Attempts to Detect Transgenic and Endogenous Plant DNA and Transgenic Protein in Muscle from Broilers Fed YieldGard[1] Corn Borer Corn

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ABSTRACT Questions regarding the digestive fate of DNA and protein from transgenic grain have been raised in regard to human consumption and trade of animal products (e.g., meat, milk, and eggs) from farm animals fed transgenic crops. Using highly sensitive, fully characterized analytical methods, fragments of transgenic and endogenous plant DNA, as well as transgenic protein, were not detected in chicken breast muscle samples from animals fed YieldGard Corn Borer Corn event MON 810 (YG). Total DNA was extracted from breast muscle samples from chickens fed for 42 d with a diet including either 55 to 60% YG grain or 55 to 60% conventional corn grain. DNA preparations were analyzed by PCR followed by Southern blot hybridization for the presence of a 211-bp fragment of the *Bacillus thuringiensis* (Bt) *cry1Ab* gene and a 213-bp fragment of the endogenous corn gene *sh2* (encoding ADP glucose pyrophosphorylase). By using 1 μg of input DNA per reaction, none of the extracted samples was positive for *cry1Ab* or *sh2* at the limit of detection for these PCR assays. A 396-bp fragment of the chicken ovalbumin (*ov*) gene, used as a positive control, was amplified from all samples showing that the DNA preparations were amenable to PCR amplification. By using a competitive immunoassay with a limit of detection of approximately 60 ng of Cry1Ab protein per gram of chicken muscle, neither the Cry1Ab protein nor immunoreactive peptide fragments were detectable in the breast muscle homogenates from chickens fed YG grain.

(*Key words*: *Bacillus thuringiensis*, Cry1Ab, enzyme-linked immunosorbent assay, polymerase chain reaction, YieldGard)

2003 Poultry Science 82:371–380

INTRODUCTION

The safety of DNA and protein introduced into genetically enhanced agricultural products is based on strong scientific principles and premarket regulatory assessments (FAO/WHO, 1991, 1996; OECD, 1998, 2000). In addition, the United Nations World Health Organization (WHO) and Food and Agriculture Organization (FAO) and the US Food and Drug Administration (FDA) and Environmental Protection Agency (EPA) have all stated that DNA, including DNA from transgenic crops, is a safe, natural component of food (US FDA, 1992; US EPA, 2001). Highly sensitive detection technologies such as those incorporating PCR can be used to assess the digestive fate of transgenic DNA from genetically enhanced crops. Questions about the digestive fate of transgenic DNA and protein have been raised in regard to human consumption and trade of animal products (e.g., meat, milk, and eggs) from farm animals fed transgenic crops.

The normal digestive process for food and feed includes constant exposure of the gastrointestinal tract to foreign DNA that is released from partially or completely digested foods or feeds. Ingested food is mechanically disrupted, and the released DNA is cleaved through acid hydrolysis and enzymatic digestion, especially by DNase I from salivary and pancreatic secretions, into small DNA fragments and free nucleotides (McAllan, 1982). The presence of various phosphatases and deaminases continue to destroy the structural integrity of any free DNA. A study with beef steers showed that plant DNA in feed is progressively degraded as it moves through the digestive tract, with over 50% degraded in the first third of the intestine and 80% having disappeared by the time the digesta reaches the terminal ileum (McAllan, 1980). DNA given directly to steers was completely degraded into

Received for publication June 27, 2002.

Accepted for publication October 31, 2002.

[1]YieldGard is a registered trademark of Monsanto Technology LLC, St. Louis, MO.

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Abbreviation Key: Bt = *Bacillus thuringiensis*; CP4 EPSPS, synthetic enzyme 5-enolpyruvylshikimate-3-phosphate synthase derived from *Agrobacterium sp.* strain CP4; *cry1Ab* = coding region for the Bt Cry1Ab protein; Cry1Ab = insecticidal protein from Bt; LOD = limit of detection; *ov* = gene encoding chicken ovalbumin; *sh2* = gene encoding maize ADP glucose pyrophosphorylase; YG = YieldGard Corn Borer Corn event MON 810.

mononucleotides in about 4 h (McAllan, 1982). More recently, studies by Schubbert et al. (1997) suggested that approximately 0.1% of purified M13 circular phage DNA ingested by mice could be detected as small fragments in white blood cells 2 to 8 h after feeding. Each mouse was fed 50 μg of phage DNA, a relatively large dose of foreign DNA. The DNA that was detected was most likely present within the white cells as part of a normal immune system scavenger process.

The digestive fate of transgenic DNA was originally studied in the mid-1970s following the advent of recombinant DNA methodologies (Maturin and Curtiss, 1977). These experiments evaluated the degradation of DNA from functionally crippled *Escherichia coli* cells and plasmids used for recombinant DNA research. It was shown that the bacterial chromosomal DNA was rapidly degraded in the small intestine of rats, attributable to stomach acids and pancreatic nucleases. Plasmid DNA was even less stable, presumably due to its smaller size. More recently, direct analysis of the stability of the transgenic DNA that encodes the *bar* gene in rapeseed demonstrated that *bar* is completely broken down into nucleotides in digestive fluids isolated from swine, chickens, and cows within 1 h at 37°C and pH 1.5 (Rasche, 1998). In addition, Chambers et al. (2002) determined that the β-lactamase gene found in transgenic corn event CG00526-176 "does not survive the digestive processes of the [chicken] stomach to pass further down the alimentary tract."

The normal digestive fate of non-allergenic proteins is such that they are rarely absorbed intact across the intestinal wall at any significant level unless specifically designed to do so, such as a special class of milk-borne immunoglobulins (IgA) that protect newborn mammals (Gardner, 1988). Digestive secretions, primarily enzymes and acid, generally prevent dietary protein from exerting biological activity within an animal. Most ingested proteins are hydrolyzed into increasingly smaller fragments in the mammalian digestive system and are either absorbed as small peptides or free amino acids, or excreted in the feces. For example, it has been shown that <0.008% of ovalbumin protein orally administered to humans is detectable in their circulation (Tsume et al., 1996). The authors concluded that the digestive tract provides a strong barrier to the absorption of macromolecular proteins into the body.

The digestive fate of each expressed protein in a transgenic crop is evaluated on a case-by-case basis as part of an overall safety assessment process outlined by the FAO/WHO (1996, 2000), OECD (1998, 2000), and regulatory agencies around the world. The digestibility of each protein introduced into a genetically modified plant is routinely assessed by an in vitro digestive fate study as part of an assessment of the allergenic potential of the protein (Metcalfe et al., 1996). To date, the proteins introduced into transgenic agricultural products approved for food use have been shown to be readily degraded in simulated gastric and intestinal studies (Metcalfe et al., 1996; Betz et al., 2000). Nonetheless, specific studies are needed to directly address whether transgenic proteins can be detected in tissue samples from animals fed transgenic crops. Ash et al. (2000) reported that an ELISA for the CP4 EPSPS protein from Roundup Ready[3] soybeans could not detect CP4 EPSPS in whole egg, egg white, liver, or feces from laying hens fed this grain.

