ORIGINAL INVESTIGATION

Fate of genetically modified maize and conventional rapeseed, and endozoochory in wild boar (Sus scrofa)

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Abstract

Feeding experiments were carried out to investigate the digestive fate of transgenic DNA and novel protein in wild boar applying polymerase chain reaction (PCR) and immunodiagnostic techniques. Furthermore, the dispersal of viable maize and rapeseed (endozoochory) was studied. A diet containing conventional rapeseed, and either genetically modified (GM) maize expressing Cry1Ab protein (Bt176) or non-GM isogenic maize was offered. By conventional and quantitative PCR both chloroplast-specific plant DNA (rubisco) and cry1Ab gene fragments were detected only in gastrointestinal content. Using an enzyme-linked immunosorbent assay (ELISA) positive signals of immunoactive Cry1Ab protein were detected in digesta samples. Analysis of endozoochory showed that excreted maize seeds retain their germination capacity only in extremely rare cases and no intact rapeseed was found in faeces. A possible dispersal of viable seeds by wild boars is highly unlikely.

Keywords: Wild boar; Bt-maize; Endozoochory; Recombinant DNA; Cry1Ab protein

Introduction

Since their commercial release in 1996, the global area of genetically modified (GM) plants has increased from 2 to 114 million ha including 35.2 million ha GM maize and 5.5 million ha GM rapeseed (James 2007). The majority of GM crops currently planted, like maize and rapeseed, have been engineered to enhance agronomic performance by transformation with genes encoding herbicide tolerance and/or pest resistance. Previously, numerous detailed studies on GM livestock feeding qualities, on nutritional evaluation and on stability and fate of recombinant DNA or proteins consumed by domestic animals, e.g. cattle, poultry and pigs have been reviewed (Flachowsky et al. 2005, 2007; Alexander et al. 2007). Studies conducted with pigs have illustrated that plant-derived and recombinant DNA as well as recombinant proteins are quickly degraded throughout the digestive tract (Chowdhury et al. 2003; Jennings et al. 2003). However, currently scarce information is available about the impact of GM crops on wildlife feeding. Guertler et al. (2008) performed
feeding experiments (maize event Bt176) with fallow deer as an example for wild ruminants. They showed a clear fragmentation of multi-copy endogenous maize genes throughout the digestive tract, but could not detect neither recombinant cry1Ab DNA nor immunoactive Cry1Ab protein in any tissue or gastrointestinal (GI) content. Regardless of these findings, it can be assumed that monogastric game animals like wild boar (Sus scrofa) consuming GM plants potentially contribute to the dispersal of transgenic DNA, novel protein and viable seeds into areas growing conventional crops. In this context it should be mentioned that home range sizes of wild boar vary according to season, food availability, sex, age class and disturbances by man between 175 and 6000 ha (summarized in Keuling et al. 2007). Although internal maize seed dispersal has not yet been studied in detail, wild boars were shown to contribute to dispersing vascular plants by endozoochory (Heinken et al. 2002; Schmidt et al. 2004). Given the recent increase of wild boar population number and range in various countries (Feichtner 1998; Goulding et al. 2003; Geisser and Reyer 2005), their adaptation to modern agricultural systems comprising 50% of nightly range in various countries (Genov 1981) and their preference of energy-rich plant food such as maize (e.g. Vassant 1997; Schley and Roper 2003; Herrero et al. 2006; Schley et al. 2008) there is considerable opportunity for wild boar to consume significant quantities of recombinant DNA and protein. Additionally, for various reasons supplemental feeding of dried maize to wild boar is common practice in many countries potentially increasing the amount of GM maize ingested (Fournier-Chambrillon et al. 1996; Hohmann and Huckschlag 2005).

