JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY



# Safety Assessment by in Vitro Digestibility and Allergenicity of Genetically Modified Maize with an Amaranth 11S Globulin

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Prospective testing for allergenicity of proteins obtained from sources with no prior history of causing allergy has been difficult to perform. Thus, the objective of this work was to assess the food safety of genetically modified maize with an amaranth globulin protein termed amarantin. Transgenic maize lines evaluated showed, in relation to nontransgenic, 4−35% more protein and 0−44% higher contents of specific essential amino acids. Individual sequence analysis with known amino acid sequences, reported as allergens, showed that none of these IgE elicitors were identified in amarantin. Amarantin was digested within the first 15 min by Simulated Gastric Fluid treatment as observed by Western blot. Expressed amarantin did not induce important levels of specific IgE antibodies in BALB/c mice, as analyzed by ELISA. We conclude that the transgenic maize with amarantin is not an important allergenicity inducer, just as nontransgenic maize.

**KEYWORDS: Food safety; transgenic maize; *Amaranthus*; amarantin; in vitro digestibility; allergenicity; animal model**

## INTRODUCTION

The food sector is now facing the problem of how to control the novel foods being produced by applying biotechnology to enhance the supply of wholesome, nutritive, tasty, and affordable food. One of the goals of genetic engineering has been to create crops tailored to provide better nutrition for humans and their domestic animals. A major target has been the improvement of the amino acid composition of seed proteins, in particular of the lysine and tryptophane content of maize and the methionine content of legume seeds (*1*, *2*). One alternative is the insertion and the expression of genes encoding essential amino acid-rich proteins, with no history of induced allergenicity, in the transgenic plant (*3*−*5*). After the transformation of a forage crop such as lupin with a methionine-rich sunflower seed albumin gene, the total sulfur amino acid increased 19%, and no changes in nitrogen levels in the seeds were observed (*6*). In the case of the transformation of soybean with the high-methionine Brazil nut protein, commercialization did not occur because of the protein's allergenic properties (*7*).

Amarantin, the 11S globulin of amaranth, is one of the most important proteins in the seed; it contains a very good balance of essential amino acids, which nearly meets the needs of human protein nutrition, in reference to protein requirements established by international health organizations (*8*).

To replenish the lysine, tryptophane, and the other amino acids deficient in maize, the cDNA of amarantin was inserted in the maize genome (*4*, *5*). Constructs contained the amarantin cDNA under the control of a tissue-specific promoter from rice glutelin-1 (osGT1) or of a tandem constitutive promoter (CaMV 35S). Total protein and some essential amino acids of the best expressing maize lines augmented 4−35% and 0−44% respectively, as compared to nontransformed maize. In addition, genetically modified maize had 0.5−1.2% of accumulated amarantin in relation to total protein content.

In view of the general concern about genetically modified organisms and their products, in particular those which serve as additives in foods and beverages, they have been thoroughly assessed for their toxicity under approaches recommended by the World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO) and other worldwide organizations (*9*, *10*). One integral part of the safety assessment of genetically modified plants is the consideration of possible human health effects, especially food allergy (*11*). The potential allergenicity of the introduced proteins can be evaluated with a decision tree developed by the International Food Biotechnology Council (IFBC) and the Allergy and Immunology Institute of the International Life Sciences Institute (ILSI). This tree focuses on the source of the gene, the homology of the newly introduced protein to known allergens, the

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10.1021/jf035487k CCC: $27.50 
Published on Web 04/08/2004

reactivity of the novel protein with IgE antibodies, the resistance of the novel protein to pepsin and trypsin in vitro digestion, and the immunoreactivity of the novel protein in appropriate animal models (*12*−*14*). Identification of allergen-specific IgE antibodies in circulation is one of the most important methods for the diagnosis of the allergenic nature of proteins (*15*). Literature search has not revealed any allergenicity associated with amaranth grain or amaranth forage. Grain amaranth has been used in many types of foods in various regions of the word, and no allergenic problems have been reported (*3*, *4*).

Worldwide maize is an important source of protein used in a large number of processed food products (*16*); thus, genetically modified maize lines should be evaluated to determine their safety. We evaluated here the in vitro digestibility and potential allergenicity of transgenic amarantin maize.

## MATERIALS AND METHODS

**Sequence Analysis of the 11S Globulin of Amaranth.** Sequence analysis of amarantin was performed in line with the suggested procedure formulated by the FAO/WHO Consultation on Assessment of the Allergenicity of Genetically Modified Foods (*13*). The sequence of amarantin was aligned with the complete Protein Information Resource (PIR database http://pir.georgetown.edu/) using FASTA (version 2.4, 2002) (*17*).

