			Cry1Ab protein content			**Cry1Ab protein conc./total protein**		
			[ng Cry1Ab g^{-1} dry weight]	**[%] of**		**[µg Cry1Ab g^{-1} total protein]**	**[%]of**	
		n		- Bt maize plant	- previous material		- Bt maize plant	- previous material
Soil entry by liquid manure	**Bt maize MON810 plant**	32	23 677 ± 1934.6	100	100	457.9 ± 46.5	100	100
	Feed (transgenic)	8	611.3 ± 64.4	2.6	2.6	10.6 ± 1.2	2.3	2.3
	Liquid manure[1]	8	204.6 ± 20.4	0.9	33.5	1.9 ± 0.4	0.4	17.9
	Liquid manure[2]	8	104.5 ± 21.5	0.4	51.1	0.9 ± 0.2	0.2	47.4
	Soil [3]	16	< CCα	-	-	-	-	-
	Isogenic maize crop[3]	16	< CCα	-	-	-	-	-
	Grass crop[3]	32	< CCα	-	-	-	-	-
Soil entry by harvest residues	**Bt maize MON810 plant**	32	23 867.7 ± 1934.6	100	100	457.9 ± 46.5	100	100
	Soil Neuhof Dec 2007	8	3.6 ± 2.0	0.02	0.02	-	-	-
	Soil[4]	136	< CCα	-	-	-	-	-

[1] week 1 of slurry storage
[2] weeks 24 and 25 of slurry storage
[3] from Non-Bt maize plots treated with liquid manure from Bt maize fed cows
[4] all soil samples collected from plots under experimental Bt maize long term cultivation (except Neuhof December 2007) were below CCα in the Cry1Ab protein ELISA
- not determinable

The major fraction of Cry1Ab protein is degraded by the processing of Bt maize plants to feed, as only 2.6 % of the novel protein determined in plant material were recovered in the transgenic feed ration. Digestive processes during the GIT passage led to a further reduction of Cry1Ab protein levels in a way that 33.5 % of the Cry1Ab protein ingested by the cow was detected in liquid manure. Half of this residual immunoactive Cry1Ab protein in slurry persisted during slurry storage for 25 weeks. When applied to biologically active agricultural soil, final degradation of this remaining Cry1Ab protein down to non detectable levels in soil is reported. The findings of the present extensive study confirm the results of earlier specific studies elucidating single degradation steps of the recombinant protein. Thus a marked reduction of Cry1Ab protein contents by ensiling and feed processing (*89, 93*) and degradation by proteolysis in the ruminant digestive tract was described earlier (*55, 58, 59, 62*). However the further and complete degradation of the residual recombinant protein in feces as a component of liquid manure has been documented in the present study for the first time.

When harvest residues are incorporated directly into soil an extremely rapid degradation of Cry1Ab protein was observed. In 128 of 140 soil samples from Bt maize plots, no Cry1Ab was detected, which implies a very efficient degradation of the recombinant protein on all field sites. Notably a complete degradation was also observed in soil samples drilled shortly after harvest on the field site Baumannshof (2008). Cry1Ab protein amounts persisting in the Neuhof soil under particular weather conditions added up to only 0.02 % of the Cry1Ab protein content in the maize crop and confirm the efficient biodegradation of the novel protein in soil. These results are consistent with the rapid biodegradation reported for Cry1Ab protein in the soil compartment by (*37*).

Cry1Ab protein concentrations calculated in relation to total protein give further information about the degradation process and elucidate the proteolytic stability of the novel protein and its immunoactive fragments compared to other proteins.

Only 2.3 % of Cry1Ab protein g^{-1} protein present in the Bt maize plant was detected in transgenic PTMR. This marked decline can be explained on the one hand by the addition of 40 % of non-transgenic components to the mixed feed ration. On the other hand ensiling and processing of maize plants to feed led to a decrease of immunoactive Cry1Ab protein levels. Apparently, heat denaturation and disintegration reduced the contents of immunoactive Cry1Ab protein to a greater extent than the total protein content.

Passage of feed through the GIT leads to a further decrease of Cry1Ab protein in relation to total protein. Thus, in liquid manure, only 18 % of the initial Cry1Ab protein g^{-1} protein in transgenic PTMR was found. A previous study (*61*) reported consistent findings with a Cry1Ab protein concentration decline of 44 % when feed containing GM-maize MON810 and freshly collected feces were analysed. The decline observed in both studies can be

explained by several reasons: first, additional protein of animal and microbial origin is added to the feed protein during the passage through the bovine GIT. Furthermore the marked reduction of Cry1Ab protein in relation to total protein might be a result of a faster degradation of Cry1Ab protein in the GIT compared to the rest of the total protein. This suggests that Cry1Ab protein exhibits no extraordinary stability compared to the entirety of other proteins in feed. This assumption is confirmed by the analysis of the Cry1Ab concentrations in slurry with increasing time of storage, where no additional protein is introduced into the system. Here, the Cry1Ab concentrations related to total protein declined to 50 % of the initial levels within 25 weeks. These findings point out that in comparison to total protein in liquid manure, Cry1Ab protein runs through a faster degradation process and can be assigned to the less stable group of proteins in liquid manure, compared to other proteins.

5.5 Amounts of Cry1Ab introduced into agricultural soil

When balancing the Cry1Ab protein amounts added to soil by the overground Bt maize plant material in this study, a Cry1Ab load of 100 ng Cry1Ab g^{-1} soil in the 30 cm top-soil layer is calculated (*64*). Thus, a calculated maximum of 462 g Cry1Ab ha^{-1} could be introduced into soil by maize crop residues. These estimates are confirmed by a maximum theoretical load of 480 g Cry1Ab ha^{-1} calculated for recombinant protein from MON810 maize (*32*). In addition to the plant material grown aboveground, a further fraction of immunoreactive Cry1Ab protein introduced into the soil compartment can be attributed to root residues (*37*). A concentration of 1.4 µg Cry1Ab g^{-1} fresh root material (*82*) and a range of 152 – 183 ng Cry1Ab g^{-1} in decomposing root residues (*37*) were reported in Bt-maize MON810 plants. The Cry1Ab detected in soil under Bt maize cultivation in this study can derive from both above ground harvest residues and root material.

Cry1Ab amounts incorporated into soil by way of liquid manure are diminutive in comparison to the amounts added to soil by Bt maize plant residues. Transgenic protein in one liquid manure application added up to minute amounts of 431 mg Cry1Ab ha^{-1} for grassland and 690 mg Cry1Ab ha^{-1} for maize fields. This Cry1Ab load, applied to agricultural fields by way of liquid manure from Bt maize fed cows, adds up to less than 0.15 % of Cry1Ab amounts introduced directly by plant residues.

5.6 *cry1Ab* DNA analysis

Another issue of the study was to assess the likelihood of introducing recombinant *cry1Ab* DNA gene fragments to agricultural fields by way of liquid manure from cows fed gm maize. For this purpose qualitative end-point PCR was applied to feed and liquid manure samples after feeding Bt-maize (MON810) to dairy cows in order to amplify fragments of the recombinant single copy *cry1Ab* gene and the plant derived control genes *rubisco* (multicopy) and the maize specific *opaque-2* (single copy) (*94*). Additionally, soil from one experimental field site under long term Bt- maize cultivation (Puch) was investigated for the presence of *cry1Ab* DNA introduced directly by Bt maize harvest residues. The LOD for *cry1Ab* and *opaque-2* DNA detection are shown in Table 6.

Table 6: Limits of detection (LOD) of the assessed gene fragments in liquid manure and soil. The LOD is presented as the lowest detectable number of copies g^{-1} sample after amplification through qualitative end-point PCR.

	cry1Ab (206 bp)	*opaque-2 (202 bp)*
Liquid manure	1,000 copies g^{-1}	100,000 copies g^{-1}
	cry1Ab (280 bp)	
Soil (Puch 0-30 cm)	330,000 copies g^{-1}	n.d.

5.6.1 Feed

DNA extraction from feed resulted in concentrations of 360 ng DNA μL^{-1} of isolate for non-transgenic and 382 ng DNA μL^{-1} for transgenic PTMR. The photometrical analysis revealed 260/280 nm ratios between 1.4 and 1.8, a DNA purity suitable for PCR analysis.

Gene fragments of the multicopy genes *rubisco* and *18S* were amplified in isolates of all PTMR samples both of non-transgenic and transgenic origin. Similarly, the 202 bp fragment of the maize specific single copy gene *opaque-2* was detected in both feed variants. The 206 bp *cry1Ab* gene fragment was present in the feed ration containing Bt-maize (MON810). It was not detected in PTMR feed samples based on non-transgenic maize (Figure 12).

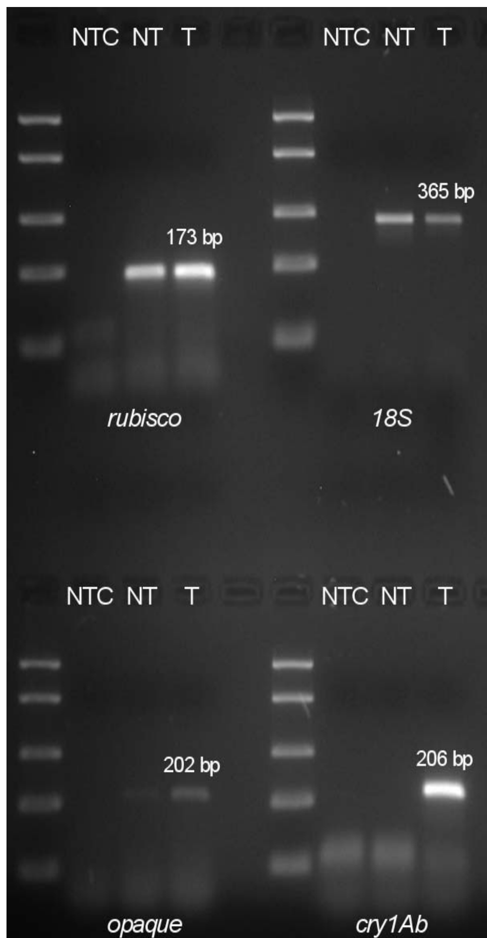

Figure 12: PCR amplification products of *rubisco* and *18S*, *opaque-2* and *cry1Ab* DNA in non-transgenic or transgenic feed ration. M: PeqLab-Low Range Marker (50, 200, 400, 850, 1500 bp), NTC: non-template control (H_2O), NT: non-transgenic PTMR, T: transgenic PTMR

5.6.2 Liquid manure

DNA extraction from liquid manure resulted in concentrations ranging from 21.3 to 86.8 ng DNA μL^{-1} isolate. The photometrical analysis revealed a DNA purity suitable for PCR analysis, as 260/280 nm ratios were between 1.4 and 1.8. Figure 13 depicts the results of PCR-analysis of liquid manure samples collected from dairy cows after long term feeding of Bt maize MON810 or non-transgenic maize. Gene fragments of the multicopy genes *18S* (365 bp amplicon) and *rubisco* (173 bp amplicon) were detected in all liquid manure samples from the first to the 29th week of storage (Fig 13 A + B). No liquid manure sample was found positive for *cry1Ab* DNA at the LOD of 1,000 copies g^{-1} in the slurry collected from cows fed transgenic or non-transgenic maize. Also the 202 bp maize specific single copy *opaque-2* control gene fragment was not detected in any slurry sample at the LOD of 100,000 copies g^{-1}.

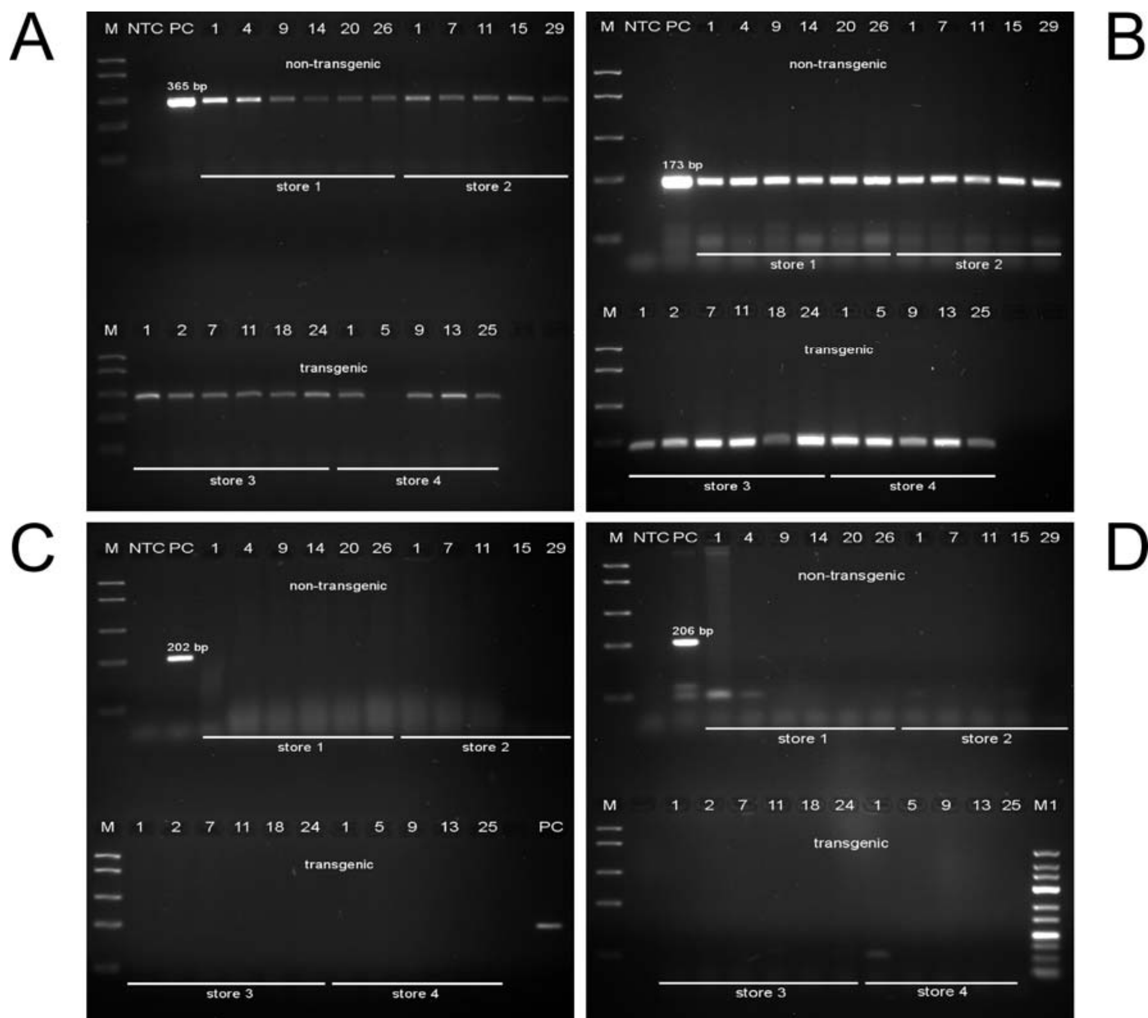

Figure 13: PCR amplification products of *18S* (A), *rubisco* (B), *opaque-2* (C) and *cry1Ab MON810* (D) in liquid manure obtained from dairy cows fed either non-transgenic or transgenic maize. In the upper part of each gel: Slurry store 1 and 2, liquid manure from control group fed non-transgenic PTMR. In the lower part of each gel: Slurry store 3 and 4, liquid manure from cows fed transgenic PTMR. Numbers indicate weeks of slurry storage before analysis. NTC: non-template control (H_2O or DNA from near-isogenic maize grain in D); PC: positive control (DNA from Bt maize MON810 grain),

Even though the small *cry1Ab* gene fragment was present in transgenic maize feed, it was not detectable in liquid manure of the cows fed the transgenic PTMR. Thus, *cry1Ab* DNA from maize MON810 was efficiently degraded during dairy cow digestion leading to non-detectable levels in slurry. These findings account for the extensive time-dependent degradation of novel DNA during the passage of feed through the bovine gastrointestinal tract reported in earlier studies (*56, 58, 95, 96*).

In the present study, the single copy *cry1Ab* and *opaque-2* gene fragments could not be amplified from slurry, whereas a 173 bp amplicon of the multicopy gene *rubisco* was detectable in all liquid manure samples. This result is in line with a report about the presence of small *rubisco* gene fragments in bovine feces samples, but absence of the 206 bp *cry1Ab*

fragment (*58*). As *rubisco* is a gene abundant in a high copy number in the plants' genome (*97*), there is a higher probability that small fragments of the *rubisco* DNA gene copies are detected by PCR than for the single copy *cry1Ab* gene. Our study confirms this suggestion, as the maize specific single copy gene *opaque-2* was also not detectable by qualitative endpoint PCR in the slurry samples. Generally, it can be assumed, that during passage through the GIT, biomolecules like protein or DNA may be protected from enzymatic degradation in heavy digestible plant material, leading to the presence of plant feed born protein or DNA fragments in feces or liquid manure. In contrast to the documented presence of insecticidal Cry1Ab protein in feces (*61*) and liquid manure (*64*), there was no accordant evidence for the presence of recombinant *cry1Ab* DNA fragments in bovine feces (*58*) or liquid manure (*70*) after feeding maize (MON810). According to these studies, there is no evidence for the application of recombinant *cry1Ab* DNA to agricultural fields by way of liquid manuring after feeding Bt maize MON810.

5.6.3 Soil

In the soil extracts from the experimental field site Puch, A_{260} values corresponding to DNA concentrations ranging from 29.7 to 52.2 ng DNA μL^{-1} isolate were obtained. The photometrical analysis revealed 260/280 nm ratios between 1.2 and 1.8.

Soil samples were collected in 2008 from the experimental field site Puch at the end of the vegetation period after nine years of Bt maize cultivation. Soil samples from Bt-maize plots and non-Bt-maize plots were tested for the presence of a 280 bp *cry1Ab* gene fragment. As depicted in Figure 14, fragments of *cry1Ab* were not detected at the LOD of 330,000 copies g^{-1} soil. However, the 173 bp fragments of the plant specific multicopy gene *rubisco* were also not detectable in the soil samples (Figure 15). The absence of recombinant single copy and plant derived multicopy gene fragments in the assessed soils can be ascribed to a fast degradation of the DNA by endogenous plant nucleases and by nucleases of microbial soil organisms. DNA molecules might also be tightly adsorbed to surface active soil particles rendering their extraction and detection of gene fragments by PCR difficult. Earlier studies showed that DNA can bind rapidly and tightly on clays and on humic acids extracted from soil (*21, 98*). Thus, for the investigated soils under long-term Bt maize cultivation, proceeding studies including a further optimization of the DNA extraction procedure for each respective soil type would be of major importance to investigate the fate of recombinant DNA from Bt maize plant debris in the soil compartment. Scientific evidence related to *cry1Ab* DNA persistence in experimental field soils is rare. Yet, the detection of the *cry1Ab* transgene in soil under Bt maize cultivation at the time point of pollination was shown (*77*). For other transgenes, for example the recombinant *CP4 epsps* gene from Roundup Ready (RR) corn and soybean, persistence in soil for up to 1 year after seeding (*99*) was

documented. Another study, accompanying the field release of transgenic rhizomania-resistant sugar beets, has shown that the DNA of the gene construct was detectable for up to six months in soil (*49*).

