

# Identification of an Abundant 56 kDa Protein Implicated in Food Allergy as Granule-Bound Starch Synthase

Hari B. Krishnan<sup>\*,†</sup> and Ming-Hsuan Chen<sup>‡</sup>

<sup>†</sup>Plant Genetics Research Unit, Agricultural Research Service (ARS), United States Department of Agriculture (USDA), University of Missouri, Columbia, Missouri 65211, United States

<sup>‡</sup>Dale Bumpers National Rice Research Center, Agricultural Research Service (ARS), United States Department of Agriculture (USDA), Stuttgart, Arkansas 72160, United States

**ABSTRACT:** Rice, the staple food of south and east Asian countries, is considered to be hypoallergenic. However, several clinical studies have documented rice-induced allergy in sensitive patients. Rice proteins with molecular weights of 14–16, 26, 33, and 56 kDa have been identified as allergens. Recently, it was documented that the 56 kDa rice allergen was responsible for rice-induced anaphylaxis. The 14–16 kDa allergens have been identified as  $\alpha$ -amylase inhibitors; the 26 kDa protein has been identified as  $\alpha$ -globulin; and the 33 kDa protein has been identified as glyoxalase I. However, the identity of the 56 kDa rice allergen has not yet been determined. In this study, we demonstrate that serum from patients allergic to maize shows IgE binding to a 56 kDa protein that was present in both maize and rice but not in the oil seeds soybean and peanut. The 56 kDa IgE-binding protein was abundant in the rice endosperm. We have purified this protein from rice endosperm and demonstrated its reactivity to IgE antibodies from the serum of maize-allergic patients. The purified protein was subjected to matrix-assisted laser desorption ionization–time of flight–tandem mass spectrometry analysis, resulting in identification of this rice allergen as granule-bound starch synthase, a product of the *Waxy* gene. Immunoblot analysis using protein extracts from a *waxy* mutant of rice revealed the absence of the 56 kDa IgE-binding protein. Our results demonstrate that the 56 kDa rice allergen is granule-bound starch synthase and raise the possibility of using *waxy* mutants of rice as a potential source of the hypoallergenic diet for patients sensitized to the 56 kDa rice allergen.

**KEYWORDS:** allergen, anaphylaxis, granule-bound starch synthase, rice

## ■ INTRODUCTION

Eight foods (milk, egg, fish, crustacean shellfish, tree nuts, peanuts, wheat, and soybeans) have been estimated to account for 90% of all food-allergic reactions.<sup>1</sup> It has been estimated that about 5% of young children and 2% of adults in the industrialized world suffer from food allergies.<sup>2</sup> World Allergy Organization's recent white book on allergy states that the allergic diseases are dramatically increasing worldwide.<sup>3</sup> This increase could also be attributed in part because of heightened awareness among doctors and patients about the allergic symptoms. Food-allergic reaction could induce symptoms such as hives, itching, wheezing, and abdominal pain. However, some individuals can develop severe allergic reaction, such as anaphylaxis, that can be life-threatening.

The majority of the global population is dependent upon rice as the main source of nutrition and energy. This is especially true in east and south Asian countries, where rice is the single most consumed cereal. In comparison to other cereals, rice is considered to be hypoallergenic and rice-induced allergies are not a common occurrence in Europe and North America.<sup>4</sup> In contrast, immunoglobulin E (IgE)-mediated hypersensitivity to rice is relatively common in Asian countries.<sup>5–7</sup> In Japan, the prevalence of rice allergy is about 10% in atopic individuals.<sup>8</sup> Interestingly, IgE-mediated hypersensitivity to rice is 6 times more common in adults than in children. This is in contrast to most other food allergies that are more common in children (5–8%) than in adults (1–2%). Exposure to rice flour or inhalation of vapors from boiling rice can induce asthma.<sup>5</sup>

Other symptoms associated with rice allergy include rhinoconjunctivitis, eczema, and atopic dermatitis.<sup>9–13</sup>