**Identity and Characteristics of the Maize Lines Analyzed.** The maize lines evaluated were the following: the nontransgenic maize was CML72 × 216, and the transgenic seeds were 30/2k, 34/C, 7/35S, and 1041/1.7k. The 30/2k maize line has 9% more protein content in relation to nontransgenic line, with specific increases of 4 and 1% in lysine (Lys) and tryptophane (Trp), respectively. The 34/C has 4% more protein and 1.6 and 2.6% of Lys and Trp, respectively. The 7/35S has 8% more protein and amino acid increases of 0−12% and specific increases of 6 and 0% in Lys and Trp, respectively, and the highly expressing tissue-specific maize line 1041/1.7k showed 35% more protein and amino acid increases of 8−44% and specific increases of 18 and 22% in Lys and Trp, respectively, and the third limiting amino acid, isoleucine (Ile) also increased by around 3% (*4*, *5*).

**Preparation of Crude Protein Extracts from both Transgenic and Nontransgenic Maize Seeds.** The pericarp and embryo from each seed were removed to eliminate the globulins present in these tissues; protein fractionation was done as described in the reference (*18*), with minor modifications. Protein fractions were obtained by grinding 2 g of endosperm, extracting with 25 mL of hexane at room temperature for 30 min, and then extracting for 1 h in 25 mL of water to recover the albumin fraction. Then, the pellet was extracted for 1 h in 25 mL of extraction buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 8.0) to recover the Glb I globulin fraction and then extraction buffer (0.1 M Tris-HCl, 0.3 M NaCl, pH 8.0) was utilized to recover the Glb II globulin fraction. Protein was determined as previously described (*19*), with bovine serum albumin as standard.

**Electrophoresis and Western Blot Analysis.** The Glb I globulin samples were concentrated by adding four volumes of cold absolute ethanol to 40−50 μg of total protein and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously reported (*20*). The gel was equilibrated in transfer buffer, and then proteins were transferred to a PVDF membrane, which were blocked with 1% BSA and washed twice. Rabbit polyclonal antibodies (kindly provided by J. Calderon, CINVESTAV-IPN) were raised against the 53-kDa amarantin purified from amaranth seeds. IgG immunoglobulins were purified from serum with an ImmunoPure column (Pierce, Rockford, IL), and to reduce background, anti-amarantin IgGs were treated with a nontransformed maize endosperm extract polypeptide-Sepharose column. Membranes were developed with BCIP (5-bromo-4-chloro-3-indolyl phosphate-toluidine salt) and NBT (*p*-nitroaziltetrazolium chloride) substrates, until color appeared.

**In Vitro Digestion of Heterologous Amarantin.** Salt soluble fractions (225 μg) from transgenic maize endosperm extracts were incubated at 37 °C with simulated gastric fluid (SGF; porcine pepsin in 0.03 M NaCl at pH 1.2) (Sigma Chemical Co. St. Louis, MO) during 60 min and then adjusted to pH 8.0 with 100 mM Tris-HCl, pH 9.5, 2 mM CaCl2 for intestinal fluid digestion (IFD; porcine trypsin and bovine chymotrypsin) (Sigma Chemical Co.) (*21*) at 1:100 ratio (digestive enzyme:protein ratio), and the reaction was stopped with buffer (50 mM Tris-HCl, 100 mM NaCl, pH 9.5). At the desired times, 20 μg aliquots were taken and reaction mixtures subjected to SDS−PAGE and then to Western blotting as already described (*20*).

**Allergenicity Evaluation of Amarantin in an Animal Model.** *Sensitization Protocol to Ovalbumin.* The allergenic potential of ovalbumin (OVA), a potent respiratory and food allergen, was evaluated as a function of serological responses (IgE antibody production) (*22*). Two groups of 6−8 week-old female BALB/C mice ($n = 5$) were used. The first group received 0.2 mL (9 mg) of aluminum potassium sulfate (adjuvant) in phosphate buffered saline (PBS). The second group received 0.2 mL of 10 μg/mL of OVA in the presence of adjuvant in PBS by intraperitoneal (ip) injection on days 1, 7, and 14. Alternatively, a group of mice ($n = 5$) received 0.2 mL of PBS alone, as control animals. Mice were exsanguined 21 days after the initiation of exposure. Individual serum samples were prepared and stored at −20 °C until analysis. Protein-specific and total IgE antibodies were detected using enzyme-linked immunosorbent assays (ELISA).