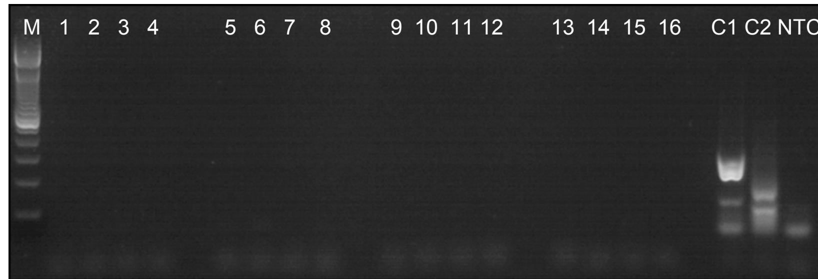

Figure 14: Absence of *cry1Ab* amplicons after amplification of a 280 bp fragment in soil samples from the experimental field site Puch cultivated with Bt maize in a long term field trial. C1: DNA maize MON810, C2: DNA near-isogenic maize, NTC: non template control, samples 1-4: 0-30 cm control plot, samples 5-8: 0-30 cm Bt-maize field, samples 9-12: 30-60 cm control plot, samples 13-16: 30-60 cm Bt-maize field

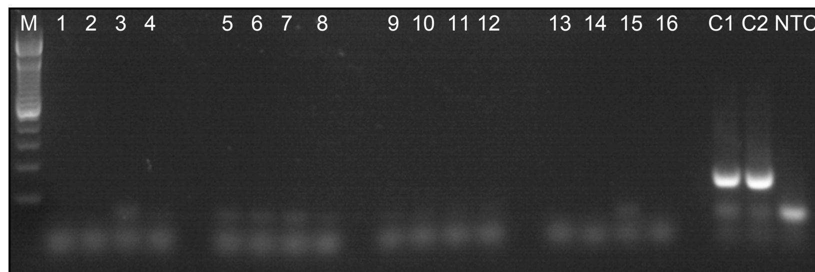

Figure 15: Absence of *rubisco* amplicons after amplification of a 173 bp fragment in soil samples from the experimental field site Puch cultivated with Bt maize in a long term field trial. . C1: DNA maize MON810, C2: DNA near-isogenic maize, NTC: non template control, samples 1-4: 0-30 cm control plot, samples 5-8: 0-30 cm Bt-maize field, samples 9-12: 30-60 cm control plot, samples 13-16: 30-60 cm Bt-maize field

6 Conclusions

This is the first field study investigating the potential entry of insecticidal Cry1Ab protein and novel DNA into soil by way of liquid manure after feeding gm maize (MON810) to cows. The study's particular feature is its overall approach covering the whole agricultural chain from the Bt-maize plant, processed feed, through the cow over liquid manure into soil and the following crop. Comparatively, the direct entry of the novel molecules into agricultural soil by plant debris from gm maize plants was studied after cultivation of Bt maize for nine growing seasons, the longest time experimentally documented so far. Thus concerns about the accumulation and persistence of Cry1Ab protein and *cry1Ab* DNA in the soil compartment were addressed. To assure the sensitive and specific detection of the recombinant molecules in the diverse sample matrices of the field trials, methods for Cry1Ab protein quantification and *cry1Ab* DNA detection were developed, optimized and validated. LODs for detection of recombinant *cry1Ab* gene fragments were determined and the ELISA for quantitative analysis of Cry1Ab protein was validated according to international guidelines laid down in the European Commission Decision 2002/657/EC.

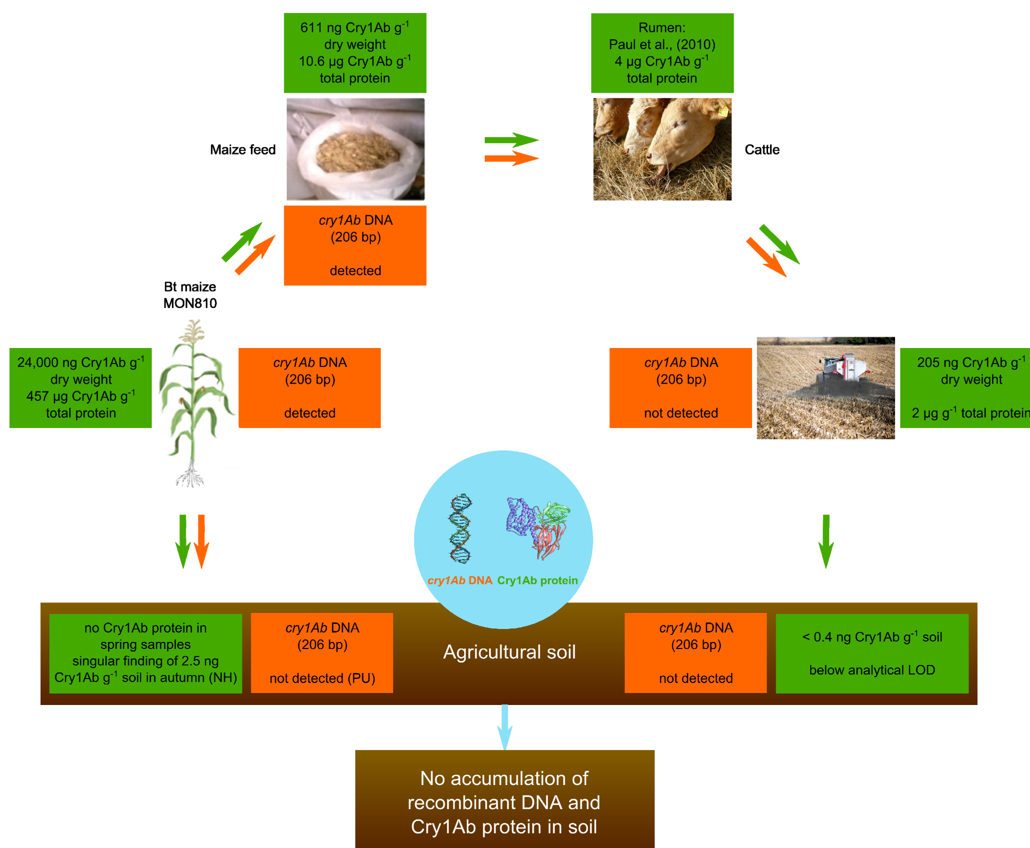

Figure 16: Schematic presentation of the results of this thesis concerning the potential entry of novel DNA and the Cry1Ab protein into soil

Surveillance of Cry1Ab protein contents in Bt maize crop, transgenic feed and liquid manure revealed a rapid degradation of the Cry1Ab protein, with a major proportion of more than 95% being degraded during food processing. Cry1Ab protein and its immunoactive fragments in feed were further degraded by digestive processes in the cows gastrointestinal tract (GIT) leading to diminutive levels in liquid manure. The calculated amount of transgenic protein in one liquid manure application to agricultural grassland or maize fields was in the range of 500 mg Cry1Ab protein ha^{-1}. This is less than 0.15% of the Cry1Ab protein introduced directly by Bt maize plant residues in the present study. Once introduced into agricultural soil, the recombinant protein was rapidly metabolized leading to non detectable levels in soil of plots cropped with Bt maize (spring samples) or treated with Cry1Ab containing slurry. No Cry1Ab protein was detected in grass or isogenic maize crop from slurry treated plots. In waterlogged soil from the Bt maize field site Neuhof temporary persistence of Cry1Ab protein was shown as 2.5 ng Cry1Ab protein g^{-1} soil was quantified six weeks after harvest. However, no transgenic protein was detected in any soil sample collected in spring from fields under long term Bt maize cultivation. Considering the Cry1Ab susceptibilities of relevant test organism species no toxicological and ecological effects on soil dwelling invertebrates are expected at the concentrations determined in slurry or soil analysed in the study. Compared to other proteins, the Cry1Ab was degraded more efficiently in the investigated feeding and manuring pathway, suggesting no extraordinary protein stability of the recombinant Cry1Ab molecule. Immunoblot analysis revealed the degradation of the 65 kDa Cry1Ab protein into smaller fragments of 42, 34 and 17 kDa. The bioactivity of these immunoactive degradation products in the environment remains to be elucidated. The presence of *cry1Ab* DNA gene fragments was proven in transgenic feed but - after digestion in the bovine GIT - the recombinant DNA was not detected in any slurry sample. Thus, no evidence for the application of novel DNA to agricultural soil by way of slurry can be drawn from this study.

In summary, the nine years lasting Bt maize cultivation trial on four independent field sites revealed no evidence for the accumulation of recombinant Cry1Ab protein or *cry1Ab* DNA in soil. The bioavailability of the novel molecules in the investigated agricultural processes is high in the living transgenic plant, decreases rapidly during feed processing and by microbial degradation of plant material in the cows GIT and is at most temporary and diminutive in soil (Figure 16). By the detailed insight into the temporary availability of the novel molecules from Bt-maize (MON810) used as bovine feed and in soil of Bt maize fields, this study provides new and scientifically secured contributions feeding the debate about the implications of gm plants in agriculture.

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9 Scientific Communications

9.1 Publications

Entire publications

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Helga Gruber, Vijay Paul, Heinrich H.D. Meyer, Martin Müller 2011: Determination of insecticidal Cry1Ab protein in soil collected in the final growing seasons of a nine-year field trial of Bt-maize MON810. Transgenic Research DOI : 10.1007/s11248-011-9509-7.

Helga Gruber, Vijay Paul, Patrick Guertler, Ales Tichopad, Hubert Spiekers, Heinrich H.D. Meyer, Martin Müller 2011: Fate of Cry1Ab protein in agricultural systems under slurry management of cows fed genetically modified maize (Zea mays L.) MON810 – a quantitative assessment. Journal of Agricultural and Food Chemistry *59* (13), pp 7135–7144, DOI: 10.1021/jf200854n

Helga Gruber, Patrick Guertler, Heinrich H.D. Meyer, Martin Müller 2011: Investigation of cow liquid manure for the presence of *cry1Ab* DNA after feeding genetically modified maize (MON810). Journal of Consumer Protection and Food Safety, submitted

Helga Gruber 2011: Nachweis von Cry1Ab-Protein im Boden nach dreijährigem Bt-Mais-Anbau. In Anbauversuche mit Bt-Mais in Sachsen. Schriftenreihe des LfULG, Heft 11/2011. Herausgeber: Sächsisches Landesamt für Umwelt, Landwirtschaft und Geologie, Dresden ISSN: 1867-2868: 94-100

9.2 Presentations

In terms of this doctoral thesis

Helga Gruber „Was passiert mit dem Bt-Protein aus GVO-Mais (MON810) in Boden?“ LfL-Jahrestagung 2008, Landshut, 04.11.08

Helga Gruber „Zum Eintrag von Cry1Ab-Protein aus Bt-Mais-Housernterückständen und Gülle Bt-Mais gefütterter Rinder in den Boden“ Fachkolloqium der LfL-Institute 2009, Freising, 27.01.09

Helga Gruber „Eintragspfade und Degradation von Bt-Protein in landwirtschaftlich genutzten Böden“ 3. Fachtagung Gentechnik des Landesamtes für Gesundheit und Lebensmittelsicherheit, Oberschleißheim, 02.12.09

Helga Gruber „Nachweis von Cry1Ab-Protein im Boden nach dreijährigem Bt-Maisanbau“ Fachkolloquium: Untersuchungen zum GVO-Anbau in Sachsen, Köllitsch Sachsen, 26.04.2010

9.3 Posters

In terms of this doctoral thesis

Gruber H., Paul V., Gellan S., Meyer H.H.D. and M. Müller (2007), Was passiert mit dem Bt-Protein aus GVO-Mais im Boden? Tag der offenen Tür der Bayerischen Landesanstalt für Landwirtschaft (LfL) Grub, 30.09.2007

Gruber H., Paul V., Meyer H.H.D. and M. Müller (2008), Validation of an enzyme immunoassay for monitoring Cry1Ab toxin in soils planted with Bt-maize (MON810) in a long-term field trial on four South German sites. 3. Workshop on Post Market Environmental Monitoring of Genetically Modified Plants. Julius Kühn-Institut, Berlin, 24. - 25.04.08

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10 Appendix

Publications in terms of this doctoral thesis

Appendix 1:

Gruber H., Paul, V., Meyer, H.H.D., and M. Müller. 2008. Validation of an enzyme immunoassay for monitoring Cry1Ab toxin in soils planted with Bt-maize (MON810) in a long-term field trial on four South German sites. Journal for Consumer Protection and Food Safety 3, Supplement 2: 22-25.

Appendix 2:

Gruber H., Paul V., Meyer H.H.D., Müller M. 2011. Determination of insecticidal Cry1Ab protein in soil collected in the final growing seasons of a nine-year field trial of Bt-maize MON810. Transgenic Research, DOI: 10.1007/s11248-011-9509-7

Appendix 3:

Gruber H., Paul V., Guertler P., Tichopad A., Spiekers H., Meyer H.H.D., Müller M. 2011. Fate of Cry1Ab protein in agricultural systems under slurry management of cows fed genetically modified maize (Zea mays L.) MON810 – a quantitative assessment. Journal of Agricultural and Food Chemistry, *59* (13), pp 7135–7144, DOI: 10.1021/jf200854n

Appendix 4:

Gruber H., Guertler P., Meyer H.H.D., Müller M. 2011. Investigation of cow liquid manure for the presence of *cry1Ab* DNA after feeding genetically modified maize (MON810). Journal for Consumer Protection and Food Safety, submitted

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Validation of an enzyme immunoassay for monitoring Cry1Ab toxin in soils planted with Bt-maize (MON810) in a long-term field trial on four South German sites

H. Gruber[1,2], V. Paul[2], H. H. D. Meyer[2], and M. Müller[1]

[1]Bavarian State Research Center for Agriculture, Freising, Germany
[2]Technische Universität München, Physiology Weihenstephan, Freising, Germany

Correspondence to: Dr. Helga Gruber, [1]Bavarian State Research Center for Agriculture,
Institute for Crop Science and Plant Breeding, Am Gereuth 8, D-85354 Freising, Germany,
Tel.: +49-(0)8161 – 714306, Fax: +49-(0)8161 – 714407, E-mail: Helga.Gruber@LfL.bayern.de

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Key words: Bt-maize (MON810), Cry1Ab protein, enzyme immunoassay, EU-Decision 2002/657/EC, soil.

Abstract: For monitoring the environmental impacts of a genetically modified crop well-established methods are a key requirement for the collection of appropriate data. With regard to the fate of Cry1Ab protein from Bt-maize MON810 released by root exudates, plant residues and pollen deposition into soil, a highly specific and sensitive sandwich enzyme immunoassay for detection and quantification of Cry1Ab in soil matrix was developed. The assay was validated according to the criteria of EU-Decision 2002/657/EC for a range of soils originating from four selected experimental field sites in South Germany, where Bt-maize MON810 has been cultivated. On three sites Bt-maize was grown permanently for eight years whereas one site was cropped with Bt-maize for one year only. The validation of the method showed that the assay fulfils the validation criteria as prescribed in the guidelines of the EU-Decision with minor restrictions. When the assay was applied to field soil samples collected from the selected four experimental sites six months after harvest, no Cry1Ab protein was detectable. By ELISA-determination and quantification of Cry1Ab protein in soils collected from these sites at further time points more profound knowledge about the long-term behaviour of Cry1Ab protein in soil will be obtained.

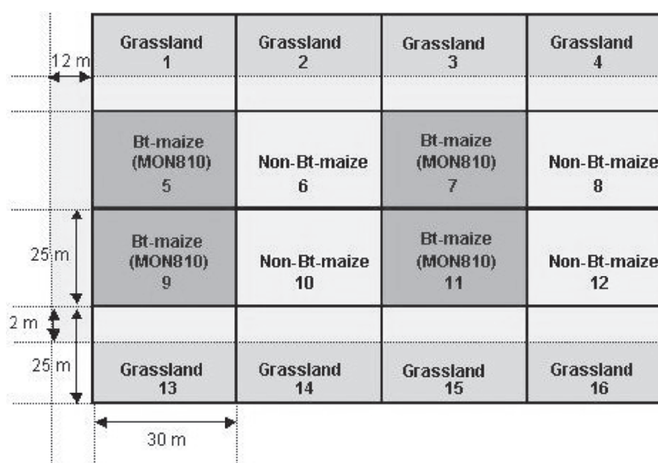

Fig. 1 Plot design on the four experimental field sites.

1. Introduction

Bt-maize MON810 expressing insecticidal endotoxin Cry1Ab from *Bacillus thuringiensis* (Bt) exhibits specific insect resistance against infestation by the European corn borer *Ostrinia nubilalis*. Thus Bt-maize cultivation decreases the need for chemical insecticides and represents an important alternative to the cropping of non genetically modified maize varieties managed with applications of conventional pesticides. Since the introduction of the Bt-maize technology the total area of agricultural land planted with Bt-maize crops has been grown up to 35 million hectares worldwide in 2007. In the European Union only Bt-maize MON810 varieties expressing the Cry1Ab toxin have been approved for commercial growth in 1998. Cry toxins are considered harmless or non-toxic to human and farm animals and there is no evidence for adverse effects of Bt maize MON810 cultivation onto nontarget invertebrates from field experiments if compared to nontransgenic fields managed with insecticides (Marvier et al., 2007). However, as Cry1Ab protein may be introduced into the soil during cultivation and harvest by root exudates, pollen deposition and incorporation of plant material (Stotzky, 2000), there is still concern about Bt

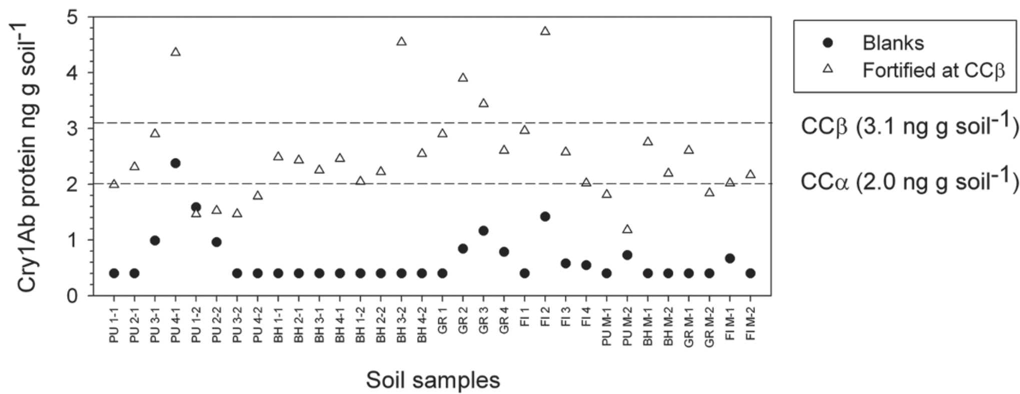

Fig. 3 Decision limit CCα and detection capability CCß of the Cry1Ab protein ELISA in soil.

maize posing a risk to the agricultural ecosystem. From the finding that Cry1Ab protein can rapidly adsorb to clay minerals and humic substances in soil the question arises whether plant produced Cry1Ab may accumulate and persist in soil (Icoz and Stotzky 2007). In order to clarify this issue a lot of studies have been performed (amongst others by Wang, 2006, Baumgarte and Tebbe, 2005, Hopkins and Gregorich, 2003) to estimate Cry1Ab protein in soils from fields cropped with Bt plants. In these field studies a great variation of Cry1Ab protein amounts

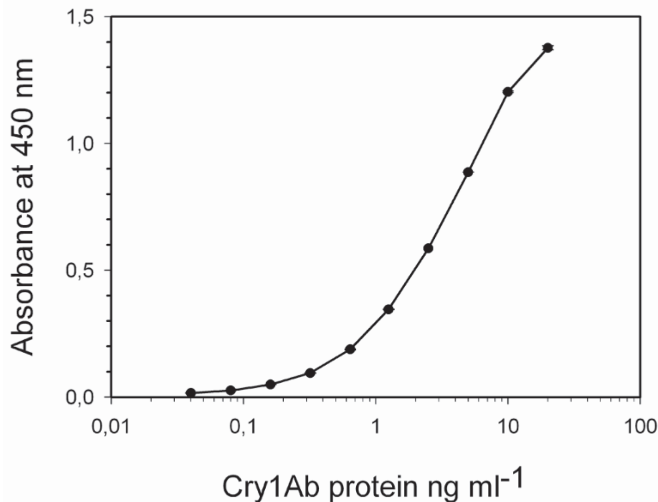

Fig. 2 Calibration curve for Cry1Ab protein quantification in soil using Cry1Ab protein standards in extraction buffer.

in bulk soil samples and of the time period Cry1Ab protein was detected after harvesting is observed. The reason for this variation can be easily explained by the various cropping systems and soil types studied and the application of different assay systems used for quantification of the Cry1Ab protein. Though the commercially available Cry1Ab ELISA kits used in these studies are reported to detect Cry1Ab protein down to 0.1 ng ml^{-1} soil extract, extensive validation of the kits before their application to soil samples has not been reported.

In the present study the development of a sensitive, specific sandwich ELISA for the detection and quantification of Cry1Ab in soil is described (Paul et al., 2008). The assay was validated as required by the guidelines of EU-Decision 2002/657/EC for a range of selected soils from experimental field sites and a first set of soils was analysed using the new method.

2. Methods

2.1 Experimental field sites and soil sampling

Experimental fields are located on four different sites in the state of Bavaria in the South of Germany. Each field contains four replicate plots planted with Bt-maize MON810 or a non Bt-maize variety (Fig. 1). At the Puch, Baumannshof and Grub sites Bt-maize was grown continuously for seven years in a long-term study before sampling. In Finsing, Bt-maize was cultivated for one year. Soil samples were collected in depths of 0–30 cm and 30–60 cm in April 2007 before sowing, sieved at 2 mm mesh-size and stored at -20 °C.