Therefore, wild boar could not only contribute to economic and ecological conflicts by transmission of diseases to domestic animals (Simpson 2002; Artois et al. 2006; Gortazar et al. 2007), damages to agricultural crops or to forests (e.g. Schley et al. 2008), but also to a distribution of transgenic maize grain and rapeseed. Thus, the aims of this study were to investigate (i) the fate of immunoreactive Cry1Ab protein and recombinant DNA after feeding transgenic maize to wild boar and (ii) the likelihood of endozoochory of viable seed.

**Material and methods**

Transgenic GM maize (Navares, Bt176) and the parental non-GM isogenic (Antares) maize (Syngenta International AG, Basel, Switzerland) were planted on experimental fields of the Bavarian State Research Center for Agriculture in Poing, Germany. Prior to feeding maize was harvested and parts of it were separated into maize kernels and the remaining plant. Conventional rapeseed (Alkido) was provided by Wagner & Sohn ÖlsaatenverarbeitungsKg, Niederviehbach, Germany and added to investigate endozoochory.

Prior to feeding seeds were tested under optimal standard conditions according to the International Rules for Seed Testing (International Seed Testing Association (ISTA) 2004). Seedlings were categorized according to ISTA Rules in ‘‘normal seedlings’’ or ‘‘abnormal seedlings’’ and ‘‘fresh’’ or ‘‘dead’’ seeds. The viability of the non-germinated ‘‘fresh’’ maize seeds was determined applying the ‘‘Topographical Tetrazolium Test’’, according to ISTA Rules. Viable seeds are defined as those that show the potential to produce ‘‘normal seedlings’’.

Seed testing revealed a germination of $74.5\pm3.7\%$ (mean $\pm$ SD) normal seedlings, $11.3\pm2.3\%$ abnormal seedlings and $14.3\pm3.3\%$ dead seeds for Antares ($n=8 \times 50$ pure-seeds); $79.3\pm5.6\%$ normal seedlings, $10.1\pm3.6\%$ abnormal seedlings and $10.6\pm2.7\%$ dead seeds for Navares ($n=8 \times 50$ pure-seeds); and $93.5\pm3.7\%$ normal seedlings, $4.5\pm2.8\%$ abnormal seedlings and $2.3\pm1.9\%$ dead seeds for rapeseed ($n=16 \times 50$ pure-seeds).

Two feeding experiments with wild boar were carried out (Table 1). In both experiments, animals were divided into two groups fed with isogenic or transgenic maize, respectively. The groups were kept separately in indoor pens to exclude any contamination. Feed was prepared by mixing fresh whole maize plants, fresh maize kernels and rapeseed. The feed mixture was weighed and provided to the respective group of animals three times per week. Minor amounts of additional

| Table 1. Experimental design and composition of diet fed<sup>a</sup> |
|-----------------|-----------------|-----------------|-----------------|
|                 | Feeding experiment 1 | Feeding experiment 2 |
| Number of animals | 3                | 2                | 3               | 5               |
| Weight of animals (kg) | 45–55           |                  | 35–40           |
| Duration of experiment (day) | 35              |                  | 35              |
| Diet<sup>b</sup> |                  |                  |                 |
| Isogenic maize    | 1.0              |                 | 1.0             |
| Isogenic maize kernels | 0.25            |                 | 0.25            |
| Transgenic maize  |                 | 1.0             |                 |
| Transgenic maize kernels |              | 0.25            |                 |
| Rapeseed         | 0.1              | 0.1             | 0.1             |

<sup>a</sup>Diets were offered as mixture to the respective group three times a week.

<sup>b</sup>Average daily feed intake (ADFI, kg fresh weight/day/animal).
fodder (wheat and potatoes) were offered and a protein–
vitamin–mineral supplement was added to the diet according to 
NRC requirements for domestic pigs. Water was available ad 
limitum. After a diet adaptation phase of 1 week, calculated 
amounts of feed were offered. Faeces were collected during all 
feeding experiments, washed using a strainer to separate maize and 
rapsedese and screened for the presence of intact grains. 

