

ORIGINAL ARTICLE

Degradation of transgenic Cry1Ab DNA and protein in Bt-176 maize during the ensiling process

B. Lutz, S. Wiedemann and C. Albrecht

Department of Physiology Weihenstephan, Technical University Munich, Weihenstephaner Berg 3, Freising, Germany

Correspondence

Christiane Albrecht, Physiology
Weihenstephan, Technical University Munich,
Weihenstephaner Berg 3, D-85354 Freising,
Germany. Tel: +49 8161 71 3994;
Fax: +49 8161 71 4421;
E-mail: christiane.albrecht@wzw.tum.de

Received: 15 September 2004;

accepted: 16 March 2005

First published online: 27 September 2005

Summary

Maize silage is commonly used as feed for farm animals. The aim of this study was to monitor the time-dependent degradation of non-recombinant chloroplast DNA (exemplified by the *rubisco* gene) in comparison with the recombinant *cry1Ab* gene in the course of the ensiling process. In parallel, the Cry1Ab protein content and fragment sizes were determined. Fragments of the *rubisco* (173, 896, 1197, 1753 and 2521 bp) and of the *cry1Ab* gene (211, 420, 727 and 1423 bp) were selected to investigate the DNA degradation process. The detection of the Cry1Ab protein was performed using an enzyme-linked immunosorbent assay (ELISA) and immunoblotting. *Rubisco* gene fragments of 173 bp were still detectable after 61 days, while fragments of 1197 and 2521 bp were detectable up to 30 days and on the first day only respectively. Polymerase chain reaction (PCR) analyses revealed that fragments of the *cry1Ab* gene with sizes of 211 and 420 bp were detectable up to 61 days, fragments with sizes of 727 and 1423 bp, 30 and 6 days respectively. The ELISA showed a decrease of the Cry1Ab protein in maize silage during the ensiling process. No marked degradation was observed during the first 43 h. Thereafter, a sharp decrease was measured. After 61 days, $23.6 \pm 0.9\%$ of the initial Cry1Ab protein was still detectable. Immunoblotting confirmed the results of the ELISA showing a positive signal of approximately 60 kDa size for 8 days of ensiling; no further immunoactive fragments were detectable by immunoblotting. In conclusion, the ensiling process markedly decreases the presence of long functional *cry1Ab* gene fragments and full size Cry1Ab protein.

Introduction

Maize is one of the most important crops worldwide. Popular not only for food, it is also widely used to produce forage. Like a variety of other plants, for example genetically modified soybean and Bt-cotton, maize has been genetically modified (GM) to control insect pathogens. The estimated global area of GM crops for 2004 was 81.0 million hectares, including approximately 19.3 million hectares of transgenic maize (James, 2004). The GM Bt-176-maize results in a higher efficiency for con-

trolling the European corn borer (*Ostrinia nubilalis*) than conventional or biological insecticides. Maize like event176 incorporates a truncated form of the *cry1Ab* gene from *Bacillus thuringiensis* (Bt); the expression is controlled by the maize-specific phosphoenolpyruvate carboxylase promoter and by the calcium-dependent protein kinase promoter (Agbios Crop Database, 2004).

Studies investigating the fate of transgenic DNA in non-target organisms showed a degradation of DNA in the gastrointestinal tract (GIT) of cows and pigs (Chowdhury et al., 2003a,b; Einspanier et al., 2004).

In pigs and chickens chloroplast DNA was detected in various tissues (Reuter and Aulrich, 2003; Tony et al., 2003; Einspanier et al., 2001). However, no traces of recombinant DNA have been detected in tissue samples of cattle fed with transgenic maize (Einspanier et al., 2001). A potential transfer of recombinant DNA to intestinal and food-associated bacteria has never been detected to date. However, under laboratory conditions, it was shown that DNA from GM plants can be transferred under laboratory conditions to bacteria if homologous sequences in the DNA of recipient cells are present (de Vries et al., 2001; de Vries and Wackernagel, 1998). Similarly to recombinant DNA, the fate and persistence of the Cry1Ab protein (event176) in non-target organisms (Chowdhury et al., 2003a,b) and in fields (Zwahlen et al., 2003) were investigated. Previous investigations in our own laboratories showed a degradation of the Cry1Ab protein in the bovine GIT (Lutz et al., 2005). However, no information about the way and rate of the degradation of Cry1Ab protein in the course of the ensiling process is available to date.

The aim of this study was to monitor the time-dependent fragmentation of the *rubisco* gene (chloroplast DNA) and recombinant *cry1Ab* gene as well as the Cry1Ab protein content of Bt-176 maize silage in the course of the ensiling process. Basic knowledge about these parameters are crucial for the risk assessment of a potential DNA transfer, e.g., into rumen bacteria of cattle and effects of the Cry1Ab protein with regard to animal health being fed with silage originating from GM plants.