The official International Union of Immunological Societies Allergen Nomenclature Subcommittee recognizes Ory s 1 and Ory s 12, two proteins isolated from rice pollen, as rice allergens.<sup>14</sup> Several other rice pollen proteins, Ory s 2, Ory s 7, Ory s 11, Ory s 13, and Ory s 23, have also been identified as putative allergens.<sup>15</sup> Immunoblot analysis using serum from patients with rice allergy has revealed IgE-reactive rice seed proteins with molecular weights of 14–16, 26, 33, and 56 kDa. The 14–16 kDa protein, which was recognized by IgE from the majority of rice-allergic patients in Japan, was identified as  $\alpha$ -amylase inhibitors.<sup>16</sup> An abundant 25 kDa rice seed globulin was also recognized by IgE from rice-allergic patients, although to a lesser frequency than the  $\alpha$ -amylase inhibitors.<sup>17</sup> The 33 kDa allergen from rice seeds have been identified as glyoxalase I.<sup>18</sup> A lipid transfer protein (LTP) has also been shown to be a potential allergen of rice-induced anaphylaxis in limited patients.<sup>19</sup>

A recent study has identified four glycosylated IgE-binding proteins with apparent molecular weights of 49, 52, 56, and 98 kDa from the serum of a German patient who had experienced frequent rice-induced anaphylaxis.<sup>4</sup> IgE binding to the 49, 52,

Received: April 1, 2013

Revised: May 13, 2013

Accepted: May 15, 2013

Published: May 15, 2013

and 98 kDa rice proteins was completely inhibited by preincubation with cross-reactive carbohydrate determinants, while the binding to the 56 kDa rice protein was not inhibited.<sup>4</sup> In addition, serum collected from additional patients reporting hypersensitivity symptoms after rice consumption also revealed IgE binding to the 56 kDa protein. These results led the authors to conclude that the 56 kDa allergen is responsible for the rice-induced hypersensitivity in the German patients. Others have also documented the reactivity of the 56 kDa rice protein to IgE antibodies from rice-allergic patients.<sup>20,21</sup> However, until now, the identity of the 56 kDa allergen has not been known.

## MATERIALS AND METHODS

**Patient's Sera.** Sera from seven patients with documented allergy to maize were obtained from IBT Laboratories (Lenexa, KS). ImmunoCAP *in vitro* quantitative assay revealed 15.5–49.9 kU/L maize-specific IgE in the sera of these patients. Two of seven maize allergic patients also contained IgE specific to rice flour, as determined by the ImmunoCAP assay. Serum from a patient with high levels of IgE against peanut allergens and serum from a health individual with no known history of allergy were also obtained from IBT Laboratories. All sera were stored at –80 °C until used.

**Plant Materials.** For our initial studies, commercial rice purchased from a local grocery store was used. Subsequently, two additional rice cultivars, Tauli and Zano No 1, were also included in our study. Tauli from GRIN (PI\_402689) (Germplasm Resources Information Network, <http://www.ars-grin.gov>) is a medium grain rice originating from Nepal. Zano No 1 (PI\_614988) is a glutinous rice (apparent amylose content of <5%, unpublished data) originating from China. These cultivars were grown at Beaumont, TX, following standard cultural management practices. After harvest, the rough rice was stored at –20 °C until use. Following the removal of the hulls, the whole grain was milled using a McGill Mill 1 (HT McGill, Houston, TX) to collect the bran and endosperm (milled rice).<sup>22</sup> Seeds of maize inbred line B73 and soybean cultivar Williams 82 were from our laboratory stocks. Peanut (jumbo Spanish peanut) was purchased from a local grocery store.

**Seed Protein Preparation.** For the isolation of total proteins, 10 mg of soybean and peanut, 20 mg of rice, and 30 mg of maize seed powder were individually extracted with 1 mL of 1× SDS sample treatment buffer [62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 30 mM Bromphenol Blue at pH 6.8, and 5% (v/v)  $\beta$ -mercaptoethanol ( $\beta$ -ME)]. The samples were placed in a 30 °C shaker for 10 min and left in a boiling water bath for 5 min. The samples were subjected to centrifugation at 15800g for 10 min, and the resulting supernatant was saved. Rice bran protein was obtained by extracting 30 mg of bran powder with 1 mL of 1× SDS sample treatment buffer as described above. Throughout the text, “rice protein” referred to protein extracted from non-glutinous rice with functional *Waxy* gene, unless specified as rice protein from a *waxy* glutinous mutant.

**Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE).** SDS–PAGE was performed using a Hoefer SE 260 minigel apparatus (Amersham Biosciences, Piscataway, NJ). Prior to electrophoretic analysis, protein samples were heated in a boiling water bath for 5 min. Electrophoresis was conducted at 20 mA for 1 h, and separated protein bands were visualized with Coomassie Blue R-250.