To exclude contamination isogenic fed wild boar groups 
were slaughtered prior to those fed transgenic maize at the end of 
both experiments. Samples for DNA and protein analyses were 
taken from GI contents (stomach, jejunum, caecum, 
colon), all visceral organs (liver, kidney, spleen, heart and lung), 
muscle, lymph node and blood, snap-frozen in liquid 
nitrogen and stored at −80 °C. To collect intact maize kernels 
and rapsedese rectal contents were visually scanned. Shortly 
before germination tests isolated seeds were washed with water and 
dried.

Using the bead-beating FastPrep technique (BIO101, 
Carlsbad, CA) samples (100 mg) were repeatedly ground with 
0.8 g of Matrix Green ceramic beads (BIO101) at 5.5 m/s for 
40 s. To improve DNA yield, samples were refrozen in liquid 
nitrogen for 10 min before being reground. The resulting fine 
powder was dissolved in 600 μl lysis buffer (C1) and 10 μl of 
RNase-A (Nucleo Spin Plant Kit; Macherey-Nagel GmbH & 
Co. KG, Düren, Germany), mixed thoroughly, and incubated 
for at least 30 min at 60 °C. All succeeding DNA purification 
steps were performed using a silica spin column following the 
manufacturer’s protocol. The DNA was finally eluted in 40 μl 
of CE-buffer (Nucleo Spin Plant Kit). Concentrations of DNA 
determined by UV absorption at 260 nm, and the DNA 
integrity was estimated by 260/280 UV absorption ratio.

All conventional polymerase chain reactions (PCRs) were 
performed in a gradient thermocycler (Biometra, Goettingen, 
Germany). Primer sequences and cycling conditions used for 
amplication of 18S ribosomal (218 bp), rubisco (173, 896, 
1197, 1753, 2521 bp) and cry1Ab DNA (211, 420, 727 bp) were 
described previously (Wiedemann et al. 2006; Guertler et al. 
2008). The reaction volume consisted of 150 ng DNA; 1 × 
PCR buffer (ABgene, Epsom, UK); 2.5 mM MgCl2 (ABgene); 
0.8 μM of both forward and reverse primers (Metabion, 
Martinsried, Germany); 4.0 mM dNTPs (ABgene) and 0.5 U 
Thermoprime Plus DNA Polymerase (ABgene). Master mixes 
for the 1753 and 2521 bp rubisco gene fragment amplication 
contained 3.5 mM MgCl2. The PCR amplicons (150 μl) were 
electrophoresed at 100 V on a 1.8% (wt/vol) agarose gel and 
visualized using an UV transilluminator. The gels were 
estimated by ELISA an immunoblotting technique as pre-
viously described by Lutz et al. (2005, 2006) was performed. 
Samples were prepared in the same manner as for the ELISA 
and loaded to SDS-PAGE on a 4–12% gradient Bis–Tris gel 
(NuPage, Invitrogen GmbH, Karlsruhe, Germany). After 
separation and transfer onto a nitro-cellulose membrane 
Cry1Ab protein was detected using a polyclonal rabbit anti-
Cry1Ab/1Ac antibody (final concentration 5 μg/ml; Agdia, Inc., 
60 min) followed by a secondary antibody solution 
(biotinylated anti-rabbit IgG in casein solution; final concen-
tration 1.5 μg/ml, 30 min). For signal amplication membranes 
were incubated for 10 min in Vectastain ABC-AmP Reagent 
(Vector Laboratories, Inc., Burlingame, USA). Isogenic maize 
served as negative control and Cry1Ab/1Ac protein included in 
the ELISA kit served as positive control. Excluding any cross-
reactions, e.g. animal or intestinal bacteria proteins, samples of 
isogenic fed animal were used as control samples.

