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Biofortification of Soybean Meal: Immunological Properties of the 27 kDa γ-Zein

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ABSTRACT: Legumes, including soybeans (*Glycine max*), are deficient in sulfur-containing amino acids, which are required for the optimal growth of monogastric animals. This deficiency can be overcome by expressing heterologous proteins rich in sulfur-containing amino acids in soybean seeds. A maize 27 kDa γ-zein, a cysteine-rich protein, has been successfully expressed in several crops including soybean, barley, and alfalfa with the intent to biofortify these crops for animal feed. Previous work has shown that the maize 27 kDa zein can withstand digestion by pepsin and elicit an immunogenic response in young pigs. By use of sera from patients who tested positive by ImmunoCAP assay for elevated IgE to maize proteins, specific IgE binding to the 27 kDa γ-zein is demonstrated. Bioinformatic analysis using the full-length and 80 amino acid sliding window FASTA searches identified significant sequence homology of the 27 kDa γ-zein with several known allergens. Immunoblot analysis using human serum that cross-reacts with maize seed proteins also revealed specific IgE-binding to the 27 kDa γ-zein in soybean seed protein extracts containing the 27 kDa zein. This study demonstrates for the first time the allergenicity potential of the 27 kDa γ-zein and the potential that this protein has to limit livestock performance when used in soybeans that serve as a biofortified feed supplement.

KEYWORDS: γ-Zein, sulfur-rich protein, allergen, IgE, transgenic soybean

INTRODUCTION

Soybeans are an excellent source of protein for both livestock and humans. However, they are deficient in the sulfur-containing amino acids cysteine and methionine. Consequently, animals (and humans) consuming mainly a grain-based diet have to be supplemented with synthetic amino acids to maintain optimal growth and development.

Numerous attempts have been made to improve the concentration of sulfur-containing amino acids in soybean.[1−4] One commonly used approach involves the expression of heterologous seed proteins rich in sulfur-containing amino acids in legumes.[5−7] Significant increase in sulfur amino acid content of soybean was achieved by the introduction of 2S albumin from Brazil nut,[1] a protein exceptionally rich in methionine.[8] However, the 2S albumin is a major Brazil nut allergen, and the transgenic soybeans expressing the 2S albumin were found to bind IgE from people who are allergic to Brazil nuts.[9] On the basis of the immunological properties on the 2S Brazil nut albumin it was suggested that the allergenicity of proteins be thoroughly tested, especially if the protein is derived from sources that are commonly allergenic, before they are introduced into feed crops.[9] Subsequently, there have been other reports of transgenic soybean plants accumulating methionine-rich storage proteins of maize.[2−4] Two maize storage proteins, δ- and γ-zeins, have been successfully introduced into soybeans by genetic engineering.[2−4] However, the immunological properties of the δ- and γ-zeins have not been investigated.

Although corn is often cited as a food containing allergens, only limited investigations have been conducted on their clinical significance. Corn proteins have been reported to cause allergies in sensitive individuals. A 9 kDa nonspecific lipid transfer protein and a 16 kDa trypsin inhibitor have been identified as major and minor corn allergens.[10] A less abundant 50 kDa protein, a component of γ-zein, has been recently identified as a major allergen.[11,12] This protein contains a sequence motif (Gln-Gln-Gln-Pro-Gln) that has been earlier identified as the IgE-binding epitope of wheat glutenin.[13] By utilizing proteomic tools several proteins including vicilin, globulin-2, 50 kDa γ-zein, endochitinase, thioredoxin, and trypsin inhibitor were identified as maize allergens.[14] Utilizing sera of Italian and Swiss patients sensitive to maize, it was observed that five patients showed IgE reactivity to the zein/prolamin fraction. These IgE-recognized proteins were identified as α-zein (22 kDa), α-zein precursor (19 kDa), and a β-zein (16 kDa). On the basis of this observation it was suggested that these proteins may possess allergenic potential that needs to be further studied.

Recently, we demonstrated that maize proteins can elicit an immunological response in young pigs. Several lines of evidence confirmed that the immunodominant protein in young pigs was the 27 kDa γ-zein.[15] However, there have been no previous papers which suggest that the 27 kDa γ-zein is a food allergen. In this study we demonstrate that the 27 kDa γ-zein binds IgE from sera obtained from human patients who are allergic to maize proteins and so may act as a potential food allergen. We also

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demonstrate that IgE bind to the maize 27 kDa γ-zein in protein extracts of soybeans expressing the maize 27 kDa γ-zein. Our results suggest that the 27 kDa γ-zein has the potential to act as a food allergen. The immunological properties of this protein thus may limit its possible use as a biofortification supplement for soybean meal.

