Safety Assessment of Recombinant Green Fluorescent Protein Orally Administered to Weaned Rats[1]

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ABSTRACT Several proposed biotechnological applications of green fluorescent protein (GFP) are likely to result in its introduction into the food supply of domestic animals and humans. We fed pure GFP and diets containing transgenic canola expressing GFP to young male rats for 26 d to evaluate the potential toxicity and allergenicity of GFP. Animals (n = 8 per group) were fed either AIN-93G (control), control diet plus 1.0 mg of purified GFP daily, modified control diet with 200 g/kg canola (*Brassica rapa* cv Westar), or control diet with 200 g/kg transgenic canola containing one of two levels of GFP. Ingestion of GFP did not affect growth, food intake, relative weight of intestine or other organs, or activities of hepatic enzymes in serum. Comparison of the amino acid sequence of GFP to known food allergens revealed that the greatest number of consecutive amino acid matches between GFP and any food allergen was four, suggesting the absence of common allergen epitopes. Moreover, GFP was rapidly degraded during simulated gastric digestion. These data indicate that GFP is a low allergenicity risk and provide preliminary indications that GFP is not likely to represent a health risk. J. Nutr. 133: 1909–1912, 2003.

KEY WORDS: • green fluorescent protein • food safety • toxicity • allergenicity • genetically modified food

Green fluorescent protein (GFP)[3] has become a valuable tool in biotechnology because it has unparalleled effectiveness as a real-time marker of promoter activity and gene expression in vivo (1,2). The potential applications for GFP are diverse; they include tracking important transgenes and monitoring their potential effect on the environment (3) and replacing the use of conventional antibiotic resistance markers in recovering transgenic plants while still providing an effective selection tool (4–6). Public debate continues concerning the safety of genetically altered products and their potential introduction into the food supply. The objective of the studies reported here was to evaluate the potential toxicity of purified recombinant GFP and transgenic canola plants (*Brassica napus* cv Westar) expressing GFP when administered orally to weaned rats for 26 d. We also evaluated the potential of GFP as a food allergen by comparing its amino acid sequence with known protein allergens and determining its stability during simulated digestion.

MATERIALS AND METHODS

Diets. AIN-93G (7,8) was used at the control diet and as the base formulation for the canola diets (Research Diets, New Brunswick, NJ). Three canola lines, the progenitor cultivar (Westar) and two transgenic lines (GFP1 and GFP2) were grown for 12 wk in a greenhouse. The transgenic material was from T_2 generation plants of homozygous T_1 individuals (4). These lines expressed ~11.8 and 5.5 μg GFP/g shoot tissue, respectively. Leaves and stems were harvested and dried for 3 d in a low temperature drying oven before powdering and inclusion into the diet. Shoot material was used because it provided a larger dose of GFP/g plant material than seeds. To accommodate the addition of 20 g/100 g powdered canola material to the diet, the AIN-93G formulation was modified by decreasing casein from 200 to 168 g/kg and cornstarch from 397.5 to 280 g/kg. The diets containing 20 g/100 g canola powder had 2 g/kg less protein, 3 g/kg less carbohydrate and 8 g/kg more fiber than the AIN-93G formulation. The AIN-93G diet and modified AIN-93G plus 200 g/kg canola diets did not contain GFP. Diets with 200 g/kg GFP1 and 200 g/kg GFP2 canola contained 2.4 ± 0.05 and 1.2 ± 0.20 μg GFP/g, respectively, as determined by ELISA (see below)

Feeding study. Four-week old, male, Long-Evans Hooded rats (Harlan Teklad, Indianapolis, IN) were divided randomly into five treatment groups consisting of eight rats each. Group 1 was fed AIN-93G (control) diet and orally given 200 μL of water containing 13.9 mmol/L sucrose via slow administration by glass dropper daily for 26 d. Group 2 received the same diet and a daily dose of 1.0 mg GFP solubilized in the sucrose solution. The addition of sucrose sweetened the solution; thus, the rats readily swallowed the dose and little, if any, regurgitation was observed. Group 3 was fed the modified AIN93G diet with 20 g/100 g progenitor canola powder. Groups 4 and 5 received the modified AIN93G diet with 20 g/100 g from one of the two independently transformed lines of GFP canola, i.e., GFP1 or GFP2. All rats in each group had free access to water and the specified diet.