Results

In both wild boar feeding experiments a total of 65 kg 
rapsedese (≈ 13.0 × 106 seeds), 98 kg Antares 
(≈ 0.35 × 106 seeds) and 114 kg Navares (≈ 0.41 × 106 
seeds) was fed but only 51 intact isogenic maize seeds and 
37 intact transgenic maize seeds were isolated from faeces. 
The germination test resulted in one normal 
and 37 intact transgenic maize seeds were isolated from faeces. 
The germination test showed that the non-
intact rapeseed could be isolated from faeces. 

PCR amplication of transgenic and isogenic diets 
revealed amplicons using rubisco primer pairs, but 
fragments of the cry1Ab gene were amplicated only in 
transgenic feed excluding any contamination of isogenic 
maize with components of the transgenic cultivar. 
Rubisco DNA fragments of 173 bp length were detected
in all digestive samples. The \textit{rubisco} gene fragments with amplicons up to 1753 bp were only generated from residual DNA extracted from samples of gastric content. A \textit{rubisco}-specific amplicon of 2521 bp could not be detected in any sample except in the transgenic maize leaf positive control. DNA fragments of the \textit{cry1Ab} gene with sizes up to 727 bp could only be amplified in samples of gastric content. Fragments of the \textit{cry1Ab} gene with sizes of 1423 bp could not be detected. Results of DNA analyses are summarized in Table 2.

Using the qPCR method, the \textit{cry1Ab} gene fragment ($\sim$100 bp) was found in samples of stomach ($n = 3$) and jejunum content ($n = 1$) from wild boar ($n = 7$) receiving diets containing transgenic ingredients. The \textit{invertase} gene fragment was also detected in gastric content ($n = 2$). All other samples were negative for both genes (Table 2).

Immunoreactive Cry1Ab protein was detected in samples of gastric content ($3.50 \pm 3.23 \text{ ng/g}; \text{mean} \pm \text{SD}$), colon content ($0.67 \pm 0.11 \text{ ng/g}$) and rectal content ($1.58 \pm 0.59 \text{ ng/g}$) of transgenic fed wild boar. In order to determine the fragment size of the Cry1Ab protein detected by ELISA, the samples were tested in an immunoblotting assay. As expected samples of transgenic maize and the positive control (Cry1Ab/1Ac protein included in the ELISA kit) showed specific bands yielding a protein with the appropriate size of 60 kDa. No sample obtained from GI content and tissue of animals fed transgenic maize showed Cry1Ab-specific bands.

### Discussion

This study was conducted to investigate the fate of recombinant DNA and protein after feeding transgenic maize to wild boar and the likelihood of the dispersal of viable maize seeds and rapeseeds. Appreciable numbers of rapeseeds are known to persist up to 4 years in normal cropping conditions and in the absence of cultivation even for over 11 years (Lutman et al. 2003). Still, we did not find intact rapeseeds in faeces of our animals. Regarding potential endozoochory we showed that excreted intact maize seeds retain their germination capacity only in extremely rare cases. Loss of germination potential is probably the result of degradation processes during digestion due to gastric acid and digestive enzymes. Nevertheless, Heinken et al. (2002) and Schmidt et al. (2004) showed dispersal, in very low numbers, of viable seeds of vascular plants after digestion in wild boar. In addition, it cannot be excluded that differences in developmental stage (juvenile vs. adult), feeding behavior, minute pulverization of seeds and/or shorter digestion time in the GI tract (GIT) due to illness, e.g. diarrhea or distinct monogastric species characteristics might result in excretion of more
viable seeds but this is thought to be highly unlikely. The ability to detect viable seeds in the faeces of wild boar may also be dependent on the part of maize plant (e.g., cob or whole plant) and the amount of maize fed. The average daily intake of whole maize plant and maize kernels added up to 1.25 kg/day. However, recommendations regarding satiation of wild boar by supplementary feeding add up to 1.5 kg of maize per wild boar per day which could potentially increase the quantity of viable seeds defecated (Vassant et al. 1987). Similarly, Vassant (1987, 1994) concluded that wild boar prefer mast to standing maize, and standing maize to dry maize and dissuasive feeding of dry maize was not effective against damage to maize in milk. Concomitantly, consumption of fresh maize varies seasonally depending on availability of preferred food source with a peak in consumption in summer (Dardailion 1987; Herrero et al. 2006; Schley et al. 2008; Giménez-Anaya et al. 2008). However, it should be noted that seed quality of Zea mays is known to be negatively impacted by freezing temperatures and the severity of frost-damage was shown to amplify with increasing moisture content (Woltz et al. 2006; DeVries et al. 2007). Therefore, the hypothetical germination of excreted moist and macerated maize kernels and following growing only takes place either in year-round warm climate conditions or during supplemental feeding of GM maize in spring.