Materials and Methods

Isogenic (Antares) and transgenic (Navares) maize (Syngenta International AG, Basel, Switzerland) were planted and grown on experimental fields of the Bavarian State Research Center for Agriculture (Poing-Grub, Germany). To minimise differences based on varying environmental conditions, Antares and Navares were cultured in close distance (approximately 1 km). At harvest, the maize plants were chopped (average size 1 cm) and processed to silage. To perform the ensiling process on a small scale for laboratory purposes, preserving jars were filled with approximately 400 g chopped maize up to the maximum capacity, pressed thoroughly and closed in an air-tight manner. Respiration of the plant materials and consumption of oxygen by aerobic micro-organisms achieved oxygen deficiency. The preserving jars were stored in the dark at a temperature of approximately 20 °C. At periodical inter-

vals, two preserving jars of each maize line (Antares, Navares) were opened, samples for DNA and protein analyses were taken and immediately frozen at -20 °C. In parallel, pH measurements were performed.

pH measurement

For pH measurement, the upper layer (approx. 5 cm ~ 100 g) of silage in the preserving jars was removed and the remaining silage was mixed well. To 50 g silage, 500 ml H₂O was added and incubated overnight. To avoid changes caused by microbial activities, Thimerosal (final concentration 0.002%; Merck, Darmstadt, Germany) was added. The pH was measured using a pH meter (WTW, Weilheim, Germany).

DNA fragment length analyses

DNA isolation

Frozen silage samples (100 mg) were ground using the FastPrep-System (BIO101, Carlsbad, CA, USA) with 0.5 g Green Matrix (5.5 m/s, 30 s) and finally dissolved in 600 µl lysis buffer (C1, Macherey-Nagel GmbH, KG, Düren, Germany). After an incubation period of at least 30 min at 60 °C, the samples were centrifuged for 1 min at 11.000 × *g*. DNA purification was performed using the Nucleo Spin Plant Kit (Macherey-Nagel GmbH, KG, Düren, Germany) according to the manufacturer's instructions. The DNA was finally eluted with 40 µl CE-buffer (Nucleo Spin Plant Kit); concentrations and integrity of the extracted DNA were determined by measuring the UV absorption at 260 nm and absorption ratios of 260/280 nm respectively.

Oligonucleotides

All primers used for the PCR amplification of the *rubisco* and *cry1Ab* genes are listed in Table 1A. Identification and fragment sizes are indicated in the name. The *rubisco* gene primer pairs were designed to amplify five fragments (173, 896, 1197, 1753 and 2521 bp) referring to the GenBank Accession No. X86563 (*Zea mays* complete chloroplast genome). The sequences of the primer pairs for *cry1Ab* fragments of 211 and 420 bp have been published elsewhere (Studer et al., 1997; Hupfer et al., 1998). For fragment sizes of 727 and 1423 bp, primers were designed referring to GenBank Accession No. I41419 (*cry1Ab* gene from patent US 5625136). Primer design was performed using the computer software PRIMER-3 (freely available at <http://www.wi.mit.edu>).

Table 1 Oligonucleotide sequences and PCR cycling conditions

| Name | Sequence | | | | |
|-----------------|---|----------------------|------------------------------------|--------|-----------------|
| (A) | | | | | |
| Cry 211F | 5'-CTC TCG CCG TTC ATG TCC GT-3' | | | | |
| Cry 211R | 5'-GGT CAG GCT CAG GCT GAT GT-3' | | | | |
| Cry 420F | 5'-CGG CCC CGA GTT CAC CTT-3' | | | | |
| Cry 420R | 5'-CTG CTG GGG ATG TTG TTG-3' | | | | |
| Cry 727F/1423F | 5'-ACG AGT GCA TCC CCT ACA AC-3' | | | | |
| Cry 727R | 5'-TAG TTG GGG AAC AGG CTC AC-3' | | | | |
| Cry 1423R | 5'-AGG TTG GTG CTC TTG GTC AG-3' | | | | |
| Rub 173F | 5'-AGC TAA TCG TGT GGC TTT AGA AGC C-3' | | | | |
| Rub 173R | 5'-TGG TAT CCA TCG CTT TGA AAC CA-3' | | | | |
| Rub 896F | 5'-AGG TAA GTT AGA AGG GGA ACG C-3' | | | | |
| Rub 896R | 5'-TGT ATG TCC CAG AAT AGG TCC C-3' | | | | |
| Rub 1197F/1753F | 5'-GGC CTA CTT CTT CAC ATT CAC C-3' | | | | |
| Rub 1197R | 5'-CAT TCT CAT CGC CTT TCT ATC C-3' | | | | |
| Rub 1753R | 5'-AAA CTC GCT ATA GTC GGC TAC G-3' | | | | |
| Rub 2521F | 5'-AAC TCA CAA CCA TTT ATG CGC-3' | | | | |
| Rub 2521R | 5'-ATG CGA CGT TTT ATT CCA GG-3' | | | | |
| Target gene | Fragment length | Initial denaturation | Amplification steps | Cycles | Final extension |
| (B) | | | | | |
| <i>cry1Ab</i> | 211 bp | 94 °C, 3 min | 94 °C 30 s; 63 °C 30 s; 72 °C 30 s | 35 | 72 °C, 2 min |
| | 420 bp | 94 °C, 3 min | 94 °C 30 s; 56 °C 30 s; 72 °C 30 s | 35 | 72 °C, 2 min |
| | 727 bp | 94 °C, 5 min | 94 °C 30 s; 56 °C 30 s; 72 °C 30 s | 35 | 72 °C, 5 min |
| | 1423 bp | 94 °C, 5 min | 94 °C 45 s; 56 °C 45 s; 72 °C 45 s | 40 | 72 °C, 5 min |
| <i>rubisco</i> | 173 bp | 94 °C, 3 min | 94 °C 30 s; 60 °C 30 s; 72 °C 30 s | 35 | 72 °C, 5 min |
| | 896 bp | 94 °C, 5 min | 94 °C 45 s; 58 °C 45 s; 72 °C 45 s | 40 | 72 °C, 5 min |
| | 1197 bp | 94 °C, 5 min | 94 °C 45 s; 58 °C 45 s; 72 °C 45 s | 40 | 72 °C, 5 min |
| | 1753 bp | 94 °C, 5 min | 94 °C 45 s; 58 °C 45 s; 72 °C 45 s | 40 | 72 °C, 5 min |
| | 2521 bp | 94 °C, 5 min | 94 °C 60 s; 64 °C 60 s; 72 °C 60 s | 40 | 72 °C, 10 min |