It is unknown whether the Cry1Ab protein of YG corn can be detected in tissues from animals fed this grain. Cry1Ab is a member of a large family of crystal (Cry) proteins produced by many different strains of the common soil bacterium *Bacillus thuringiensis* (Bt). Bt-based products have been widely used as microbial pesticides since 1961 (McClintock et al., 1995) because Cry proteins are specifically effective in controlling certain orders and species of insect pests and are typically harmless to nontarget organisms (Höfte and Whiteley, 1989).

The present study was conducted to address two related questions. First, can extremely sensitive DNA analytical methods detect *cry1Ab* or *sh2* (corn endogenous) gene fragments in broiler muscle from chickens fed YG grain? PCR assays for the *cry1Ab* and *sh2* genes are described that use a relatively high amount of input chicken DNA per reaction. Assay products are then analyzed by a radioisotopic Southern blot detection method that allows these assays to have a limit of detection (LOD) approaching one 2C genome equivalent of DNA (approximately 5 pg of nuclear DNA per diploid corn cell; Arumuganathan and Earle, 1991). Second, can a competitive immunoassay detect Cry1Ab protein fragments in broiler muscle from chickens fed YG grain? The immunoassay described below for the Cry1Ab Bt protein uses a competitive ELISA format to detect both intact and immunoreactive fragments of the protein, if present.

MATERIALS AND METHODS

Samples and Processing

All chickens were raised as part of a feeding study to assess composition and animal feed performance of chickens fed YG and conventional corn grain (Taylor et al., 2003). These experiments were conducted in accordance with principles and guidelines for the care and use of agricultural animals in research (FASS, 2001). Each chicken was labeled with a wing tag number prior to the feeding trial, and the birds were randomly assigned to feeding groups of either YG or conventional corn. Birds from each feeding group were selected in a nonsystematic manner at the end of the 42-d feeding period for tissue collection. Breast muscle samples from 10 chickens fed YG grain and 10 chickens fed conventional corn grain were collected for DNA and protein analyses. The wing tag numbers were used to track tissue samples, resulting in a blinded sequence of samples that was maintained throughout the subsequent analyses. Tissues were collected at time of slaughter; placed in individual plastic

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bags with a label indicating the bird number, sample collection date, and tissue type; and immediately frozen and shipped to the laboratory on dry ice.

Tissue samples were further processed using aseptic techniques. All subsampling occurred in a laboratory where no PCR products or plasmids were handled. While samples were still frozen, the surfaces of each breast were removed with a butcher knife on a cutting board, both of which were cleaned with a 10% (vol/vol) bleach solution prior to use, to obtain a block of muscle tissue. The process was then repeated on a second clean cutting board with a second clean knife, thus ensuring that the muscle tissue sample was free from surface contamination prior to subsampling for DNA extraction. The frozen block of tissue was transferred to a third clean cutting board and was finally subsampled into small frozen tissue cubes using a clean knife. Tissue cubes were placed into sterile 50-mL polypropylene tubes that had been pretreated with ultraviolet light in a clean hood. Each person involved in tissue subsampling wore a clean disposable lab coat and surgical gloves. All cutting surfaces, knives, and processing equipment were cleaned with a 10% (vol/vol) bleach solution between each sample, and gloves were changed between samples. Samples were maintained on dry ice during subsampling and then stored in a freezer at –80°C until analyzed.

DNA Analysis

DNA Extraction. Chicken breast muscle samples (3.4 to 8.4 g) were homogenized on ice in four volumes of lysis solution [10 m*M* Tris-HCl, pH 8.0, 50 m*M* EDTA, 1% (wt/vol) SDS] with a Polytron PT 10/35 homogenizer.[4] A 0.6-mL aliquot of each homogenized tissue sample was digested overnight (~18 h) with 0.1 mg of Proteinase K[5] at 55°C. The homogenate was then treated with 3 μL of RNase A[5] for 30 min at 37°C. Ammonium acetate was added to the homogenate to a final concentration of 1.9 *M*, and the mixture was centrifuged at 12,000 × *g* for 3 min to pellet precipitated proteins and cellular debris. DNA was pelleted in isopropanol, washed with 70% (vol/vol) ethanol, air-dried, and resuspended in 100 μL of TE buffer (10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA). To serve as positive controls for the extraction procedure, approximately 100 and 250 pg of purified YG genomic DNA were spiked into a subset of tissue homogenates containing 120 to 150 mg of starting tissue immediately prior to the start of the DNA extraction procedure. Every chicken breast muscle sample was extracted in duplicate. Each extract was used in duplicate PCR assays for both *cry1Ab* and *sh2*.

PCR and Southern Blot Analysis. Extreme caution was used when handling tissue samples and DNA extracts so as not to contaminate them with previously amplified DNA, plasmids containing transgenes, or any other source that might contain plant or transgenic DNA. Extracted DNA was quantitated using Hoefer's DyNA Quant 200 fluorometer[6] with Hoechst dye. Preliminary experiments with the *cry1Ab* PCR assay were conducted with purified YG genomic DNA in chicken genomic DNA matrix to determine that 1 μg of chicken DNA could be used in the *cry1Ab* assay without detectable matrix effects. Therefore, the PCR assays were conducted with 1 μg of genomic DNA in a total reaction volume of 50 μL. Pipetters dedicated for PCR setup were used with sterile, aerosol-resistant tips. Each reaction was performed in a PTC-225 DNA Engine Tetrad[7] thermocycler and contained 2.5 m*M* $MgCl_2$, 0.2 μ*M* of each primer, 200 μ*M* of each dNTP, and 2 U of *Taq* DNA polymerase in 1× reaction buffer. The PCR cycling conditions for the coding sequence of *cry1Ab* were as follows: 1 cycle at 94°C for 1 min; 35 cycles at 96°C for 30 s, ramp to 72°C at 1°C/s, 72°C for 1 min; 1 cycle at 72°C for 5 min. The PCR cycling conditions for the endogenous corn gene, *sh2* (ADP glucose pyrophosphorylase, GenBank Accession No. M81603), were 1 cycle at 94°C for 3 min; 35 cycles at 96°C for 30 s, 55°C for 30 s, 72°C for 45 s; 1 cycle at 72°C for 5 min. The sequences (5′ to 3′) of the forward and reverse primers, respectively, for *cry1Ab* amplification were as follows: CCT GGA GCG CGT CTG GGG CCC TGA TTC T; GGC GCT GCC CCT GAA GCT ACC GTC GAA GTT CT. The sequences (5′ to 3′) of the forward and reverse primers, respectively, for *sh2* amplification were as follows: TTC GGG AGG CAA GTG TGA TTT CG; GTC GGC AAG AAT GGA GCA ATT C. Primer sequences were selected using DNASTAR PrimerSelect software based on the coding regions of the respective genes. Each set of PCR assays included both positive and negative controls. The positive controls were reactions containing 100 ng of purified YG genomic DNA. Additional positive controls were the DNA extracts from samples spiked with YG genomic DNA as described above. Negative PCR controls did not contain any template DNA.

