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IDENTIFICATION OF A BRAZIL-NUT ALLERGEN IN TRANSGENIC SOYBEANS

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Abstract *Background.* The nutritional quality of soybeans (*Glycine max*) is compromised by a relative deficiency of methionine in the protein fraction of the seeds. To improve the nutritional quality, methionine-rich 2S albumin from the Brazil nut (*Bertholletia excelsa*) has been introduced into transgenic soybeans. Since the Brazil nut is a known allergenic food, we assessed the allergenicity of the 2S albumin.

Methods. The ability of proteins in transgenic and nontransgenic soybeans, Brazil nuts, and purified 2S albumin to bind to IgE in serum from subjects allergic to Brazil nuts was determined by radioallergosorbent tests (four subjects) and sodium dodecyl sulfate–polyacrylamide-gel electrophoresis (nine subjects) with immunoblotting and autoradiography. Three subjects also underwent skin-prick testing with extracts of soybean, transgenic soybean, and Brazil nut.

Results. On radioallergosorbent testing of pooled serum from four subjects allergic to Brazil nuts, protein extracts of transgenic soybean inhibited binding of IgE to Brazil-nut proteins. On immunoblotting, serum IgE from eight of nine subjects bound to purified 2S albumin from the Brazil nut and to proteins of similar molecular weight in the Brazil nut and the transgenic soybean. On skin-prick testing, three subjects had positive reactions to extracts of Brazil nut and transgenic soybean and negative reactions to soybean extract.

Conclusions. The 2S albumin is probably a major Brazil-nut allergen, and the transgenic soybeans analyzed in this study contain this protein. Our study shows that an allergen from a food known to be allergenic can be transferred into another food by genetic engineering. (N Engl J Med 1996;334:688-92.)

ALLERGIES to nuts are among the most common food allergies,[1] and allergies to the Brazil nut are well documented.[2] Concern has been expressed about the introduction of allergenic proteins into food plants by genetic engineering.[3-5] The Food and Drug Administration (FDA) has directed developers of new plant varieties to consider the allergenic potential of donor organisms in assessing the safety of foods derived from genetically engineered plants.[6] If there is insufficient information to demonstrate that the introduced protein could not cause allergic reactions in a susceptible population, then the food would require a label to alert consumers to this fact.

The nutritional quality of legumes, including soybeans (*Glycine max*), for both humans and animals is compromised by a deficiency of methionine in the protein fraction of the seeds.[7] As a result, diets for domestic animals that are based on soybean meal must be fortified with methionine or protein sources of this essential amino acid. For the same reason, human vegetarians must carefully balance their diets to ensure an adequate intake of methionine. There have been many attempts to manipulate the balance of essential amino acids in important crops by traditional methods of plant breeding, but improvements in nutritional quality have often come at the expense of agronomic properties such as yield or grain quality. The introduction of genes encoding sulfur-rich proteins from other plants into soybeans through recombinant-DNA techniques is a promising strategy for improving the nutritional quality of soybeans without adversely affecting agronomic performance. The 2S albumin from the seeds of the Brazil nut (*Bertholletia excelsa*) is ideal for this purpose because it is composed of 18 percent and 8 percent, respectively, of the sulfur amino acids methionine and cysteine.[8] The 2S albumin gene from the Brazil nut has been introduced into soybeans,[9] tobacco, oilseed rape (*Brassica napus*), the legume *Vicia narbonensis*, and beans (*Phaseolus vulgaris*).[10-14]

Our objective was to determine whether the 2S albumin from the Brazil nut as expressed in transgenic soybeans was able to bind IgE from people who are allergic to Brazil nuts. Proteins that bind IgE from people with allergies are likely to be allergens, since the mechanism of immediate hypersensitivity is the cross-linking of specific proteins and specific IgE on the surface of sensitized mast cells, causing them to degranulate and release histamine and other mediators of allergic disease.

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Supported by a grant from Pioneer Hi-Bred International, Inc.