**Immunoblot Analysis.** Immunoblot analysis was performed following the procedure described by Krishnan et al.<sup>23</sup> Briefly, proteins fractionated by SDS–PAGE were electrophoretically transferred to nitrocellulose membranes (Protran, Schleicher & Schuell, Inc., Keene, NH). The nitrocellulose membrane was incubated with 5% milk in Tris-buffer saline (TBS, pH 7.3) for 1 h, followed by incubation with serum from patients allergic to maize or peanut at room temperature with gentle rocking. Non-specific binding was eliminated by washing the membrane 4 times with TBS containing 0.05% Tween-20 (TBST) for 10 min for each wash. Specifically bound

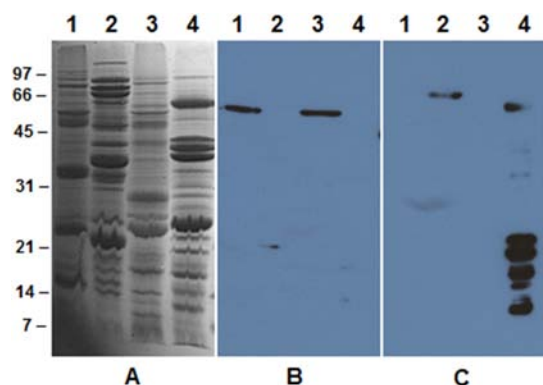
antibodies were detected by incubating the membrane with 1:5000 dilution of goat anti-human IgE–horseradish peroxidase conjugate antibody (Biosource, Camarillo, CA) for 2 h. Immunoreactive polypeptides were visualized by incubation of the membrane with an enhanced chemiluminescent substrate (Super Signal West Pico Kit, Pierce Biotechnology, Rockford, IL).

**Purification of the 56 kDa Rice Allergen.** Finely ground rice seed powder (100 mg) was extracted with 1 mL of 50 mM Tris-HCl at pH 6.8 and 1 mM ethylenediaminetetraacetic acid (EDTA) in a 30 °C shaker for 30 min. The slurry was centrifuged at 15800g for 10 min, and the supernatant that contains the albumins was discarded. The pellet was extracted with 1 mL of 50 mM Tris-HCl at pH 6.8, 1 mM EDTA, and 0.5 M NaCl as before to remove the globulins. Following the centrifugation step, the pellet was extracted with 50% isopropanol and 5%  $\beta$ -ME. The slurry was centrifuged as above, and the resulting pellet was extracted with 1× SDS sample treatment buffer. The slurry was boiled for 5 min and used for purification of the 56 kDa protein. Proteins were separated by preparative SDS–PAGE and briefly stained with Coomassie Blue, and the band corresponding to the 56 kDa protein was excised. The protein was recovered from the gel pieces as described earlier.<sup>24</sup> The gel-eluted 56 kDa protein was resolved on 13.5% SDS–PAGE, stained with Coomassie Blue, and subjected to matrix-assisted laser desorption ionization–time of flight (MALDI–TOF) mass spectrometry.

**MALDI–TOF Mass Spectrometry Analysis of 56 kDa Rice Protein.** SDS–PAGE-purified 56 kDa protein from rice endosperm, shown to react with serum from maize-allergic patients, was fractionated on a 10% gel and stained with Coomassie Blue G-250. The abundant 56 kDa protein was excised from the gel, cut into smaller sections, and destained completely. Following this, the gel pieces were reduced with dithiothreitol and alkylated with iodoacetamide for mass analysis. The protein was further treated with trypsin in an overnight digest. The solution recovered from the gel pieces was frozen with liquid nitrogen and then lyophilized dry. The dried digest was reconstituted with 5  $\mu$ L of 990:10 (v/v) water/88% formic acid and desalted on a C18 micro-Ziptip. The final mixture of peptides was eluted from the Ziptip in 3  $\mu$ L of 700:290:10 (v/v/v) acetonitrile/water/88% formic acid. A 1  $\mu$ L aliquot was combined with an equal volume of  $\alpha$ -cyano-4-hydroxycinnamic acid (ACHC) matrix, and a portion of the mixture was analyzed in the positive-ion mode on an Applied Biosystems Q-Star XL quadrupole-TOF tandem mass spectrometer. Spectra were acquired for the eight most intense ions in the MS spectrum of each digest sample. MALDI–TOF/TOF spectra were processed/formatted in the “combined MS and MS/MS” mode with Applied Biosystems GPS Explorer software. Results were submitted to Matrix Science’s Mascot program ([www.matrixscience.com](http://www.matrixscience.com)) for database searches.