2.2. ELISA

A highly specific and sensitive sandwich enzyme immunoassay based on immunoaffinity purified polyclonal antibody raised against 65 kDa active Cry1Ab toxin in rabbit was developed for the detection and quantification of Cry1Ab toxin in soil. Duplicates of 50 µl Cry1Ab protein calibrators (0.04 to 20 ng ml^{-1}) and soil extracts were used in ELISA and a Cry1Ab protein calibration curve was constructed. Cry1Ab toxin concentrations were determined in unknown samples by interpolation.

2.3. Validation

The assay validation for calculation of CCα and CCβ values for the Cry1Ab toxin ELISA was performed according to the guidelines of EU-Decision 2002/657/EC on 32 soil samples collected from the surroundings of experimental fields. CCα value was calculated from the Cry1Ab protein background noise level in the blanks and CCβ value was determined by fortifying the same samples at the concentration level of CCα value.

Tab. 1 Cry1Ab protein determination in soil samples collected six months after harvest. Data are expressed as ng Cry1Ab protein g^{-1} soil and represent mean values (four replicates).

Field site	Puch		Baumannshof		Grub	Finsing
Soil depth	0–30 cm	30–60 cm	0–30 cm	30–60 cm	0–30 cm	0–30 cm
Non-Bt-maize	< 0.40	< 0.40	< 0.40	< 0.40	< 0.40	< 0.40
Bt-maize (MON810)	< 0.40	< 0.40	< 0.40	< 0.40	< 0.40	0.45

2.4. Recovery

Soil samples with non-detectable levels of Cry1Ab protein were collected from experimental fields in spring. Recovery rates for Cry1Ab toxin were investigated by spiking four replicate soil samples per site and depth at concentration levels of 5, 20 and 60 ng Cry1Ab protein per g of fresh soil.

3. Results

3.1. Assay sensitivity for Cry1Ab toxin

A calibration curve used for Cry1Ab determination in soil is shown in Fig. 2. The analytical range of the assay is 0.04 to 20 ng Cry1Ab mL^{-1} extraction buffer which corresponds to 0.4 ng to 200 ng Cry1Ab protein g^{-1} soil and allows determination of Cry1Ab down to the analytical limit of 0.4 ng Cry1Ab protein g^{-1} soil.

3.2. Decision limit and detection capability

The background values for Cry1Ab protein in soil determined by analysis of 32 blank samples ranged from 0.4 to 2.37 ng g^{-1} with a mean value of 0.65 ng g^{-1}. The decision limit CCα calculated from the mean value of blank samples was 2.0 ng Cry1Ab protein g^{-1} soil. After the 32 samples were fortified at the concentration level of CCα values ranged from 0.70 to 3.27 ng g^{-1} (mean 1.95 ng g^{-1}; S.D. 0.65 ng g^{-1}). The calculated detection capability CCß was 3.1 ng Cry1Ab protein g^{-1} soil (Fig. 3).

3.3. Recovery rates for Cry1Ab protein in soil

When blank soil samples from experimental field sites were fortified with Cry1Ab protein at concentration levels of 5, 20 and 60 ng Cry1Ab protein g^{-1} soil recoveries ranged from 45% to 91%. The recovery rates showed a strong dependence to the origin of soil. The highest recovery was reached in the upper layer of Baumannshof soil (mean of three spike concentrations 89%) whereas in the lower layer of the Puch soil recovery averaged 49%.

3.4. Surveillance of Cry1Ab protein in experimental field soils

In all soil samples collected from experimental fields six months after harvest Cry1Ab protein concentrations were far below the decision limit CCα (Tab. 1). Neither on Bt-maize MON810 nor on non-Bt-maize plots Cry1Ab protein was detectable in these soil samples.

4. Conclusions

A sandwich ELISA based on an immunoaffinity purified polyclonal antibody has been developed for the Cry1Ab protein determination in soil. It was validated for a selected set of four soil types corresponding to four experimental Bt-maize field sites. For these sites the assay meets the validation criteria of the EU-Decision 2002/657/EC, but as a result of low Cry1Ab protein recovery rates in some soil types the required 5% limit for β-error (percentage of samples lying below CCα when fortified at CCß) was exceeded. As some soils exhibit physicochemical properties interfering with the ELISA for Cry1Ab protein detection further method development and validation has to be carried out independently for various soil types. When the validated Cry1Ab protein ELISA was applied to field soil samples collected six months after harvest no Cry1Ab protein was detected on either of the sites though three of the surveilled experimental fields were planted with Bt maize MON810 continuously for seven years before sampling. In the future both the validated assay system and the long-term field experiment will allow for further studies to gain profound knowledge about the short- and long-term behaviour of Cry1Ab protein in agricultural soils.

5. Acknowledgement

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Transgenic Res
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ORIGINAL PAPER

Determination of insecticidal Cry1Ab protein in soil collected in the final growing seasons of a nine-year field trial of Bt-maize MON810

Helga Gruber · Vijay Paul · Heinrich H. D. Meyer · Martin Müller

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Abstract Cultivation of genetically modified maize (Bt-maize; event MON810) producing recombinant δ-endotoxin Cry1Ab, leads to introduction of the insecticidal toxin into soil by way of root exudates and plant residues. This study investigated the fate of Cry1Ab in soil under long-term Bt-maize cultivation in an experimental field trial performed over nine growing seasons on four South German field sites cultivated with MON810 and its near isogenic non Bt-maize variety. Cry1Ab protein was quantified in soil (<2 mm size) using an in-house validated ELISA method. The assay was validated according to the criteria specified in European Commission Decision 2002/657/EC. The assay enabled quantification of Cry1Ab protein at a decision limit (CCα) of 2.0 ng Cry1Ab protein g^{-1} soil with analytical recovery in the range 49.1–88.9%, which was strongly correlated with clay content. Cry1Ab protein was only detected on one field site at concentrations higher than the CCα, with 2.91 and 2.57 ng Cry1Ab protein g^{-1} soil in top and lower soil samples collected 6 weeks after the eighth growing season. Cry1Ab protein was never detected in soil sampled in the spring before the next farming season at any of the four experimental sites. No experimental evidence for accumulation or persistence of Cry1Ab protein in different soils under long-term Bt-maize cultivation can be drawn from this field study.

Keywords Soil · Genetically modified maize · Bt-maize MON810 · Long-term field trial · Cry1Ab protein · Biosafety · ELISA

H. Gruber (✉) · M. Müller
Bavarian State Research Center for Agriculture, Institute for Crop Science and Plant Breeding, Am Gereuth 8, 85354 Freising, Germany
e-mail: hgruber@wzw.tum.de

H. Gruber · V. Paul · H. H. D. Meyer
Physiology Weihenstephan, Technische Universität München, Weihenstephaner Berg 3, Freising, Germany

Present Address:
V. Paul
National Research Centre on Yak, (ICAR), Dirang, West Kameng District, Arunachal Pradesh 790101, India

Present Address:
H. Gruber
Bavarian Health and Food Safety Authority, Veterinärstr. 2, 85764 Oberschleissheim, Germany

Introduction

Bacillus thuringiensis (Bt) is a spore-forming bacterium that produces proteinaceous, crystalline inclusions during sporulation. These inclusions may contain one or more types of insecticidal crystal proteins (ICPs). The ICPs are solubilized and hydrolysed in the midgut of larvae of susceptible insects, leading to the release of polypeptide toxins which can

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cause larval death by forming receptor-mediated cation-selective ion channels in the membrane of epithelial cells, disruption of the midgut epithelial barrier, and osmotic cell lysis (Schnepf et al. 1998; Hoefte and Whiteley 1989). Numerous distinct crystal protein (*cry*) genes coding for insecticidal Cry proteins of different amino acid sequence, size, and structure have been identified (Crickmore et al. 1998). Each class of Cry proteins is characterized by a narrow range of susceptible target organism species offering very target-oriented pest-management by application of selected Cry-proteins as insecticides. In the early 1990s genetically modified (gm) Bt-plants expressing insecticidal Cry proteins were generated and followed by the first approval and commercialized release of gm maize varieties in 1996 (Mendelsohn et al. 2003). A broad assortment of Bt-maize varieties is now distributed in gm maize-growing countries, including a variety of Cry proteins, which may be present singularly, in combination, or combined with other traits in stacked events. Most Bt-maize varieties carry a modified *cry1Ab* gene in their plant genome (CERA-GM Crop Database 2010) and express a truncated version of the Cry1Ab protein which is highly active against Lepidoptera and widely used to combat the European corn borer (*Ostrinia nubilalis* Hübner), a major pest in maize fields (Koziel et al. 1993). A benefit of Bt-crops is the reduced need for application of broad-spectrum chemical pesticides, because the toxins are produced continuously within these plants and have high specificity for insect pests (Roush 1997; Ferré and Van Rie 2002). On the other hand, there is some concern that Bt-crops may pose a risk to natural and agricultural ecosystems, because non-target air and soil-borne organisms are also exposed to the recombinant Bt-toxin during the growing season. A substantial amount of the Bt-protein will be introduced into soil through root exudates and root debris during the growth of the plant according to Saxena et al. 2002. After harvest and field management additional toxin enters the soil from plant residues and might persist and accumulate when maize is planted after maize (Tapp and Stotzky 1998; Stotzky 2000). A minor input from pollen can be assumed (Losey et al. 1999).

Potential effects of recombinant Cry proteins introduced into the agricultural environment have been investigated in a wide range of studies on non-target organisms, for example earthworms, nematodes, and soil microbiota (Saxena and Stotzky 2001, Icoz and Stotzky 2008), on beneficial insects (predators and parasites of insect pests; Hilbeck et al. 1999; Harwood et al. 2006; Priestley and Brownbridge 2009), and on the potential selection and enrichment of toxin-resistant target insects (Tabashnik et al. 1997; Van Rie et al. 1990; Kaiser-Alexnat et al. 2005). According to current knowledge, however, there is no scientific evidence from these studies that Bt-maize poses a severe hazard to any non-target organism (Marvier et al. 2007; Mendelsohn et al. 2003).

With regard to the biosafety of a new gm crop it is still important to assess the potential persistence of the transgenic protein introduced into the soil. In general, persistence depends on the amounts of toxin introduced into the soil, the rate of consumption and inactivation by insect larvae, the rate of microbial degradation, and its abiotic inactivation (Saxena and Stotzky 2003). If the input of Cry1Ab protein into the soil exceeds its degradation, the toxin could accumulate particularly in soil under long-term cultivation of Bt-maize. The possibility of persistence and accumulation of Cry1Ab protein in soil was addressed in earlier studies on the binding of the proteinaceous toxin to surface-active particles, for example clay minerals and humic substances. Adsorption and tight binding of purified Cry1Ab protein to clay minerals has been reported in several laboratory studies (Pagel-Wieder et al. 2007; Crecchio and Stotzky 2001; Stotzky 2000, 2002). In natural soil, this binding can reduce the availability of the transgenic protein to microbes and thus inhibit microbial degradation and lead to the persistence of clay-bound toxin (Koskella and Stotzky 1997). Furthermore, clay bound toxin retained its larvicidal activity in these studies (Tapp and Stotzky 1995; Saxena and Stotzky 2000).

Qualitative and quantitative analysis of the Bt-protein content of agricultural soil derived from experimental fields has been performed in a considerable range of studies using different immunological detection methods. Maximum concentrations of Cry1Ab protein reported in bulk soil by Baumgarte and Tebbe (2005) were in the range 3 ng Cry1Ab protein g^{-1} soil, whereas Hopkins and Gregorich (2003) found 4.4 ng Cry1Ab protein g^{-1} soil in the soil fraction >2 mm size but only traces

(below 0.1 ng g^{-1}) in fine soil <2 mm size. Saxena and Stotzky (2000) qualitatively determined Cry1Ab protein in Bt-maize rhizosphere soil using Lateral Flow Quickstix (EnviroLogix detection limit <10 ppb corresponding to 10 ng Cry protein g^{-1} soil) and assessed all soil samples positive for the presence of the Bt-toxin. Head et al. (2002) reported no Cry1Ac protein accumulation in soil under Bt-cotton cultivation. Likewise, Wang et al. (2006) could not detect Cry1Ab protein from transgenic rice (KMD) in rhizosphere soil.

In these experimental field trials, the maximum time of Bt-crop cultivation was five years. Thus, the issue of a potential accumulation of Cry1Ab protein in soil as a result of continuous entry of Bt-maize plant residues over more than five vegetation periods has not been addressed in these studies. For this reason experimental Bt-maize field plots established on four different sites in the South of Germany in the year 2000 (Bavarian Research Center for Agriculture—Bayerische Landesanstalt für Landwirtschaft 2005) were of a special interest for obtaining further knowledge about the long-term behaviour of the Bt-toxin in soil, because Bt-maize MON810 producing the δ-endotoxin Cry1Ab was grown without interruption on these field sites.

Highly specific and sensitive analytical methods are essential to trace and quantify the minor amounts of Cry1Ab protein which could be detected in soil after entry of Bt-maize plant residues. The most common and reliable technique for sensitive detection and quantification of Cry1Ab protein is enzyme-linked immunosorbent assay (ELISA). In this study we applied an in-house developed and standardized ELISA based on a highly specific immunoaffinity purified polyclonal antibody for Cry1Ab protein determination in animal liquids and animal by-products (Paul et al. 2008, 2010). The assay was successfully validated and has been used for surveillance of transgenic protein in feed, and in the faeces, blood plasma, milk, and urine of cows (Guertler et al. 2009, 2010). This method was adopted and validated according to the guidelines for analytical method validation cited in European Commission Decision 2002/657/EC (Byrne 2002) for quantitative analysis of Cry1Ab protein in the soil matrix (Gruber et al. 2008). By application of this validated assay, quantities of Cry1Ab protein in soil samples from different field sites and with distinct physicochemical properties can be measured.

The objective of this study was to quantify and compare the amounts of Cry1Ab protein and its degradation behaviour in agricultural soils from four different experimental field sites during the eighth and ninth Bt-maize MON810 growing seasons in a nine-year continuous Bt-maize planting study. The main focus of the study was to investigate any possibility of persistence or accumulation of the recombinant Cry1Ab protein in soil after long-term Bt-maize cropping.

Materials and methods

Experimental field sites, maize cultivars and soil sampling

The four experimental field sites Baumannshof, Puch, Neuhof, and Grub are located in Bavaria in the South of Germany. The main features of the field sites and their soil characteristics are given in Table 1. Each field contained four replicate plots cultivated with the Bt-maize variety Kuratus (event MON810) and four replicate plots planted with the near isogenic maize variety Gavott (both cultivars were from KWS Saat,

Table 1 Experimental sites and soil characteristics in the field trial investigating Bt-maize long-term cultivation over a period of nine growing seasons

Field site	Absolute altitude (m)	FAO soil type	pH $CaCl_2$	Soil texture 0–30 cm (% clay/% silt/% sand)	Mean temperature (°C)	Average rainfall (mm/year)
Baumannshof	365	Cambisol	4.8	6/7/87	7.8	636
Puch	550	Rendzina	5.8	18/70/12	8.0	920
Grub	525	Luvisol	7.0	30/42/28	7.4	967
Neuhof	516	Planosol, gleysol	6.8	21/6/18	7.6	764

Einbeck, Germany). MON810 cultivars were chosen because they constituted the only genetically modified crops approved for cultivation in the European Union at the time of the field trial. The size of the replicate plots was 25 m × 30 m. A detailed view of the plot design is given in Gruber et al. (2008). Growing of gm (MON810) and non-gm maize cultivars was started in 2000 and continued without interruption until the end of the maize growing season in 2008. Whole maize plants were harvested for grain maize according to good agricultural practice. Chopped plant residues, maize stubble, and root material remained on the field plots. Soil samples analysed in this study were collected from experimental field plots in 2007, 2008, and 2009. Soil samples were collected from each plot at a depth of 0–30 cm and 30–60 cm at ten sampling points by using a drilling core (diameter 4.5 cm). Ten individual drilling core soil samples were mixed and combined to obtain one sample per plot and depth. The soil samples were stored under cold conditions until further processing. In the laboratory, soil samples were mixed well and sieved to 2 mm mesh size. Aliquots of the fraction <2 mm were stored at −20°C until analysed for Cry1Ab protein. In soil samples from all analysed field sites collected in the spring before seeding, no maize plant litter particles >2 mm were retained after sieving. All residual small-sized organic material of decomposing Bt-maize plants was included in the Cry1Ab protein analysis of spring samples.

Extraction and quantification of Cry1Ab protein

Reagents used were of analytical grade and supplied by Merck (Darmstadt, Germany) unless stated otherwise. HPLC-purified trypsin-activated Cry1Ab protein for standard preparation was provided by Dr William J. Moar, Auburn University, USA. Highly specific polyclonal antibodies against Cry1Ab protein were raised in rabbits, immunoaffinity purified, and labelled with biotin as described elsewhere (Paul et al. 2008).

For protein extraction, 200 mg (wet weight) fresh soil was mixed with 2 ml ice-cold extraction buffer (8 mM NaH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 2.5 mM EDTA, 0.1% Tween 20; pH 7.4) containing 0.5% skim milk and extracted by vigorous horizontal shaking (220 movements per min) at ambient temperature. Soil suspensions were centrifuged at 4,500 rpm, 15 min, 4°C. A 400-µl aliquot of the supernatant was centrifuged at 15,000 rpm and 4°C for 15 min and the clear sample extract (supernatant) was directly used for Cry1Ab protein quantification in the ELISA.

ELISA procedure and assay validation

A previously developed and optimized sandwich ELISA based on highly specific immunoaffinity purified anti-Cry1Ab protein rabbit polyclonal antibody (Paul et al. 2008) was validated according to the guidelines of European Commission Decision 2002/657/EC for quantification of Cry1Ab protein in soil extracts (Gruber et al. 2008). The assay enabled analysis of Cry1Ab protein in soil with a decision limit (CCα value) of 2.0 ng Cry1Ab protein g^{-1} wet soil.

In brief, the ELISA procedure was performed in 96-well microtiter plates (Maxisorp, Nunc, Denmark) coated with immunoaffinity purified anti-Cry1Ab antibody at a concentration of 0.025 µg $well^{-1}$ in 100 µl 50 mM sodium carbonate/bicarbonate buffer pH 9.6 and blocked with 300 µl 1% BSA in PBST (Serva, Heidelberg, Germany) $well^{-1}$. Aliquots of 50 µl Cry1Ab protein standards (2–1,000 pg per 50 µl extraction buffer) and unknown sample extracts were added in duplicate to respective wells of the microtiter plate followed by addition of 50 µl PBST. Plates were incubated overnight at 6–8°C while shaking and washed six-times with PBST the next day. The amount of antibody-captured Cry1Ab protein in the respective wells was determined by applying 0.5 ng biotin-labelled anti-Cry1Ab detection antibody in 100 µl PBST to each well, followed by Streptavidin–horseradish peroxidase enzyme conjugate (Roche Diagnostics, Germany; 100 µl, diluted 1:20,000 in PBST) and subsequent incubation with 150 µl 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma, Germany) for 35 min. The reaction was stopped by adding 50 µl 2 M sulfuric acid. Absorbance was read at 450 nm in a microplate reader (Sunrise, Tecan, Germany) and Cry1Ab protein concentrations in unknown samples were interpolated from the Cry1Ab protein calibration curve generated using online Magellan V6.1 software (Tecan, Austria).

Analytical recovery

Analytical recovery of Cry1Ab protein in soil samples was determined in field site-representative soil samples taken at a minimum distance of 300 m from the Bt-maize field plots in 2007 before sowing. Additional soil samples from two Bavarian experimental field sites (Schwarzenau and Finsing) were included in the recovery study to obtain more information about the analytical performance of the assay. Eight independent replicate blank soil samples (200 mg) for each field site and soil depth were spiked with Cry1Ab protein at concentrations of 1.0, 4.0, 12.0 ng Cry1Ab protein in 100 µl PBS (8 mM NaH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4; pH 7.4) and incubated on ice for 20 min to enable adsorption and binding of the protein to surface-active soil particles. A control sample was incubated with 100 µl PBS only. Cry1Ab protein was quantified in spiked soil samples using the Cry1Ab protein ELISA and analytical recovery calculated in relation to the amount of Cry1Ab protein determined by ELISA in the spiking solutions.