Products of PCR were separated on a 2% (wt/vol) agarose gel in 1× TBE (89 m*M* Tris, pH 8.4; 89 m*M* boric acid; 2 m*M* EDTA) by electrophoresis and were visualized by ethidium bromide staining under ultraviolet illumination. After photography, the DNA in the gel was denatured, neutralized, transferred to a nylon membrane, and cross-linked under ultraviolet light by standard techniques (Sambrook and Russell, 2001). Approximately 25 ng of each probe template was labeled with ^{32}P-dCTP[6] by a random priming method (RadPrime DNA Labeling System[8]). Probe templates were purified amplicon of the 211-bp fragment of the *cry1Ab* gene or the 213-bp fragment of the *sh2* gene. Prehybridization and hybridization also followed standard techniques (Sambrook and Russell, 2001), and each membrane was washed in an aqueous solution of 0.1% (wt/vol) SDS at 65°C. Blots were exposed to Kodak BioMax MS-2 film in conjunction with one BioMax MS intensifying screen.[9]

As described above, appropriate positive and negative controls were included in all the analyses to ensure the

[4]Brinkmann Instruments, Inc., Westbury, NY.
[5]Qiagen Inc., Valencia, CA.
[6]Amersham Pharmacia Biotech Inc., Piscataway, NJ.
[7]MJ Research, Inc., Watertown, MA.
[8]Life Technologies, Rockville, MD.
[9]Sigma Chemical Co., St. Louis, MO.

sensitivity and reproducibility of the DNA extractions and PCR assays. In addition, all DNA extracts were analyzed by PCR for a 396-bp region of the chicken ovalbumin (*ov*) gene (GenBank Accession No. V00383) to ensure the quality of the extracted DNA and its suitability for PCR. Reaction and cycling conditions for the *ov* PCR were identical to those used for the *sh2* PCR (see above) except each reaction contained 1.5 m*M* $MgCl_2$, 1 U of *Taq* DNA polymerase, and 500 ng of template DNA. The sequences (5′ to 3′) of the forward and reverse primers, respectively, for *ov* amplification were as follows: TGA AGA TGG AGG AAA AAT ACA ACC; TGC AGC AGA TAA CAT ACT TTT CAT. Products of *ov* amplification were visualized on an ethidium bromide-stained agarose gel.

Protein Analysis

Protein Extraction. Tissue samples were weighed and then lyophilized overnight (~14 h) in 50-mL sterile polypropylene conical tubes. To each lyophilized sample was added 10 to 12 chrome-steel beads (0.25-inch diameter)[10] followed by vigorous shaking in a Harbil 5G-HD mixer[11] for about 6 min to mechanically disrupt the tissue. Ten volumes of extraction buffer were added to each sample based on the fresh weight of the tissue prior to lyophilization. Extraction buffer consisted of 81 m*M* $Na_2HPO_4 \cdot 7H_2O$, 15 m*M* KH_2PO_4, 1.5 *M* NaCl, 27 m*M* KCl, 1 m*M* PMSF, and Protease Inhibitor Cocktails 1 and 2.[9] The homogenates were again shaken in the presence of buffer for 6 min to thoroughly resuspend the powdered tissue. The samples were incubated overnight (~14 h) at 4°C followed by centrifugation at 14,000 × *g* for 10 min. The supernatants were dispensed into single-use aliquots and stored at –80°C.

Competitive Immunoassay. Analysis of tissue samples for the presence of the Cry1Ab protein was performed using a competitive ELISA. Purified, recombinant Cry1Ab protein was diluted to 1.5 μg/mL in coating buffer (50 m*M* carbonate buffer, pH 9.6), and 100 μL was dispensed per well into 96-well microtiter plates. The Cry1Ab protein was immobilized to the microtiter plates by incubation overnight at 4°C. After immobilization, each well was aspirated and washed three times with PBST washing buffer [10 m*M* phosphate buffer, pH 7.4, 0.8% (wt/vol) NaCl, 0.05% (vol/vol) Tween-20]. Sera from goats, which were immunized with purified recombinant Cry1Ab protein, were purified on a Cry1Ab immuno-affinity column[12] to obtain antibodies specific for Cry1Ab protein. The specificity of these antibodies was confirmed by Western blot analysis. The Cry1Ab-specific immuno-affinity purified antibodies were pre-incubated overnight at 4°C with either 1) tissue sample extracts or 2) protein standards in the presence of an equivalent concentration of tissue extract. Into each well of the Cry1Ab protein-coated microtiter plate was transferred 100 μL of the antibody/extract mixture.

The Cry1Ab ELISA was a two-step procedure consisting of 100 μL of standard or test sample being incubated on the plate for 1 h at 37°C. This incubation was followed by aspirating and washing each well three times with PBST washing buffer (~300 μL/well). In the second step, 100 μL/well of a 1:5,000 dilution of rabbit anti-goat polyclonal antibody, conjugated to horseradish peroxidase (HRP) and prepared in stabilizing buffer (Stabilzyme HRP Conjugate Stabilizer[13]), was incubated in the assay plate for 1 h at 37°C. After aspiration and washing of each well three times with PBST washing buffer (~300 μL/well), 100 μL of the enzyme substrate 3,3′,5,5′-tetramethylbenzidine (TMB) was added per well and allowed to develop for approximately 10 min at room temperature. The enzymatic reaction was terminated by addition of 3 *M* phosphoric acid at 100 μL per well. Immediately after the enzymatic reaction was stopped, absorbance readings at 450 nm were taken using a dual wavelength plate reader with a reference wavelength of 650 nm. The concentration of Cry1Ab protein was determined in each sample by interpolation against a serially diluted eight-point standard curve, with values that ranged from 0.25 to 4.0 ng/mL. The standard curve was determined by a quadratic curve-fitting model (Microsoft Excel). The LOD for this assay was determined to be approximately 60 ng of Cry1Ab protein per gram of chicken muscle tissue (~60 ppb).

RESULTS

Testing for cry1Ab and sh2 DNA Fragments

A PCR followed by Southern blot hybridization was used to analyze DNA extracted from chicken breast muscle samples for a 211-bp region of the Bt *cry1Ab* gene and a 213-bp region of the endogenous corn gene *sh2* (encoding ADP glucose pyrophosphorylase). Both assays were shown to be capable of detecting as little as 1 pg of purified genomic DNA from YG grain (Figures 1 and 2). Routinely, however, the LOD was approximately 5 pg, or roughly one diploid genome equivalent of corn DNA (Arumuganathan and Earle, 1991). It is difficult to visualize the low amounts of PCR product generated from picogram amounts of genomic template using merely ethidium bromide staining. For example, product from the *sh2* assay is not clearly visible on a stained agarose gel (Figure 2) unless more than 10 pg of purified template DNA is used in the reaction. However, when coupled to Southern blot analysis with specific ^{32}P-labeled probes, the presence of amplicon is unequivocal for both *cry1Ab* and *sh2* assays (Figures 1 and 2). Not only is the sensitivity of each assay increased by Southern blotting, but also the clear bands obtained on an autoradiograph alleviate some of the uncertainty inherent in the interpretation of a gel result. It

[10]NN Ball and Roller, Mountain City, TN.
[11]Fluid Management, Wheeling, IL.
[12]Pierce, Rockford, IL.
[13]SurModics, Eden Prairie, MN.