MATERIALS AND METHODS

Patients' Sera. Sera from seven patients allergic to maize were obtained from IBT Laboratories (Lenexa, KS). ImmunoCAP in vitro quantitative assay demonstrated the presence of maize-specific IgE (17.5−49.9 IgE ku/L) in the sera of these patients. Human sera containing high levels of IgE against soybean allergen (Gly m Bd 28 kDa) was a generous gift from Dr. Michael Zeece, University of Nebraska.

Plant Material and Protein Preparation. Transgenic soybeans expressing the maize 27 kDa γ-zein[4] and nontransformed control plants were grown in a greenhouse with 16 h day length and 30/18 °C day/night temperatures. Seeds of maize inbred line B73 were kindly provided by Dr. Sherry Flint-Garcia, USDA-ARS, Columbia, MO. Total seed proteins from 10 mg of soybean and 30 mg of maize inbred line B73 were obtained by extraction with 1 mL of 1× SDS sample treatment buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 30 mM Bromphenol Blue, pH 6.8) in a 30 °C shaker for 30 min. After centrifugation (15800*g*, 10 min), 5% (v/v) β-mercaptoethanol (β-ME) was added to the supernatant and boiled for 5 min. Aliquots (10 μL) of the supernatant were loaded onto a 13.5% acrylamide gel and separated by SDS-PAGE. Gels were stained overnight with Coomassie Blue G-250.

Immunoblot Analysis. Immunoblot analysis was carried out as described earlier.[15]. Briefly, proteins electrophoretically transferred to nitrocellulose membranes (Protran, Schleicher & Schuell Inc., Keene, NH) were blocked with 5% milk in Tris-buffered saline (TBS, pH 7.3) for 1 h and incubated in sera from patients allergic to maize at 1:500 dilution or with maize γ-zein polyclonal antibody at 1:10000 dilution overnight at room temperature with gentle rocking. After four washings with TBS containing 0.05% Tween-20 (TBST) for 10 min each, the membrane was incubated for 2 h in either 1:5000 dilution of goat anti-human IgE−horseradish peroxidase conjugate antibody (Biosource, Camarillo, CA) or affinity-purified goat anti-rabbit IgG−horseradish peroxidase (HRP) conjugate (Bio-Rad Laboratories, Hercules, CA) at 1:3000 dilution. Immunoreactive polypeptides were detected with an enhanced chemiluminescent substrate (Super Signal West Pico Kit; Pierce Biotechnology, Rockford, IL) according to the manufacturer's protocol.

Purification of the 27 kDa Maize γ-Zein. Prolamin fraction enriched in γ-zein was obtained by following the procedures described previously[16] with some modifications. Briefly, 100 mg of dry seed powder was sequentially extracted in a 30 °C shaker for 30 min each with 1 mL of 50 mM Tris-HCl, pH 6.8, and 1 mM EDTA (albumin), then 50 mM Tris-HCl, pH 6.8, and 1 mM EDTA and 0.5 M NaCl (globulin), then 50% isopropanol (α- and β-zeins), and then 50% isopropanol and 5% β-ME (γ-zeins). Each extraction was followed by centrifugation for 10 min at 15800*g* in a microcentrifuge, the supernatant was removed, and the remaining pellet was re-extracted with the next solution. Three volumes of ice-cold acetone was added to the supernatant from the γ-zein-enriched fraction and stored at −20 °C overnight. Precipitated proteins were recovered by centrifugation at 15800*g* for 20 min. The protein pellets were air-dried and resuspended in a small volume of SDS sample buffer and fractionated by preparative SDS-PAGE. The 27 kDa γ-zein was eluted from the preparative gels as described previously.[17] The identity of the purified protein was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.[15]

Bioinformatic Analysis. Maize 27 kDa γ-zein sequence (GenBank AAL16977) was subjected to FASTA[18,19] search of the Food Allergy Research and Resource Program (FARRP) Protein AllergenOnline Database (www.allergenonline.org/index.shtml), which includes sequences of 1471 allergens or putative allergens. This sequence search program, in addition to performing the full-length FASTA, also scanned each possible 80 amino acid segment of the 27 kDa γ-zein against the AllergenOnline database, looking for matches of at least 35% identity.[20,21]