*Sensitization Protocol to Heterologous Amarantin.* The potential allergenicity of wild-type maize and genetically modified maize with heterologous amarantin was analyzed by oral and ip administration. The globulin fractions (Glb I and Glb II) of nontransformed maize (CML72 × 216) or transgenic maize lines 7/35S and 1041/1.7K were used. Groups of BALB/c mice ($n = 5$) received a daily dose by gavage of 0.2 mL of 0.25 and 1% w/v globulin fraction in PBS; control animals received an equal volume of $H_2O/H_2O$ (*22*, *23*). Mice were exsanguined 42 days after the initiation of exposure. The serum samples were prepared and stored at −20 °C until analysis. For ip administration, groups of mice ($n = 5$) received 0.2 mL of 0.25 or 1% globulin fraction of nontransformed or transgenic maize lines in PBS; control animals received an equal volume of PBS alone. The animals were ip injected on days 1, 7, and 14 and were exsanguined 21 days after the start of exposure; sera were prepared and stored as described above. Amarantin specific and total IgE antibodies were quantitated using ELISA.

**Measurement of Anti-OVA and Anti-Amarantin IgE Antibodies.** ELISA techniques were used to measure total and specifically induced serum IgE antibodies for OVA and the recombinant amarantin expressed in maize. For the quantitation of OVA-specific IgE antibodies, 96-well microtiter plates (Dynex, Technologies, VA) were coated overnight at 4 °C with 100 μL/well with a 5 μg/mL solution of OVA (Sigma A-5503) or with a 100 μg/mL solution of globulin fraction of nontransformed or transgenic maize in carbonate buffer pH 9.6 (50 mM of carbonate/bicarbonate). The plates were washed three times with 100 μL/well of PBS containing 0.1% Tween 20. This was followed by addition of 100 μL/well of blocking buffer (PBS containing 1% skim milk). After 1 h of incubation at room temperature, the plates were washed, and 1:10 dilutions of mice serum in blocking buffer were added to the well and incubated for 1 h at room temperature. After washing, 100 μL/well of 2 μg/mL biotinylated anti-mouse IgE (PharMigen Bioscience, CA) in blocking buffer was added. After incubation for 1 h at room temperature, the plates were washed again, and 100 μL/well of streptavidin-horseradish peroxidase (SAv-HRP) conjugate (PharMigen Bioscience, CA) diluted 1:1000 in blocking buffer was added and incubated at room temperature for 30 min. The plates were washed six times, and 100 μL/well of substrate abts 2,2-azino-bis (Sigma) was added. The plates were developed at room temperature for 5−10 min. Finally, for end-point assays, the reaction was stopped with 100 μL/well of 1% SDS. Optical density was read spectrophotometrically at 405 nm with an ELISA plate reader. For specific quantitation of IgE content, a standard curve was made with purified mouse IgE standard (PharMigen Bioscience, CA). For the detection of total IgE, 100 μL/well of 2 μg/mL anti-mouse IgE capture mAb (PharMigen Bioscience, CA) were added in coating buffer (PBS, pH 7.4) and analyzed as above.

## RESULTS AND DISCUSSION

With the development of genetically modified crop plants, there has been a growing interest in the approaches available

**Table 1.** Sequence Similarity of Amarantin with Every Allergen in the Database[a]

| highest no. of contiguous amino acids | matching sequence | protein | accession no. | maximum no. of contiguous amino acids after digestion |
|---|---|---|---|---|
| 6 | GLLLPS | legumin-like prot (*Fagopyrum esculentum*) | BAA21760 | 2 |
| | HQKIRH | 11S legumin-like protein (*Corylus avellana*) | AAL73404 | 3 |
| | RFYLAG | glycinin subunit G3 (*Glycine max*) | CAA33217 | 2 |
| | EFRCAG | legumin-like prot (*Fagopyrum esculentum*) | BAA21760 | 4 |
| | VFDEEL | allergen Arah3/Arah4 (*Arachis hypogea*) | AAM46958 | 4 |
| | HQKIRH | A5A4B3 subunit precursor (*Glycine max*) | CAA26478 | 2 |
| | VPQNFA | glycinin subunit G3 (*Glycine max*) | CAA33217 | 4 |
| 7 | VVPQNFA | allergen Arah3/Arah4 (*Arachis hypogea*) | AAM46958 | 5 |
| | PHYNLNA | glycinin subunit G3 (*Glycine max*) | CAA33217 | 2 |
| | IPGCPET | legumin-like prot (*Fagopyrum esculentum*) | BAA21760 | 6 |
| | IPGCPET | A5A4B3 subunit precursor (*Glycine max*) | CAA26478 | 6 |
| 8 | APELIYIE | 11S legumin-like protein (*Corylus avellana*) | AAL73404 | 4 |
| | VIRRTIEP | 11S legumin-like protein (*Corylus avellana*) | AAL73404 | 4 |