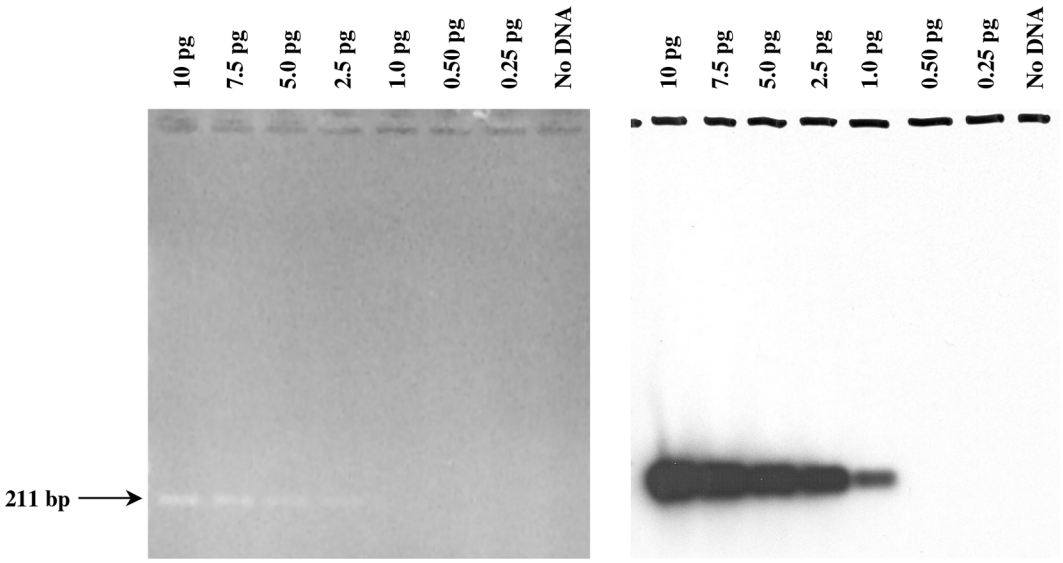

FIGURE 1. PCR amplification and Southern blot analysis of genomic DNA from YieldGard Corn Borer Corn for the *cry1Ab* gene fragment. Purified genomic DNA from YieldGard grain was used in PCR assays designed to amplify a 211-bp region of the *cry1Ab* coding region. Included as a negative PCR control was a reaction without template DNA (no DNA). These assays were conducted to determine the limit of detection for the *cry1Ab* assay. Twenty microliters of each 50-μL reaction were separated by agarose gel electrophoresis, blotted onto a nylon membrane, and probed with ^{32}P-labeled *cry1Ab* amplicon. The ethidium bromide-stained gel under ultraviolet illumination is shown on the left, and its corresponding autoradiograph is shown on the right. The amount of template DNA in each reaction is listed at the top of each lane.

is sometimes difficult to visually distinguish the specific target PCR product from spurious amplification products, especially if the product of interest is small in size and in very low in abundance. In addition, as the number of cycles used in PCR is increased to achieve greater assay sensitivity, the possibility of producing a non-specific or questionable PCR amplification product increases.

Representative gels and blots of the tissue analyses for *cry1Ab* and *sh2* are shown in Figures 3 and 4, respectively. As described above, if we were to rely on gel analysis alone, then the results in Figure 4 would suggest the possibility that this single-copy endogenous plant gene is present in all muscle samples because a faint amplification product was obtained on the gel at approximately the correct molecular weight of 200 bp. When coupled to Southern blot analysis, however, the results were strikingly clear with no hybridization of the *sh2* probe to the 200-bp band, showing that this band was a non-specific amplification product. In fact, the sensitivity of the Southern method was so great, as measured by the specific binding of radiolabeled probe, that exposures of less than 1 h, such as those shown in Figures 1 to 4, were usually

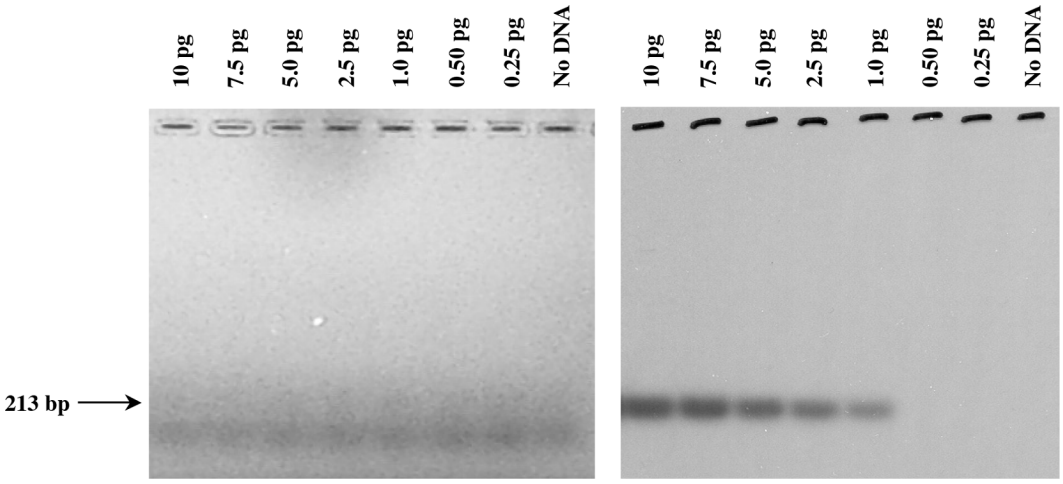

FIGURE 2. PCR amplification and Southern blot analysis of genomic DNA from YieldGard Corn Borer Corn for the *sh2* gene fragment. Purified genomic DNA from YieldGard grain was used in PCR assays designed to amplify a 213-bp region of the *sh2* coding region. Included as a negative PCR control was a reaction without template DNA (no DNA). These assays were conducted to determine the limit of detection for the *sh2* assay. Twenty microliters of each 50-μL reaction were separated by agarose gel electrophoresis, blotted onto a nylon membrane, and probed with ^{32}P-labeled *sh2* amplicon. The ethidium bromide-stained gel under ultraviolet illumination is shown on the left, and its corresponding autoradiograph is shown on the right. The amount of template DNA in each reaction is listed at the top of each lane.