The feeding trial was 26 d. Food intake was recorded daily, and body weight was measured twice weekly. At the end of the study, the rats were deprived of food overnight before killing by CO_2 asphyxiation. Two randomly selected rats from both group 1 and 2 were orally administered sucrose water without or with 1.0 mg of GFP, respectively, 1 h before killing. The UNCG Campus Animal Care

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[3] Abbreviations used: ALT, alanine aminotransferase; AST, aspartate aminotransferase; FER, food efficiency ratio; GFP, green fluorescent protein; HRP, horseradish peroxidase.

and Use Committee approved all experiments and procedures. The following organs were collected, rinsed with saline to remove adherent blood and weighed: heart; left lung; liver; spleen; stomach; left kidney; small intestine (duodenum to ileocecal sphincter); large intestine (cecum and entire colon); and left testis. Samples of the luminal contents from stomach and small and large intestines were collected. Also, feces were collected from two randomly selected rats in each dietary group during the 24-h period before overnight food deprivation. To determine GFP content of luminal contents and feces, soluble protein was extracted using a protocol optimized for GFP extraction (4) and quantified by ELISA. Similarly, livers were homogenized with a motor-driven Teflon pestle in a glass vessel with 50 mL of PBS at 4°C. Homogenate was centrifuged (12,000 × *g* for 20 min at 4°C) to obtain a fraction for analysis of immunoreactive GFP. Blood was collected via cardiac puncture immediately after killing from two randomly selected rats in each group. Coagulated samples were centrifuged at 1000 × *g* for 10 min at 4°C to prepare serum for analysis of GFP and activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (Sigma Diagnostics, St. Louis, MO).

Sandwich ELISA assay. Samples and standards were added to Reacti-Bind Anti-GFP plates (Pierce Biotechnology, Rockford, IL) coated with polyclonal GFP goat antibodies and incubated for 1 h. The samples were diluted to a volume of 100 μL with carbonate buffer (0.8 g/L Na_2CO_3, 1.5 g/L $NaHCO_3$, pH 9.5). Each sample was analyzed in triplicate. After the wells were washed with PBS (pH 7.4; 4 mmol/L Tween 20), a rabbit anti-GFP polyclonal antibody (Molecular Probes, Eugene, OR) at a 1:10,000 dilution was added as the primary antibody and incubated for 30 min. After washing, goat polyclonal antibodies against rabbit immunoglobulin G conjugated to the horseradish peroxidase (HRP) enzyme (Sigma-Aldrich, St. Louis, MO) at a 1:10,000 dilution were added and then incubated for 30 min. The wells were washed a final time in PBS. Absorbance at 450 nm was measured for each well after the addition of the HRP substrate (Pierce Biotechnology).

Epitope detection. The amino acid sequence of the mGFP5ER protein was compared with 405 accession numbers from the protein allergen sequence database (9,10). The searches were conducted with two alignment programs, FASTA (11) and ClustalW (12), to identify sequential amino acid matches. Because of the interest in finding small regions of identity, the search parameters were set for local alignment with an initial match of two amino acids and no gap penalties.