[a] From: http://us.expasy.org/tools/peptidecutter/

to assess the potential allergenicity of novel gene products. To provide assurance that a novel protein is not a potential allergen, approaches have been proposed that are based on a decision tree (*10*, *12*, *13*). The key features of such a decision tree are that it takes into consideration multiple features of the protein in question which, taken together, enable a judgment to be made on the probability of a protein being allergenic. In this report, the amarantin expressed in maize has been assessed using the most recent FAO/WHO decision tree (*12*−*14*). Here, we have focused on the source of the protein, sequence similarity with known allergens, resistance to pepsin hydrolysis, and animal model allergenicity.

**Amarantin Sequence Similarity.** The amarantin sequence was analyzed and compared with each member sequence in a database containing known allergen sequences (*17*). The number of exact matches obtained with octamers, heptamers and hexamers was 2, 4, and 7, respectively. Most of the matches obtained with at least six contiguous amino acids identified by the FASTA program were from 11S legumin-related proteins and one from *Arachis hypogea*; however, due to large sequence similarity among globulins, large amino acid similarity could be expected (*24*). Matches with sequences of contiguous amino acids in other proteins are listed in **Table 1**. Careful individual analysis of the octamers, heptamers, and hexamers from exact matches with amino acid sequences, reported as known allergenicity inducers, showed that none of these matches were identified in amarantin amino acid sequence as Ig-E dependent allergens. Moreover, sequence digestions derived by the program "Peptide Cutter at http://us.expasy.org/tools/peptidecutter/" showed that all exact peptides-matches within amarantin contains at least one digestion site, resulting in smaller peptides not inducing allergenicity. More importantly, more than eight contiguous amino acids or more are required to elicit IgE-dependent allergenicity (*13*).

**Immunodetection of Heterologous Amarantin in Maize Endosperm.** Under nonreducing conditions, a band was detected by Western blot in the transformed endosperm (**Figure 1**, Lanes 1−4) and native amarantin extracts with expected molecular weight of 53 kDa (**Figure 1**, Lane 6, whereas no signal was detected in no transformed endosperm of maize (**Figure 1**, Lane 5). Under reducing conditions, one very light band of 53 kDa (**Figure 2**, Lanes 1−5) was detected that corresponds to proamarantin, and another polypeptide of 34 kDa was observed, corresponding to the acidic subunit of amarantin. The heterologous amarantin polypeptide comigrates with the subunits of the partially purified protein from amaranth seed. Antiamarantin

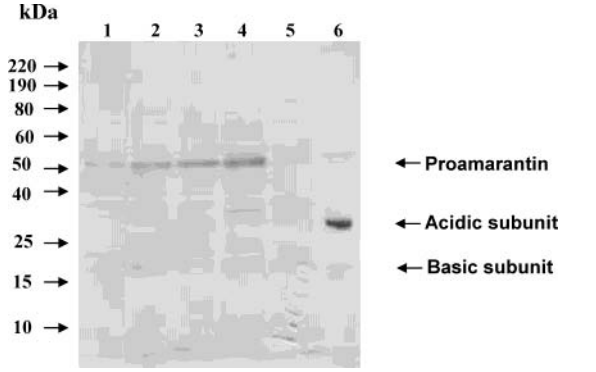


**Figure 1.** Immunoblot analysis of Glb I globulin fraction was resolved by SDS-PAGE and analyzed with rabbit antibody raised against amarantin under nonreducing conditions. Lane 1, 30/2k; lane 2, 34/C; lane 3, 7/35S; lane 4, 1041/1.7k; lane 5, nontransgenic maize; lane 6, amarantin as a positive control (reducing conditions). In lanes 1–5, 40 $\mu$g was loaded into each lane.