Soil texture analysis

Soil texture analysis was performed on aliquots of the eight replicate field site soil samples used in the recovery study. Air-dried soil particles <2 mm in diameter were assessed for particle size distribution in accordance with DIN ISO 11277 by sedimentation in a Sedimat 4–12. H_2O_2 pretreatment to remove organic matter was omitted.

Cry1Ab protein quantification in experimental field soils

Immunoreactive Cry1Ab protein was quantified in bulk soil samples from four experimental field sites in the eighth and ninth growing seasons of consecutive cultivation of Bt-maize MON810 (variety Kuratus) and the near isogenic Non-Bt-maize variety (Gavott). Soil was also collected in spring after nine years of Bt-maize cropping and analysed for Cry1Ab protein determination. For each sampling date, soil samples from four replicate plots cropped with Bt-maize or Non-Bt-maize were investigated. The sandwich ELISA used for Cry1Ab protein analysis was validated on the experimental field soils. The validated assay enabled Cry1Ab protein determination and quantification at a decision limit, CCα, of 2.0 ng Cry1Ab protein g^{-1} wet soil. When EU validation guidelines for an analytical method are applied, soil samples below the decision limit (2.0 ng Cry1Ab protein g^{-1} wet soil) will be classified as negative for the presence of analyte (Cry1Ab protein) whereas values higher than the decision limit (2.0 ng Cry1Ab protein g^{-1} wet soil) may indicate the presence of immunoreactive Cry1Ab protein. The analytical detection limit given by the minimum concentration of Cry1Ab protein calibrator measurable in the ELISA corresponds to 0.4 ng Cry1Ab protein g^{-1} soil.

Statistics

Final data are presented as ng Cry1Ab protein g^{-1} wet weight soil sample. Student's *t* test was used to compare the means of Cry1Ab concentrations in soil samples considering a *P* value below 0.05 as significant.

Results

Recovery of Cry1Ab protein from experimental field site soil

Analytical recovery of purified trypsin-activated Cry1Ab protein from representative soil samples of the experimental Bt-maize fields ranged from 49.1 to 88.9% (Table 2). The lowest recovery was achieved for Puch (30–60 cm) and the highest recovery for Baumannshof (0–30 cm). The percentage of Cry1Ab protein recovered from the different soils fortified with known amounts of Cry1Ab protein was strongly correlated ($R = 0.923$) with the clay content of the soils assessed (Fig. 1). High clay content was associated with low recovery whereas low clay content resulted in high recovery of Cry1Ab protein from soil. Thus, for the sandy soil from Baumannshof containing less than 6% clay, analytical recovery was higher than 85% Cry1Ab protein. On the other hand, when the clay content exceeded 25%, as for the lower depth soils of Puch, Neuhof, Schwarzenau and Grub field sites, recovery was in the range 49–60%.

Table 2 Analytical recovery achieved for Cry1Ab protein determination in spiked soil samples

Amount of Cry1Ab protein added ($ng\ g^{-1}$ soil)	Puch		Baumannshof		Neuhof		Grub
	0–30 cm	30–60 cm	0–30 cm	30–60 cm	0–30 cm	30–60 cm	0–30 cm
5	75.9 ± 3.6	44.7 ± 9.8	90.0 ± 5.1	82.7 ± 4.0	79.2 ± 5.4	56.4 ± 6.6	72.0 ± 4.0
20	71.3 ± 6.2	49.5 ± 7.0	89.9 ± 4.3	86.6 ± 2.2	69.6 ± 2.9	53.5 ± 3.9	61.7 ± 1.2
60	78.0 ± 8.6	53.1 ± 9.7	86.9 ± 5.5	91.4 ± 5.1	62.2 ± 4.5	48.0 ± 4.4	46.2 ± 3.9
Mean recovery (%)	75.1	49.1	88.9	86.9	70.3	52.6	60.0

Data are presented as mean values ± SD (four replicates) as percentages of the amount of Cry1Ab protein measured in the spike solution

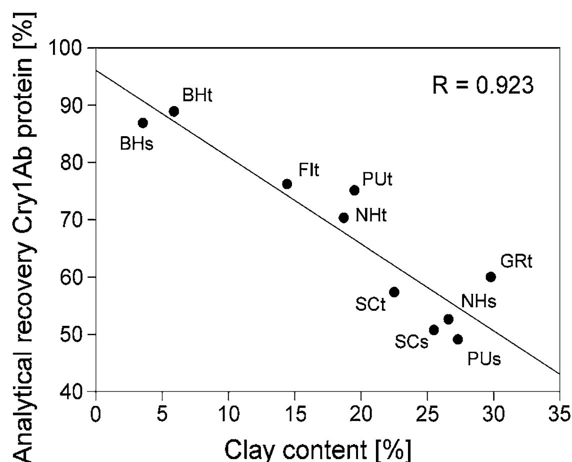

Fig. 1 Analytical recovery of Cry1Ab protein determined in spiking experiments, and clay content of experimental field site soils including two independent additional Bt-maize field sites (SC and FI). *Dots* represent mean values for each field site (*PU*, Puch; *BH*, Baumannshof; *NH*, Neuhof; *GR*, Grub; *SC*, Schwarzenau; *FI*, Finsing) and soil depth (*t*, topsoil 0–30 cm; *s*, subsoil 30–60 cm) calculated from eight replicate samples analysed

Quantification of Cry1Ab protein in soils of experimental field sites

Quantification of immunoreactive Cry1Ab protein in bulk soil samples of four experimental field sites revealed that the recombinant Cry1Ab protein was not detected in any of the soil samples taken from the control plots cultivated with non-transgenic maize. For none of these samples did amounts of Cry1Ab protein exceed the decision limit, CCα, of 2.0 ng Cry1Ab protein g^{-1} soil (Fig. 2). Also, in Bt-maize MON810 planted plots, immunoreactive Cry1Ab protein was never detected in bulk soil samples collected in the spring before seeding. Furthermore, no transgenic protein was determined in any of the soil samples from the field sites Baumannshof (Fig. 2a) and Grub (Fig. 2d).

For all soil samples from Puch field plots amounts of Cry1Ab were below the CCα of 2.0 ng Cry1Ab protein g^{-1} soil. However, in 2008, in soil (0–30 cm depth) collected in Puch from transgenic plots directly after the harvest mean Cry1Ab protein values were 1.17 ± 0.30 ng Cry1Ab protein g^{-1} soil, which is below the CCα but significantly elevated ($P < 0.05$) compared with soil samples from non-Bt-maize plots, in which recombinant protein levels were below the analytical detection limit of 0.4 ng Cry1Ab protein g^{-1} soil. Soil from Bt-maize plots of the experimental field site at Neuhof (collected in December 2007, 6-weeks after harvest) were unequivocally assessed to be positive for the presence of the recombinant protein, because Cry1Ab protein values exceeded 2.0 ng g^{-1} soil (Fig. 2c). Throughout the study, soil samples collected from Neuhof experimental field site in December 2007 were the only samples in which Cry1Ab concentrations were higher than 2.0 ng Cry1Ab protein g^{-1} soil. Mean Cry1Ab protein levels were 2.91 ± 1.54 ng Cry1Ab protein g^{-1} soil in top soil and 2.57 ± 1.59 ng Cry1Ab protein g^{-1} soil in the lower soil. However, Cry1Ab protein determination in the corresponding top soil samples (0–30 cm) collected after harvest in November of the following year, 2008, revealed mean values of 1.50 ± 0.79 ng Cry1Ab protein g^{-1} soil on Bt-maize plots versus 0.65 ± 0.22 ng Cry1Ab protein g^{-1} soil on non-Bt-maize control plots. Cry1Ab protein values were significantly higher in soil of the Bt-maize plots than in the non-Bt-maize control plots. As stated above, similar tendencies were reported in top soil samples collected from Puch in October 2008.

Finally, in all soil samples taken in spring 2009, Cry1Ab protein concentrations were close to or

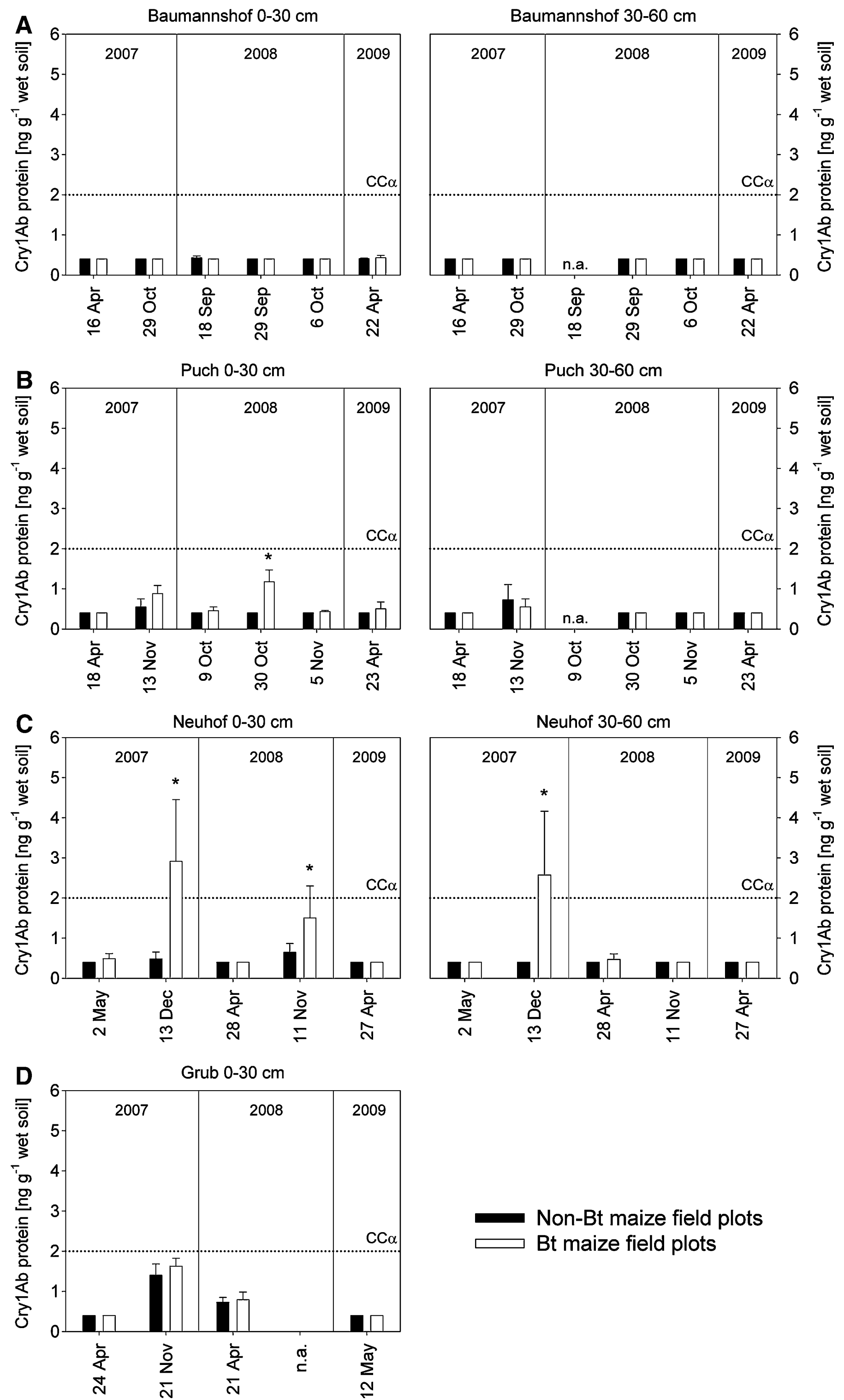

Fig. 2 Cry1Ab protein concentrations in bulk soil samples collected at depths of 0–30 and 30–60 cm on the experimental field sites Baumannshof (**a**), Puch (**b**), Neuhof (**c**) and Grub (**d**) cultivated with Bt-maize MON810 (*white bars*) and a near isogenic non-Bt-maize variety (*black bars*) in the eighth (2007) and ninth (2008) growing seasons of consecutive Bt-maize cultivation. Data from 2009 are Cry1Ab protein concentrations in soil samples collected terminally in spring after nine years long-term cultivation of Bt-maize MON810. The *dotted line* marks the decision limit CCα at 2.0 ng Cry1Ab protein g^{-1} wet soil. *Asterisks* indicate statistically significant differences ($P < 0.05$) in Cry1Ab protein quantities in soil from Bt-maize or non-Bt-maize field plots. *n.a.* not analysed

below the analytical detection limit of 0.4 ng Cry1Ab protein g^{-1} soil with a maximum value of 0.5 ng Cry1Ab protein g^{-1} soil. Thus, no Cry1Ab protein was detected in soil on any of the four investigated experimental field sites in spring after nine consecutive Bt-maize growing seasons (Fig. 2).

Discussion

Analytical assay performance

The sensitive and specific sandwich ELISA method used to investigate the fate of the Cry1Ab endotoxin in soil was validated according to European Commission Decision 2002/657/EC (Gruber et al. 2008). This validated assay enabled comparative quantitative analysis of a broad range of soils from different field sites with different matrix properties because of their distinct physicochemical soil properties. The analytical performance of the enzyme immunoassay was assessed by spiking blank soil matrix samples from different field sites with the Cry1Ab protein and determining the recovery of the protein from these samples. No Cry1Ab concentration-dependent adsorption saturation was observed in the tested low concentration range between 5 and 60 ng Cry1Ab protein g^{-1} soil. Clay adsorption studies similarly did not show saturation when clay minerals were incubated with Cry1Ab protein in a concentration range up to 8,000 ng g^{-1} clay (Pagel-Wieder et al. 2004) or at lower concentrations of 400–900 ng g^{-1} clay (Pagel-Wieder et al. 2007). Recovery of Cry1Ab protein from the soil matrix in the range 49.1–88.9% compares favourably with extraction efficiencies reported in earlier studies for Cry proteins. Earlier studies reported recovery of Cry1Ab protein from soil between 27 and 60% by use of a specific extraction buffer (Badea et al. 2010; Baumgarte and Tebbe 2005; Palm et al. 1994). Whereas amounts of Cry1Ab protein desorbed from soil using double-distilled water were 10% or less (Chevallier et al. 2003; Pagel-Wieder et al. 2004) even lower recovery, less than 0.1%, when using only water for extraction was reported for recombinant Cry3Bb1 (Meissle and Romeis 2009). The major reason for incomplete analytical recovery of Cry proteins in the soil matrix lies in adsorption of the proteinaceous recombinant endotoxins by surface-active soil particles, as was intensively studied in previous work (Stotzky 2005; Pagel-Wieder et al. 2004). Accordingly, in this study, analytical recovery differed for the various soils indicating that physical and chemical properties of the soils had a strong effect on adsorption of the Cry1Ab protein. When recovery was analysed with regard to soil particle size distribution, a strong correlation with the clay content of the investigated soil was shown. This is consistent with clay minerals being the major compounds responsible for the Cry1Ab adsorption in these soils and in accordance with results reported for other soils in previous studies (Chevallier et al. 2003; Tapp et al. 1994; Pagel-Wieder et al. 2004, 2007).

It is important to mention, that reduced analytical recovery of Cry1Ab protein from the soil matrix may result in the lack of detection of a fraction of bound Cry1Ab protein, particularly for field soils with high clay content. In our study, this applies to subsoils of the experimental field sites Neuhof and Puch and to the topsoil from the site Grub. Cry1Ab protein concentrations around the decision limit CCα may result in measured ELISA concentrations below CCα, indicating the absence of the Cry1Ab protein in a sample as per the EU-decision guideline. Thus in particular for soils with high clay content, results need to be interpreted cautiously with regard to analytical recovery. However, the potential accumulation of the recombinant protein in any of the investigated soils would be revealed by the applied ELISA method. If the transgenic protein was accumulated in soil, Cry1Ab protein quantities would be in a higher concentration range, where the described matrix effects are overcome by the high efficiency of the extraction method and the sensitivity of the assay (Paul et al. 2008).

Study design

The issue of potential accumulation of insecticidal recombinant Cry1Ab protein was addressed in this study by investigating four independent experimental field sites under Bt-maize MON810 cultivation for a period of nine years. Of reported literature on field studies with Bt-maize, this is the longest duration of Bt-maize cultivation in consecutive growing seasons in a field for experimental analysis of the Cry1Ab protein content of soil. Previously reported studies of the persistence of Cry1Ab protein in soil were

followed up for a maximum of five growing seasons (Baumgarte and Tebbe 2005; Hopkins and Gregorich 2003). Binding and degradation of the recombinant Cry proteins from gm maize plants in soil depends strongly on soil characteristics; for example clay content, organic matter content, water-holding capacity, pH, etc. (Stotzky 2000; Pagel-Wieder et al. 2004). Environmental factors, for example regional weather conditions, will lead to various microclimates in different field sites with regard to soil temperatures and soil moisture contents. The combination of crop variety, soil type, and environmental factors results in the variable degradation times observed for Cry proteins, as reviewed by Icoz and Stotzky (2008). Important environmental factors were considered in this study by including four different sites in the experiment to assess the potential accumulation of transgenic Bt-protein under various agricultural field conditions.

Cry1Ab protein content of soil

The ELISA method used here enables the determination of immunoreactive Cry1Ab protein at a decision limit, CCα, of 2.0 ng Cry1Ab protein g^{-1} soil, which is in the range of Cry1Ab protein concentrations theoretically to be expected in soil. Reported quantities of Cry1Ab protein in Bt-maize MON810 plant material examined concurrently in the course of the study were 6.7–8.4 μg Cry1Ab protein g^{-1} fresh weight Bt-maize chaff (Gruber et al. unpublished results). Clark et al. (2005) reported similar average Cry1Ab protein expression levels of 4.65 μg Cry1Ab protein g^{-1} plant material. It has been shown that a major fraction of immunoreactive Cry1Ab protein introduced into soil in agricultural practice can be attributed to root residues (Baumgarte and Tebbe 2005). A concentration of 1.4 μg Cry1Ab protein g^{-1} fresh root material (Nguyen and Jehle 2007) and a range of 152–183 ng Cry1Ab protein g^{-1} in decomposing root residues (Baumgarte and Tebbe 2005) were reported in Bt-maize MON810 plants. As maize stubble usually remains on the field after harvest, this root material has to be regarded as a substantial source of Cry1Ab protein entering the soil. Depending on the soil tillage system, a proportion of the root material will remain enclosed in the soil where it is subsequently degraded. A few published estimates of the amount of Cry proteins added to soil by transgenic crops have been reviewed by Clark et al. (2005). These estimates range from 1.58 μg Cry1Ac kg^{-1} soil under Bt-cotton given in the Biopesticides Registration Action Documents (US Environmental Protection Agency Office of Pesticide Programs 2001) to 580–2,410 μg of Cry3Bb1 kg^{-1} soil for maize MON863. The maximum theoretical load calculated for recombinant Cry1Ab protein from MON810 maize adds up to 208 ng Cry1Ab protein g^{-1} soil, assuming incorporation into a 15-cm furrow slice (Clark et al. 2005). These estimates are confirmed by balancing Cry1Ab protein amounts determined in the course of this study leading to a calculated Cry1Ab protein load of 100 ng Cry1Ab protein g^{-1} soil in the 30 cm topsoil layer. The Cry1Ab protein assay developed and validated in this study is very suitable for estimation of these calculated amounts of Cry1Ab protein introduced into soil under Bt-maize cultivation.