Polymerase chain reaction (PCR)

All amplification reactions were carried out on a gradient thermocycler (Biometra, Göttingen, Germany). The reaction volume consisted of 150 ng DNA; 1x PCR reaction buffer (ABgene, Epsom, UK); 2.5 mM MgCl₂ (ABgene); 0.4 μM primers (Metabion, Martinsried, Germany); 0.2 mM dNTPs (ABgene) and 0.5 units Thermoprime Plus DNA Polymerase (ABgene). The master mixes for the 1753 and 2521 bp amplification included 3.5 mM MgCl₂. Cycling conditions are listed in Table 1B. The positive control represents DNA isolated from transgenic maize leaves. DNA extracted from isogenic maize leaves served as negative control for *cry1Ab* gene analyses. All PCR analyses for the *rubisco* and *cry1Ab* gene were performed at least twice.

Agarose gel electrophoresis

A total of 15.0 μl of each amplification reaction was separated on 1.8% agarose gel stained with ethidium bromide and visualised using an UV transillumina-

tor. The gels were digitised using a video documentation system (Vilber Lourmat, Marne-la-Vallée Cedex 1, France).

Sequencing

The PCR products were commercially sequenced to confirm nucleotide sequence identity (Medigenomix, Martinsried, Germany).

Cry1Ab protein measurement (ELISA)

Estimation of the Cry1Ab protein was carried out using a commercially available ELISA kit according to the manufacturer's instructions (Agdia, Elkhart, IN, USA). To pulverise the samples, 0.2 g material was ground using the FastPrep-System (BIO101) with 0.8 g Green Matrix at 6 m/s for 40 s. The procedure was repeated until the sample was pulverised; the finally obtained powder was dissolved in 1000 μl Multi-Event Buffer (provided in the kit). The control Cry protein, provided by the supplier,

was diluted and used in following concentrations to create a standard curve: 0.015, 0.03, 0.06, 0.125, 0.25, 0.5, 1.0 and 1.6 ng/ml. The results were expressed as Cry1Ab protein (ng) per g wet sample. All samples were measured in duplicates.

Immunoblotting

The samples were prepared as described above (Cry1Ab protein measurement), except that PBS (pH 7.4) with protease inhibitors (Merck KGaA, Darmstadt, Germany) was used as extraction buffer. A quantity of 17.75 μ l of extracted protein, 1.0 μ l 1,4-dithiothreitol (1 mM; Merck) and 6.25 μ l SDS sample buffer (4 \times) were applied to SDS-PAGE on a 4–12% gradient Bis-Tris gel (NuPage, Invitrogen, Karlsruhe, Germany). 2-Morpholinoethanesulfonic acid was used as running buffer. After separation, the polypeptides were transferred onto a nitro-cellulose membrane (pore size 0.45 μ m; Schleicher & Schuell BioScience, Keene, NH, USA). The membranes were blocked with TBS-Tween20-Buffer (0.05 M Tris, 0.15 M NaCl pH 7.6; 1% Tween20) + 1% (w/v) non-fat dried milk overnight at 4 °C; subsequently, the membranes were incubated for 60 min in TBS-Tween20-Buffer supplemented with a polyclonal rabbit anti-Cry1Ab/1Ac antibody (final concentration 5 μ g/ml; Agdia). The membranes were washed four times with casein solution and incubated for 30 min in secondary antibody solution (biotinylated antirabbit IgG in casein solution; final concentration 1.5 μ g/ml). After three additional washing steps with casein solution for 10 min, membranes were incubated in Vectastain ABC-AmP Reagent (Vector Laboratories, Burlingame, CA, USA) for 10 min. Membranes were washed three more times (casein solution, 10 min) and incubated for 5 min with chemiluminescent substrate (DuoLuX, Vector Laboratories). Following two final washing steps (0.1 M Tris, pH 9.3, 5 min), the membranes were exposed to a chemiluminescent detection film (Roche, Mannheim, Germany) for 15 min. All washing and incubation steps were performed at room temperature unless indicated otherwise. Isogenic maize (Antares) was used as negative control; Cry1Ab/1Ac protein included in the ELISA kit served as positive control.