## RESULTS AND DISCUSSION

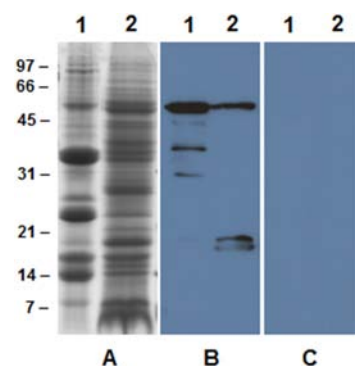
**IgE from Patients Allergic to Maize Cross-react against a 56 kDa Rice Protein.** Previously, we have identified the maize 27 kDa  $\gamma$ -zein as a potential allergen to humans and early weaned pigs.<sup>25,26</sup> During the course of these studies, we observed that IgE antibodies from two of seven patients with maize allergy cross-reacted against a 56 kDa protein from both maize and rice. To examine the occurrence of 56 kDa IgE-reactive protein in other crops, we conducted an immunoblot analysis using protein extracts from rice, soybean, maize, and peanut. Serum from a patient with maize allergy revealed strong IgE binding to a 56 kDa protein. This reactivity was detected only from protein extracts from rice and maize (Figure 1). This cross-reactivity suggests that the 56 kDa protein from maize and rice are homologous and can be considered as panallergen. It has been well-established that IgE antibodies raised against a given allergen can cross-react against similar proteins originating from related plant species.<sup>27</sup> Previous studies have also shown that serum from patients



**Figure 1.** IgE immunoblotting of rice, soybean, maize, and peanut seed proteins with sera from maize- and peanut-allergic patients. (A) Total seed proteins from rice (lane 1), soybean (lane 2), maize (lane 3), and peanut (lane 4) were fractionated on 13.5% SDS-PAGE and visualized with Coomassie Blue R-250. Proteins from two gels similar to the one shown in panel A were transferred to nitrocellulose membranes and incubated with (B) serum from a maize-allergic patient or (C) serum from a peanut-allergic patient. IgE-binding polypeptides were identified using anti-human IgE-horseradish peroxidase conjugate antibody followed by chemiluminescent detection. Protein molecular weight markers in kilodaltons are included on the left of the figure.

with rice-induced rhinitis and asthma contain IgE antibodies that react against rice proteins with molecular weights of 14–16, 33, 56, and 60 kDa.<sup>12</sup> On the basis of our immunoblot analysis, we speculate that the 56 kDa rice allergen found in our study may be the same IgE-binding protein that has been described in previous studies.<sup>4,12,16</sup> Our immunoblot analysis revealed that the serum from the maize-allergic patient revealed no detectable IgE binding to any proteins from soybean and peanut (Figure 1). When the nitrocellulose membrane containing the protein extracts from these crops was challenged with serum from a patient with peanut allergy, no IgE binding to the 56 kDa was seen (Figure 1). However, the serum from the peanut-sensitive patient revealed strong IgE reaction to several proteins from the peanut extract with apparent molecular weights of 68, 17, 16, 15, and 14 kDa. Faint IgE reaction against a 35 kDa protein was also seen. These IgE-reactive proteins presumably represent the well-characterized peanut allergens.<sup>28</sup> Thus far, eight peanut allergens, Ara h 1 (63–68 kDa), Ara h 2 (17 kDa), Ara h 3 (57 kDa), Ara h 4 (35.9 kDa), Ara h 5 (14 kDa), Ara h 6 (14.5), Ara h 7 (15.8 kDa), and Ara h 8 (19.9 kDa) have been officially recognized.<sup>28</sup> Most of these allergens are storage proteins belonging to vicillin, conglutin, and glycinin protein families, with the exception of Ara h 5 and Ara h 8, which belong to profilin and the pathogenesis-related protein family PR-10, respectively.<sup>28</sup> Serum from the peanut-allergic patient also exhibited IgE binding to a 72 kDa soybean protein (Figure 1). We have previously identified this soybean allergen as the  $\alpha$  subunit of  $\beta$ -conglycinin.<sup>23,29</sup>