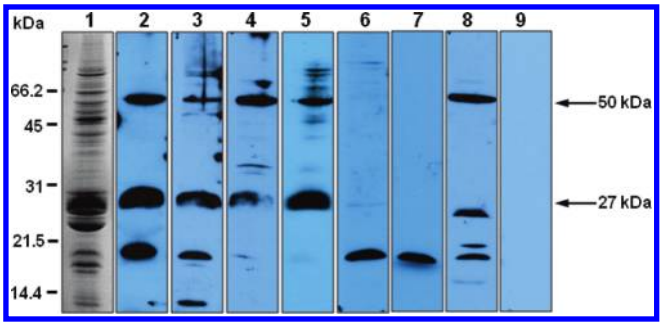

Figure 1. IgE immunoblotting of maize seed proteins with sera from maize-sensitive patients. Total seed proteins from B73 maize fractionated by SDS-PAGE were transferred to nitrocellulose membranes, cut into strips, and incubated individually with sera from seven maize-sensitive patients (lanes 2−8) or serum from an individual with no history of maize allergy (lane 9). Immunoreactive proteins were detected using anti-human IgE−horseradish peroxidase conjugate antibody followed by chemiluminescent detection. Lane 1 represents total maize proteins visualized with Coomassie Blue R-250; lanes 2−8 were incubated with sera from patients 1−7, respectively. The positions of the protein molecular weight markers in kDa are shown on the left. The arrows point to the 50 and 27 kDa immunoreactive maize proteins.

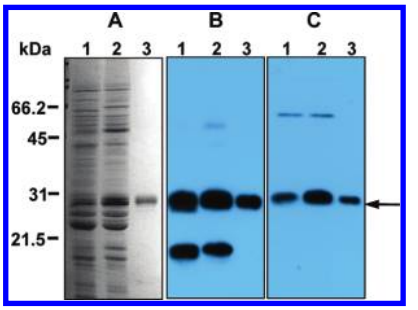

Figure 2. Immunoblot confirmation of the 27 kDa IgE-binding maize protein as γ-zein. Total proteins (lane 1), prolamin fraction (lane 2), and purified γ-zein fraction (lane 3) were fractionated by SDS-PAGE on a 13.5% gel and stained with Coomassie Blue (panel A). Proteins shown in panel A were transferred to nitrocellulose membranes and probed with antibodies raised against the purified γ-zein (panel B) or serum from a maize-sensitive patient (panel C). Immunoreactive proteins were identified using either anti-rabbit IgG−horseradish peroxidase conjugate antibody (panel B) or anti-human IgE−horseradish peroxidase conjugate antibody (panel C) followed by chemiluminescent detection. The positions of the protein molecular weight markers in kDa are shown on the left. The arrow on the right side of the figure points to the 27 kDa γ-zein.

RESULTS

IgE from Serum from Patients That React Immunologically to Maize Bind to the 27 kDa γ-Zein. Sera obtained from seven patients who tested positive by ImmunoCAP assay for elevated IgE to maize proteins were used in the immunoblot blot analysis. IgE from five of the seven subjects (patients 1−4 and 7) sensitive to dietary maize bound to a 50 kDa protein from a total