Comparing the four experimental field sites (Fig. 2), immunoreactive Cry1Ab protein could only be detected and quantified on the Neuhof field site on one sampling date—after harvest in 2007. The presence of the recombinant protein in the topsoil and subsoil even after 6-weeks of harvest could be attributed to the extreme weather conditions after harvest in this trial year. Continuous rainfall led to soil water-logging on this field site in such a way that the harvest residues and root material were kept in a poorly aerated environment at low temperatures after harvest. The rate of decomposition of plant material will be greatly reduced under such field conditions, because efficient aerobic microbial degradation of organic material is inhibited. As a consequence, persistence of the recombinant Cry1Ab protein for a few months after harvest is possible in exceptional and extreme environmental circumstances, leading to temporary prevention of degradation of plant material. However, by the next seeding date, complete degradation of Cry1Ab protein was shown on the respective field site (Neuhof) in our field trial. In topsoil samples collected from the field sites Neuhof and Puch within 1 week after harvest in 2008, Cry1Ab protein quantities determined by ELISA were slightly but significantly higher in Bt-maize plots than in non-Bt-maize plots. But the estimated values were below the CCα decision limit of 2.0 ng Cry1Ab protein g^{-1} soil. In this case, the presence of minimum amounts of Cry1Ab protein of approximately 1 ng Cry1Ab

protein g^{-1} soil must be assumed as revealed by statistical assessment of the data. Here it becomes obvious that the definition of a decision limit, CCα, of the ELISA according to European Commission Decision 2002/657/EC results in safe assessment of samples regarding unspecific soil matrix effects. Samples taken in November at the Grub field site with high background absorbance can be appropriately classified as not suspicious for the presence of Cry1Ab protein. Beyond that, if particular sets of soil samples with less unspecific background absorbance are analysed, a more sensitive assay could be achieved, as demonstrated for soil samples collected in Puch and Neuhof after harvest in 2008.

The quantities of Cry1Ab determined in bulk soil samples under cultivation of Bt-maize MON810 in this study are in agreement with data reported after previous studies of the soil fraction <2 mm. Hopkins and Gregorich (2003) found trace amounts <0.1 ng of Cry1Ab protein g^{-1} soil, and a maximum concentration of 3 ng Cry1Ab protein g^{-1} soil was reported by Baumgarte and Tebbe (2005).

Our findings suggest that on any of the four sites the overall climatic and field site conditions led to complete degradation of the Bt-maize plant material containing the recombinant Cry1Ab protein by the following growing season. No persisting immunoreactive Cry1Ab protein was detected in any soil shortly before the next seeding over the experimental period of three years, which comprised the last third of nine years of Bt-maize planting. Accordingly, no evidence for accumulation of the recombinant Cry1Ab protein from Bt-maize MON810 in agricultural soils was obtained in this study. The reported results further suggest that the recombinant Cry1Ab protein has no extraordinary stability in soil. Glasshouse experiments showing rapid degradation of Bt-protein from potted Bt-maize MON810 plants in three different soil types (Badea et al. 2010) are consistent with our results from field-scale experiments.

Toxicological relevance of Cry1Ab protein in soil

When immunological assay systems are used to quantify Cry1Ab protein in soil samples two important methodological aspects have to be considered in interpretation of the collected data. First, Cry1Ab protein bound by strong interactions to the soil matrix will possibly not be captured by the ELISA leading to underestimation of the Cry1Ab concentration in the assessed samples. Second, the ELISA enables quantification of Cry1Ab and its immunoreactive degradation products (Paul et al. 2010) but gives no information about the remaining biological activity of the Cry1Ab protein molecules. Both the presence of bound toxin and its insecticidal activity can only be determined by use of sensitive organism species in biotoxicity assays.

Appropriate test organisms used in earlier studies are the target organism *Ostrinia nubilalis*, other lepidopteran species, or susceptible soil dwelling invertebrates. The Cry1Ab protein susceptibility of the target organism *O. nubilalis* was described by a LC_{50} ranging from 0.10 to 0.34 µg of Cry1Ab per gram of diet in a study performed by Saeglitz et al. (2006). Accordingly, *O. nubilalis* larvae fed on biogas reactor effluent containing Cry1Ab protein concentrations of 2.67 ng g^{-1} and below—the concentration range in the Neuhof autumn 2007 soil in this study—showed no increased mortality compared with a group receiving control effluent without Cry1Ab (Rauschen and Schuphan 2006). Both free and humic acid-bound Cry1Ab protein was insecticidal to larvae of the lepidopteran tobacco hornworm *Manduca sexta* with LC_{50} values between 215 and 304 ng 100 μl^{-1} (Crecchio and Stotzky 1998). A study in which solutions of recombinant Cry1Ab protein were fed to the soil-dwelling nematode *Caenorhabditis elegans* revealed verifiable effects on growth and reproduction at minimum concentrations of 118 and 41 mg Cry1Ab protein l^{-1} nutrient solution, respectively (Hoess et al. 2008). This corresponds to Bt-toxin concentrations approximately 10,000 times higher than those measured in the experimental field soils in this study. The cited nematode study of the test organism *C. elegans* identified deleterious effects of rhizosphere and bulk soil samples from fields with Bt-maize (MON810) containing 0.51 and 1.31 ng Cry1Ab g^{-1} soil dry weight. However, although the observed effects correlated with concentrations of the Cry1Ab protein in soil they could not be explained by the direct toxicity of the Cry1Ab protein. These results from the nematode study point out that in addition to quantification of Cry1Ab protein in soil samples additional assessment of Bt toxin using biotoxicity assays should gain importance regarding further studies.

If the Cry1Ab susceptibilities of test organisms are compared with the concentrations of Cry1Ab protein determined in the field soils in this study no toxicological and ecological effects on soil dwelling invertebrates should be expected. These assumptions are in accord with current knowledge of the effects of Cry proteins on soil-dwelling organisms as summarized by Icoz and Stotzky (2008). For most soil organisms no toxic effects were reported. Studies on the effects on soil nematodes show different findings, though with most of the results revealing no effects.

In conclusion the major finding of this study is rapid degradation of Cry1Ab protein from plant residues remaining on the field after harvest and no accumulation or persistence of Cry1Ab protein after nine successive years of Bt-maize MON810 cultivation.

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Fate of Cry1Ab Protein in Agricultural Systems under Slurry Management of Cows Fed Genetically Modified Maize (*Zea mays* L.) MON810: A Quantitative Assessment

Helga Gruber,[†,‡,§] Vijay Paul,[‡,∥] Patrick Guertler,[‡,§] Hubert Spiekers,[⊥] Ales Tichopad,[‡,#] Heinrich H. D. Meyer,[‡] and Martin Müller*,[†]

[†]Institute for Crop Science and Plant Breeding, Bavarian State Research Center for Agriculture, Am Gereuth 8, Freising, Germany

[‡]Physiology Weihenstephan, Technische Universität München, Weihenstephaner Berg 3, Freising, Germany

[⊥]Institute for Animal Nutrition and Feed Management, Bavarian State Research Center for Agriculture, Prof.-Dürrwächter-Platz 3, Poing, Germany

ABSTRACT: The objective of the study was to track the fate of recombinant Cry1Ab protein in a liquid manure field trial when feeding GM maize MON810 to dairy cows. A validated ELISA was applied for quantification of Cry1Ab in the agricultural chain from GM maize plants, feed, liquid manure and soil to crops grown on manured fields. Starting with 23.7 μg of Cry1Ab g^{-1} dry weight GM maize material, a rapid decline of Cry1Ab levels was observed as 2.6% and 0.9% of Cry1Ab from the GM plant were detected in feed and liquid manure, respectively. Half of this residual Cry1Ab persisted during slurry storage for 25 weeks. After application to experimental fields, final degradation of Cry1Ab to below detectable levels in soil was reported. Cry1Ab exhibited a higher rate of degradation compared to total protein in the agricultural processes. Immunoblotting revealed a degradation of the 65 kDa Cry1Ab into immunoreactive fragments of lower size in all analyzed materials.

KEYWORDS: GM maize, MON810, Cry1Ab protein, dairy cow, liquid manure, soil, *Zea mays*

1. INTRODUCTION

The global area planted with biotech crops comprised 148 million hectares in 2010 with genetically modified (GM) maize accounting for a major proportion as 47 million hectares were cultivated with GM maize.[1] The majority of GM maize crops used at present exhibit resistance against insect pests by expression of insecticidal proteins derived from delta-endotoxins of *Bacillus thuringiensis* (Bt). These Bt proteins are summarized as crystal (Cry) proteins as they are stored in crystalline inclusion bodies in the bacterial cell.[2,3] Each class of Cry proteins is characterized by a narrow range of susceptible target organism species as the toxin needs proteolytic activation in the gut and binding to selective receptors localized in the midgut epithelial cells of larvae to unfold its toxic effects.[4] This defined spectrum of insecticidal activity allows for target specific pest management and reduced application of insecticides when growing Bt crops.[5]

MON810 is a transgenic maize event based on the introduction of a modified *cry1Ab* gene into the plant genome enabling plants to synthesize a truncated form of the protein Cry1Ab. Cry1Ab is highly active against Lepidoptera and widely used to combat the European corn borer (*Ostrinia nubilalis* Hübner), a major pest in maize fields.[6] MON810 cultivars have been registered and commercialized in 1996 in the United States, followed by their approval in other countries all over the world including countries of the European Union in 1998.[7]

Since the development and application of the Bt-maize technology, safety concerns have been raised concerning potential adverse effects on human and animal health and the environment, though an extensive environmental risk assessment is prescribed and performed before the approval of any GM crop.[8,9] For Cry1Ab expressing maize these concerns were met by a broad range of studies dealing with the toxicity and allergenic potential of Cry proteins,[10−12] transfer of Cry1Ab from livestock feed into food for human consumption,[13] impact on nontarget organisms[14,15] and entry of Cry1Ab from Bt-maize plants into agricultural soils.[16−18] Although the current scientific knowledge about Bt crops gives no evidence for any significant risk to the environment or to human and animal health,[7,19] commercial Bt-maize (MON810) cropping was banned in some EU countries in the face of low consumer acceptance when GM crops are employed in food and feed production.[20]

Regarding the fate and metabolism of the insecticidal Cry1Ab protein in dairy cows fed Bt-maize, results from a 25 month long-term feeding study[21] revealed that immune detective Cry1b protein fragments are not transferred to body fluids as urine, blood plasma and milk.[22] However, as the protein is not completely degraded by digestive processes in the gastrointestinal tract (GIT), the Cry1Ab is still detectable in feces of Bt-maize fed cattle.[23] Considering these findings in view of manuring practice on dairy farms, the use of a mixture of feces and urine as liquid manure for fields implicates the introduction of the recombinant Cry1Ab protein into the agricultural environment, particularly the soil compartment, provided the fact that it is not totally degraded during liquid manure storage. Whereas the entry of Cry1Ab into soil via maize plant residues has been extensively studied,[18,24−27] the entry via liquid manure of Bt-maize fed cattle

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Table 1. Time Schedule for Application of Liquid Manure to Experimental Field Lots and Collection of Samples for Assessing Cry1Ab Protein Contents in the Field Trial Carried Out in 2007

	date of liquid manure treatment or sampling	
	Grub[a]	Finsing[a]
1st manure application grass	Apr 3	Apr 3
1st manure application maize field	Apr 19	Apr 18
1st grass cut	May 2	May 4
2nd manure application grass	May 3	May 4
2nd manure application maize field	May 15	Jun 12
2nd grass cut	May 31	Jun 4
3rd manure application grass	Jun 4	Jun 4
3rd grass cut	Jul 16	Jul 18
4th manure application grass	20-Jul	20-Jul
4th grass cut	Aug 24	Aug 27
5th manure application grassland	Aug 28	Aug 28
sampling of maize plants	Sep 19	Sep 19
soil sampling	Oct 8	Oct 9

[a] Experimental field site.

has not been examined so far. To address this issue, an extensive field trial was performed in close association with a Bt-maize MON810 feeding study[13,21] to track the further fate of Cry1Ab in liquid manure.

In order to provide a set of quantitative Cry1Ab protein data reflecting the entire agricultural manuring process, this study investigated liquid manure storage, its soil entry and harvest of maize and grass crops from fields after application of manure from Bt-maize fed cows. Consequently, to meet the analytical requirements of the trial, an appropriate detection method to evaluate the presence of Cry1Ab in a range of samples with different matrix properties was necessary. We took advantage of a highly specific immunoaffinity purified polyclonal antibody based ELISA system for Cry1Ab toxin determination in animal liquids and animal byproducts,[23,28] that had been validated and successfully used for surveillance of transgenic protein in feces and urine of cows.[22] By adopting this ELISA system to other matrices and validating the detection system for the sample material investigated in the present liquid manure field trial, we aimed to keep track of the insecticidal Cry1Ab protein through different agricultural environments under comparable analytical conditions.

In the course of feed processing and passage through the GIT of the cow the Cry1Ab undergoes various proteolytic processes leading to the breakdown of the insecticidal protein into smaller fragments.[28−31] In agricultural manuring practice, further degradation may take place during the storage of liquid manure and by physicochemical breakdown and proteolytic microbial activity when liquid manure is applied to agricultural soil. Analysis of the fragmentation patterns of the Cry1Ab protein in the samples collected in a field trial could give important additional information considering the stability of the insecticidal protein and its fragments in the described processes.

Therefore, the objectives of this study were to develop, optimize and standardize extraction and immunological detection methods for Cry1Ab in liquid manure, soil, maize and grass crop samples. The methods were then applied to samples of a liquid manure field trial after feeding cows maize MON810 in order to assess the persistence and degradability of the insecticidal Cry1Ab protein in different stages of agricultural manure management.

2. MATERIALS AND METHODS

2.1. Feeding Experiment and Liquid Manure Field Trial. All handling of the dairy cows was performed under the approval of the Bavarian State Research Center of Agriculture (LfL, Grub, Germany) institutional animal care and use committee. A detailed description of the Bt-maize feeding experiment and of the feed composition in the daily diet of the cows is given in refs 21 and 13.

Five Simmental cows of the target group were fed a partial total mixed ration (PTMR) containing 63% (dry matter basis) GM maize MON810 whereas the control group received a substantially equivalent ration based on near-isogenic maize components. Cows were kept in a stable equipped with special slurry sampling facilities for a time period of six days. The entire stall manure of each group was collected and pooled. Storage was carried out according to agricultural practice at ambient temperature for further experimentation (stores 1 and 2, near-isogenic feed ration; stores 3 and 4, GM feed ration).

The liquid manure field trial took place in 2007 on two experimental field sites in Grub and Finsing, both located in Southern Bavaria, Germany. Whereas the grassland was under common permanent agricultural cultivation, maize fields were under experimental maize cultivation before the liquid manure field trial for seven years and one year, respectively. Experimental maize cultivation continued during the liquid manure trial in 2007 by growing the GM maize hybrid Kuratus (KWS-Saat, Einbeck, Germany) representing the transformation event MON810 and its near-isogenic maize hybrid Gavott (KWS-Saat, Einbeck, Germany) as control on four replicate plots of 750 m^2 size.

Liquid manure of the Bt-maize fed group and the control group was applied to four replicate small plots of 6 m^2 on grassland and within maize fields according to the time schedule shown in Table 1. On the maize fields, liquid manure of transgenic and non-transgenic origin was applied each to GM maize and near-isogenic maize plot, resulting in a total of 16 small plots of manure application. The manure was evenly applied with a watering can in practice-oriented maximum admissible amounts of 2.5 L/m^2 on grassland and 4 L/m^2 on maize fields.

2.2. Sampling Procedures. Representative PTMR feed samples based on GM maize and near-isogenic maize were collected daily within the period of liquid manure retrieval from the cows and were subsequently pooled and ground finely in liquid nitrogen using mortar and pestle.

Liquid manure samples were collected from slurry stores at the beginning of storage and at each date of manuring the experimental field lots (Table 1). Each sample was pooled from six subsamples taken with a 35 mm diameter sampling tube after exhaustive homogenization of the slurry from the tank.

At four time points, depicted in Table 1, the entire grass cut from each plot was harvested. Grass from each plot was mixed, one aliquot drawn from the core of the crop, chopped to small pieces and ground in liquid nitrogen.

For Western blot analysis, one set of maize plants of the varieties Kuratus (GM) and Gavott (near-isogenic) were grown in the glasshouse at 22 °C with a weekly nitrogen phosphorus potassium (NPK) treatment. The leaves of four plants of each variety were collected in the BBCH growth stages[32] of BBCH14 (5 leaves) and BBCH69 (end of tasseling) omitting the uppermost and undermost leaves. Leaves were immediately reduced to small pieces using a scalpel, ground in liquid nitrogen and frozen at −20 °C. Mature maize plants at growth stage of BBCH85 (ripening stage) were harvested by cutting with a sickle from each experimental field plot treated with liquid manure. Ten representative plants were selected and chaffed by a crop chopper to pieces of

approximately 5 cm size in diameter. Aliquots of the well-mixed chaffed material were ground in liquid nitrogen.

Finally, soil samples were taken at the end of the vegetation period in depths of 0−30 cm and 30−60 cm from maize field plots and 0−15 cm from grassland plots as mixed samples comprising ten drilling cores. Soil was homogenized and sieved at 2 mm mesh size. All sample material was stored at −20 °C until analyzed.

2.3. Analysis of Substantial Equivalence: Influence of Different Types of Liquid Manure on Heterogeneous Parameters of Maize and Grass Crop. Maize plants, grass crop and liquid manure were assessed for substantial equivalence by determining fourteen maize parameters (fresh matter, dry matter, starch, enzyme soluble organic substance, crude fiber, crude protein, crude fat, sugar, neutral soluble fiber, acid soluble fiber, acid soluble lignin, organic neutral soluble fiber, organic acid soluble fiber, in vitro digestible organic matter), six grass crop parameters (fresh matter, dry matter, crude fiber, crude protein, crude fat, crude ash) and nine parameters for liquid manure (dry matter, pH of liquid manure, pH of liquid manure extract, total nitrogen, NH_4−N, P_2O_5, K_2O, MgO, CaO; samples taken over the entire manure storage period). Statistical analysis investigating the influence of the two types of liquid manure (transgenic origin/non-transgenic origin) on various maize and grass parameters was performed by using ANOVA. As there were 14 or 6 individual tests conducted (one for each parameter), the threshold level of α was adjusted to multiple comparison to prevent the overall probability of type 1 error to be larger than 5%. The Bonferroni correction method was applied according to the formula $1 - (1 - \alpha)^{1/n}$. Only effects with obtained *p*-values <0.0037 for maize and <0.0083 for grass are considered significant. In addition, a multivariate analysis of variance (MANOVA) model was created using liquid manure as explanatory variable with the same set of response variables as in the ANOVA model. This test studied the effect of the factor liquid manure on all response variables simultaneously, using the overall probability of type one error set to 5% (no significant overall effect with the Wilks lambda statistic significance $p = 0.5181$).

2.4. Reagents. All reagents were of analytical grade and supplied by Merck (Darmstadt, Germany) unless otherwise stated. HPLC-purified trypsin-activated Cry1Ab protein was used as standard and for validation procedures. Development, purification and biotin-labeling of highly specific polyclonal anti-Cry1Ab antibodies were described in detail in ref 23. Assay buffer in the ELISA was phosphate-buffered saline (PBS; 8 mM NaH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 pH 7.4) containing 0,1% Tween 20.

2.5. Protein Extraction. *2.5.1. Protein Extraction of Maize and Maize Feed (PTMR).* Protein was extracted by homogenization of ground maize plant and feed samples in ice cold extraction buffer (EB: 8 mM NaH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.1% Tween 20 pH 7.4) containing protease inhibitor (Roche, Germany) using a Fast Prep FP120 (MP Biomedicals, Germany) homogenization machine. Samples (100 mg) were weighed in FastPrep tubes filled with 200 mg of beads (Lysing Matrix D; MP Biomedicals, Germany) and homogenized five times at 5 m s^{-1} for 20 s with a 5 min cooling step on ice in between each homogenization cycle. Protein from the samples was extracted by horizontal shaking at 225 rpm for 30 min at room temperature. The homogenate was then centrifuged at 15000*g* at 4 °C for 15 min, and 500 μL of the supernatant was subjected to a second centrifugation to receive a clear sample extract. Before Cry1Ab analysis, maize and PTMR extracts were diluted in EB in a ratio of 1:100 and 1:5 respectively.

2.5.2. Protein Extraction of Liquid Manure. Samples of 100 mg of thawed liquid manure were extracted by the FastPrep procedure as described above, with the exception of using 1 mL of ice cold EB containing protease inhibitors and 1.5% skim milk as extractant. In extracts prepared for total protein determination, skim milk was omitted in the extraction buffer.

2.5.3. Protein Extraction of Grass. Protein from finely ground grass samples was extracted applying the procedure stated above but using 400 mg of sample and 4 mL of ice cold EB containing protease inhibitors and 1.5% skim milk in a FastPrep-24 (MP Biomedicals, Germany) homogenization machine. The homogenate was centrifuged at 4,500 rpm for 15 min at 4 °C and 500 μL of the supernatant clarified by a second centrifugation step (15000*g*, 15 min, 4 °C) before ELISA analysis.