**IgE-Binding 56 kDa Protein Is Enriched in Rice Endosperm.** To investigate the distribution of the 56 kDa rice allergen in rice caryopsis, we first obtained protein preparations from bran and the endosperm and resolved them by SDS-PAGE (Figure 2). Examination of the Coomassie-stained gel revealed several abundant proteins with apparent molecular weights of 34–39, 21–23, and 14 kDa. Previous studies have shown that the 34–39 and 21–23



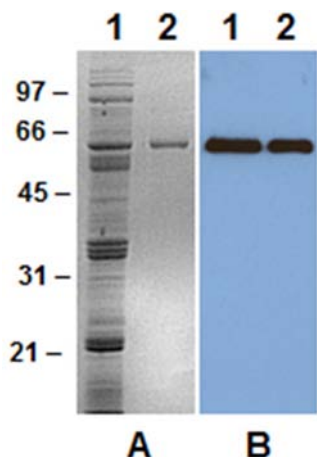
**Figure 2.** IgE reactivity with total proteins from rice endosperm and bran. Total seed proteins from rice endosperm (lane 1) and bran (lane 2) were fractionated on 13.5% SDS-PAGE and visualized with Coomassie Blue R-250. Proteins from two gels similar to the one shown in panel A were transferred to nitrocellulose membranes and incubated with (B) serum from a patient with documented maize allergy or (C) serum from a healthy individual with no known history of allergy. IgE-binding polypeptides were identified using anti-human IgE-horseradish peroxidase conjugate antibody followed by chemiluminescent detection. Protein molecular weight markers in kilodaltons are included on the left of the figure.

kDa polypeptides are the acidic and basic subunits of glutelins, respectively, the abundant seed storage proteins of rice.<sup>30,24</sup> The 14–16 kDa proteins are the prolamins and represent the second abundant class of rice seed storage proteins.<sup>31</sup> In addition to these proteins, 25 and 16 kDa globulins were found in the rice endosperm fraction (Figure 2). The rice bran fraction contained several proteins whose molecular weights ranged from 5 to 100 kDa. Proteins from rice bran and endosperm were transferred to a nitrocellulose membrane and challenged with sera from two patients with maize allergy. The immunoblot results showed that the sera from both patients contained IgE antibodies against the rice 56 kDa protein. Serum from patient I had lower IgE reactivity against the 56 kDa protein (data not shown), while the patient II serum in addition to a strong reaction against the 56 kDa protein also revealed weak IgE binding to 33 and 40 kDa proteins from the endosperm and 16 and 18 kDa proteins from the bran fraction (Figure 2). On the basis of the IgE-binding, it is evident that the 56 kDa rice allergen is present in both the endosperm and the bran fraction. However, it appears that the 56 kDa protein is present at a higher concentration in the endosperm than bran. Serum from a healthy individual with no known history of allergy revealed no IgE reactivity against rice proteins (Figure 2). It should be pointed out that IgE binding observed by immunoblot analysis using commercially obtained sera may not be clinically relevant. It is possible that one could detect strong IgE reactivity *in vitro* immunoblotting studies, yet the patient can be clinically tolerant. Several factors such as family history, geographical location, genetic factors, and cultural and dietary habits can influence the *in vitro* IgE-binding immunoassays.

**Purification of the Rice 56 kDa Allergen and Its Identification as Granule-Bound Starch Synthase by MALDI-TOF-MS/MS.** To facilitate the identification of the rice allergen, we first obtained a protein fraction that was enriched in the 56 kDa protein by sequentially removing the albumins and globulins. This protein fraction was subjected to preparative gel electrophoresis, and the prominent band corresponding to the 56 kDa protein was excised from the gel. A single band was observed when the gel-eluted protein



was resolved by SDS–PAGE (Figure 3). To confirm that the purified protein corresponds to the same protein that binds IgE



**Figure 3.** IgE reactivity to the purified 56 kDa rice protein. (A) Rice endosperm total protein (lane 1) and the purified 56 kDa protein (lane 2) were fractionated on 13.5% SDS–PAGE and visualized with Coomassie Blue R-250. (B) Proteins shown in panel A were transferred to nitrocellulose membrane and incubated with serum from a maize-allergic patient. IgE-binding polypeptides were identified using anti-human IgE–horseradish peroxidase conjugate antibody followed by chemiluminescent detection. Protein molecular weight markers in kilodaltons are included on the left of the figure.