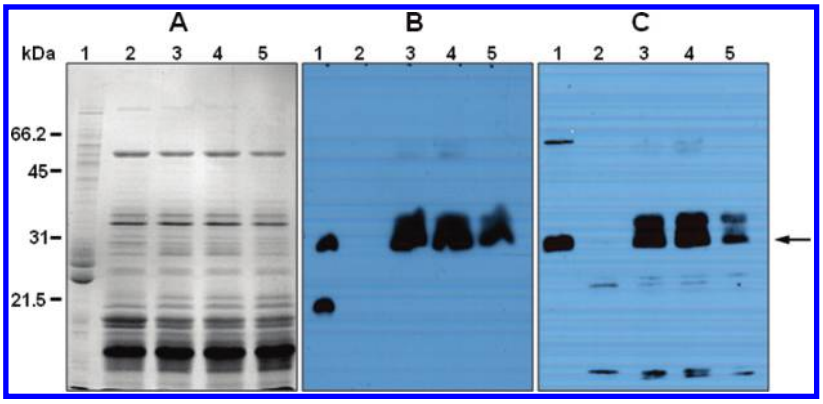

Figure 3. Immunoblot demonstration of transfer of the 27 kDa maize γ-zein to transgenic soybean. Panel A represents Coomassie blue stained SDS-PAGE gel showing total B73 maize seed proteins (lane 1), nontransgenic soybean extract (lane 2), and seed extracts from independent transgenic soybean lines (lanes 3−5). Proteins shown in panel A were transferred to nitrocellulose membranes and probed with antibodies raised against the purified γ-zein (panel B) or serum from a maize-sensitive patient (panel C). Immunoreactive proteins were identified using either anti-rabbit IgG−horseradish peroxidase conjugate antibody (panel B) or anti-human IgE−horseradish peroxidase conjugate antibody (panel C) followed by chemiluminescent detection. The γ-zein antibody and the serum from a maize-sensitive patient react against the proteins of similar molecular weights in extracts from maize (lane 1) and transgenic soybeans (lanes 3−5), but do not react with nontransgenic soybean extract (lane 2). The arrow on the right side of the figure points to the 27 kDa γ-zein.

maize seed protein extract (Figure 1). Sera from four of these subjects (patients 1−4) also showed strong binding to a 27 kDa protein. IgE from the sera of two subjects (patients 5 and 6) failed to react against either the 27 or 50 kDa maize protein but bound to a protein of approximately 18 kDa. The 18 kDa maize protein was also recognized by the sera from three other subjects (patients 1, 2, and 7). In contrast, immunoblot analysis using human sera containing high levels of IgE against the soybean allergen Gly m Bd 28[22] or serum from an individual with no history of maize sensitivity revealed no cross-reactivity against any of the maize proteins (Figure 1, lane 9). We have shown earlier that the 27 kDa protein, which elicits strong immunogenic reaction in piglets, was a γ-zein by immunoblot analysis and MALDI-TOF-MS.[15] To confirm that the 27 kDa protein recognized by human sera is the γ-zein, we performed immunoblot analysis using purified 27 kDa γ-zein Because the 27 kDa γ-zein was purified from preparative SDS-PAGE gels, it is possible other proteins having a molecular weight similar to that of γ-zein may also been copurified by the procedure. The purified 27 kDa γ-zein was recognized by the human IgE, demonstrating that humans allergic to maize react at least in part to the 27 kDa γ-zein (Figure 2).

IgE Binding to the 27 kDa γ-Zein in Soybean Expressing the Maize 27 kDa γ-Zein Gene. To increase the sulfur amino acid content of soybean we had earlier generated soybeans expressing the maize 27 kDa γ-zein.[4] We isolated alcohol-soluble proteins from three soybean lines expressing the maize 27 kDa γ-zein and one nontransformed soybean line. These samples were analyzed with SDS-PAGE and Western blot (Figure 3). Examination of the Coomassie-stained gel revealed no obvious changes in the protein profile between soybean lines expressing maize 27 kDa γ-zein and the nonexpressing soybean line (Figure 3A). Western blot analysis using antibodies generated against the purified γ-zein revealed cross-reactivity against 27 and 16 kDa proteins (Figure 3B). Maize γ-zein antibodies showed cross-reactivity against 32 and 27 kDa proteins from the extracts of soybean plants expressing maize 27 kDa γ-zein, but no immunoreactive proteins were detected in nontransformed soybean (Figure 3B). The immunoreactive 32 kDa protein detected in transgenic soybeans is likely an unprocessed form of the γ-zein. IgE from the serum of those subjects sensitive to maize proteins also bound to the 32 and 27 kDa protein, and only from the extracts of soybeans expressing maize 27 kDa γ-zein (Figure 3C). Weak binding to low molecular weight proteins was detected in all soybean protein extracts (Figure 3C).