2.5.4. Protein Extraction of Soil. Soil samples (<2 mm) of 200 mg were extracted using 2 mL of EB containing 2.5 mM EDTA and 0.5% skim milk in plastic tubes by horizontal shaking at RT for 30 min at 225 rpm. A clear extract was gained after centrifugation at 4,500 rpm for 15 min at 4 °C followed by centrifugation at 15000*g* for 15 min at 4 °C.

2.6. BCA Assay. Total protein concentrations in PTMR, liquid manure and maize extracts was measured by bicinchoninic acid (BCA) assay[33] using BSA (SERVA, Heidelberg, Germany) in a concentration range of 0 to 1000 μg/mL PBST as protein standard.

2.7. Cry1Ab Protein ELISA. For the determination of the Cry1Ab concentration in the protein extracts, a previously developed and well-described sandwich ELISA system[23] was used whereas the incubation time of calibrators and unknown samples was changed from 3 to 15 h.

2.8. Assay Validation. A separated validation of the Cry1Ab protein ELISA was carried out for the matrices of plant material (maize and grass), soil and liquid manure according to the criteria specified in the recently adopted European Commission Decision 2002/657/EC34 for the performance and validation of screening and confirmatory analytical methods. A detailed description of the general standardization procedure is given in ref [23].

2.8.1. Decision Limit (CCα) and Detection Capability (CCβ). The validation procedure was carried out for the plant material by using 32 discrete grass and 16 near-isogenic maize samples (blanks) derived from sections of the experimental fields, where neither GM maize was planted nor liquid manure from GM maize feeding was applied. A number of 32 blank liquid manure samples, collected from cows fed the diet based on near-isogenic maize, were taken for standardization of the Cry1Ab ELISA in liquid manure. Accordingly 32 independent blank soil samples were drilled at least 300 m distant of GM maize cultivation on four experimental field sites including Grub and Finsing for validation of the Cry1Ab ELISA in soil.

These blank samples were analyzed using the Cry1Ab ELISA to demonstrate the range of blank matrix effects and to determine the decision limits (CCα) and the detection capabilities (CCβ) in the different sample materials. According to the EC guideline 2002/657/EC, CCα is defined as three times the average signal-to-noise level measured in the assay for Cry1Ab in the blanks. CCβ was determined by use of the equation $CC\beta = CC\alpha + 1.64 \times SD_s$, SD_s being the standard deviation obtained for the blank samples fortified at the spike concentration level of CCα. Whereas the α-error is the percentage of blank samples exceeding the CCα-value, β-error describes the percentage of blank samples spiked at Cry1Ab concentration of CCβ and falling below the CCα-value.

2.8.2. Precision and Recovery. Precision of the Cry1Ab-ELISA was expressed by intra-assay and interassay coefficients of variation (CV) calculated from the analysis of Cry1Ab control samples dissolved in EB containing 1.5% skim milk at three concentrations of 0.3, 1.2, and 5.0 ng mL^{-1} in nine independent assays.

The mean analytical recovery of Cry1Ab in each matrix was determined by spiking eight of the blank samples collected for assay validation at three different concentration levels of Cry1Ab (5, 20, and 60 ng g^{-1} sample) and measuring Cry1Ab concentrations in the spiked samples by applying the Cry1Ab ELISA.

2.8.3. Statistics. Final data are presented as ng of Cry1Ab protein g^{-1} wet weight sample and as μg of Cry1Ab protein per g total protein. Student's *t* test was used to compare the means of Cry1Ab concentrations in maize plants, PTMR, and liquid manure of transgenic and non-transgenic origin, considering a *P*-value below 0.05 as significant.

2.9. Immunoblotting Procedure. The fragmentation of Cry1Ab protein from GM maize MON810 was recorded by immunoblot analyses of protein extracts from maize plants, PTMR samples and liquid

301 manure samples. Amounts of 5 (maize plants), 50 (PTMR) or 220 (liquid manure) μg total protein were applied to 12% reducing SDS−polyacrylamide gels, and proteins were resolved by gel electrophoresis at 120 V for 150 min, followed by blotting of the proteins onto nitrocellulose membranes (Protran AB 85, Whatman, Dassel, Germany). Immunodetection with polyclonal Cry1Ab specific antibodies (rabbit 0.1 μg/mL) and visualization of immunoactive Cry1Ab fragments was performed according to the procedures described earlier.[28] HPLC purified trypsin activated Cry1Ab protein (65 kDa) was used as a positive control for the presence of Cry1Ab in the respective samples. 310

311 3. RESULTS AND DISCUSSION

312 **3.1. Analysis of Substantial Equivalence: Influence of Different Types of Liquid Manure on Heterogeneous Parameters of Maize and Grass Crop.** The analysis of nine chemical parameters of the two types of liquid manure resulted in the following mean differences: dry matter, 5% ($n = 7$); pH of liquid 316 manure, 1% ($n = 6$); pH of liquid manure extract, 3% ($n = 6$); total nitrogen, 2% ($n = 7$); NH_4−N, 8% ($n = 8$); P_2O_5, 4% ($n = 5$); K_2O, 4% ($n = 5$); MgO, 4% ($n = 5$); CaO, 0% ($n = 5$). These differences are minor regarding the substantial equivalence of the applied liquid manure. Statistical analysis of 14 nutrient and energy parameters in all maize samples and of six respective parameters in all grass samples revealed no significant effect of transgenic or non-transgenic liquid manure treatment onto these parameters in maize and grass crop (data not shown). The MANOVA revealed no significant overall effect of the factor liquid manure on all response variables simultaneously. These results are in close accordance with the Bt maize long-term feeding study where the compositional equivalence with respect to nutrient concentration of GM and near-isogenic maize fed to the dairy cows was demonstrated.[21] 331

332 **3.2. Analytical Assay Performance.** *3.2.1. Assay Precision.* Calibration curves of Cry1Ab standards in the respective extraction buffers allowed quantification of Cry1Ab in the extracts over the dynamic range from 0.04 to 20 ng mL^{-1}. The analytical limit of 0.04 ng mL^{-1} of extract corresponds to 0.4 ng of Cry1Ab protein g^{-1} wet sample for all the analyzed matrices. Cry1Ab control samples in concentrations of 0.3, 1.2, and 5.0 ng mL^{-1} in nine independent assays indicated a good assay precision, with a mean intra-assay coefficient of variation of 5.9% and a mean interassay coefficient of variation of 14.6% (Table 2). 341 T2

342 *3.2.2. Assay Standardization.* The assay standardization was performed according to the European Commission Decision 2002/657/EC[34] and resulted in distinct analytical limits for the different sample materials (Table 3). According to the European Commission Decision false compliant rates (β-error: samples below CCα, when spiked at a concentration level of CCβ) of 347 T3

Table 2. Analytical Precision for ELISA in Cry1Ab Protein Control Samples: Coefficients of Variation at Three Different Concentrations (C1−C3) of Cry1Ab Protein (Three Determinants per Assay) in Nine Independent Assays

	Cry1Ab protein controls			
CV[a]	C1: 0.3 ng/mL	C2: 1.2 ng/mL	C3: 5.0 ng/mL	mean CV
intra-assay (%)	11.0	2.2	4.6	5.9
interassay (%)	17.1	14.0	11.9	14.3

[a] Coefficient of variation.

Table 3. Decision Limits (CCα), Detection Capabilites (CCβ) and Recoveries Achieved for Cry1Ab Protein Determination in Spiked Samples of the Different Matrices Investigated

sample material	CCα [ng g^{-1} wet wt]	CCβ [ng g^{-1} wet wt]	α-error [%]	β-error [%]	mean recovery[a] [%]
feed (PTMR)	4.02	5.96	0	0	78.9 ± 13.2[b]
liquid manure	1.20	1.41	0	0	71.2 ± 5.1
soil	2.00	3.06	0[c]	0[c]	68.1 ± 9.9[c]
plant material	1.38	2.00	0	3.1	98.8 ± 7.2

[a] Mean recovery ± SD after spiking with three concentrations of Cry1Ab protein (eight replicates per spike concentration). [b] As determined by Guertler et al., 2010. [c] Validation soil samples of the experimental field sites Grub and Finsing.

Table 4. Cry1Ab Protein Concentration in Soil Samples Collected in the Field Trial[a]

field site	cultivation	origin of maize feed and liquid manure[b]	Cry1Ab protein [ng g^{-1} wet wt]
Grub	GM maize MON810	NT	0.40 ± 0.00
	GM maize MON810	T	0.40 ± 0.00
	maize near-isogenic	NT	0.40 ± 0.00
	maize near-isogenic	T	0.40 ± 0.00
Finsing	GM maize MON810	NT	0.45 ± 0.09
	GM maize MON810	T	0.50 ± 0.14
	maize near-isogenic	NT	0.40 ± 0.00
	maize near-isogenic	T	0.40 ± 0.01
Grub	grassland	NT	1.56 ± 0.32
	grassland	T	1.68 ± 0.21
Finsing	grassland	NT	2.45 ± 1.95
	grassland	T	3.42 ± 1.62

[a] Mean values and standard deviation of soil samples drilled from four replicate plots are shown. [b] T: feed and liquid manure of transgenic origin. NT: feed and liquid manure of non-transgenic origin.

Table 5. Quantitative Degradation of Cry1Ab Protein in Agricultural Processes Relevant for Liquid Manure Management[a]

		Cry1Ab protein content		Cry1Ab protein concn/total protein	
	n	[ng of Cry1Ab g^{-1} dry wt]	[%] of Bt maize plant	[μg of Cry1Ab g^{-1} of total protein]	[%] of Bt maize plant
maize near-isogenic	32	<CCα[b]	–[c]	–	–
GM maize MON810	32	23 677 ± 1934.6	100	457.9 ± 46.5	100
feed (NT[d])	8	<CCα	–	–	–
feed (T)	8	611.3 ± 64.4	2.6	10.6 ± 1.2	2.3
liquid manure[e] (NT)	8	<CCα	–	–	–
liquid manure[e] (T)	8	204.6 ± 20.4	0.9	1.9 ± 0.4	0.4
liquid manure[f] (NT)	8	<CCα	–	–	
liquid manure[f] (T)	8	104.5 ± 21.5	0.4	0.9 ± 0.2	0.2
soil[g] (NT)	16	<CCα	–	–	
soil[g] (T)	16	<CCα	–	–	–
near-isogenic maize crop (NT)	16	<CCα	–	–	–
near-isogenic maize crop (T)	16	<CCα	–	–	–
grass crop (NT)	32	<CCα	–	–	
grass crop (T)	32	<CCα	–	–	–

[a] Cry1Ab concentrations are given in mean values ± standard deviation. Columns 3 and 4 show Cry1Ab contents on dry weight basis. In columns 5 and 6 Cry1Ab in relation to total protein contents is depicted. The quantitative decrease of the recombinant protein by each degradation step is shown by the percentages of Cry1Ab protein recovered in a sample in relation to Cry1Ab quantities in the primary GM maize plant material. [b] <CCα: any sample analyzed revealed Cry1Ab measurements below the decision limit CCα determined fpr the respective sample matrix. [c] –: not calculable as Cry1Ab was not detected in the samples. [d] T: feed and liquid manure of transgenic origin. NT: feed and liquid manure of non-transgenic origin. [e] Week 1 of slurry storage. [f] Weeks 24 and 25 of slurry storage. [g] Maize field plots.

<5% are prescribed for screening assays. Similarly, <5% false compliant results (α-error: blanks exceeding the CCα value) are accepted. As depicted in Table 3, the 5% limit for α-error and β-error was under-run in all investigated matrices. Thus, the CCα and CCβ values satisfy the criteria for the performance and validation of screening and quantitative analytical methods of this guideline in the sample materials of PTMR, liquid manure, soil and plant material collected in the liquid manure field trial.

3.2.3. Analytical Recovery. For each of the different sample materials analyzed, the analytical performance of the assay was assessed by spiking matrix samples of non-transgenic origin with Cry1Ab protein. The immunoassay operated well for the investigated matrices with mean analytical recovery rates of 68% for soil; 71% for liquid manure, 79% for PTMR and 99% for plant material (Table 3). Recovery of 68% Cry1Ab protein from the soil matrix is in the range of 49−89% shown for different soils[16] and compares favorably with extraction efficiencies between 27 and 60% reported in earlier studies for Cry proteins.[18,27,35] For PTMR 79% Cry1Ab recovery was documented earlier by ref 22.

3.3. Cry1Ab Protein Contents. *3.3.1. Cry1Ab Protein Contents in Feed (PTMR).* Transgenic and non-transgenic feed samples (PTMR) given to the dairy cows during slurry collection were analyzed for their Cry1Ab contents by applying the validated Cry1Ab immunoassay. As depicted in Table 5, no Cry1Ab was detected in the non-transgenic feed samples of the control group. In the transgenic PTMR, Cry1Ab was reported in amounts of 278 ± 29 ng of Cry1Ab g^{-1} wet feed sample. This corresponds well with amounts of 246 ng of Cry1Ab g^{-1} feed sample reported for samples analyzed in course of the associated Bt-maize feeding study.[28]

3.3.2. Cry1Ab Contents in Liquid Manure. During the 29 weeks of the liquid manure field trial, slurry samples of control manure (cows fed on non-transgenic ration) and the manure of cows fed a transgenic ration were collected with proceeding time of storage from the slurry stores. In total, 22 samples were analyzed for the presence of Cry1Ab using the validated ELISA system. No liquid manure sample of the cows fed a non-transgenic ration was positive for the presence of Cry1Ab at the decision limit CCα of 1.2 ng of Cry1Ab g^{-1} wet slurry (Figure 1). In the stores 3 and 4 filled with slurry from the transgenic ration-fed cows, Cry1Ab was detected in all samples taken at different time points with initial concentrations of 23.5 ± 0.9 and 18.2 ± 1.0 ng of Cry1Ab g^{-1} slurry in stores 3 and 4, respectively. After feed ingestion, microbial activity in the rumen leads to degradation of feed protein, but a fraction of protein passes through the rumen and is partly degraded in the small intestine. Thus, immunoactive Cry1Ab protein fragments were found in contents of the GIT and in the feces of cows and pigs fed GM feed.[28,31,36,37] Previous work reported the presence of Cry1Ab in feces, but the absence of Cry1Ab in urine of maize MON810 fed dairy cows.[22] Thus in liquid manure, as a mixture of both, the Cry1Ab detection can be attributed to poorly digested GM maize plant feed material in the feces. Apparently, the Cry1Ab protein was protected from degradation by proteolytic enzymes in the GIT when enclosed in the heavy digestible parts of the maize plant. In contrast easily accessible purified Cry1Ab, isolated from GM maize plants, was shown to be very rapidly degraded by pepsin in in vitro studies.[38]

During further storage, Cry1Ab concentrations in samples taken from the tanks continuously decreased to Cry1Ab contents of 9.2 ± 1.1 and 8.6 ± 2.1 ng g^{-1} slurry in stores 3 and 4. This implies a significant decrease ($P < 0.01$) in Cry1Ab concentration to a mean of 51% of the initial values within 25 weeks of storing liquid manure. Thus, Cry1Ab derived from transgenic feed is further degraded in the liquid manure during storage. Still, a proportion of Cry1Ab will persist in liquid manure within the time period of slurry storage in common agricultural practice. Thus, it has to be assumed that minor amounts of Cry1Ab will be applied to agricultural fields by slurry management of dairy farms feeding GM maize MON810. In the described field experiment, transgenic protein in one liquid manure application added up to

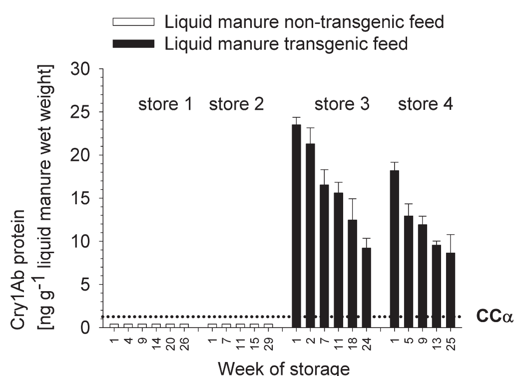

Figure 1. Detection and quantification of Cry1Ab protein in liquid manure samples derived from cows fed partial total mixed ration containing near-isogenic maize (store 1, store 2) or GM maize MON810 (store 3, store 4). Samples were analyzed with proceeding time of storage in the slurry stores. The data are presented as mean ± SD values (n = 4).

diminutive amounts of 431 mg of Cry1Ab ha^{-1} for grassland and 690 mg of Cry1Ab ha^{-1} for maize fields. Evidently, these amounts are very small in comparison to the amounts of Cry proteins added to soil by GM maize plant residues remaining on the fields after harvest.[16] From the Cry1Ab amounts determined in GM maize chaff analyzed in this study, a calculated maximum of 462 g of Cry1Ab ha^{-1} could be introduced into soil by maize crop residues. These estimates are confirmed by a maximum theoretical load of 480 g of Cry1Ab ha^{-1} calculated for recombinant protein from GM maize MON810 by ref 39. Thus, the theoretical Cry1Ab protein load applied to agricultural fields by way of liquid manure from GM maize fed cows adds up to less than 0.15% of Cry1Ab protein introduced directly by plant residues.

Cry1Ab biotoxicity studies provide a basis to estimate the toxicological relevance of the Cry1Ab concentrations arising in liquid manure. The Cry1Ab protein susceptibility of the target organism *Ostrinia nubilalis* was described by a LC_{50} ranging from 0.10 to 0.34 μg of Cry1Ab per gram of diet.[40] Both free and humic acid bound Cry1Ab protein was insecticidal to larvae of the lepidopteran tobacco hornworm *Manduca sexta* with LC_{50} values between 215 and 304 ng of 100 μL^{-1}.[41] Thus the Cry1Ab concentrations detected in this study were clearly below any concentration that would cause a direct toxic effect on nontarget organisms such as *Manduca sexta* or *Caenorhabditis elegans*.[42]

3.3.3. Cry1Ab Contents in Soil. At the experimental field sites Grub and Finsing, 32 soil samples in total were collected from maize field and grassland lots treated with liquid manure obtained from cows receiving non-transgenic or transgenic feed rations. In the soil samples collected from maize lots, no Cry1Ab was detectable as ELISA analysis revealed concentrations below the CCα of 2.0 ng of Cry1Ab g^{-1} of soil (Table 4). For the maize field, this gives evidence for degradation of the insecticidal Cry1Ab protein when introduced into soil by way of liquid manuring. Cry1Ab values of soil under grassland management at the field site Grub were below CCα of 2.0 ng of Cry1Ab g^{-1} of soil in both manuring variants showing that the transgenic protein applied with the slurry is degraded to nondetectable levels in this soil. For soil samples collected at the Finsing field site, values exceeding CCα of 2.0 ng of Cry1Ab g^{-1} of soil were determined on the lots treated with slurry from non-transgenic and transgenic origin. This can be due to a cross reaction of the polyclonal antibody used in the ELISA with organic substances in this soil horizon, which is rich in fine grass roots and organic material. Natural organic matter and in particular humic substances were identified as commonly interfering substances when environmental samples were analyzed by ELISA before.[43−45] Beyond the finding that there is no significant difference in soil samples of Finsing undergoing each slurry treatment, further methodological development is necessary to give final evidence for the presence or absence of Cry1Ab protein in this special soil matrix rich in organic substances. In summary the absence of detectable Cry1Ab in the soil samples indicates that, after their release from the conserving environment in liquid manure,[46] Cry1Ab in undigested GM plant residues will undergo a fast degradation in the well-aerated and microbially active soil matrix. These results are consistent with the rapid biodegradation reported for Cry1Ab introduced directly into the soil compartment by Bt maize plant residues after harvest.[16,18]

3.3.4. Cry1Ab Contents in Grass. Grass crop was sampled at four cutting dates keeping a minimum time interval of 28 days to the preceding manure application as commonly practiced in grassland management. All grass samples analyzed were below CCα of 1.4 ng of Cry1Ab g^{-1} of plant material investigated in this study (Table 5). Thus, no Cry1Ab was detected in any of the grass samples collected from grassland lots treated with slurry from cows fed GM maize or from the control group receiving non-transgenic feed.