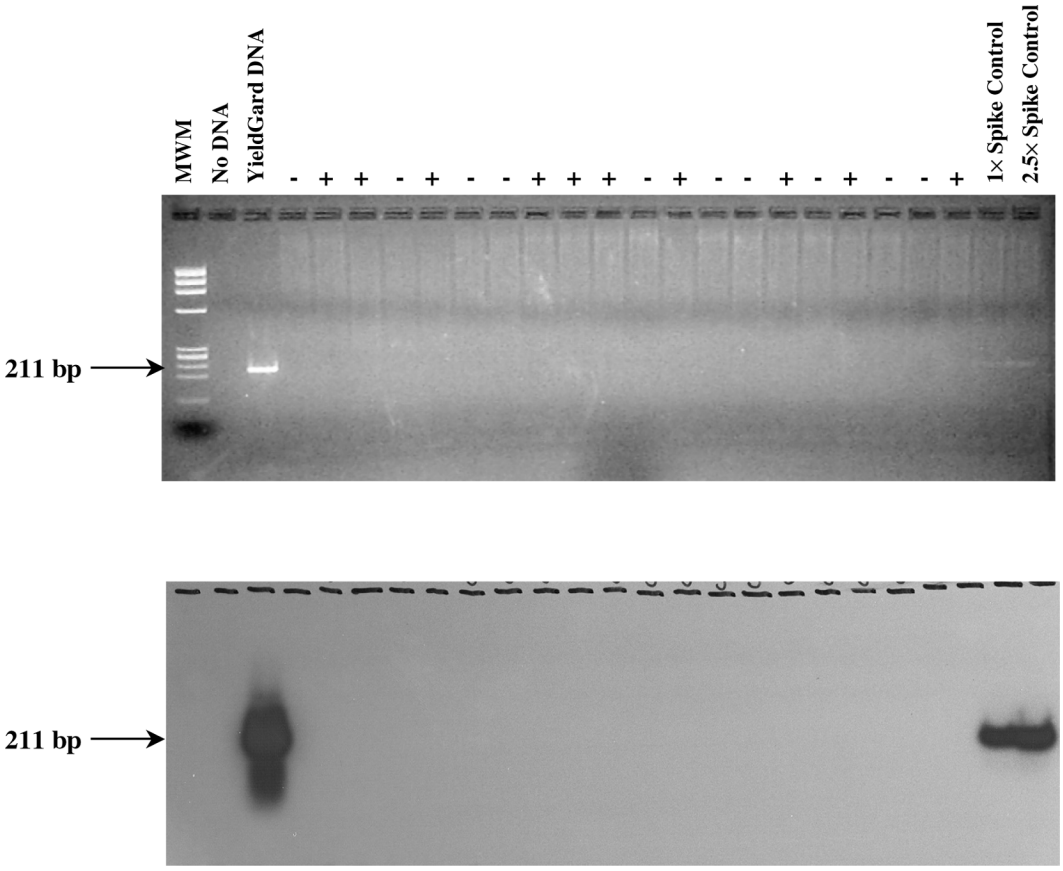

FIGURE 3. PCR amplification and Southern blot analysis of DNA extracts from chicken breast muscle samples for the *cry1Ab* gene fragment. DNA extracts from animals fed YieldGard (+) or conventional (–) corn grain were used in PCR assays designed to amplify a 211-bp region of the *cry1Ab* coding region. Included as PCR controls were reactions without template DNA (no DNA) and reactions with purified genomic DNA from YieldGard grain. Picogram amounts of YieldGard genomic DNA were spiked into a subset of chicken muscle homogenates (at approximately 0.7 and 2 pg of DNA per milligram of tissue) prior to extraction to serve as positive extraction controls (1× and 2.5× spike controls). Twenty microliters of each 50-μL reaction were separated by agarose gel electrophoresis (top), blotted onto a nylon membrane, and probed with ^{32}P-labeled *cry1Ab* amplicon (bottom). MWM = molecular weight marker.

sufficient to visualize a PCR product. Blots, however, were purposefully overexposed to X-ray film (typically twice the optimum exposure) to ensure that no product was present in samples considered true negatives.

Given the sensitivity of the PCR assays (i.e., able to detect the equivalent amount of DNA found in a single diploid corn cell), it is not surprising that false positives can result from contamination of the extract or PCR with template DNA. Equally important is to have certainty in negative PCR results. In addition to conducting the analyses in a sample-blinded manner, well-defined criteria for accepting "true positive" and "true negative" PCR results were established. Before a sample could be confirmed as having undetectable levels of plant or transgenic DNA, the following criteria had to be met. First, samples were always extracted in duplicate, and both *cry1Ab* and *sh2* PCR assays were conducted in duplicate, too. The results had to be consistent between duplicate reactions and duplicate extracts. For a Southern blot to be considered valid, both positive and negative control reactions had to produce the expected results. As a positive control for both DNA extraction and PCR, each of two spiked samples had to produce positive results on the Southern blot. Finally, to ensure that the DNA was amenable to PCR and did not contain significant inhibitors, each tissue extract was used in a PCR to amplify a fragment of the endogenous chicken *ov* gene. None of the extracted DNA from chicken breast muscle samples was positive according to the criteria described above for *cry1Ab* or *sh2*, and, in fact, all samples met the criteria for being true negative results.

Testing for Cry1Ab Protein

An immunoassay for Cry1Ab protein was described by Stave et al. (2000) that involves capture of the protein by immobilized antibody, followed by detection of the captured protein with a second Cry1Ab antibody preparation (i.e., a sandwich ELISA). A critical component of the safety assessment of proteins introduced into transgenic crops for food and feed use is the demonstration that these introduced proteins are readily digested in simulated gastric models (Metcalfe et al., 1996). Because Cry1Ab protein was shown to be rapidly digested in simulated gastric models, it was highly unlikely that intact Cry1Ab protein would be found in samples of muscle