27 kDa γ-Zein Exhibits Sequence Similarity to Known Allergens in the Database. A key component in evaluating the immunological properties of a protein is comparison of the putative allergenic sequences with those of known allergens using a bioinformatics approach. We compared the maize 27 kDa γ-zein amino acid sequence by a FASTA search[18,19] against the Food Allergy Research and Resource Program (FARRP) Protein Allergen Online Database (www.allergenonline.org). Examination of the full-length FASTA search revealed significant homology with several known or putative allergenic sequences. The maize 27 kDa γ-zein showed 33.2% identity (52.0% similarity) in a 229 amino acid overlap to wheat low molecular weight glutenin and 30.7% identity (54.2% similarity) in a 238 amino acid overlap to wheat γ-gliadin B precursor, respectively (Figure 4A). The 27 kDa γ-zein also had sequence similarity to 2S albumin seed storage proteins (Figure 4B) from *Sesamum indicum* (31.7% identity; 53.8% similarity in a 104 amino acid overlap), *Juglans nigra* (33.3% identity; 55.6% similarity in a 99 amino acid overlap), *Pistacia vera* (28.4% identity; 60.8% similarity in a 74 amino acid overlap), *Anacardium occidentale* (32.4% identity; 63.4% similarity in a 71 amino acid overlap), and *Bertholletia excelsa* (27% identity; 57% similarity in a 100 amino acid overlap). A scan of each possible 80 amino acid segment of the 27 kDa γ-zein against the Allergen Online database, searching for matches of at least 35% identity, retrieved 37 protein sequences. With the exception of two 80 amino acid segments, the remainder of the protein sequences matched sequences that belong to the wheat low molecular weight glutenin or γ-gliadin, two known wheat allergens. A search for shorter identical segments of 8 contiguous amino acids was also performed. This analysis revealed a segment, RQQCCQQLRQ, present in the 27 kDa γ-zein was similar to regions in the ω-5 gliadin and LMW glutenin 3.[13]

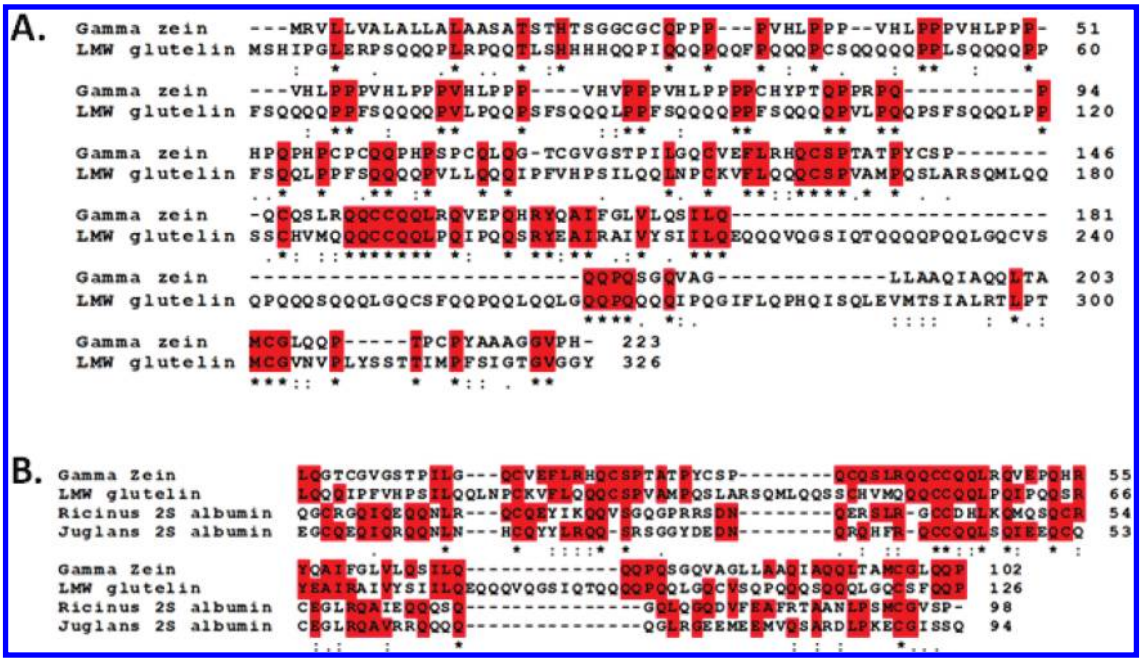

Figure 4. (Panel A) Amino acid sequence alignment of maize 27 kDa γ-zein with wheat low molecular weight glutelin. (Panel B) Partial amino acid sequence alignment of maize 27 kDa γ-zein with wheat low molecular weight glutelin and *Ricinus* and *Juglans* 2S albumins. The accession numbers of the sequences are as follows: maize 27 kDa γ-zein, AAL16977; wheat low molecular weight glutelin, CAI79052; *Ricinus communis* 2S albumin, PO1089; *Juglans nigra* 2S albumin, AAM54365. Positions of amino acid identity are boxed and denoted with asterisks; similar amino acids are indicated with colons. Multiple sequence alignment was performed using the CLUSTAL W program from UniProt Knowledgebase (http://www.uniport.org/).