METHODS

Subjects

For radioallergosorbent assays, a serum was pooled from four subjects with a history of allergic reactions to Brazil nuts that included oropharyngeal swelling and itching, facial swelling, laryngeal edema, and bronchospasm with wheezing. All four had positive skin-prick tests in response to extracts of Brazil nut and positive radioallergosorbent tests, with binding to Brazil-nut protein in the solid phase that was 9 to 38 times greater than binding with control serum from subjects with no history of food allergies. For experiments using sodium dodecyl sulfate–polyacrylamide-gel electrophoresis (SDS-PAGE), serum was obtained from five additional subjects who either indicated that they had similar symptoms or reported a history of avoiding all nuts. These five also had positive skin-prick tests and positive radioallergosorbent tests, with binding to Brazil-nut protein in the solid phase that was 4 to 64 times greater than binding with control serum from subjects with no history of food allergies. As a control, serum was pooled from eight subjects without food allergies. Skin-prick tests with transgenic material were performed on three subjects with histories of sensitivity to Brazil nuts and no history of sensitivity to soybeans and three subjects (one with atopy) with no history of sensitivity to Brazil nuts or soybeans. All subjects gave informed consent as stipulated by

the University of Nebraska or the University of Wisconsin institutional review board or by Plasma Labs, International (Everett, Wash.).

Radioallergosorbent Assay

Transgenic and nontransgenic soybeans were obtained from Pioneer Hi-Bred International (Johnston, Iowa). Raw Brazil nuts were obtained from Open Harvest Grocery (Lincoln, Nebr.). The Brazil nuts, transgenic soybeans, and nontransgenic soybeans were defatted with acetone and ethyl ether.[15] Extracts were prepared (1:10 wt/vol) with 0.01 M potassium phosphate–buffered saline, pH 7.4, containing 0.02 percent sodium azide and stirred at room temperature for two hours. The extracts were clarified by ultracentrifugation for one hour at 82,000×*g*. The protein content of the extracts was determined by the Lowry–Folin method.[16] Brazil-nut protein in the solid phase was prepared according to the method of Adolphson et al.[17] with microcrystalline cellulose and 10 mg of protein from defatted Brazil-nut extract.

For the radioallergosorbent assay,[17] serial dilutions of Brazil-nut and soybean extracts were prepared with potassium phosphate–buffered saline containing 0.02 percent sodium azide as the diluent and incubated overnight with Brazil-nut protein in the solid phase and pooled serum from four subjects allergic to Brazil nuts. The solid phase was then washed and incubated overnight with antihuman IgE labeled with iodine-125 (Pharmacia and Upjohn, Diagnostics Division, Columbus, Ohio). Excess labeled antibody was removed by washing, and the radioactivity of the solid phase was measured with a sodium iodide scintillation detector. The percent inhibition of IgE binding was calculated with the use of values from samples without inhibitor protein as a measure of maximal binding. For the radioallergosorbent test,[17] serum samples were allowed to react directly with the solid phase without inhibitor protein, and bound IgE was measured as described above. Scores for the radioallergosorbent test were calculated as a multiple of the score obtained with pooled serum from subjects without food allergies.

Electrophoresis and Immunoblotting

Extracts of Brazil nut, transgenic soybean, and nontransgenic soybean were prepared as described above, except that the pH of potassium phosphate–buffered saline containing 0.02 percent sodium azide was 7.2 and single soybean seeds were used for extraction. The Brazil nuts and soybeans were not defatted. The purified 2S albumin from the Brazil nut[18] was diluted in potassium phosphate–buffered saline containing 0.02 percent sodium azide, pH 7.2. Proteins from these extracts and the 2S albumin were separated by SDS-PAGE with minigels with gradients of 10 to 20 percent (Bio-Rad Laboratories, Hercules, Calif.). The wells were loaded with 15 μg of protein from Brazil-nut and soybean extracts and 3.7 μg of 2S albumin. The proteins were transferred from gels to nitrocellulose by electroblotting as described by Towbin et al.[19] Nitrocellulose blots were incubated with control serum or individual serum samples from nine subjects allergic to Brazil nuts. IgE binding was detected by autoradiography after incubation of the washed blots with ^{125}I-labeled antihuman IgE.[20] Alternatively, gels obtained with SDS-PAGE were stained with Coomassie blue to visualize the proteins.[21] α-Lactalbumin (14.4 kd), soybean trypsin inhibitor (20.1 kd), carbonic anhydrase (30 kd), ovalbumin (43 kd), bovine serum albumin (67 kd), and rabbit-muscle phosphorylase *b* (94 kd) served as molecular-weight markers.