antibodies from maize-allergic patients, we performed western blot analysis. The results of immunoblot analysis confirmed that the purified protein revealed strong IgE reactivity. To confirm the identity of the 56 kDa rice allergen, the purified protein was subjected to MALDI–TOF mass spectrometry. This analysis revealed a significant sequence homology to the granule-bound starch synthase (GBSSI) of *Oryza sativa*, *Oryza glaberrima*, *Oryza rufipogon*, and *Oryza barthii*. Eight peptides from the 52 kDa protein gave statistically significant protein scores for the matches with granule-bound starch synthase, with MOWSE scores above the 95% confidence level (Table 1). Amino acid sequence comparison between the rice and maize granule-bound starch synthase revealed 82% sequence identity between these two cereals. Thus, the IgE-binding maize protein (Figure 1B) is likely also a granule-bound starch synthase.

To examine if the IgE-reactive rice GBSSI shares any sequence homology to known allergens, we subjected the entire protein sequence of rice granule-bound starch synthase to

FASTA analysis against The Food Allergy Research and Resource Program (FARRP) Protein AllergenOnline Database ([www.allergenonline.org/index.shtml](http://www.allergenonline.org/index.shtml)). Bioinformatic analysis of full-length or overlapping 80 amino acid segment of rice granule-bound starch synthase revealed no homology to any of the known allergens deposited in the database.

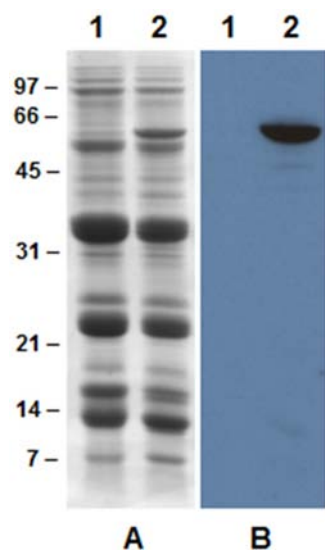
**Waxy Mutants of Glutinous Rice Do Not Accumulate IgE-Reactive 56 kDa Allergen.** Rice starch is composed of amylose and amylopectin, and the relative concentration of amylose plays an important role in determining the cooking, processing, and edible properties of starch.<sup>32</sup> Low amylose content rice is the most preferred rice in countries like Japan, Korea, and China. Granule-bound starch synthase, encoded by the *Waxy* (*Wx*) gene, is responsible for the synthesis of amylose.<sup>33–36</sup> The *waxy* mutants of glutinous rice are available, and these mutants do not accumulate the *Waxy* protein, GBSSI.<sup>37</sup> Because MALDI–TOF mass spectrometry analysis of the 56 kDa rice allergen revealed strong homology to granule-bound starch synthase, we wanted to verify this observation by immunoblot analysis of protein samples obtained from a glutinous rice *waxy* mutant. An examination of the total protein profile of a *waxy* mutant was very similar to that of a non-glutinous rice cultivar, with the exception of an abundant 56 kDa protein (Figure 4). The 56 kDa protein, which is abundant in non-glutinous rice cultivar, was not detected in the *waxy* mutant line (Figure 4). To verify if this protein is indeed the protein that reacted against the serum from rice-allergic patients, we performed immunoblot analysis. An IgE-specific reaction was seen against the 56 kDa protein from the non-glutinous rice cultivar, but no signal was detected from the protein extract from the *waxy* mutant (Figure 4). Our results confirm that the abundant rice protein eliciting a strong immune response is indeed the granule-bound starch synthase.