DISCUSSION

Approximately 5% of young children and 2% of adults in the industrialized world suffer from food allergies.[23] Allergies to milk, eggs, and peanuts are common in young children, whereas peanuts, tree nuts, and shellfish are the most common allergens in adults.[24] Although maize is a major component of a wide range of foods including breakfast cereals, tortilla chips, corn snacks, and corn flour-based soups and sauces, incidence of food hypersensitivity reactions to maize is less common.[25,26] However, reports of severe reactions to maize have been reported from southern Europe and Mexico, where maize is widely consumed.[27] Even though a previous study has noted IgE reactivity to α- and β-zeins,[28] until now no studies identified the 27 kDa γ-zein as a food allergen. We have earlier demonstrated that the 27 kDa γ-zein induces strong immunological response in young pigs and could be a potential allergen.[15] In this study, we demonstrate that the same protein may also be a potential food allergen. By employing full-length FASTA search against the AllergenOnline database, we were able to detect sequence homology between maize 27 kDa γ-zein and several known allergens (Figure 4). This bioinformatic analysis enabled us to ascertain the potential risk of allergenic cross-reactivity of 27 kDa γ-zein. Interestingly, the 27 kDa γ-zein contains a peptide (RQQCCQQLRQ) that has been identified as an IgE binding epitope of high molecular weight glutenin, a known allergen.[13] Additionally, using sera from individuals sensitive to maize, we were able to demonstrate specific IgE binding to the 27 kDa γ-zein. Collectively, these results underscore the allergenic potential of the 27 kDa γ-zein.

The γ-zein has been successfully expressed in several important crops such as soybean, barley, and alfalfa.[4,29,30] The maize γ-zein, which codes for a sulfur amino acid-rich protein, has also been introduced into soybean to improve its nutritional quality.[4] A phenylalanine-free γ-zein protein has also been expressed in transgenic soybeans to address the protein needs of phenylketonuria patients as well as to provide an alternative market for soybean through the production of a value-added trait (Trick, unpublished data). Similarly, the 27 kDa γ-zein has also been introduced into alfalfa to improve its nutritional quality for wool production.[30] In the case of barley, the 27 kDa γ-zein was expressed for the purpose of altering the grain soft texture to hard-textured endosperm.[29] Additionally, chimeric protein (zeolin) composed of phaseolin, the seed storage protein of common beans and 89 amino acids of γ-zein, has been expressed in tobacco and alfalfa.[31,32] The zeolin, which accumulated to very high levels in tobacco, was localized in the ER-located protein bodies. The success of this approach has prompted the use of γ-zein to stably produce value-added proteins in plants.[33] However, the potential allergenicity of the γ-zein may limit the usefulness of this approach for the biofortification of soybeans.

Biofortification of crops has become a major focus of crop improvement efforts as food security has become a significant global issue and rising food and feed prices affect the nutritional quality of human and livestock diets. In those countries where incomes are on the rise, the demand for protein in the form of meat increases, and thus the pressure on livestock production intensifies.[34] With the pressure to improve the performance of livestock comes the need for improved strategies for the biofortification of feed to supply nutrients normally deficient in standard feed stocks. One of the strategies to biofortify feed is to introduce novel genes, which direct the synthesis of limiting nutrients, into the plants that form the bulk of livestock diets. In those countries where poverty is common, rising food prices force people to switch to cheaper staple foods that fill the stomach but do not provide the necessary nutrients to maintain or improve health.[35] Thus, there is a push to biofortify staple food crops, as with feed crops, by the introduction of novel genes into

the crop to improve the nutritive value of the diet. However, for a biofortified food or feed crop to be effective, and gain regulatory approval, it must meet certain criteria.[36] One of these criteria is that the introduced gene does not produce a protein that would elicit an allergic response in the consumer, whether the consumer is livestock or human.[37−40] Apart from the obvious risk to human or animal health, introduced allergens negate the desired benefits of the biofortification effort because the induced immune response to foodborne allergens limits growth performance and negatively affects health, in some cases severely.[41] The source of the gene, sequence identity to known allergens, ability to bind IgE from patients allergic to the introduced protein, and resistance of the introduced protein to digestion to pepsin are some of the key factors that can be used to predict the allergenicity potential of a protein.[40] On the basis of these criteria it is evident that the 27 kDa γ-zein is a potential allergen. Thus, the use of the 27 kDa γ-zein as a means of improving the nutritional value of soybean protein, by improving the amino acid profile of the meal, appears to be severely limited, and other ways of achieving this goal need to be pursued.

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