Skin-Prick Testing with Transgenic Material

Extracts for skin-prick (epicutaneous) testing were prepared from Brazil nuts, transgenic soybeans, and nontransgenic soybeans obtained from the sources described above. These materials were not defatted. They were prepared (1:10 wt/vol) with 0.01 M phosphate-buffered saline, pH 7.4, and rocked overnight at 4°C. The extracts were clarified by ultracentrifugation at 82,000×*g* for one hour and filtered through a series of 0.45-μm and 0.2-μm sterile filters. The filtrates were collected in sterile vials containing an equal volume of sterile glycerol, mixed, and stored at 4°C until use. Immediately before use, serial dilutions of sterile extracts (1:100 to 1:1,000,000 vol/vol) as well as a 1:50 dilution were prepared with a saline diluent (0.9 percent sodium chloride, 0.03 percent albumin, and 0.04 percent phenol; Miles Laboratories, Spokane, Wash.).

Skin-prick testing was performed according to the method of Norman.[22] A histamine base solution (1.8 mg per milliliter and 50 percent glycerol wt/vol; Allermed, San Diego, Calif.) and saline diluent were used as positive and negative controls, respectively. Titration was conducted starting with the most dilute extracts (1:1,000,000) and progressing through serial dilutions until a positive response (3-mm wheal) was observed in subjects allergic to Brazil nuts. Subjects acting as negative controls underwent skin-prick testing with full-strength extracts and negative- and positive-control solutions as described above. The diameter of the wheal-and-flare response was measured 10 minutes after the skin was pricked. The subjects were asked to refrain from taking antihistamines or other medications that could interfere with skin-test responses for a minimum of 48 hours before testing.

Results

An extract of transgenic soybean competed effectively with Brazil-nut proteins that were bound to solid-phase, microcrystalline cellulose for binding to IgE from the serum of subjects allergic to Brazil nuts (Fig. 1). The degree of inhibition observed with the extract of transgenic soybean was similar to that observed with an extract of raw Brazil nut (Fig. 1). No inhibition was encountered with an extract from nontransgenic soybean that has the same genetic background as the transgenic plant and similar levels of protein. This result indicates that the inhibition by the transgenic-soybean extract was not due to normal soybean proteins or to the nonspecific binding of ^{125}I-labeled antihuman IgE to soy-

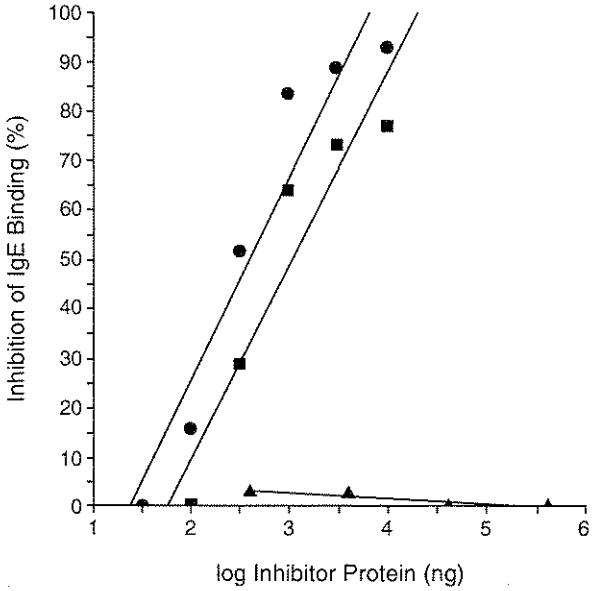

Figure 1. Results of Radioallergosorbent Assays with Extracts of Nontransgenic Soybean (▲), Transgenic Soybean (■), and Brazil Nut (●).

The logarithm of the number of nanograms of total protein in each dilution of extract was plotted against the percent inhibition of IgE binding as calculated by the following formula: (counts without inhibitor protein − counts with inhibitor protein) × 100 ÷ counts without inhibitor protein. The slope of the inhibition curve for the Brazil-nut extract was 40.8, whereas the slope of the curve for the transgenic-soybean extract was 39.4.