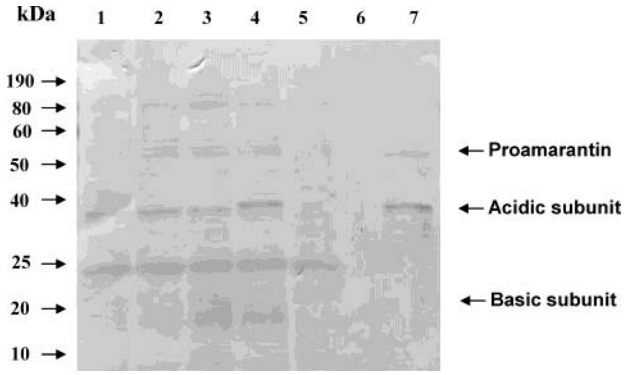


**Figure 2.** Immunoblot analysis of Glb I globulin fraction was resolved by SDS-PAGE and analyzed with rabbit antibody raised against amarantin under reducing conditions with 2-mercaptoethanol. Lane 1, 30/2k; lane 2, 34/C; lane 3, 7/35S; lane 4, 1041/1.7k; lane 5, nontransgenic maize; Lane 7, Amarantin as a positive control. In lanes 1–4, 40 $\mu$g was loaded into each lane.

antibody reacts weakly against amarantin basic subunit of 22 kDa due to low immunogenicity profile; this is in agreement with the behavior of soybean glycinin expressed in rice (*25*). Differences in protein level accumulation in seeds in transgenic plants may be attributed to tissue-specific posttranscriptional effects of amarantin gene transcripts. However, considerable amounts of the unprocessed precursor molecule were also

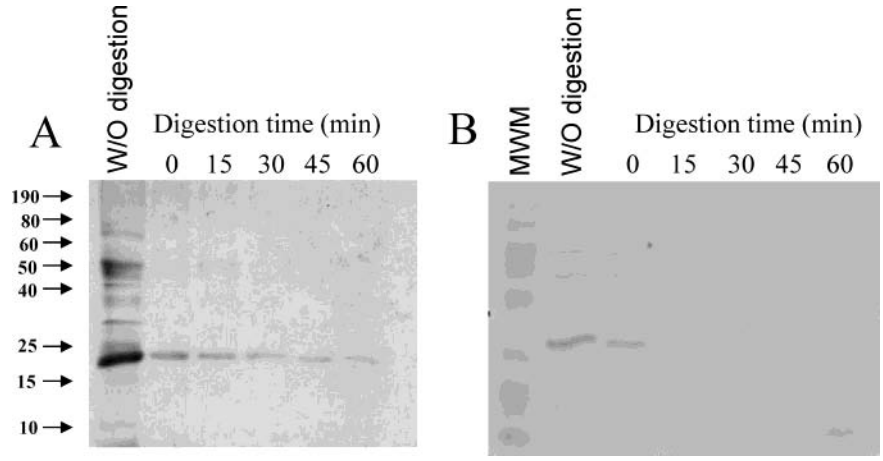


**Figure 3.** Gel staining (**A**) and immunoblot analysis (**B**) of enriched globulin fraction (GlbI) digested with simulated gastric fluid (SGF) and Intestinal gastric fluid (IGF) of transgenic plant 7/35S. Lane 1, fraction without digestion; lane 2, 0 min of digestion; lane 3, 15 min after digestion; lane 4, 30 min after digestion; lane 5, 45 min after digestion, lane 6, 60 min after digestion.

**Table 2.** Total and Specific IgE[a–c] of Mice Groups Immunized Intraperitoneally with Ovalbumin and Globulin Fraction of Transgenic Maize

| ovalbumin | | | golbulin | | | | |
|---|---|---|---|---|---|---|---|
| | OVA | | | amarantin (Glb I and Glb II fractions) | | | |
| | | | | 0.25% | | 1.0% | |
| treatment[d] | total IgE | specific IgE | maize samples and PBS | total IgE | specific IgE | total IgE | specific IgE |
| **adjuvant** | 55.9 ± 3.3 | ND | **CML72X216** | 67.3 ± 4.3[ab] | ND | 99.7 ± 4.2[a] | ND |
| **OVA** | 364.1 ± 57 | 302 ± 53 | **7/35S** | 66.1 ± 3.4[b] | ND | 100.2 ± 4.4[a] | ND |
| **PBS** | 16 ± 2.6 | ND | **1041/1.7k** | 77.8 ± 8.0[a] | ND | 111.8 ± 27.8[a] | ND |
| | | | **PBS** | 16.2 ± 3.3[c] | ND | 16.9 ± 3.4[b] | ND |

[a–c] ng/ml, ND = not detected; each value is the mean of five repetitions ± SE. Values with unlike superscripts are significantly different ($P < 0.05$). [d] Adjuvant, aluminum potassium sulfate; OVA, ovalbumin; PBS, phosphate buffered saline.

present in maize endosperm extracts, indicating that the efficiency of processing may be lower in the heterologous system (*4*).