Results

pH in the course of the ensiling process

Monitoring of the pH-value during the course of the ensiling process served as an indicator of the progress and the quality of the fermentation reac-

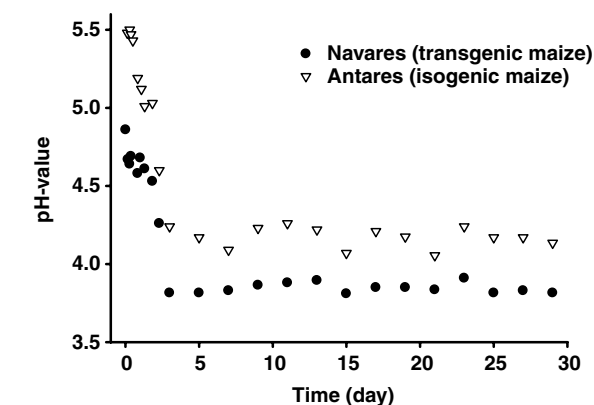


Fig. 1 pH of isogenic (Antares) and transgenic (Navares) maize silage in the course of the ensiling process.

tion, in which enteric and lactic acid bacteria are involved. At the beginning of the ensiling process, the pH of Antares was 5.5; it declined after 2 days to 4.2 and reached the steady state with a final pH of approximately 4.1 (Fig. 1). Navares started with a pH value of 4.8 at the beginning of the fermentation process and reached a final pH of 3.8 after 2 days (Fig. 1).

DNA fragment length analyses

Rubisco DNA with sizes of 173 bp was detectable in all samples (Fig. 2a, upper panel). After 30 days, DNA fragments with sizes of 1197 bp were not detectable (Fig. 2a, middle panel). PCR amplifications of the *rubisco* gene with 2521 bp were only possible in samples of the initial 24 h (Fig. 2a, lower panel). Fragment length analyses of 896 bp and 1753 bp were identical to 1197 bp and 2521 bp, respectively, thus the figures are not shown.

DNA fragments of the *cry1Ab* gene with sizes of 211 bp and 420 bp were detectable up to 61 days, 727 bp and 1423 bp fragments were found up to 30 and 6 days respectively. Representative gel images of PCR analyses with different fragment sizes of the *cry1Ab* gene are shown in Fig. 2b. The maximally detectable fragment lengths of the *rubisco* and *cry1Ab* genes are summarised in Fig. 2c.

Cry1Ab protein degradation

Cry1Ab protein was analysed using ELISA and immunoblotting technique. No marked degradation of immunoactive Cry1Ab protein was observed during the initial 43 h (Fig. 3a/inset). Thereafter, a marked decrease of the Cry1Ab signal was measured