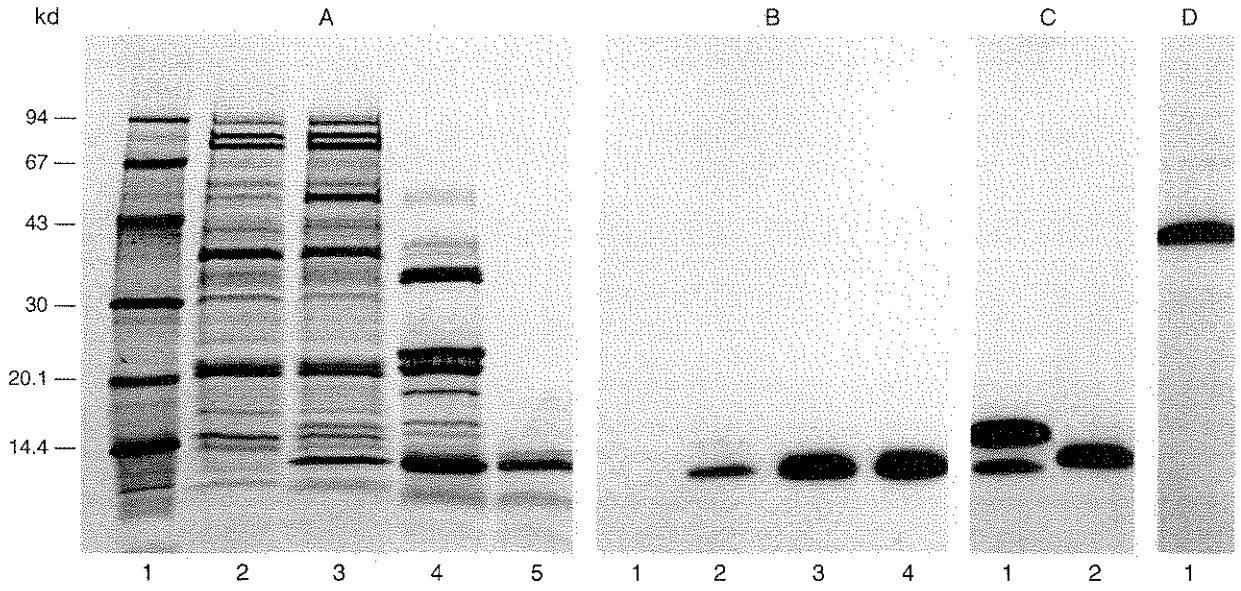

Figure 2. Results of SDS–PAGE and Autoradiography.

In Panel A, SDS–PAGE gels (gradient, 10 to 20 percent) were stained with Coomassie blue. Lane 1 shows the molecular-weight standards; lane 2, nontransgenic-soybean extract; lane 3, transgenic-soybean extract; lane 4, Brazil-nut extract; and lane 5, purified 2S albumin from the Brazil nut. Panels B, C, and D show autoradiographic results of IgE binding. In Panel B, IgE in serum from a subject allergic to Brazil nuts bound to the 9-kd 2S albumin Brazil-nut protein (lane 4) and proteins of similar molecular weights in extracts from Brazil nut (lane 3) and transgenic soybean (lane 2), but did not react with an extract of nontransgenic soybean (lane 1). In Panel C, IgE in serum from a subject allergic to Brazil nuts bound to the 9-kd 2S albumin in Brazil-nut extract (lane 2) and to the 9-kd 2S albumin and 12-kd processed intermediate in transgenic soybean (lane 1). In Panel D, IgE in serum from a subject allergic to Brazil nuts bound to a 42-kd protein in Brazil-nut extract (lane 1) but did not bind detectably to the 2S albumin in transgenic-soybean extract.

bean extract. The parallel inhibition slopes obtained with extracts of transgenic soybean and Brazil nut indicate considerable similarity between the IgE-binding epitopes in these two extracts.