**In Vitro Digestibility of the Expressed Amarantin.** The digestibility of the amarantin expressed in the transgenic maize endosperm was confirmed by using SGF and IGF. Protein staining and immunoblotting (**Figure 3**, parts **A** and **B**) indicated that high molecular weight proteins, including heterologous amarantin, were completely digested within 15 min. These results suggest that this protein may not show allergenic activity (*11*, *26*). In the evaluation of the resistance of the protein to digestion by pepsin and comparison with reference proteins, the latter proteins behaved as previously reported (*11*) when exposed to pepsin. Bovine serum albumin underwent rapid hydrolysis ($t < 15$ min), and OVA showed pronounced resistance to hydrolysis at $t > 60$min (data not shown). Proteins that are rapidly hydrolyzed to single amino acid and peptides smaller than 3.5 kDa by pepsin are considered less likely to be allergenic (*9*, *26*). In the case of amarantin, digestive tract enzymes hydrolyzed the protein readily, resulting in 90% loss of the protein assessed by both SDS-PAGE and Western blot.

The important food allergen Brazil nut 2S albumin is as stable to digestion as is sunflower seed 2S albumin, determined by the resistance to thermally and enzymatic induced denaturation (*27*). Preheating increased the digestibility of some protein in SGF and SIF, and the digestion pattern, including fragment formation, significantly changed (*28*).

The ability of food allergens to reach the intestinal mucosa is a prerequisite to allergenicity. This ability necessarily implies survival to gastric digestion by pepsin secreted into the stomach. A protein that is stable to the proteolytic and acidic conditions of the digestive tract has an increased probability of reaching the intestinal mucosa where absorption can occur.

**Allergenicity in an Animal Model.** *Anti-OVA IgE System.* Intraperitoneal administration of 10 $\mu$g/mL of OVA with adjuvant resulted in OVA-specific IgE responses in all mice. The mean concentrations are summarized in **Table 2**; remarkable differences were found in the contents of total IgE between the groups that were immunized with OVA (364 ng/mL) and adjuvant (56 ng/mL). OVA-specific IgE was detected in the group immunized with OVA (302 ng/mL). Anti-OVA IgE antibodies were undetectable in the mice group that received immunization with adjuvant. These results suggest that the majority of total IgE in the group sensitized intraperitoneally with OVA was due to OVA. This system was implemented with the goal of standardizing the experimental conditions, and we concluded that BALB/c strain mice elicit serological responses (IgE antibody production). In addition, OVA induced strong IgE responses when administered ip, in the BALB/c mice. However, oral exposure to this protein was associated with low grade IgE production in previous reports (*22*, *29*).

**Evaluation of Anti-Heterologous Amarantin IgE Production.** *Intraperitoneal Administration.* Intraperitoneal administration of enriched fractions (Glb I and Glb II) from transgenic maize endosperm and from nontransgenic maize were analyzed. No statistical differences were observed in total IgE levels between the groups immunized with transgenic maize or nontransgenic maize in each dosage treatment (0.25 and 1.0%) (**Table 2**). We found statistical differences between the levels of total IgE between both transgenic and nontransgenic maize and those immunized with PBS, maybe due to biological induction by protein contained in maize extracts. These results

**Table 3.** Mean Concentrations of Total IgE[a–c] Produced by BALB/c Mice Treated by Oral Administration with Globulin Fraction from Non-Transgenic Maize, Transgenic Maize, and a Control Group ($H_2O$)

| | amarantin (Glb I and Glb II fractions) | | | |
|---|---|---|---|---|
| | 0.25% | | 1.0% | |
| treatment | total IgE | specific IgE | total IgE | specific IgE |
| CML72X216 | $23.1 \pm 4.2^{c}$ | ND | $35.6 \pm 6.0^{bc}$ | ND |
| 7/35S | $25.3 \pm 3.1^{bc}$ | ND | $43.4 \pm 3.0^{ab}$ | ND |
| 1041/1.7k | $34.8 \pm 3.4^{a}$ | ND | $46.5 \pm 14.5^{a}$ | ND |
| $H_2O$ | $32.2 \pm 5.3^{ab}$ | ND | $32.2 \pm 5.3^{c}$ | ND |

[a–c] ng/ml, ND = not detected, each value is the mean of five repetitions ± SE. Values with unlike superscripts are significantly different ($P < 0.05$).

suggest also that the ip administration per se was not responsible for IgE induction. More importantly, no specific anti-amarantin IgE were detected by ELISA assay from transgenic maize endosperm (**Table 2**).