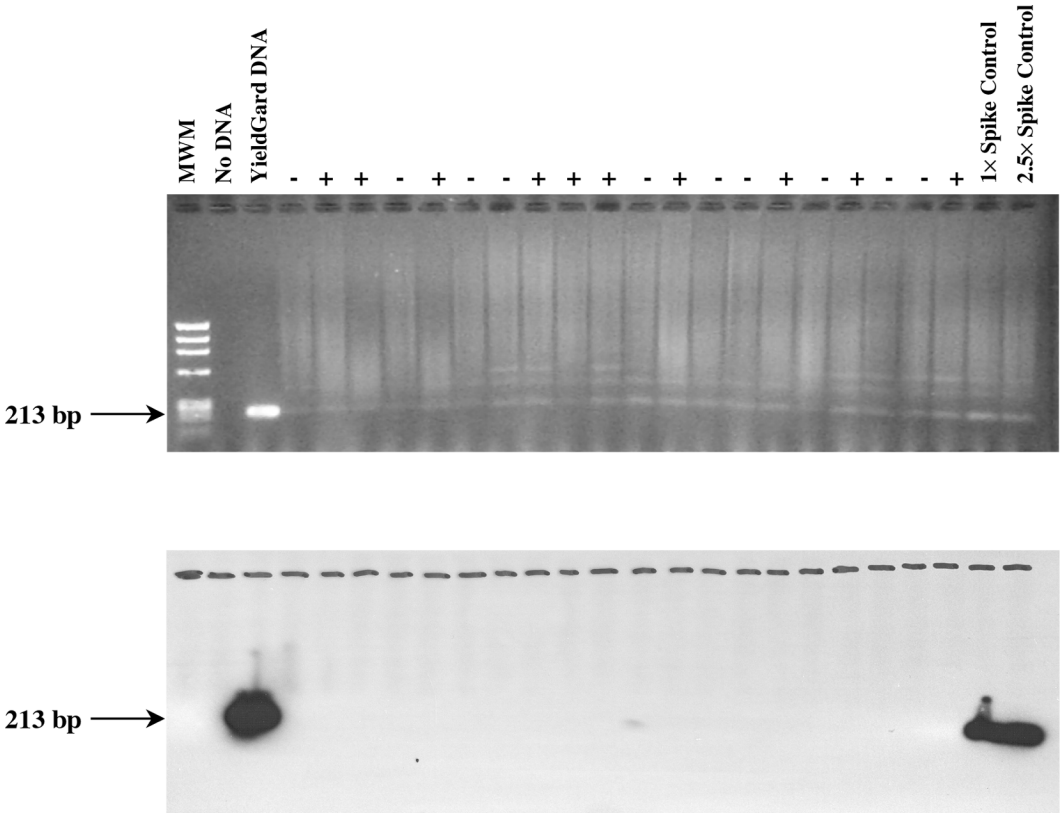

FIGURE 4. PCR amplification and Southern blot analysis of DNA extracts from chicken breast muscle samples for the *sh2* gene fragment. DNA extracts from animals fed YieldGard (+) or conventional (–) corn grain were used in PCR assays designed to amplify a 213-bp region of the *sh2* gene. Included as PCR controls were reactions without template DNA (no DNA) and reactions with purified genomic DNA from YieldGard grain. Picogram amounts of YieldGard genomic DNA were spiked into a subset of chicken muscle homogenates (at approximately 0.7 and 2 pg of DNA per milligram of tissue) prior to extraction to serve as positive extraction controls (1× and 2.5× spike controls). Twenty microliters of each 50-μL reaction were separated by agarose gel electrophoresis (top), blotted onto a nylon membrane, and probed with ^{32}P-labeled *sh2* amplicon (bottom). MWM = molecular weight marker.

tissue from chickens fed YG grain. Therefore, a competitive ELISA was developed that had potential to detect partially digested fragments of Cry1Ab protein in addition to the intact protein. The principle of the competitive ELISA is as follows: incubation of the standard protein or test sample with anti-Cry1Ab antibody in solution allows a binding equilibrium to be reached between the Cry1Ab protein or immunoreactive fragments of Cry1Ab in solution and Cry1Ab immobilized to the assay plate. Fragments of Cry1Ab protein that can still bind anti-Cry1Ab antibodies will be detected in this assay configuration, whereas these fragments would most like go undetected in a sandwich assay configuration due the requirement that at least two accessible antibody epitopes be present on the protein to bridge between immobilized and labeled antibodies. The enhanced sensitivity of the competitive assay for fragments of protein was demonstrated by digesting Cry1Ab protein in US Pharmacopia simulated gastric fluid (~15 U/mL of pepsin in a pH 1.3 solution) for ~20 s and then analyzing the digested protein in the sandwich or competitive ELISA. The sandwich ELISA demonstrated no reactivity with the digested Cry1Ab protein up to 16 ng/mL [i.e., all optical densities (OD) in sample wells were less than or equal to the OD of buffer-only wells; data not shown]. However, the competitive assay successfully detected Cry1Ab protein in the preparation digested in vitro at ≥ 4 ng/mL (data not shown).

The concentration of Cry1Ab protein was determined in each sample by interpolation against a serially diluted eight-point standard curve (Figure 5) with values that ranged from 0.25 to 4 ng/mL in the presence of 10% (wt/vol) tissue extract. Because the tissue matrix could impact the ability of the antibody to bind the protein fragments in the sample extract, the standard curve was generated in the same concentration of tissue homogenate as the test samples to normalize for the effect of tissue matrix. The curve was generated using a quadratic curve-fitting model. The LOD for this assay is about 60 ng of Cry1Ab protein per gram of chicken muscle tissue (~60 ppb). Over a period of 3 d with two independent runs per day conducted by two analysts, the CV of the ELISA was 25% at the LOD in the presence of 10% (wt/vol) chicken matrix. None of the samples tested was above the LOD of the assay. In addition, there were no statistical differences ($P < 0.05$) in measurements between homogenates of chicken

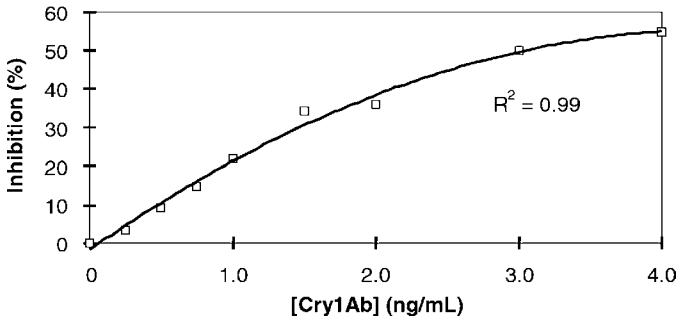

FIGURE 5. Standard curve for competitive ELISA to detect Cry1Ab protein in the presence of chicken tissue extract. The concentration of Cry1Ab protein in chicken extracts was measured by interpolation against an eight-point standard curve with values ranging from 0.25 to 4.0 ng/mL Cry1Ab protein in the presence of 10% (wt/vol) chicken extract. The standard curve shown in this figure was generated using a quadratic curve-fitting model. The limit of detection for this assay is about 60 ng of Cry1Ab protein per gram of chicken muscle tissue (~60 ppb).

breast samples derived from YG or conventional corn fed animals when analyzed by the competitive immunoassay.

DISCUSSION

It is accepted that ingested DNA and protein (endogenous or transgenic) cannot be assimilated to any significant extent in animal tissues including meat, milk, or eggs (Beever and Kemp, 2000; Jonas et al., 2001). Limited data exist, however, on whether highly sensitive PCR and ELISA analytical methods can detect fragments of ingested DNA or protein, respectively, in animal tissues. In addition, there is a need for well-characterized molecular and immunodiagnostic methods suitable for analysis of animal tissue samples for the presence or absence of DNA and protein from diets containing transgenic plant material.