IgE from eight of the nine subjects allergic to Brazil nuts bound to purified 2S albumin from the Brazil nut (molecular weight, 9000)[18] and to a 9-kd protein in an extract of whole Brazil nut (Fig. 2A and 2B). IgE from seven of the nine subjects allergic to Brazil nuts also bound to a transgenic-soybean protein that migrated with the 2S albumin and that was absent from nontransgenic-soybean extract (Fig. 2B). Serum from two of these subjects showed very weak binding to proteins in nontransgenic soybeans, but these proteins did not migrate with the 2S albumin. No binding was observed with serum from control subjects. IgE from the serum of five of the nine subjects allergic to Brazil nuts also bound to a protein of approximately 12 kd that was present in some transgenic-soybean extracts (Fig. 2C). The two bands seen in lane 1 of Figure 2C are most likely the 12-kd protein intermediate and the mature 9-kd subunit of the 2S albumin.[8]

Table 1. Results of Skin-Prick Tests in Three Subjects Allergic to Brazil Nuts.

Subject No.	Maximal Wheal Diameter/Maximal Flare Diameter (mm)				
	Diluent	Histamine	Nontransgenic-soybean extract	Transgenic-soybean extract*	Brazil-nut extract*
1	0/0	6/10	2/5	15/35 (1:1000)	10/30 (1:1000)
2	1/0	7/19	2/0	9/29 (1:10,000)	7/26 (1:10,000)
3	0/0	5/20	2/5	14/41 (1:1,000,000)	8/54 (1:1,000,000)

*The dilutions of the extracts are given in parentheses.

In addition to the 2S albumin, the transgenic soybean contained proteins encoded by two bacterial marker genes: neomycin phosphotransferase and β-glucuronidase. Purified commercial preparations of neomycin phosphotransferase and β-glucuronidase denatured with sodium dodecyl sulfate did not bind IgE from the serum of subjects allergic to Brazil nuts. IgE from the serum of six of these nine subjects bound to other Brazil-nut proteins. The identities of these other proteins are not known. In one case, IgE did not bind detectably to the 2S albumin but instead recognized a 42-kd protein (Fig. 2D). In another case, IgE bound to both the 2S albumin and the 42-kd protein (data not shown), although the binding to the 2S albumin was much stronger; this subject's serum also recognized minor IgE-binding proteins of 21, 25, 39, and 57 kd, with comparatively much weaker binding, in Brazil nuts. In all other cases, binding to other Brazil-nut proteins was not very strong, and there was no consistent pattern to these individual responses.

Three subjects with histories of hypersensitivity to Brazil nuts and no history of hypersensitivity to soy-

beans underwent skin-prick testing. All three had positive reactions to the transgenic-soybean and Brazil-nut extracts at dilutions ranging from 1:1,000,000 to 1:1000 (Table 1). A representative reaction is shown in Figure 3. All three had positive responses to the histamine control and no response to the saline diluent and the nontransgenic-soybean extract. Three control subjects had no reaction to full-strength extracts of Brazil nut, transgenic soybean, and soybean or to saline diluent and had a positive response to the histamine control.

Discussion

The 2S albumin from the Brazil nut is very likely a major allergen. Major allergens are proteins that bind substantially to IgE from more than 50 percent of the patients with that specific allergy. In this case, serum from eight of nine subjects allergic to Brazil nuts recognized the 2S albumin as a major IgE-binding protein of the Brazil nut, and this protein was by far the strongest IgE-binding protein in seven of the eight subjects. IgE from these seven subjects also bound to the 2S albumin in transgenic soybeans, which carry the Brazil-nut 2S albumin gene. Furthermore, skin-prick tests with extracts of transgenic soybean were positive in the three subjects allergic to Brazil nuts who were tested, whereas tests with nontransgenic-soybean extract were negative. Oral challenges would be necessary to confirm the allergenicity of the 2S albumin; however, they would pose a risk to this group of subjects, most of whom experience life-threatening symptoms on inadvertent consumption of Brazil nuts.