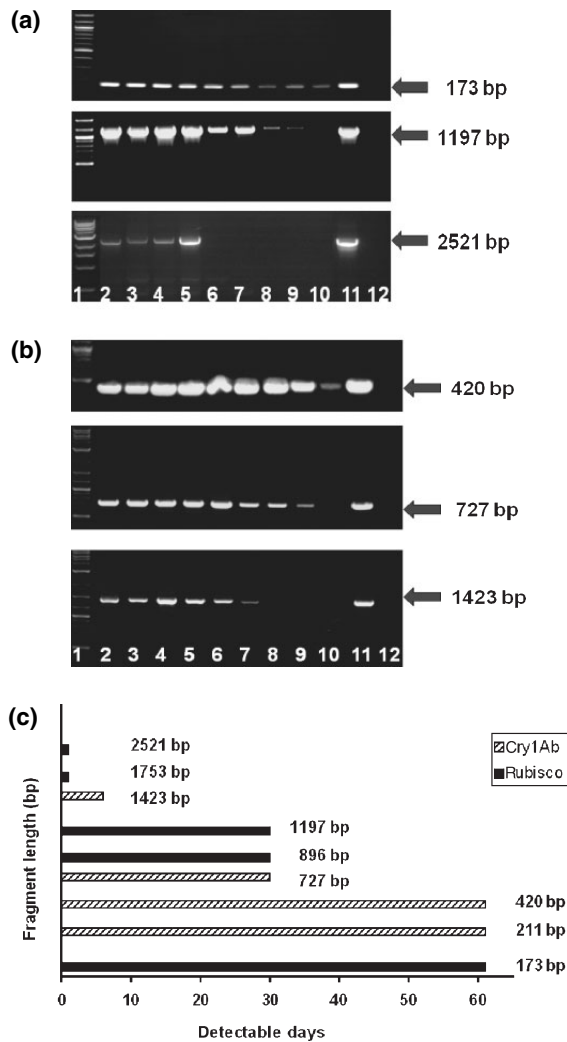


Fig. 2 Agarose gel electrophoresis of representative PCR products. (a) *rubisco* gene. Primer pairs for detection of 173, 1197 and 2521 bp were used. Fragment length analyses of 896 bp and 1753 bp were identical to 1197 bp and 2521 bp respectively. Lanes 1–12: 1 = marker, 2 = 0 h, 3 = 4 h, 4 = 10 h, 5 = 1 day, 6 = 2 days, 7 = 6 days, 8 = 14 days, 9 = 30 days, 10 = 61 days, 11 = positive control (transgenic maize leaves), 12 = negative control (H₂O). (b) *cry1Ab* gene. Primer pairs for detection of 420, 727 and 1423 bp were used. Fragment analyses of 211 bp were identical to 420 bp. Lanes 1–12: 1 = marker, 2 = 0 h, 3 = 4 h, 4 = 10 h, 5 = 1 day, 6 = 2 days, 7 = 6 days, 8 = 14 days, 9 = 30 days, 10 = 61 days, 11 = Antares, 12 = positive control (transgenic maize leaves), 13 = negative control (H₂O). Arrows indicate the size of the PCR amplification products. Shown are the maximally detectable fragment sizes. (c) Summary of the maximally detectable fragment length of the *rubisco* and *cry1Ab* gene.

(Fig. 3a). After 20 and 61 days, $56 \pm 0.2\%$ (7.4 ± 0.02 ng/g) and $23.5 \pm 0.9\%$ (3.1 ± 0.12 ng/g), respectively, of the initial immunoactive Cry1Ab protein (13.2 ± 0.2 ng/g) were still detectable.

In order to get information about the time course and potential degradation or fragmentation of the Cry1Ab protein, an immunoblotting technique using a commercial polyclonal Cry1Ab antibody was established. The detection limit of the immunoblotting assay was determined in a serial dilution experiment with fresh transgenic maize leaves (Navares) and corresponded to 50 mg wet sample, equivalent to approximately 2.0 ng Cry1Ab protein per gram of wet sample according to ELISA concentration measurements (Lutz et al., 2005).

Immunoblotting confirmed the results of the ELISA. No differences in the intensity of the bands could be detected for the samples of the first 2 days (Fig. 3b, left panel). In contrast, in samples which were subjected to the ensiling process for longer than 2 days degradation of the Cry1Ab protein was clearly visible. A faint band for the full size protein still occurred after 8 days whereas no immunoactive Cry1Ab protein could be observed after 12 days. No additional bands were detected.

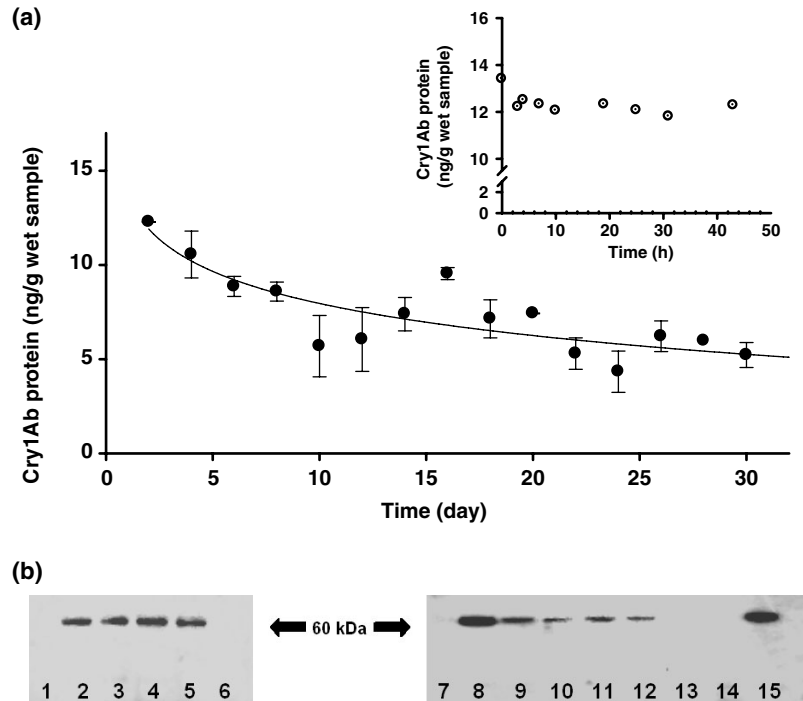
Discussion

To our knowledge, this is the first study investigating the degradation of the Cry1Ab protein combined with detailed fragment analyses of chloroplast and recombinant DNA during the ensiling process. This study was accomplished to estimate the likelihood of an *in vivo* uptake of functional and recombinant DNA into micro-organisms of farm animals and to assess a potential activity of the full size Cry1Ab protein.