Testing for Transgenic DNA

The present study was conducted to assess whether a DNA detection method, optimized for sensitivity and reliability, could detect fragments of transgenic DNA from YG grain in the meat from chickens fed this genetically modified product. Highly sensitive methods were needed because the amount of transgenic DNA that an animal would consume per day is very small, especially compared to the total amount of DNA consumed each day. For example, it has been calculated that a 600-kg dairy cow, fed Bt corn grain as 40% of the diet and Bt silage as 20% of the diet, will consume about 600 mg of total plant DNA per day with less than 3 μg per day being transgenic Bt DNA (Beever and Kemp, 2000).

In the current study, DNA fragments of the Bt transgene, *cry1Ab*, and the endogenous corn gene, *sh2*, were not detected by exceedingly sensitive methods using 1 μg of input DNA for PCR followed by Southern blot hybridization. The sensitivity of these PCR-Southern methods is essentially at the theoretical LOD because these methods can detect a single 2C genome equivalent of corn DNA. The appropriate positive and negative DNA extraction and PCR controls, LOD of the assays, and data acceptance or rejection criteria are included in the present study to characterize and confirm the results.

Klotz and Einspanier (1998) showed that a 200-bp portion of the CP4 EPSPS gene encoding the introduced protein that confers tolerance to Roundup[3] herbicide was not detected by PCR and Southern blot analysis of blood and milk of dairy cows fed Roundup Ready soybeans. However, in their publication it was reported that a fragment of a highly abundant chloroplast gene was detectable in the white blood cells, but not in milk, of these dairy cows. A simple explanation of these results is that the chloroplast gene fragment was detected because of its significantly greater abundance in soybean cells compared to the single copy of the coding region for CP4 EPSPS in Roundup Ready soybean. Similarly, the coding region of *cry1Ab* in YG corn is a single copy event, so based on the data of Klotz and Einspanier (1998) one would not expect to detect *cry1Ab* DNA fragments in chicken muscle samples, consistent with the present results.

Recently, data were published from a study in which dairy cows, beef steers, and broiler chickens were fed either conventional corn grain or grain from another transgenic insect-protected corn product, event BT176[14] (Aumaitre et al., 2002; Einspanier et al., 2001). The investigators evaluated two DNA detection technologies (conventional PCR and LightCycler[15] real-time PCR). Although LightCycler PCR showed advantages for detecting Bt corn in feed, for animal tissue samples, this technique did not provide additional sensitivity beyond standard PCR methods. The presence of even a small portion of the coding region of the Bt *cry1Ab* gene was not detectable by either conventional or LightCycler PCR in any samples from the cows, steers, or chickens fed BT176 corn. Similar to the data generated by Klotz and Einspanier (1998), however, a small portion of the coding region of a highly abundant chloroplast gene ($tRNA_{leu}$) was detected in lymphocytes of dairy cows and possibly in their milk by conventional PCR. The $tRNA_{leu}$ gene fragment was also detected in muscle, liver, spleen, and kidney samples of chicken but not in any tissue sample from steers. It has been estimated that the number of copies of a single plastid gene can vary from approximately 500 to 10,000 per single root or leaf cell, respectively (Bendich, 1987), whereas the *cry1Ab* coding region is a single-copy event in the nuclear genome of insect-protected corn. Therefore, the high abundance of the $tRNA_{leu}$ gene may account for detection of fragments of this gene in animal tissues in which the single copy Bt *cry1Ab* gene was undetectable.

The present study showed that a small sized fragment (211 bp) of the *cry1Ab* coding region was not detected in

[14]Syngenta Seeds, Inc., Research Triangle Park, NC.
[15]Roche Diagnostics Corp., Indianapolis, IN.

breast meat from chickens fed YG grain. The LOD for the routine detection of the *cry1Ab* gene fragment in the present study was approximately 5 pg of purified YG genomic DNA per reaction. The LOD for this PCR-Southern method is essentially at the theoretical limit of the assay by detecting a single 2C genome equivalent of corn DNA (Arumuganathan and Earle, 1991). Our results confirm the recent results of broiler chicken studies in Thailand (Khumnirdpetch et al., 2001). The Thai study used real-time PCR to test whether transgenic DNA was detectable in meat, skin, duodenal, or liver samples from broilers fed genetically modified soybean meal. The investigators concluded that "PCR results of the broiler samples during 1 to 7 wk are all negative" and "the GMOs [genetically modified organisms] content in SBM [soybean meal] has been metabolized in the gut of the broiler and show no residue in the meat." Similarly, fragments of the *cry1Ab* gene and an endogenous, single-copy corn gene (*sh2*) were not detectable in DNA extracted from pork loin samples fed YG or conventional corn (Weber and Richert, 2001). Likewise, Phipps et al. (2001) did not detect transgenic DNA in milk from dairy cows receiving 18.5% of their diet as YG grain.

Testing for Transgenic Protein

Just as the DNA detection methods were optimized for detection of small fragments of transgenic DNA in chicken breast muscle matrix, the present study also describes a competitive immunoassay method that was specifically developed to determine whether the Cry1Ab protein or immunoreactive fragments of this protein could be detected in breast muscle samples from broilers fed YG grain. The Bt Cry1Ab protein is readily degraded under simulated gastric digestion conditions and, therefore, it is highly unlikely that intact Cry1Ab protein would be present in muscle tissue samples from YG-fed broiler chickens. The present study demonstrated that a preparation of Cry1Ab protein digested in vitro was detectable at concentrations ≥4 ng/mL, whereas a more conventional sandwich ELISA showed no reactivity with the digested Cry1Ab preparation up to 16 ng/mL. The Cry1Ab competitive ELISA had an LOD of approximately 60 ng of Cry1Ab protein per gram of chicken muscle tissue extract, and yet no immunoreactive fragments of Cry1Ab were detected in the breast muscle homogenates from chickens fed YG grain.

Summary

Using highly sensitive PCR assays coupled to Southern blot detection of specific amplicons, small fragments of the *cry1Ab* and *sh2* genes were not detected in breast meat samples from chickens fed to 42 d of age a diet highly enriched in YG grain. Additionally, using the same muscle tissue samples from YG-fed broilers, neither intact nor immunoreactive fragments of the Cry1Ab protein were detected by a sensitive competitive Cry1Ab ELISA. Although it is conceivable that a more sensitive DNA detection technology may someday allow for the specific detection of a very small fragment of a low-abundance ingested gene in animal tissues, this is highly improbable. The assays used in the present study were optimized to the extent that these methods reached the theoretical LOD for the tested genes. The present results showing the absence of detectable levels of transgenic DNA and protein fragments are consistent with the rapid and effective degradation of macromolecules in animal digestive tracts.

ACKNOWLEDGMENTS

We extend a special thanks to Roy L. Fuchs and Mark E. Holland for their continuous support of this project and reviews of the manuscript. We also acknowledge Shana B. Kays and the Monsanto Quality Assurance Unit for their critical reviews of the data.

REFERENCES

Arumuganathan, K., and E. D. Earle. 1991. Nuclear DNA content of some important plant species. Plant Mol. Biol. Rep. 9:208–218.