Recently, Melo et al.[23] concluded on the basis of experiments in animals that the 2S albumin from the Brazil nut was not a major allergen. However, the ability of a protein to induce an IgG1 response in animals is not always a good indicator of the ability of that protein to induce an IgE response in humans. We consider that our evidence justifies the designation of the 2S albumin from the Brazil nut as a major Brazil-nut allergen, *Ber e* 1.[24] Food derived from new plant varieties, including soybeans, that are likely to contain *Ber e* 1 should be appropriately labeled to alert consumers, in compliance with FDA policy. Our findings demonstrate the transfer of a major food allergen during the development of improved crop varieties through genetic engineering.

We also detected some binding of IgE to other Brazil-nut and soybean proteins in tests with serum from one or more of the subjects who were allergic to Brazil nuts. Binding to a 12-kd protein from the transgenic soybean (Fig. 2C) can be explained by the fact that the 2S albumin in Brazil nuts is known to be composed of 9-kd and 3-kd subunits that are processed from a 17-kd precursor protein by means of a 12-kd intermediate.[8] In transgenic soybeans, this processing is often incomplete, resulting in the accumulation of the 12-kd intermediate (unpublished data). The 42-kd protein from the Brazil nut was the primary allergen for one of the nine subjects who were allergic to Brazil nuts. However, this allergen is not a major Brazil-nut allergen because most subjects allergic to Brazil nuts who were tested in our study did not have IgE specific for this protein. With other plant foods, such as peanuts and soybeans, numerous IgE-binding proteins are detected,[20,25] and the identification of the major IgE-binding proteins can be problematic. In the case of animal foods, such as shrimp and cod, multiple IgE-binding proteins are detected, but one predominant IgE-binding protein is usually easily identifiable.[26,27]

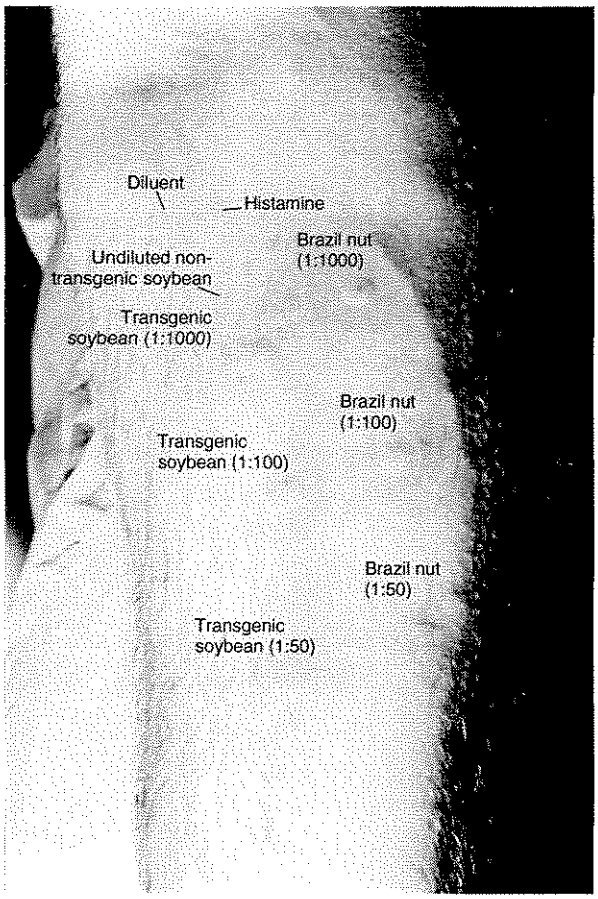

Figure 3. Reactivity on Skin-Prick Testing to Extracts of Transgenic Soybean, Nontransgenic Soybean, and Brazil Nut in a Subject Allergic to Brazil Nuts.
The dilutions are given in parentheses.

It is prudent to assess the allergenicity of proteins in transgenic foods if those proteins have been derived from sources that are commonly allergenic. The use of currently available animal models alone to predict allergenicity in humans does not produce accurate results. Techniques such as radioallergosorbent tests, SDS-PAGE with immunoblotting, and skin-prick testing can identify IgE-binding proteins that are probable allergens in transgenic foods derived from sources known to be allergenic. This strategy will not be useful in assessing the allergenicity of transgenic foods in which the allergenicity of the source of donor genetic material

is unknown. Most current applications in the field of plant biotechnology fall into the latter category.

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