A variety of primers were used to amplify fragments of the high copy *rubisco* gene in sizes of 173, 896, 1197, 1753 and 2521 bp. The 173 bp *rubisco* fragment was detected in all samples of the experimental period. In contrast, higher-sized products of 1197 and 2521 bp were only detectable up to 30 days and 24 h. In order to compare the detectability and time-dependent degradation of the high copy *rubisco* gene with the single copy *cry1Ab* gene, *cry1Ab* gene fragments of 211, 420, 727 and 1423 bp were analysed.

Similarly to the 173 bp *rubisco* fragment, 211 and 420 bp *cry1Ab* fragments were detected in all samples of the experimental period. Fragments of the *cry1Ab* gene with sizes of 727 bp were amplifiable over the same period of time as the 896 and 1197 bp *rubisco* fragments, while the 1423 bp *cry1Ab* fragment was only detectable up to 6 days. The 1753 and 2521 bp *rubisco* fragments could only be amplified up to 24 h. Although having a higher copy number and there-

Fig. 3 Immunoactivity of the Cry1Ab protein. (a) Measurements of the Cry1Ab protein using ELISA from day 2 to day 30; Inset: Degradation of the Cry1Ab protein within the initial 43 h. (b) Immunoblotting of Cry1Ab protein in silage samples. The arrow indicates the Cry1Ab protein-specific size of approximately 60 kDa. (Left panel) Lanes 1–6: 1 = Antares; 2 = Navares (0 h); 3 = Navares (7 h); 4 = Navares (12 h); 5 = Navares (3 days); 6 = Navares (12 days); (Right panel) Lanes 7–15; 7 = Antares; 8 = Navares (0 h); 9 = Navares (2 days); 10 = Navares (4 days); 11 = Navares (6 days); 12 = Navares (8 days); 13 = Navares (12 days); 14 = Navares (16 days); 15 = positive control (Cry-protein provided in the ELISA Kit).



fore suggesting a higher probability of detection, the *rubisco* gene showed a similar degradation pattern as the *cry1Ab* gene. The results of this study are comparable with those of Aulrich et al. (2004), where DNA degradation in isogenic and transgenic ensiled corn was investigated, and Hupfer et al. (1999), where fragments of the *invertase* gene and the *cry1Ab* gene in maize silage were analysed. All studies showed a degradation of plant and recombinant DNA during the ensiling process.

DNA degradation processes during ensiling are common consequences of the mechanical treatment and the concomitant disruption of cell walls and membranes. Furthermore, low pH conditions in the silage, endogenous nucleases of the plant and/or exogenous nucleases of the microflora are contributing factors to DNA degradation. However, during the first 24 h of the ensiling process, the DNA fragments obtained are theoretically still long enough to represent a full-sized gene. For example, the prokaryotic *bla* gene, encoding a β -lactamase which confers resistance to ampicillin has a size of 861 bp (Bolivar et al., 1977).

Important in this context are several studies showing transformations by which bacteria can acquire foreign DNA (Nielson et al., 2000; Kay et al., 2002; de Vries and Wackernagel, 2002). Kharazmi et al. (2003a) showed by marker rescue transformation that plasmid and chromosomal DNA of bacteria as

well as DNA of transgenic potatoes were transferred to bacteria under laboratory conditions. An *in vivo* transfer has not been detected under experimental conditions but cannot be excluded as the hypothetical transformation rate of transgenic DNA to *B. subtilis* was calculated to be equal to 8.5×10^{-19} and 1.2×10^{-27} for homologous and non-homologous recombination respectively (Kharazmi et al., 2003b). However, hereby it has to be considered that foreign DNA fragments have to be rescued by homologous recombination or, as demonstrated for *Streptococcus pneumoniae*, by homology-directed illegitimate recombination during transformation (Prudhomme et al., 2002).

In parallel to the detection of DNA degradation with PCR techniques, the present study also monitored the degradation of the Cry1Ab protein in the silage samples using immunoblotting techniques. Using ELISA, no significant degradation of the Cry1Ab protein during the first 43 h was observed. Thereafter, a clear reduction of the Cry1Ab content was found. To exclude that the ELISA detected only immunoactive fragments, as previously shown in samples of bovine rumen and GIT content (Lutz et al., 2005), an immunoblotting method using a polyclonal antibody was applied. The immunoblotting showed a time-dependent decreasing signal intensity for the full size Cry1Ab protein (60 kDa). In contrast to earlier studies testing bovine GIT sam-

ples, no smaller immunoactive fragments were detected.