Ash, J. A., E. Scheideler, and C. L. Novak. 2000. The fate of genetically modified protein from Roundup Ready® soybeans in the laying hen. Poult. Sci. 79(Suppl. 1):26. (Abstr.)

Aumaitre, A., K. Aulrich, A. Chesson, G. Flachowsky, and G. Piva. 2002. New feeds from genetically modified plants: substantial equivalence, nutritional equivalence, digestibility, and safety for animals and the food chain. Livest. Prod. Sci. 74:223.

Beever, D. E., and C. F. Kemp. 2000. Safety issues associated with the DNA in animal feed derived from genetically modified crops. A review of scientific and regulatory procedures. Nutr. Abstr. Rev. Ser. B Livest. Feeds Feeding 70:175–182.

Bendich, A. J. 1987. Why do chloroplasts and mitochondria contain so many copies of their genome? BioEssays 6:279–282.

Betz, F. S., B. G. Hammond, and R. L. Fuchs. 2000. Safety and advantages of *Bacillus thuringiensis*-protected plants to control insect pests. Regul. Toxicol. Pharmacol. 32:156–173.

Chambers, P. A., P. S. Duggan, J. Heritage, and J. M. Forbes. 2002. The fate of antibiotic resistance marker genes in transgenic plant feed material fed to chickens. J. Antimicrob. Chemother. 49:161–164.

Einspanier, R., A. Klotz, J. Kraft, K. Aulrich, R. Poser, F. Schwägele, G. Jahreis, and G. Flachowksy. 2001. The fate of forage plant DNA in farm animals: A collaborative case-study investigating cattle and chicken fed recombinant plant material. Eur. Food Res. Technol. 212:129–143.

FAO/WHO. 1991. Strategies for assessing the safety of foods processed by biotechnology. Report of a joint FAO/WHO consultation. WHO, Geneva, Switzerland.

FAO/WHO. 1996. Biotechnology and food safety. Report of a joint FAO/WHO consultation. FAO, Food and Nutrition Paper 61. WHO, Geneva, Switzerland.

FAO/WHO. 2000. Safety aspects of genetically modified foods of plant origin. Report of a joint FAO/WHO consultation. WHO, Geneva, Switzerland.

FASS. 2001. Guidelines for the Care and Use of Agricultural Animals in Research and Teaching. Federation of Animal Science Societies, Savoy, IL.

Gardner, M. L. G. 1988. Gastrointestinal absorption of intact proteins. Ann. Rev. Nutr. 8:329–350.

Höfte, H., and H. R. Whiteley. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. 53:242–255.

Jonas, D. A., I. Elmadfa, K.-H. Engel, K. J. Heller, G. Kozianowski, A. König, D. Müller, J. F. Narbonne, W. Wackernagel, and J. Kleiner. 2001. Safety considerations of DNA in food. Ann. Nutr. Metab. 45:235–254.

Khumnirdpetch, V., U. Intarachote, S. Treemanee, S. Tragoonroong, and S. Thummabood. 2001. Detection of GMOs in the broilers that utilized genetically modified soybeanmeals as a feed ingredient. Abstract 585 in Plant and Animal Genome IX Conference, San Diego, CA. http://www.intl-pag.org/pag/9/abstracts/P08_38.html. Accessed Feb. 2003

Klotz, A., and R. Einspanier. 1998. Detection of "novel-feed" in animals? Injury of consumers of meat or milk is not expected. Mais 3:109–111.

Maturin, L., and R. Curtiss. 1977. Degradation of DNA by nucleases in intestinal tract of rats. Science 196:216–218.

McAllan, A. B. 1980. The degradation of nucleic acids and the removal of breakdown products from the small intestines of steers. Br. J. Nutr. 44:99–112.

McAllan, A. B. 1982. The fate of nucleic acids in ruminants. Prac. Nutr. Soc. 41:309–317.

McClintock, J. T., C. R. Schaffer, and R. D. Sjoblad. 1995. A comparative review of the mammalian toxicity of *Bacillus thuringiensis*-based pesticides. Pestic. Sci. 45:95–105.

Metcalfe, D. D., J. D. Astwood, R. Townsend, H. A. Sampson, S. L. Taylor, and R. L. Fuchs. 1996. Assessment of the allergenic potential of foods derived from genetically engineered crop plants. Crit. Rev. Food Sci. Nutr. 36:165–186.

OECD. 1998. Report of the OECD workshop on the toxicological and nutritional testing of novel foods. OECD, Paris, France.

OECD. 2000. Report of the OECD task force for the safety of novel foods and feeds. OECD, Okinawa, Japan.

Phipps, R., D. Beever, and A. Tingey. 2001. Detection of transgenic DNA in bovine milk: Results for cows receiving a TMR containing corn grain modified for insect protection (MON 810). J. Anim. Sci. 79:476.

Rasche, E. 1998. Rapeseed with tolerance to the nonselective herbicide glufosinate ammonium. Pages 211–221 in Plant Oils and Fuels: Present State of Science and Future Developments. N. Martini and J. Schell, ed. Springer, New York.

Sambrook, J. and D. W. Russell. 2001. Molecular Cloning: A Laboratory Manual. 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schubbert, R., D. Renz, B. Schmitz, and W. Doerfler. 1997. Foreign (M13) DNA ingested by mice reaches peripheral leukocytes, spleen, and liver via the intestinal wall mucosa and can be covalently linked to mouse DNA. Proc. Natl. Acad. Sci. USA 94:961–966.

Stave, J., K. Magin, H. Schimmel, T. Lawruk, P. Wehling, and A. Bridges. 2000. AACC collaborative study of a protein method for detection of genetically modified corn. Cereal Food World 45:497–501.

Taylor, M. L., G. F. Hartnell, B. George, and M. A. Nemeth. 2003. Comparison of broiler performance when fed diets containing grain from YieldGard (MON 810), YieldGard × Roundup Ready (GA21), non-transgenic control, or commercial corn. Poult. Sci. 82:(in press).

Tsume, Y., Y. Taki, T. Sakane, T. Nadai, H. Sezaki, K. Watabe, T. Kohno, and S. Yamashita. 1996. Quantitative evaluation of the gastrointestinal absorption of protein into the blood and lymph circulation. Biol. Pharm. Bull. 19:1332–1337.

US EPA. 2001. Bt plant-pesticides risks and benefits assessments: Insect resistance management. SAP Report No. 2000-07a. http: //www.epa.gov/oscpmont/sap/2000/october/octoberfinal.pdf. Accessed Oct. 2002.

US FDA. 1992. Statement of policy: Foods derived from new plant varieties. Notice, Fed. Regist. 57:22984–23005.

Weber, T. E., and B. T. Richert. 2001. Grower-finisher growth performance and carcass characteristics including attempts to detect transgenic plant DNA and protein in muscle from pigs fed genetically modified "Bt" corn. Abstract 162 in Midwestern Section ASAS and Midwest Branch ADSA Meeting, Des Moines, IA. ASAS, Savoy, IL.