3.3.5. Cry1Ab Contents in Maize. In the field trial, GM maize Kuratus and the corresponding near-isogenic variety Gavott were grown and manured twice during the vegetation period using slurry from feeding with non-transgenic or transgenic feed ration. Analysis of chopped plant material revealed that all near-isogenic maize samples were below the CCα of 1.4 ng of Cry1Ab g^{-1} of plant material irrespective of the origin of liquid manure applied to the plants (Figure 3). This implies that no residual traces of the transgenic protein from undigested GM maize plant material in liquid manure were dispersed to and persisted during the growth of the maize plants. According to these findings conventional maize plants harvested from fields manured with slurry containing traceable amounts of Cry1Ab originating from GM maize feed can be regarded as free of Cry1Ab toxin. These results are important regarding concerns about Cry1Ab protein remains on crops grown in soils, in which Bt-maize has been cultivated before. It was demonstrated earlier that Cry1Ab released to soil in root exudates of Bt-maize, from the degradation of the biomass of Bt-maize, or as purified protein, was not taken up from soil or from hydroponic culture by conventional maize plants.[47]

Analysis of the GM maize plants revealed mean Cry1Ab concentrations ranging from 6.7 to 8.4 μg of Cry1Ab g^{-1} of plant material. This is consistent with data on Cry1Ab protein contents of GM maize MON810 plants reported before.[48]

3.4. Degradation of Cry1Ab Protein in Relation to Total Protein Contents. Cry1Ab concentrations of PTMR, liquid manure and maize plants of transgenic origin were also calculated on the base of the respective total protein contents to investigate the degradation of Cry1Ab in comparison to the entirety of other proteins.

Total protein contents of the chaffed maize plants were found to be between 16.2 and 18.2 mg of total protein g^{-1} of crop wet weight without significant differences between field sites, GM and near-isogenic variety and origin of liquid manure applied to maize lots (P = 0.05). The mean Cry1Ab concentrations in maize plants were in the range of 391 to 499 μg of Cry1Ab g^{-1} of total

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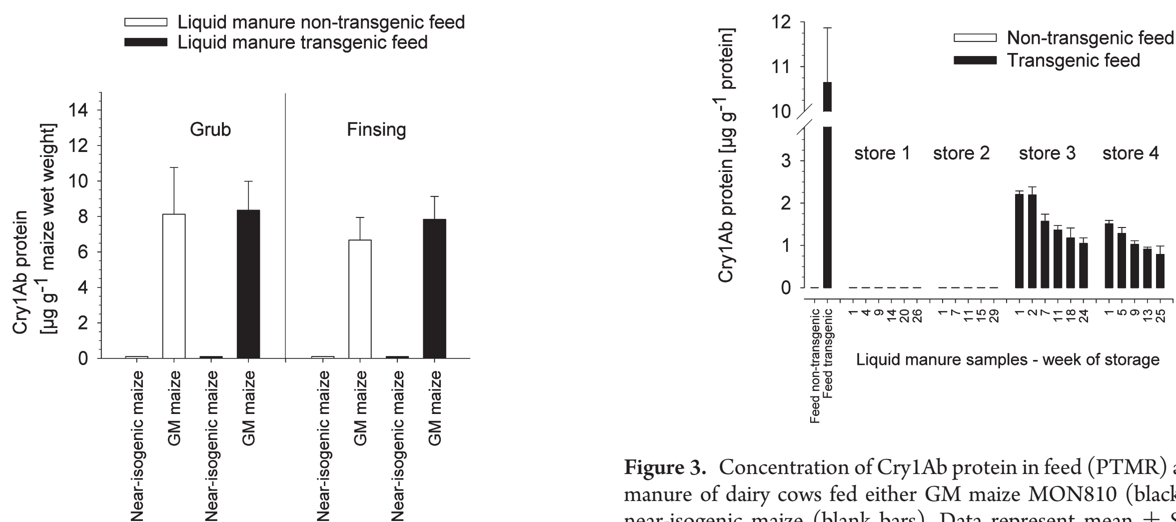

Figure 2. Cry1Ab contents in GM maize and near-isogenic maize plants harvested from experimental field lots under slurry management when feeding near-isogenic maize (blank bars) and GM maize MON810 (black bars).

Figure 3. Concentration of Cry1Ab protein in feed (PTMR) and liquid manure of dairy cows fed either GM maize MON810 (black bars) or near-isogenic maize (blank bars). Data represent mean ± SD values ($n = 4$) in μg of Cry1Ab protein g^{-1} of total protein in PTMR and liquid manure after successive time of storage.

protein with a mean level of 458 μg of Cry1Ab g^{-1} of total protein (Table 5). The corresponding concentration in the transgenic feed ration was 10.6 μg of Cry1Ab g^{-1} of total protein in the PTMR. Thus, in relation to the initial concentrations of the transgenic protein in GM maize MON810 plants, only 2.3% of Cry1Ab g^{-1} of protein was present in transgenic PTMR. This decline can be explained both by the addition of up to 40% of conventional feed components to the mixed feed ration[22] and by the ensiling and processing of maize plants to feed leading to heat denaturation and disintegration of the Cry1Ab protein.[30,49,50]

In liquid manure, mean levels of 2.0 μg of Cry1Ab g^{-1} of total protein were observed at the time point of filling the slurry stores (Figure 3). Accordingly, the Cry1Ab concentration in liquid manure is decreased to 18% of the initial value of 10.6 μg of Cry1Ab g^{-1} of total protein reported in the transgenic PTMR. A previous study[28] reported consistent findings with a Cry1Ab concentration decline of 44% when feed containing GM maize MON810 and freshly collected feces were analyzed. The decline observed in both studies can be explained by two reasons: First, additional protein of animal and microbial origin is added to the feed protein during the passage through the bovine gastrointestinal tract (GIT). Second, the marked reduction of Cry1Ab protein in relation to total protein points to a faster degradation of Cry1Ab in the GIT compared to the rest of the total protein, suggesting that Cry1Ab exhibits no greater stability compared to the entirety of other proteins in feed. Analysis of the Cry1Ab concentrations in liquid manure samples with increasing time of storage showed a further decline of Cry1Ab g^{-1} of total protein (Figure 3). Within 23 weeks of storage decreasing concentrations of 2.20, 2.19, 1.57, 1.36, 1.18, and 1.05 were reported in slurry store 3 and a decline over 1.51, 1.28, 1.02, 0.91, and 0.79 μg of Cry1Ab g^{-1} of total protein within 24 weeks in slurry store 4. On average, the Cry1Ab concentrations were 50% of the initial levels of Cry1Ab g^{-1} of total protein after 23 or 24 weeks of storage. Compared to this, total protein levels, averaged over the four slurry tanks, declined from 12.4 ± 1.3 to 9.3 ± 1.1 mg of protein g^{-1} wet slurry corresponding to a mean loss of 25% of the total protein during storage. These findings point out that, in comparison to total protein in liquid manure, Cry1Ab protein undergoes an even faster degradation process and can be assigned to the less stable group of proteins in liquid manure.

3.5. Quantitative Balance of Cry1Ab Protein Turnover in Feeding and Liquid Manure Management. The data collected in the study allow for balancing of Cry1Ab contents over the whole pathway from GM maize MON810 crop over feed and liquid manure into soil and the following crop. The quantitative turnover of the recombinant Cry1Ab protein and its immunoreactive fragments is summarized in Table 5. The major fraction of Cry1Ab is degraded by the processing of the GM maize plants to feed, as only 2.6% of the novel protein determined in plant material was recovered in the transgenic feed ration. Digestive processes during the GIT passage led to a further reduction of Cry1Ab levels in a way that 0.9% of the Cry1Ab content in the GM maize plant was detected in liquid manure. Half of this residual immunoactive Cry1Ab in slurry persisted during slurry storage for 25 weeks. When applied to agricultural soil, final degradation of Cry1Ab protein to below detectable levels in soil was reported. Cry1Ab concentrations in relation to total protein give further information about the degradation process and elucidate the proteolytic stability of the novel protein and its immunoactive fragments compared to other proteins. The overall decline of the Cry1Ab concentrations related to total protein (Table 5, column 6) suggests that Cry1Ab protein exhibits a lower stability compared to the entirety of other proteins in the GM maize plant and the feed components.

3.6. Western Blot Analysis. By Western blot analysis, no Cry1Ab protein specific protein bands could be detected in maize plants of the near-isogenic variety. The investigation of GM maize plant material, transgenic feed and liquid manure by immunoblot analyses revealed a fragmentation of the full-sized (65 kDa) Cry1Ab protein into smaller immunoreactive fragments in each of the analyzed materials.

In all of the GM maize MON810 plant material, Cry1Ab protein fragments of 42 kDa, 34 kDa and 17 kDa are present in addition to the full-sized 65 kDa protein (Figure 4). However, the smallest 17 kDa fragment was not detected in leaves of BBCH14 plants, and signals for this fragment detected in leaves of BBCH69 and mature BBCH85 plants were weak compared to the more prominent bands of higher molecular weight Cry1Ab

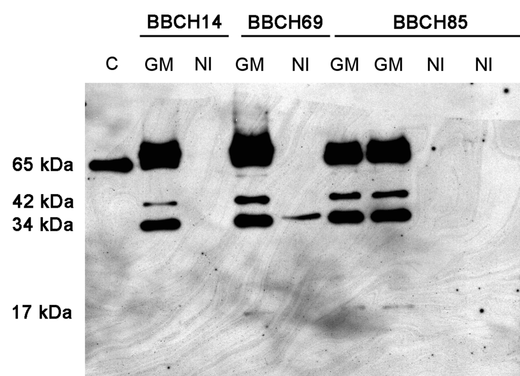

Figure 4. Western blot analysis of Cry1Ab protein in maize plants. Immunoreactive Cry1Ab protein fragments of genetically modified (GM) and near-isogenic (NI) maize plants in successive developmental stages of BBCH14, BBCH69 (plant leaves) and BBCH85 (chaffed maize crop) are presented. 500 pg of trypsin activated HPLC purified Cry1Ab protein served as positive control (C).

fragments. The fragmentation pattern looks similar in the developmental stages of BBCH14, BBCH69 and BBCH86 suggesting a continuous synthesis and degradation of Cry1Ab in the GM plant. These findings are in accordance with studies using Cry1Ab purified from GM maize plants for in vitro degradation experiments. There, a fragmentation of the recombinant Cry1Ab plant protein into fragments of molecular size between 20 and 70 kDa was reported before addition of pepsin as the proteolytic agent.[38] The observation that Cry1Ab produced by the GM plant is partially degraded in any stage of development indicates a continuous breakdown of the recombinant Cry1Ab protein by endogenous plant proteases.

In transgenic feed, the full sized Cry1Ab protein and immunoactive fragments of 42 kDa, 34 kDa and 17 kDa were detected by immunoblot analyses (Figure 5), whereas none of these fragments were recorded in non-transgenic feed. The closely related pattern of Cry1Ab protein fragmentation in mature transgenic maize plants and processed feed indicates that the degradation of the recombinant Cry1Ab during feed processing affects immunoactive fragments of any size. Accordingly, the 65 kDa protein and the 42 kDa, 34 kDa and 17 kDa immunoactive fragments were detected by blot analyses in transgenic maize kernels and transgenic maize cobs,[28] which were components of the total mixed ration fed to the cows in the long-term feeding study using GM maize MON810. However, in this study, no 17 kDa could be detected in PTMR as the concentrations of this fragment in the PTMR were too low to be detected by Western blot analyses.

Immunoblot analysis of liquid manure obtained from cows fed GM maize MON810 revealed Cry1Ab protein fragments in the size of 65 kDa and 34 kDa (Figure 6), whereas the 42 kDa fragment detected in GM plant and feed samples was not recorded in liquid manure. These findings indicate a progressive microbial and enzymatic proteolysis of the Cry1Ab protein fragments in the bovine digestive tract. Previous reports of Cry1Ab degradation in the bovine digestive tract are in accordance with the Cry1Ab fragmentation patterns shown in the present study.[28,29,31] During slurry storage further degradation of the 65 kDa and 34 kDa immunoactive Cry1Ab protein fragments takes place as can be deduced from decreasing band intensities with proceeding time of storage. After 24 weeks of storage the

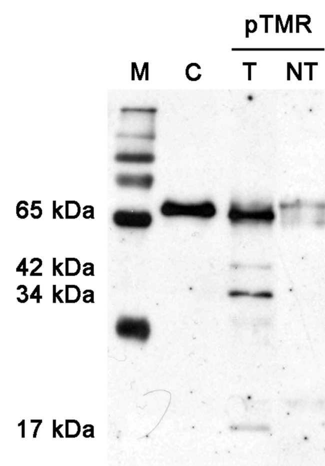

Figure 5. Western blot showing immunoactive Cry1Ab protein fragments in feed. Protein extracts of partial total mixed ration of transgenic (T) and non-transgenic (NT) origin were analyzed. Trypsin treated and HPLC purified Cry1Ab protein (100 pg) was included as a positive control (C).

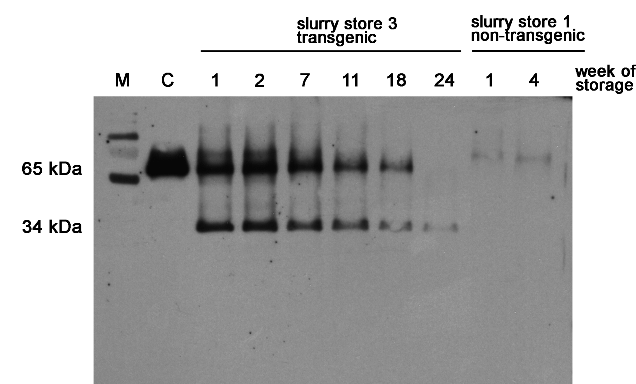

Figure 6. Western blot showing immunoreactive Cry1Ab protein fragments in liquid manure from GM maize MON810 fed cows with proceeding time of storage. Samples from slurry store 3 show a degradation of Cry1Ab protein in liquid manure of the GM maize fed animals from week 1 to 24. Liquid manure samples from the control group fed non-transgenic ration (store 1) was analyzed at weeks 1 and 4. As a positive control (C), 500 pg of trypsin treated and HPLC purified Cry1Ab protein was used.

65 kDa Cry1Ab protein was not detected any more, suggesting a gradual degradation into the smaller 34 kDa fragment. This 34 kDa fragment was reported to be the most prominent immunoactive fragment in transgenic feed, digesta and feces before,[28] which could be a sign of a higher proteolytic stability of this degradation product. The fragmentation pattern of the Cry1Ab protein in addition to Cry1Ab quantity is an important criterion regarding effects of Cry1Ab introduced into the environment by agricultural processes. It should be considered that the insecticidal pathway of the recombinant Cry1Ab is closely related to the presence of all functional domains of the whole trypsin resistant 65 kDa protein.[5,51−53] To our knowledge toxic activity of Cry1Ab fragments smaller than 65 kDa has not been reported so far. Finally, the presence of Cry1Ab or Cry1Ab fragments exhibiting insecticidal activity can only be determined using sensitive organism species in biotoxicity assays at present.

In conclusion, the field trial keeps record about the extensive and, compared to other proteins, rapid degradation of recombinant Cry1Ab protein during processes in the liquid manure management of a dairy farm feeding GM maize (event MON810), leading to nondetectable levels in soil and the following crop.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +49. 8161 714082. Fax: +49. 8161,715227. E-mail: martin.mueller@lfl.bayern.de.

Present Addresses

[§]Bavarian Health and Food Safety Authority, Veterinärstr. 2, Oberschleissheim, Germany.
[‖]National Research Centre on Yak, (ICAR), Dirang-790101, West Kameng District Arunachal Pradesh, India.
[#]Biotechnology Institute, Czech Academy of Science, Prague, Czech Republic.

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2 Investigation of cow liquid manure for the presence of *cry1Ab* DNA after feeding genetically modified maize
3 (MON810)

4

5 (*cry1Ab* DNA - liquid manure)

6

7 Helga Gruber[a,b,2], Patrick Guertler[b,2], Heinrich H.D. Meyer[b], Martin Müller[a,*]

8

9 [a]Bavarian State Research Center for Agriculture, Institute for Crop Science and Plant Breeding, Am Gereuth 8,
10 85354 Freising, Germany

11 [b]Physiology Weihenstephan, Technische Universität München, Weihenstephaner Berg 3, 85354 Freising,
12 Germany

13 [2]Present address: Bavarian Health and Food Safety Authority, Veterinärstr. 2, 85764 Oberschleißheim, Germany

14

15

16

17

18 *Corresponding author and present address: Martin Müller, Bavarian State Research Center for Agriculture,
19 Institute for Crop Science and Plant Breeding, Am Gereuth 8, Freising, Germany, Phone: +49 8161 714082, Fax:
20 +49. 8161,715227, martin.mueller@lfl.bayern.de

21

22 Key words

23 Bt-maize MON810, *cry1Ab* DNA, dairy cow, liquid manure, PCR

24

25

Abstract

The potential entry of *cry1Ab* DNA fragments into soil by liquid manure from cows fed transgenic maize MON810 was addressed in the present study. Five cows each were fed a partial total mixed ration containing maize MON810 or nearly isogenic non-genetically modified maize. Slurry produced was collected, stored and analysed for the presence of plant derived *rubisco* and maize specific *opaque-2* DNA fragments, as well as a 206 bp *cry1Ab* gene fragment by qualitative endpoint PCR. The limit of detection (LOD) for the single copy genes *opaque-2* and *cry1Ab* was determined by spiking experiments. Analysis of 22 liquid manure samples revealed the presence of a 173 bp fragment of the multicopy gene *rubisco*, but absence of *opaque-2* at the LOD in all samples. The 206 bp *cry1Ab* fragment was present in transgenic feed but not detectable in any of the slurry samples at the LOD of 1,000 copies of *cry1Ab* DNA g^{-1} liquid manure.

Introduction

One of the most common genetically modified (GM) plants grown worldwide is Bt-maize, engineered to express insecticidal crystal (Cry) proteins by insertion of *cry* genes from *Bacillus thuringiensis* (Whiteley and Schnepf 1986) into the plants' genome. Bt-maize (MON810) cultivars exhibit specific insect resistance to the European corn borer (*Ostrinia nubilalis*, Hübner) whose larvae are affected by the Cry1Ab protein when feeding on Bt-maize plants (Koziel et al. 1993). Since the commercial release of those GM plants into the feed market in 1996, public concerns are expressed about the fate of the recombinant DNA and the insecticidal Cry1Ab protein, when Bt maize is fed to livestock for human consumption. Furthermore the release of recombinant DNA and protein from GM plants into the environment, leading to the exposition of target and non-target organisms to these novel molecules and potential impacts onto these organisms are discussed controversially (Marvier et al. 2007; Mendelsohn et al. 2003; Rauschen 2010; Schmidt et al. 2009).

Regarding the entry of GM plant DNA into the soil compartment, a potential horizontal gene transfer of transgenic plant DNA to soil borne bacteria is widely discussed (Nielsen et al. 2001). So far, no evidence for such a putative transfer exists from field investigations using marker genes from transgenic plants and indigenous soil and plant-associated bacteria (Becker et al. 1994; Gebhard and Smalla 1998; Paget et al. 1998). However, horizontal gene transfer of transgenes is basically possible, as shown in laboratory studies exposing naturally transformable *Acinetobacter* sp. as recipient bacteria to purified DNA of transgenic plants such as sugar-beet, tomato, potato or oilseed rape. In these studies, bacterial capture of plant DNA fragments was reported, though under artificial conditions and at very low frequencies (de Vries and Wackernagel 1998; Gebhard and Smalla 1998; Nielsen et al. 2000).

The digestive fate of *cry1Ab* DNA in the cows gastrointestinal tract (GIT) has been addressed in a range of studies (Chowdhury et al. 2004; Einspanier et al. 2001; Einspanier et al. 2004; Wiedemann et al. 2006). These studies give report about the progressive and rapid degradation of plant-derived and transgenic DNA throughout the digestive tract leading to non-detectable levels of recombinant DNA in fecal samples. The findings were further confirmed after long term feeding of Bt maize (event MON810), as none of the dairy cows fecal and urine samples was found positive for the presence of novel DNA (Guertler et al. 2010).

The entry, accumulation and persistence of the recombinant Cry 1Ab protein into soil through maize cultivation has been analyzed before and it can be stated that even nine year subsequent Bt maize cropping does not lead to

accumulation and persistence of the Bt protein in soil (Gruber et al. 2011a). To further assess the putative entry of recombinant Cry1Ab protein molecules or recombinant DNA fragments into agricultural soil by way of liquid manure under field conditions, an associated study was performed investigating liquid manure obtained from the cows of the above mentioned long term feeding trial. Regarding the recombinant protein, minor levels of the Cry1Ab protein were detected in the slurry and were consequently applied to agricultural fields, where its rapid degradation in the soil compartment could be observed, as no Cry1Ab protein was detected in the manured soil (Gruber et al. 2011b submitted).