The distinct degradation pattern of the Cry1Ab protein in different environments such as bovine rumen on one side and silage on the other side could be associated with the differing physiological and biochemical conditions involved in the degradation process. One important factor herein is the pH value. In the rumen of cattle, the physiological pH value ranges between 5.5 and 6.5 while in maize silage values of approximately 4.0 are found due to the production of lactic acid in the anaerobic fermentation process.

In addition, the diversity in the composition of the microbial community in both environments could contribute to the differences found. In relation to the predominantly strictly anaerobic bacteria in the bovine rumen, with the main purpose of digestion of whole plant material including protein, the lactic acid bacteria in silage ferment mainly the watersoluble carbohydrates. In the course of the ensiling process, the bacterial population changes. Starting with a variable mixed population, the steadily declining pH value results in a selection and survival of only lactic acid tolerable bacteria. Accordingly, a rapid decrease of the Cry1Ab protein was mainly observed after 43 h. As in the acidic and anaerobic environment most protein consuming bacteria are supposed to be erased and, after the consumption of the supply of carbohydrates, even the lactic acid tolerable bacteria do not survive, the degradation process of the protein is reduced. This might suggest that the Cry1Ab protein degradation rate in the silage is dependent both of the low pH value and of the bacterial composition. Though our results indicate that significant degradation of the Cry1Ab protein during the ensiling process occurs, it was apparently possible to detect the immunoactive Cry1Ab protein with ELISA and immunoblotting technique in different parts of the GIT after feeding silage to cattle and pigs (Chowdhury et al., 2003a,b). However, independent of the degradation pattern of the Cry1Ab protein, there is no indication for an interaction between the Cry1Ab protein and the bovine rumen or epithelial cells of the GIT of cattle and pigs; solely Cry3Ac protoxin was shown to bind to surface proteins in the mouse small intestine (Fares and El-Sayed, 1998; Vasquez-Padron et al., 2000).

In summary, a degradation process of DNA and Cry1Ab protein is detectable during the ensiling process. Up to 8 days of ensiling, approximately 65% of the full size Cry1Ab protein is ingested by domestic

animals fed GM maize. After 61 days only approximately 23% of the initial ELISA signal was measurable and only DNA fragments with sizes of 420 bp were detectable. In conclusion, the ensiling process markedly reduces the presence of long *cry1Ab* gene fragments and full size Cry1Ab protein.

Acknowledgements

This study was supported by the German Federal Ministry of Research and Technology (BMBF grant No. 0312631D) and the Federal Agency for Nature Conservation (BfN grant No. 20767432). We thank Tamara Stelzl, Inge Celler, and Bastian Weiss for technical assistance. We are indebted to the staff of the Bavarian State Research Center for Agriculture, Poing-Grub for organisation, logistics and technical assistance during the maize cultivation.

References

- Agbios Crop Database. 2004: *Essential Biosafety*, 2nd edn. Merrickville, Ontario, Canada. <http://www.essentialbiosafety.info>.
- Aulrich, K.; Pahlow, G.; Flachowsky, G., 2004: Influence of ensiling on the DNA-degradation in isogenic and transgenic corn. *Proceedings of the Society of Nutrition Physiology* **13**, 112 [Abstract].
- Bolivar, F.; Rodriguez, R. L.; Betlach, M. C.; Boyer, H. W., 1977: Construction and characterization of new cloning vehicles. I. Ampicillin-resistant derivatives of the plasmid pMB9. *Gene* **2**, 95–113.
- Chowdhury, E. H.; Shimada, N.; Murata, H.; Mikami, O.; Sultana, P.; Miyazaki, S.; Yoshioka, M.; Yamanaka, N.; Hirai, N.; Nakajima, Y., 2003a: Detection of Cry1Ab protein in gastrointestinal contents but not visceral organs of genetically modified Bt11-fed calves. *Veterinary and Human Toxicology* **45**, 71–75.
- Chowdhury, E. H.; Kuribara, H.; Hino, A.; Sultana, P.; Mikami, O.; Shimada, N.; Guruge, K. S.; Saito, M.; Nakajima, Y., 2003b: Detection of corn intrinsic and recombinant DNA fragments and Cry1Ab protein in the gastrointestinal contents of pigs fed genetically modified corn Bt11. *Journal of Animal Science* **81**, 2546–2551.
- De Vries, J.; Wackernagel, W., 1998: Detection of nptII (Kanamycin resistance) genes in genomes of transgenic plants by marker-rescue transformation. *Molecular and General Genetics* **257**, 606–613.
- De Vries, J.; Wackernagel, W., 2002: Integration of foreign DNA during natural transformation of *Acinetobacter* sp. by homology-facilitated illegitimate recombination. *Proceedings of the National Academy of Science of the United States of America* **99**, 2094–2099.