To assess the fate of the cry1Ab gene the degradation of transgenic DNA in the GIT of wild boar was investigated. Due to the higher number of copies of each gene per genome the endogenous chloroplast rubisco gene was used as a model to analyze the degradation of ingested DNA. Endogenous fragments, which included sequences from the rubisco (173–1753 bp) and invertase (~100 bp) were amplifiable depending on the origin of the contents and primer set, but no amplicon was detected in tissue samples or blood. These data agree with results of studies performed with domestic pigs fed insect-resistant maize (Chowdhury et al. 2003). However, in our study the cry1Ab gene was detectable up to sizes of 420 bp in gastric content, Chowdhury et al. (2003) only tested cry1Ab amplicons of 110 bp which they found in contents of all parts of the GIT. Phipps and Beever (2000) concluded that the enzymatic activity of the gut ensures extensive degradation of nucleic acids contained in feed. These data are comparable with results summarized by Flachowsky et al. (2005). Unlike our results, Reuter and Aulrich (2003) showed that rubisco gene fragments were detectable outside the GIT and concluded that cry1Ab DNA may cross the GI barrier if present in enough quantity. However, 72 h after feeding a diet containing transgenic ingredients they did not even detect cry1Ab gene fragments throughout the GIT.

Our results indicate that feed-ingested DNA is partially resistant to mechanical, chemical and enzymatic activities of the GIT of wild boar and is not completely degraded. It has to be noted that maize kernels were still visible within faeces and DNA detected near the end of the digestive tract was protected within undigested maize residue, but biologically relevant functional activity of that DNA is highly unlikely. However, any small polynucleotide DNA fragment that might enter the body and blood may then be phagocytized by mononuclear leukocytes and further degraded by cellular enzymes and nucleases in different tissues (Doerfler 2000).

Positive signals of the Cry1Ab protein in samples from stomach, colon and rectum of wild boar fed transgenic maize were measured using ELISA. None of the isogenically fed wild boar showed positive reaction to the Cry1Ab protein excluding any cross-reactions with maize-specific components. Immunoblotting yielded no significant bands of the Cry1Ab protein of approximately 60 kDa in samples from both groups. For the transgenic fed group which was founded in low initial Cry1Ab protein concentrations for immunoblotting analyses.

The ELISA data are in agreement with a study by Chowdhury et al. (2003) who measured the Cry1Ab protein in various GI contents of Bt11-fed pigs. Unlike our results they found that immunoblotting of gastric content samples resulted only in a weak band of 65 kDa mass which could be the result of lower Cry1Ab protein amounts in offered feed, reduced gastric emptying time due to all fed feedstuff or different protein degradation of the two different maize varieties. However, no Cry1Ab protein was detectable either in tissue samples of wild boar (this study) or of domestic pigs.

These data suggest that no bioactive Cry1Ab protein will leave the GIT of wild boar. Any potential impact of the Cry1Ab protein on mammalian cells would possibly be confined to epithelial cells of the GIT. Potential immunological reactions of epithelial cells caused by exposure to the Cry1Ab protein should be subject of further investigations. Uncontrolled dispersal of viable maize kernels and rapeseed is extremely low in wild boar.

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References