Digestive stability. An in vitro protocol (13) for simulated gastric and small intestinal phases of digestion was modified as follows. Test samples included either 4 g applesauce only, 4 g applesauce containing 25 μg/g purified GFP, 8 g intact canola leaves from progenitor plants only and 8 g transgenic canola (GFP2) leaves. Three independent samples were prepared for digestion. Samples were suspended in 30 mL of saline and homogenized for 2 min. Aliquots (1.0 mL) were collected to quantify GFP content of starting materials. The remaining homogenate was subjected to simulated gastric digestion by the addition of HCl to decrease pH to 2.0 and porcine pepsin (2 g/L) with incubation in a shaking (95 rpm) waterbath at 37°C. Aliquots were collected after 5, 10 and 20 min and pH increased to 6.0 by the addition of 1 mol/L $NaHCO_3$. Sample remaining after 20 min was neutralized and subjected to simulated small intestinal digestion (13) with aliquots removed for analysis after 5, 10 and 20 min. A separate experiment was conducted to evaluate stability of GFP at pH 2.0 in the absence of pepsin. Starting homogenate and samples collected at indicated times during gastric and small intestinal phases of digestion were centrifuged at 1000 × *g* for 5 min to prepare the supernatant for analysis of GFP by Western analysis (4).

Statistical analysis. Body weight, food intake, food efficiency ratio, AST and ALT serum activity, and relative organ weights were analyzed by ANOVA general linear model (SAS, Cary, NC). Significant differences ($P < 0.05$) were determined using Tukey's means comparisons.

RESULTS

Feeding trial. Food intake did not differ among the groups (**Table 1**). Final body weight and food efficiency ratio (FER; g body weight gained/g total food intake) were slightly, but significantly ($P < 0.05$), lower for rats fed the modified AIN-93G diet containing 20 g/100 g canola powder compared with control AIN93G diets (Table 1). The differences in weight gain and FER were independent of the GFP content of the diet. The slightly lower FER for groups fed the canola-containing diets may be due to increased fiber and lower protein and carbohydrate content of diets containing powdered canola. Relative weights (mg wet tissue/g body) of small and large intestines, heart, liver, lung, stomach, kidney, spleen and testis also were independent of diet type and oral administration of pure GFP (data not shown). AST and ALT activities in serum did not differ among the groups (data not shown), indicating that ingestion of GFP was not associated with hepatic or cardiac damage.

GFP was not detected in the small and large intestinal contents, feces, liver homogenate or serum of rats chronically fed diets with transgenic canola. GFP fluorescence was evident in the small intestinal contents of rats orally administered 1.0 mg GFP 1 h before killing. Similarly, fecal pellets from rats administered 1 mg pure GFP daily fluoresced green (**Fig. 1**). ELISA of the small intestinal contents and feces indicated the presence of 1.04 ± 0.18 (~0.1% of the dose) and 0.34 ± 0.01 μg GFP (0.034% daily dose), respectively.

Epitope detection. Amino acid sequence analysis was sufficiently sensitive to identify homology between the endoplasmic reticulum targeting sequence on the engineered mGFP5ER and the secretory targeting sequences from a number of allergen proteins (**Fig. 2**). However, there were no matches greater than four consecutive amino acids between

TABLE 1

Body weight gain, food intake and food efficiency ratio (FER) of rats fed the AIN-93G diet or that diet plus varying amounts of green fluorescent protein (GFP) or canola[1]

Dietary treatment	Initial body weight	Final body weight	Total food intake	Total GFP intake	FER
	g			mg	g gain/g feed
AIN-93G (control)	76 ± 11	271 ± 11[a]	409 ± 38	0.0	0.49 ± 0.03[a]
AIN-93G + oral GFP	78 ± 8	277 ± 17[a]	411 ± 29	26.0	0.48 ± 0.03[a]
Modified AIN-93G (20 g/100 g Westar canola)	85 ± 8	255 ± 17[b]	414 ± 28	0.0	0.41 ± 0.02[b]
Modified AIN-93G (20 g/100 g GFP1 canola)	84 ± 5	253 ± 12[b]	407 ± 18	0.98 ± 0.04	0.41 ± 0.02[b]
Modified AIN-93G (20 g/100 g GFP2 canola)	86 ± 9	262 ± 11[b]	419 ± 31	0.50 ± 0.04	0.42 ± 0.01[b]

[1] Values are means ± SD, $n = 8$. Means in a column without a common letter differ, $P < 0.05$.