*Oral Administration.* Oral administration of allergens resembles natural-occurring sensitization. Oral administration of transgenic and nontransgenic maize led to a marked reduction in total IgE production in BALB/c mice. No significant differences were observed in total IgE between the groups immunized with transgenic maize 7/35S maize and nontransgenic maize at both levels (**Table 3**), whereas significant differences were detected when transgenic plant 1041/1.7k was compared with nontransgenic maize. However, specific anti-amarantin IgE antibodies were undetectable in both treatments (0.25 and 1%).

The result obtained from transgenic maize indicated that amarantin level expressed in maize endosperm did not evoke specific IgE antibodies, whereas high levels of specific anti-OVA IgE levels were found. In another study expressing albumin protein from amaranth in potatoes, the authors showed that the hypersensitivity tests in an animal model did not evoke any IgE response, which negates the possibility that this albumin protein could be allergenic (*3*).

Oral administration by gavage may be considered to reflect more accurately the relevant route of human exposure; the data summarized here indicate that this approach may not possess the sensitivity or reliability to provide an initial assessment of allergenic potential. Oral exposure to ovalbumin is associated with a low grade IgE production (*22*). The utility of the ip exposure model for the purpose of hazard identification and characterization is currently being explored more thoroughly with a wider range of test proteins. The development of a predictive animal model is, in this context, often indicated to be of major importance in improving the currently used ILSI/IFBC decision tree. In particular, if transferred genes, coding for new proteins, are derived from products with an unknown history of allergenicity or if proteins show one or more physicochemical characteristics of known allergens, the ultimate proof of the presence/absence of sensitizing activity of the novel proteins can be established in a predictive animal model (*22*, *23*, *30*). The studies focused on the development of protocols to evaluate the allergenic potential of amarantin expressed in maize demonstrated that OVA induced strong total and specific IgE responses when administered ip and that BALB/c mice may provide an appropriate model for the identification and characterization of protein allergens (*22*, *29*). In brief, our studies indicate that amarantin was digested with SGF, which indicates its low probability to induce allergenicity. In addition, our results suggest that expressed amarantin in transgenic maize did not induce important levels of specific IgE antibodies in BALB/c mice, which means that these proteins contained in maize are not important allergenicity inducers.

## ACKNOWLEDGMENT

We thank F. Guevara-Lara for critical reading of this manuscript and Yolanda González for technical help.