To complete this work with regard to the recombinant DNA, the present study refers to the analysis of transgenic and non-transgenic feed and the corresponding liquid manure for the presence of plant derived and transgenic *cry1Ab* DNA.

In Bt maize (MON810) plants, the *cry1Ab* gene sequence is present in one copy per haploid genome (Holck et al. 2002). For assessment of the degradational behaviour of this single copy *cry1Ab* gene fragment, the maize plant derived zein regulatory gene *opaque*-2 (O2) (Maddaloni et al. 1989) was chosen as a single copy control gene. Comparatively, fragments of the plant specific multicopy gene *rubisco* (ribulose-1,5-bisphosphate carboxylase/Oxygenase) were detected to follow the fate of plant derived multicopy gene DNA when it is degraded in the digestive tract and in the obtained slurry stored for use as liquid manure. Small DNA gene fragments in the range of 200 bp were chosen for specific detection of the target genes. By this both potential full-sized functional genes and their degradation products are captured in the analysis.
The main objective of this study was to eludicate the likelihood of introducing recombinant *cry1Ab* DNA gene fragments to agricultural fields by way of liquid manure from cows fed gm maize. For this purpose qualitative end-point PCR was applied to feed and liquid manure samples after feeding Bt-maize (MON810) to dairy cows in order to amplify fragments of the recombinant *cry1Ab* gene and plant derived genes.

Materials and Methods

Animals and feeding

The study was performed under the approval of the Bavarian State Research Center (LfL, Grub, Germany). Ten dairy cows out of a long-term GM maize feeding experiment were used for the presented experimental liquid manure trial. The detailed design of the long-term GM maize feeding experiment and of the feed composition in the daily diet of the dairy cows is outlined in previous studies (Guertler et al. 2009; Steinke et al. 2010). Shortly, the target group was fed on a partial total mixed ration (PTMR) containing 63 % genetically modified corn (MON810, Kuratus, as grain, corn stem pellets and silage), whereas the control group was fed on a substantially equivalent PTMR based on the non-transgenic variety (Gavott). For the collection of liquid manure, five cows each of each group were kept in a stable equipped with special slurry sampling facilities for a time period of six days. The entire stall manure of each group was collected and pooled. Storage was carried out in slurry stores according to agricultural practice at ambient temperature for further experimentation (store 1 and 2: control group, near isogenic feed ration; store 3 and 4: target group fed transgenic feed ration).

Sampling procedures

Representative PTMR feed samples of the transgenic and the non-transgenic diet were collected daily within the period of liquid manure retrieval from the cows, were subsequently pooled and ground finely in liquid nitrogen using mortar and pestle.

Liquid manure samples were collected from slurry stores at the beginning of storage and at defined time intervals of slurry storage (Table 1). Each sample was pooled from six subsamples taken with a 35 mm diameter sampling tube after exhaustive homogenization of the slurry from the tank.

PTMR feed and liquid manure samples were stored at −20 °C until used for DNA extraction.

Table 1

DNA extraction

The extraction procedure developed by (Guertler et al. 2009) was slightly modified and applied to isolate genomic DNA from all liquid manure and PTMR feed samples. Liquid manure or ground feed (300 mg fresh weight) was used for DNA extraction and filled into FastPrep® tubes containing 300 mg matrix beads (Lysing Matrix D, MP Biomedicals, Eschwege, Germany). After addition of CTAB extraction buffer, the mixture was homogenized by applying the bead beating FastPrep® technique (FP120, MP Biomedicals, Eschwege, Germany) at 5.5 m s^{-1} for 30 s twice. 40 µL Proteinase K (20 mg mL^{-1}) were added and the samples were incubated on a shaker over night at 60 °C. Further, a centrifugation step at 3,000×g and 4 °C for 5 min was performed and the supernatant was transferred into a new tube. After RNA digestion by addition of 5 µl RNase A (10 mg mL^{-1}), incubation for 15 min at 60°C on a shaker and subsequent centrifugation for 1 min at 16,000xg, the supernatant was filled into a new tube. Chloroform (600 µL) was added, followed by vigorous shaking and centrifugation for 10 min at 16,000×g and 4 °C. The upper watery phase was applied to 500 µL 2-propanol (80%) and 2 µL glycogen (20 mg mL^{-1}, Sigma, Munich, Germany). The samples were incubated for 1 h at room temperature and subsequently centrifuged at 16,000×g and 4°C for 10 min. The DNA pellet was washed in 500 µl EtOH (70%) and loaded on a Wizard® SV Column (Promega, Mannheim, Germany). The column was centrifuged and washed using 2-propanol (80%). Finally, 50 µL of prewarmed Tris–HCl (10 mM, pH=9.0, 70 °C) was used for DNA elution.

DNA concentration of all samples was determined by using a Nanodrop 1000 (Thermo Scientific). Additionally, the DNA purity was assessed by using the UV absorption ratio of 260/280 nm.

PCR analysis

Qualitative end-point PCR was performed using 100 ng genomic DNA to test for the presence of a 206 bp fragment of *cry1Ab* (Accession No. AY326434) in PTMR and liquid manure samples. As a positive DNA extraction control, a 365 bp fragment of ribosomal *18S* RNA was amplified. Additionally a 173 bp fragment of chloroplast specific *rubisco* (Accession No. X86563) and a 202 bp fragment of the maize specific single copy *opaque-2* gene (Accession No. X15544) was amplified as a positive control. Primer sequences are given in table 2. Cycling conditions for amplification of the *opaque-2* gene fragment were 95°C-5 min; 30 cycles (95°C-30 sec; 55°C-30 sec;72°C-30 sec) 72°C-5 min. The cycling conditions for amplification of the other three gene fragments are summarized in (Guertler et al. 2008). Isolated DNA from GM maize grain (MON810) served as a

144 positive control for *cry1Ab*, *opaque-2* and *rubisco*. The PCR products were separated by gelelectrophoresis and
145 visualized by staining with ethidiumbromide.
146
147 Table 2
148
149 Spiking procedure for determination of limits of detection
150 Liquid manure samples from cows fed non-transgenic maize were used in spiking experiments by adding
151 different concentrations of genomic DNA isolated from Bt maize MON810 grain to check for PCR inhibition
152 and to determine the limit of detection (LOD). The added DNA corresponded to a range of 1 copy to 100,000
153 copies *opaque-2* or *cry1Ab* DNA per 10 mg of liquid manure. One liquid manure sample was assessed without
154 adding genomic Bt-maize DNA and used as a non-template control (NTC). The LOD is presented as the lowest
155 detectable number of copies g^{-1} liquid manure after amplification through qualitative end-point PCR.
156
157 Results
158 Feed analysis
159
160 DNA extraction from feed resulted in concentrations of 360 ng DNA μL^{-1} of isolate for non-transgenic and 382
161 ng DNA μL^{-1} for transgenic PTMR. The photometrical analysis revealed 260/280 nm ratios between 1.4 and 1.8,
162 a DNA purity suitable for PCR analysis.
163 The results of the qualitative endpoint PCR analysis of the PTMR samples are shown in figure 1. Gene
164 fragments of the multicopy genes *rubisco* and *18S* were amplified in isolates of all PTMR samples both of non-
165 transgenic and transgenic origin. Similarly, the 202 bp fragment of the maize specific single copy gene *opaque-2*
166 was detected in both feed variants. The 206 bp *cry1Ab* gene fragment was not detected in PTMR feed samples
167 based on non-transgenic maize. In the feed ration containing Bt-maize (MON810) the *cry1Ab* DNA fragment
168 was clearly present as shown by the specific 206 bp amplicon on the gel.
169
170 Figure 1
171
172 Liquid manure analysis
173 DNA extraction from liquid manure resulted in concentrations ranging from 21.3 to 86.8 ng DNA μL^{-1} isolate.
174 The photometrical analysis revealed a DNA purity suitable for PCR analysis, as 260/280 nm ratios were between
175 1.4 and 1.8.
176 For the limit of detection (LOD) of assessed target genes in liquid manure samples, only copy numbers of single
177 copy genes were determined by means of qualitative endpoint PCR. In the control liquid manure sample assessed
178 without adding genomic Bt-maize DNA, neither the *cry1Ab* gene fragment nor the maize specific gene fragment
179 of the single copy gene *opaque-2* was amplified. In the spiking experiment, a LOD of 1,000 copies of *cry1Ab*
180 DNA g^{-1} liquid manure and 100,000 copies of *opaque-2* DNA g^{-1} liquid manure were determined (data not
181 shown).
182 The plant specific multicopy gene *rubisco* and the eukaryotic multicopy gene *18S* were detected in all samples
183 including the unspiked control.

184 Figure 2 depicts the results of PCR-analysis of liquid manure samples collected from dairy cows after long term
185 feeding of Bt maize MON810 or non-transgenic maize. The samples were collected and analysed with
186 proceeding time of storage. Gene fragments of the multicopy genes *18S* (365 bp amplicon) and *rubisco* (173 bp
187 amplicon) were detected in all liquid manure samples from the first to the 29th week of storage (Fig 2 A + B).
188 The 202 bp maize specific single copy *opaque-2* gene fragment was not detected in any slurry sample at the
189 LOD of 100,000 copies g^{-1} (Fig 2 C). No liquid manure sample was found positive for *cry1Ab* DNA at the LOD
190 of 1,000 copies g^{-1} in the slurry collected from cows fed transgenic or non-transgenic maize (Fig 2 D).
191

192 Figure 2

193

194 Discussion

195 The present study was accomplished to test liquid manure derived from GM maize MON810 fed cows for the
196 presence of plant-specific and GM maize specific *cry1Ab* gene fragments. The trial was performed to eludicate
197 the possibility of recombinant *cry1Ab* DNA being applied to agricultural fields by the liquid manuring practice
198 of a GM maize feeding dairy farm.

199 Liquid manure samples were analysed for the presence of a small *cry1Ab* gene fragment of 206 bp size using a
200 sensitive and well-established conventional PCR method. Its suitability for the investigated slurry matrix was
201 proven by determination of the LOD for the 206 bp *cry1Ab* gene fragment, which is in line with its LOD
202 reported earlier for bovine feces (Guertler et al. 2010).

203 In our study, the small *cry1Ab* gene fragment was present in transgenic maize feed but was not detectable in
204 liquid manure of the cows fed the transgenic PTMR. The same finding was revealed for the maize specific
205 *opaque-2* gene fragment. Together this confirms earlier studies giving report about extensive time-dependent
206 degradation of plant specific as well as novel DNA during the passage of feed through the bovine gastrointestinal
207 tract (Chowdhury et al. 2004; Einspanier et al. 2001; Guertler et al. 2010; Wiedemann et al. 2006).

208 The *cry1Ab* and *opaque-2* gene fragments could not be amplified from slurry, whereas a 173 bp amplicon of the
209 multicopy gene *rubisco* was detectable in all liquid manure samples. This result is in line with Guertler *et al.*
210 2010 giving report about the presence of small *rubisco* gene fragments in bovine feces samples, but absence of
211 the 206 bp *cry1Ab* fragment.

212 Basically, the ability to detect fragments of plant genes in matrices of the gastrointestinal tract mainly depends
213 on their copy number, provided that other detection parameters, e.g. primer quality, are comparable. According
214 to Artim *et al.* (2001) the copy number of a multicopy gene may add up to the 10,000 fold of the single-copy of
215 e.g. the *opaque-2* gene or the *cry1Ab* transgene present per cell (Artim et al. 2001; Phipps et al. 2003). As
216 *rubisco* is a gene abundant in a high copy number in the plant genome (Gutteridge and Gatenby 1995), there is a
217 higher probability that small fragments of the *rubisco* DNA gene copies are detected by PCR in liquid manure
218 than for the single copy *cry1Ab* gene. Our study confirms this suggestion, as the maize specific single copy gene
219 *opaque-2* was also not detectable by qualitative endpoint PCR in the slurry samples. Generally, it can be
220 assumed, that during passage through the GIT, biomolecules like protein or DNA may be protected from
221 enzymatic degradation in heavy digestible plant material, leading to the presence of plant feed born protein or

222 DNA fragments in feces or liquid manure. Regarding the insecticidal Cry1Ab protein from maize (MON810)
223 feed, it was shown that the protein is detectable at low levels in feces and liquid manure (Gruber et al. 2011b
224 submitted; Paul et al. 2010). In contrast to this, there was no accordant evidence for the presence of recombinant
225 *cry1Ab* DNA fragments in bovine feces (Guertler et al. 2010) or liquid manure (this study) after feeding maize
226 (MON810).

227 In conclusion, the results of the present study indicate that *cry1Ab* DNA from maize MON810 is efficiently
228 degraded during dairy cow digestion leading to non-detectable levels in liquid manure applied to agricultural
229 fields.

230

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Table 1: Slurry samples collected with proceeding storage time in course of the experimental liquid manure field trial

origin of feed	non-transgenic maize ration											transgenic maize ration										
store	**1**						**2**					**3**						**4**				
sample nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
storage week	1	4	9	14	20	26	1	7	11	15	29	1	2	7	11	18	24	1	5	9	13	25

Table 2. Primer sequences as used for the DNA analysis and fragment size of the amplicons

target gene	fragment size (bp)	primer sequences	reference
cry1Ab	206	For: 5′-CCT GGA GAA CTT CGA CGG TA-3′ Rev: 5′-TCG TGC CGT AGA GAG GAA AG-3′	(Guertler et al. 2008)
rubisco	173	For: 5′-AGC TAA TCG TGT GGC TTT AGA AGC C-3′ Rev: 5′-TGG TAT CCA TCG CTT TGA AAC CA-3′	(Wiedemann et al. 2006)
opaque-2	202	For: 5’-CTGGAGAAAGAGTGCCCTTG -3’ Rev: 5’-TGGCTAACCGGTTTACGTTC-3’	this study
18S	365	For: 5′-AAG TCT TTG GGT TCC GGG-3′ Rev: 5′-GGA CAT CTA AGG GCA TCA CA-3′	(Guertler et al. 2008)

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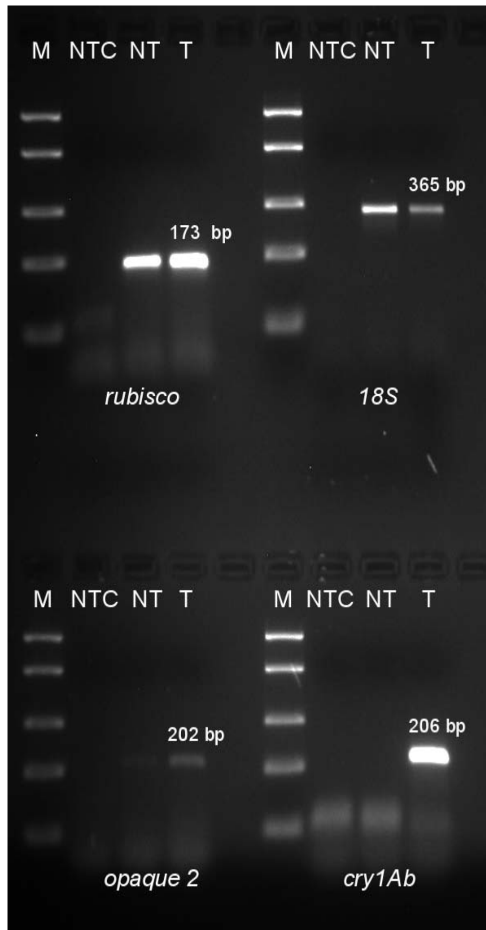

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323 Figure 1: PCR amplification products of *rubisco* and *18S, opaque-2* and *cry1Ab* DNA in non-transgenic or
324 transgenic feed ration. M: PeqLab-Low Range Marker (50, 200, 400, 850, 1500 bp), NTC: non-template control
325 (H_2O), NT: non-transgenic PTMR, T: transgenic PTMR.

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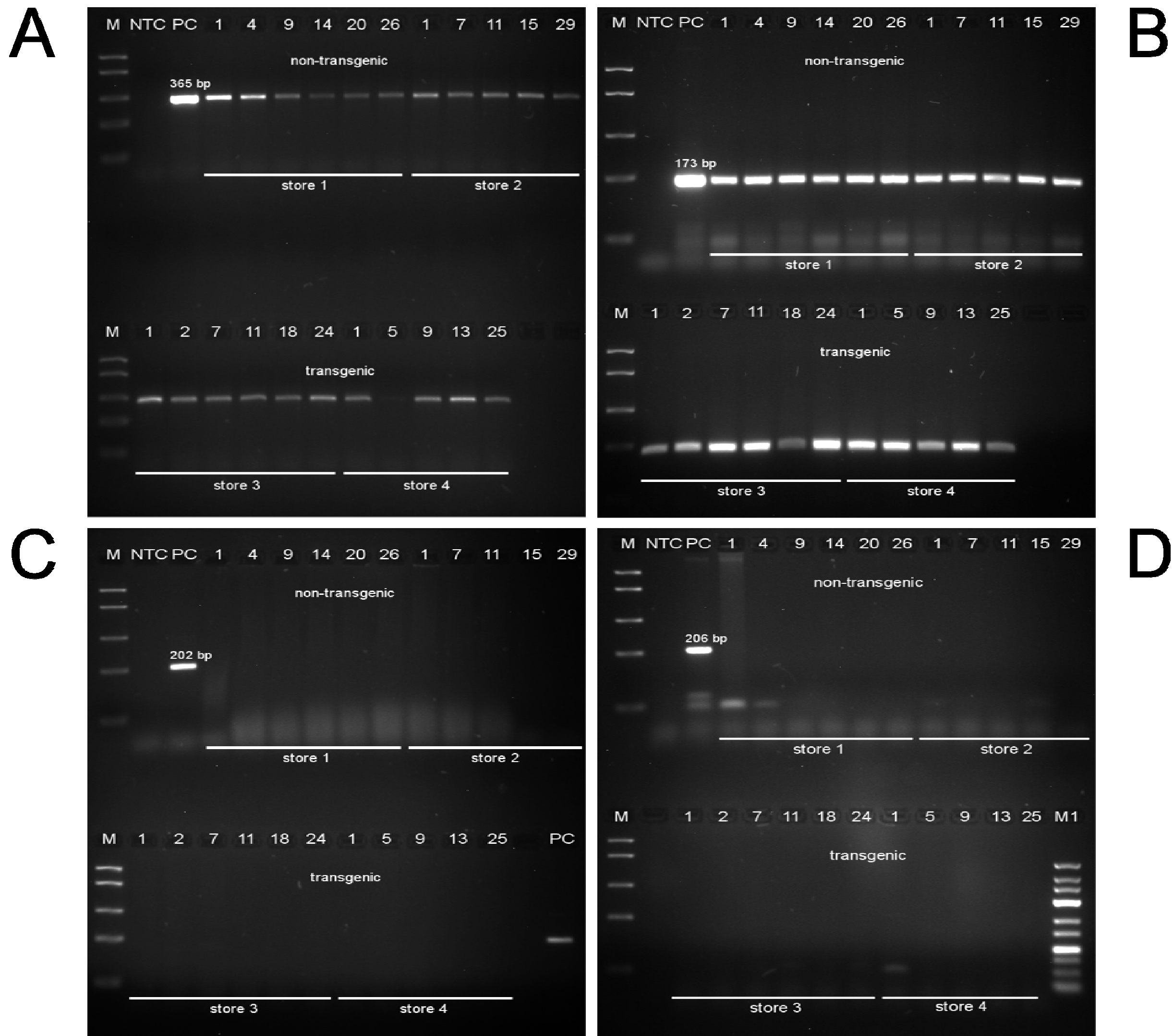

Figure 2: PCR amplification products of *18S* (A), *rubisco* (B), *opaque-2* (C) and *cry1Ab MON810* (D) DNA in liquid manure obtained from dairy cows fed either non-transgenic or transgenic maize. In the upper part of each gel: Slurry store 1 and 2, liquid manure from control group fed non-transgenic PTMR. In the lower part of each gel: Slurry store 3 and 4, liquid manure from cows fed transgenic PTMR. Numbers indicate weeks of slurry storage before analysis. M: 50 - 1,500 bp PeqLab-Low Range Marker, NTC: non-template control (H_2O or DNA from near isogenic maize grain in D); PC: positive control (DNA from Bt maize MON810 grain), M1: 25 - 700 bp PeqLab-Ultra Low Range Marker.