FIGURE 1 Green fluorescent protein (GFP) is present in feces of rats fed purified GFP. Fecal material from rats orally administered 1 mg purified GFP daily fluoresced green when exposed to UV light (indicated by the arrow), whereas feces from control rats autofluoresced red.

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mGFP5ER     M K T N L F L F F S L L L S L S S A
Agglutinin  M K P - F C V F L T F S L L L A A S S K
AA Identity * *           * *   * * * * *     * *

E F S K G E E L F T G V V P I L V E L D G D V N G H
K V D S - A E T V S F N F N S F S E G N P A I N F Q
          *                     *             *

- K F S V S G E G E G D A T Y G K L T L K F I C T T
G D V T V L S N G N I Q L T N L N K V N S V G R V L
        *       *       *

G K L P V P - W P - - - - - - - T L V T T F S Y G V
Y A M P V R I W S S A T G N V A S F L T T F S F E M
      * *     *                   * * * *

Q C F S R Y P D H M K R H D F F K S - - - - A M P E
K D I K D Y - D P A D G I I F F I A P E D T Q I P A
          *   *               * *                 *

G Y V Q E R T I F F K D D G N Y K T R A E V K F E G
G S I G G G T L G V S D T K G A G H F V G V E F - -
*           *           *               *   *

D T L V N - R I E L K G I D - F K E D G N I L G H K
D T Y S N S E Y N D P P T D H V G I D V N S V D S V
* *     *                   *       *   *

L E Y N Y N S H N - V Y I M A D K Q K N G I K V N F
K T V P W N S V S G A V V K V T V I Y D S S T K T L
          * *

K I R H N I E D G S V Q L A D H Y Q Q N T P I G D G
S V A V T N D N G D I T - T - - I A Q - - - V V D L
              *                   *           *

P V L L P D N H Y L S T Q S A L S K D P N E
K A K L P E R V K F G F S A S G S L G G - -
      * *                       *
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FIGURE 2 Amino acid sequence comparison between mGFP5ER and food allergens does not indicate a common epitope. The upper line of this figure is the mGFP5ER protein sequence. The sequence of peanut agglutinin, a food allergen, is shown on the lower line. Asterisks on the third line indicate matches of amino acids between the two sequences. The first highlighted region indicates homology between the endoplasmic reticulum targeting sequence from mGFP5ER and the secretory signaling sequence on the allergen. This peptide is cleaved before folding of the mature protein. The second highlighted region indicates an area of identity limited to four amino acids.

the structural residues of GFP and any of the proteins listed in the database. When considering sequences of high similarity, the longest match for GFP and one region of peanut agglutinin was four consecutive amino acids (Fig. 2), a length of homology that is insufficient to suggest an epitope match.

Digestive stability. When purified GFP in applesauce was subjected to simulated gastric and intestinal digestion, immunoreactive GFP was not detected as early as 5 min after initiation of the gastric phase (**Fig. 3**, lanes 3 and 4). Purified GFP was stable throughout the 20-min gastric phase of digestion when pepsin was absent (lane 8), indicating its susceptibility to peptic hydrolysis at low pH. GFP in transgenic canola leaves also was hydrolyzed during simulated gastric digestion (lanes 6 and 7). Moreover, the protein in the plant material was degraded during the gastric phase even in the absence of pepsin (lane 9). As expected, there were no detectable bands when gels containing samples of applesauce alone and progenitor canola powder before or after digestion were probed with anti-GFP.