## LITERATURE CITED

(1) Tabe, L.; Higgins, T. J. Engineering plant protein composition for improved nutrition. *Trends Plant Sci.* **1998**, *3*, 282−286.
(2) Habben, J.; Larkins, B. Genetic modification of seed proteins. *Curr. Opin. Biotechnol.* **1995**, *6*, 171−176.
(3) Chakraborty, S.; Chakraborty, N.; Datta, A. Increasing nutritive value of transgenic potato by expressing nonallergenic seed albumin gene from *Amaranthus hypochondriacus*. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 3724−3729.
(4) Rascón-Cruz, Q.; Sinagawa-García, S. R.; Osuna-Castro, J. A.; Bohorova, N.; Paredes-López, O. Accumulation, assembly, and digestibility of amarantin expressed in transgenic tropical maize. *Theor. Appl. Genet.* **2004**, *108*, 335−342.
(5) Rascón-Cruz, Q. Expresión de la proteína de reserva 11S de amaranto en maíz. Ph.D. Thesis. Centro de Investigación y de Estudios Avanzados del IPN. 2003, Irapuato, Gto. México.
(6) Molving, L.; Tabe, L.; Eggum, B.; Moore, A.; Craig, S.; Spencer, D.; Higgins T. Enhanced methionine levels and increased value of seeds of transgenic lupins (*Lupinus angustifolius L.*) expressing a sunflower seed albumin gene. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 8393−8398.
(7) Nordlee, J.; Taylor, S.; Towsend, J.; Thomas, J.; Bush, R. Identification of Brazil-nut allergen in transgenic soybean. *N. Eng. J. Med.* **1996**, *334*, 688−692.
(8) Barba de la Rosa, A.; Herrera-Estrella, A.; Utsumi, S.; Paredes-López, O. Molecular characterization, cloning, and structural analysis of a cDNA enconding an amaranth globulin. *J. Plant Physiol.* **1996**, *149*, 527−532.
(9) FAO/WHO. Report of a joint FAO/WHO Expert Committee on Allergenicity of Foods Derived from Biotechnology. Rome, Italy. FAO/WHO 22−25 January, 2001.
(10) WHO. Safety aspects of genetically modified foods of plant origin. Report of a joint FAO/WHO Expert Consultation. World Health Organization, Geneva, 2000.
(11) Astwood, J.; Leach, J.; Fuchs, R. Stability of food allergens to digestion in vitro. *Nature Biotechnol.* **1996**, *14*, 1269−1273.
(12) Metcalfe, D.; Astwood, J.; Townsend, R.; Sampson, H.; Taylor, S.; Fuchs, R. Assessment of allergenic potential of foods derived from genetically engineered crop plants. *Crit. Rev. Food Sci. Nutr.* **1996**, *36*, S165−S186.
(13) FAO/WHO. Biotechnology and Food Safety Report of Joint FAO/WHO. Rome, 2001.
(14) Taylor, S. L. Protein allergenicity assessment of food produced through agricultural biotechology. *Annu. Rev. Pharmacol. Toxicol.* **2002**, *42*, 99−112.
(15) Kim , T. E.; Park, S. W.; Cho, N. Y.; Choi, S. Y.; Yong, T. S.; Nahm, S. L.; Lee, S.; Noh, G. Quantitative Measurement of Serum Allergen-Specific IgE on protein Chip. *Exp. Mol. Med.* **2002**, *34*, 152−158.
(16) Paredes-López, O.; Serna-Saldívar, S. O. y Guzmán-Maldonado S. H. Los Alimentos Mágicos de las Culturas Indígenas de México- El Caso de la Tortilla. 2000, Cap. 3. Composición y características nutrimentales del grano de maíz. El Colegio de Sinaloa, México, 17−21.
(17) Gendel, M. The use of amino acid sequence alignments to assess potential allergenicity of protein used in genetically modified foods. *Adv. Food Nutr. Res.* **1999**, *42*, 45−61.
(18) Chen, S.; Paredes-López, O. Isolation and characterization of the 11S globulin from amaranth seed. *J. Food Biochem.* **1997**, *21*, 53−65.
(19) Bradford, M. M. A rapid sensitive method for the quantitation of microgram quantity of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(20) Laemmli, U. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(21) Roesler, K.; Rao, A. Rapid gastric fluid digestion and biochemical characterization of engineered proteins enriched in essential amino acids. *J. Agric. Food Chem.* **2001**, *49*, 3443−3415.
(22) Dearman, R. J.; Kimber, I. Determination of protein allergenicity: studies in mice. *Toxicol. Lett.* **2001**, *120*, 181−186.
(23) Dearman, R. J.; Skinner, R. A.; Herouet, C.; Labay, K.; Debruyne, E.; Kimber, I. Induction of antibody responses by protein allergens: interlaboratory comparisons. *Food Chem Toxicol.* **2003**, *41*, 1509−1516.
(24) Shewry, P.; Halford, N. Cereal seed storage proteins: Structures, properties, and role in grain utilization. *J. Exp. Bot.* **2002**, *53*, 947−958.
(25) Katsube, T.; Kurisaka, N.; Ogawa, M.; Maruyama, N.; Ohtsuka, R.; Utsumi, S.; Takaiwa, F. Accumulation of soybean glycinin and its assembly with the glutelins in rice. *Plant Physiol.* **1999**, *120*, 1063−1073.
(26) Fu, T.; Abbott, U.; Hatzos, C. Digestibility of food allergens and nonallergenic proteins in simulated gastric and simulated intestinal fluid−a comparative study. *J. Agric. Food Chem.* **2002**, *50*, 7154−7160.
(27) Murtagh, G. J.; Archer, D. B.; Durmoulin, M.; Ridout, S.; Matthews, S.; Arshad, S. H.; Alcocer, M. J. C. In vitro stability and immunoreactivity of the native and recombinant plant food 2S albumins Ber e 1 and SFA-8. Clinical & Experimental Allergy. **2003**, *33*, 1147−1153.
(28) Takagi, K.; Teshima, R.; Okunuki, H.; Sawada, J. Comparative study of in vitro digestibility of food protein and effect of preheating on the digestion. *Biol. Pharm. Bull.* **2003**, *26*, 969−973.
(29) Kimber, I.; Betts, C. J.; Dearman, R. J. Assessment of the allergenic potential of proteins. *Toxicol. Lett.* **2003**, *11*, 140−141:297−302.
(30) Penninks, A.; Knippels, M. Determination of protein allergenicity: studies in rats. *Toxicol. Lett.* **2001**, *120*, 171−180.

**Received for review December 18, 2003. Revised manuscript received February 23, 2004. Accepted February 27, 2004. Financial support and scholarships to Sugey R. Sinagawa-García, Q. Rascón-Cruz, A. Valdez-Ortiz, and S. Medina-Godoy provided by CONACyT-México. S.R.S.G. and Q.R.C. received scholarships from CONCyTEG.**

JF035487K