- De Vries, J.; Meier, P.; Wackernagel, W., 2001: The natural transformation of the soil bacteria *Pseudomonas stutzeri* and *Acinetobacter* sp. by transgenic plant DNA strictly depends on homologous sequences in the recipient cells. *FEMS Microbiology Letters* **195**, 211–215.
- Einspanier, R.; Klotz, A.; Kraft, J.; Aulrich, K.; Poser, R.; Schwägele, F.; Jahreis, G.; Flachowsky, G., 2001: The fate of forage plant DNA in farm animals: A collaborative case-study investigating cattle and chicken fed recombinant plant material. *European Food Research and Technology* **212**, 129–134.
- Einspanier, R.; Lutz, B.; Rief, S.; Berezina, O.; Zverlov, V.; Schwarz, W.; Mayer, J., 2004: Tracing residual recombinant feed molecules during digestion and rumen bacterial diversity in cattle fed transgenic maize. *European Food Research and Technology* **218**, 269–273.
- Fares, N. H.; El-Sayed, A. K., 1998: Fine structural changes in the ileum of mice fed on delta-endotoxin-treated potatoes and transgenic potatoes. *Natural Toxins* **6**, 219–233.
- Hupfer, C.; Hotzel, H.; Sachse, K.; Engel, K. H., 1998: Detection of the genetic modification in heat-treated products of Bt-maize by polymerase chain reaction. *Zeitschrift für Lebensmitteluntersuchung und Forschung* **206**, 203–206.
- Hupfer, C.; Mayer, J.; Hotzel, H.; Sachse, K.; Engel, K. H., 1999: The effect of ensiling on PCR-based detection of genetically modified Bt maize. *European Food Research and Technology* **209**, 301–304.
- James, C., 2004: Preview: Global status of commercialised transgenic crops: 2004. *International Service for the Acquisition of Agri-biotech Applications (ISAAA) Briefs* **32**, 1.
- Kay, E.; Vogel, T. M.; Bertolla, F.; Nalin, R.; Simonet, P., 2002: In situ transfer of antibiotic resistance genes from transgenic (transplastomic) tobacco plants to bacteria. *Applied and Environmental Microbiology* **25**, 3345–3351.
- Kharazmi, M.; Sczesny, S.; Blaut, M.; Hammes, W. P.; Hertel, C., 2003a: Marker rescue studies of the transfer of recombinant DNA to *Streptococcus gordonii* in vitro, in foods and gnotobiotic rats. *Applied and Environmental Microbiology* **69**, 6121–6127.
- Kharazmi, M.; Bauer, T.; Hammes, W. P.; Hertel, C., 2003b: Effect of food processing on the fate of DNA with regard to degradation and transformation capability in *Bacillus subtilis*. *Systematic and Applied Microbiology* **26**, 495–501.
- Lutz, B.; Wiedemann, S.; Albrecht, C., 2005: Degradation of Cry1Ab-protein from genetically modified maize in the bovine gastrointestinal tract. *Journal of Agricultural and Food Chemistry* **53**, 1453–1456.
- Nielson, K. M.; van Elsas, J. D.; Smalla, K., 2000: Transformation of *Acinetobacter* sp. strain BD413 (pFG4A ntpII) with transgenic plant DNA in soil microorganisms and effects of kanamycin on selection of transformants. *Applied and Environmental Microbiology* **66**, 1237–1242.
- Prudhomme, M.; Libante, V.; Claverys, J. P., 2002: Homologous recombination at the border: insertion-deletions and the trapping of foreign DNA in *Streptococcus pneumoniae*. *Proceedings of the National Academy of Science of the United States of America* **19**, 2100–2105.
- Reuter, T.; Aulrich, K., 2003: Investigations on genetically modified maize (Bt-maize) in pig nutrition: fate of feed-ingested foreign DNA in pig bodies. *European Food Research and Technology* **216**, 185–192.
- Studer, E.; Dahinden, I.; Lüthy, J.; Hübner, P., 1997: Detection of the genetically engineered Maximizer-maize using the polymerase chain reaction (PCR). *Mitteilung auf dem Gebiete der Lebensmittel Hygiene* **88**, 515–524.
- Tony, M. A.; Butschke, A.; Broll, H.; Grohmann, L.; Zagon, J.; Halle, I.; Dänicke, S.; Schauzu, M.; Hafez, H. M.; Flachowsky, G., 2003: Safty Assessment of Bt 176 maize in broiler nutrition: Degradation of maize-DNA and its metabolic fate. *Archives of Animal Nutrition* **57**, 235–252.
- Vasquez-Padron, R. I.; Gonzales-Cabrera, J.; Garcia-Tovar, C.; Neri-Bazan, L.; Lopez-Revilla, R.; Hernandez, M.; Moreno-Fierro, L.; de la Riva, G. A., 2000: Cry1Ac protoxin from *Bacillus thuringiensis* sp. Kurstaki HD73 binds to surface proteins in the mouse small intestine. *Biochemical and Biophysical Research Communications* **271**, 54–58.
- Zwahlen, C.; Hilbeck, A.; Gugerli, P.; Nentwig, W., 2003: Degradation of the Cry1Ab protein within transgenic *Bacillus thuringiensis* corn tissue in the fields. *Molecular Ecology* **12**, 765–775.