DISCUSSION

Oral administration of 1.0 mg purified GFP/d for 26 d was not toxic for growing male rats. Although there was a slight decline in weight gain and the FER of rats fed the modified AIN-93G diet containing 200 g/kg canola, food intake, relative organ weights, and enzyme markers in serum indicative of liver and heart damage did not differ among the dietary groups receiving control and GFP-containing diets. Several other studies have found that transgenic plant foods do not represent a health risk and provide the same nutritional value as the progenitor crop (14–17). Our results are consistent with these findings. Moreover, transgenic animals that constitutively express GFP have been reported as being healthy (18–20). This may differ from dietary exposure because the GFP in transgenic animals is located intracellularly and toxicity or allergenicity may function differently than when exposed extracellularly. Nevertheless, these data do suggest that GFP is minimally toxic, a conclusion that is supported by this feeding study.

Identification of epitopes with the potential to induce allergenic responses in proteins being introduced into the food supply via genetically engineered plants represents an impor-

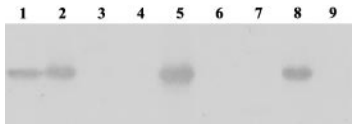

FIGURE 3 Protein blot of an in vitro digest of green fluorescent protein (GFP). Purified and recombinantly expressed GFP are degraded during simulated gastric digestion. Purified GFP in an applesauce matrix and transgenic canola expressing GFP (GFP 2) were subjected to simulated gastric and small intestinal phases of digestion as described in Materials and Methods. For the representative blot presented, proteins in starting material (homogenate) and samples removed 5 min after initiation of the gastric phase and the intestinal phase of digestion were separated by SDS-polyacrylamide gel electrophoresis and probed with anti-GFP. Lane 1 contains 100 ng of GFP standard. Lanes 2, 3 and 4 contain the starting homogenate, gastric phase and intestinal phase of purified GFP in applesauce, respectively. Lanes 5, 6 and 7 contain the homogenate, gastric phase and intestinal phase of GFP2 canola, respectively. Lane 8 contains the gastric phase of purified GFP without pepsin, and lane 9 contains the gastric phase of GFP2 canola without pepsin. Blots for samples collected 10 and 20 min after initiation of gastric digestion were the same as for data shown in this figure.

tant consideration for the assessment of food safety (21). A significant match is considered to be six consecutive identical amino acids, because the hypothesized minimal epitope size is seven amino acids, and the presence of five or fewer consecutive amino acids has a significant likelihood to occur by chance (9,10). When such epitopes are present, additional testing of the protein using sensitized serum, skin-prick tests or double-blind placebo challenges is required (22). Our analysis showed that GFP does not share sufficient homology in amino acid sequence with any known food allergen to suggest the presence of a common epitope. This result is not surprising because the β-can structure (23) of GFP is novel and unlike that of proteins found in common foods. Also, the similarity of relative weight of the small and large intestines of rats either orally administered pure GFP or fed diets containing transgenic canola suggests that GFP is not an allergen or irritant (24).

GFP was readily degraded during simulated gastric digestion. Furthermore, chronic consumption of GFP transgenic plants did not result in detectable quantities of GFP in the digestive tract. Even oral administration of a dose of purified GFP 26-fold greater than the transgenic canola diets resulted in most of the protein being digested by the rats. These data indicate that GFP is not stable during digestion. This result is important because stability during digestion is a common characteristic of food allergens (25,26). It is noteworthy that the protein in the transgenic plant was rapidly destroyed at pH 2.0 even in the absence of pepsin, suggesting the susceptibility of GFP to acid-stable proteases in the homogenized plant tissue (27). Because GFP does not share any identifiable allergen epitopes or characteristic digestive stability of known allergens, it seems appropriate to classify GFP as a low allergen risk (22,28).

The results of our studies suggest that GFP represents a minimal risk for the food supply. Further support for this preliminary conclusion requires a long-term feeding study of both male and female rodents and detailed compositional analysis of the transgenic plant to address concerns that transgene insertion may induce unforeseen effects on nutrient composition and availability (29).

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