Genes encoding granule-bound starch synthase in plants are highly conserved and are grouped into two families, GBSSI and GBSSII.<sup>38</sup> The GBSSI gene is expressed mostly in storage tissues, while the GBSSII gene is expressed in non-storage tissues.<sup>39</sup> Genomic sequence analyses have shown that GBSSI and GBSSII genes in both eudicots and monocots are highly conserved and presumably evolved from a common ancestor.<sup>38</sup> The strong reactivity of the GBSSI gene with IgE antibodies from the serum from maize-allergic patients confirms that these two proteins share a close structural relationship. This reactivity is to be expected because a previous study has shown that granule-bound starch synthase from rice, barley, corn, wheat, and foxtail millet share structural and immunological

**Table 1.** Identification of Immunogenic Rice Protein as 56 kDa Starch Synthase by MALDI–TOF/TOF Mass Spectral Analysis

protein identified	accession number NCBIInr	MOWSE (50 ppm)	sequence coverage (%)	theoretical molecular weight (Da)	experimental molecular weight (Da)	peptides matched
granule-bound starch synthase ( <i>Oryza sativa</i> )	gil7798551	324	16	66928	56000	K.TGGLGDLVGLPPAM <sup>a</sup> AANGHR.V (98–118)
	gil83375792					R.VFIDHPSFLEK.V (163–173) R.ILNLNPNPYFK.G (211–221) R.FAFEDYPELNLSER.F (272–285) R.KIRLIAFIGR.L (399–408) K.IRLIAFIGR.L (400–408) K.FNAPLAHLIM <sup>a</sup> AGADVLAVPSRF (463–483) R.FEPCGLIQQGMRY (484–496)

<sup>a</sup>Oxidized methionine.



**Figure 4.** *Waxy* mutant of glutinous rice does not accumulate the 56 kDa allergen. (A) Total seed proteins from a *waxy* mutant (lane 1) and a non-glutinous rice line (lane 2) were fractionated on 13.5% SDS-PAGE and visualized with Coomassie Blue R-250. (B) Proteins shown in panel A were transferred to nitrocellulose membrane and incubated with serum from a maize-allergic patient. IgE-binding polypeptides were identified using anti-human IgE-horseradish peroxidase conjugate antibody followed by chemiluminescent detection. Note the absence of IgE reactivity against the 56 kDa protein from the *waxy* rice mutant extracts. Protein molecular weight markers in kilodaltons are included on the left of the figure.

homologies.<sup>40</sup> In our present study, we did not observe any IgE reactivity to proteins from soybean and peanut. Recent X-ray structure analysis of rice GBSSI has demonstrated a disulfide bond between Cys337 and Cys529.<sup>41</sup> Interestingly, Cys337 is conserved in the Poaceae family (rice, wheat, rye, barely, corn, and sorghum) but is replaced by valine in dicot family members (soybean, kidney bean, pea, cassava, *Arabidopsis*, and potato). Further studies are required to examine if the conserved disulfide bond between Cys337 and Cys529 plays a role in cross-reactivity between rice and maize GBSSI.

Several strategies have been employed to create hypoallergenic rice. Novel processing technologies have been developed and commercialized in Japan to produce hypoallergenic rice.<sup>42,43</sup> However, hypoallergenic rice produced by processing technology is not cost-effective and has an unintended effect on the quality of the rice product. Molecular biological approaches have resulted in development of transgenic rice with reduced levels of rice allergens.<sup>44,45</sup> Because of the reluctance in accepting genetically modified foods in some Asian countries, the best option is to find null mutants for rice allergens. Mutants for two of the rice allergens, 25 and 14–16 kDa proteins, have been identified and characterized.<sup>46,47</sup> However, even in these null mutants, the complete elimination of allergens is not possible because some of them are encoded by multigene families. In the case of the 56 kDa allergen, it is encouraging to note that GSSI is encoded by a single-copy gene<sup>48</sup> and there are currently several well-characterized null mutants available. Because the 25, 33, and 56 kDa rice allergens are encoded by single-copy genes in the rice genome, it should be desirable to combine the mutations into a single rice cultivar by plant breeding. Successful creation of a rice cultivar with

mutations in the 25, 33, and 56 kDa allergens should be a valuable source of hypoallergenic rice for rice-allergy patients.

## AUTHOR INFORMATION

### Corresponding Author

\*Phone: 573-882-8151. Fax: 573-884-7850. E-mail: hari.krishnan@ars.usda.gov.

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

The authors thank Jeong Sun-Hyung for excellent technical assistance, Joel Nott at the Protein Facility of the Iowa State University for MALDI-TOF-MS/MS analysis, and Joyce Davis (IBT Laboratories) for the generous gift of serum from maize- and peanut-allergic patients. Product names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may be suitable.

## REFERENCES

- (1) U.S. Food and Drug Administration (FDA). *Food Allergies: What You Need to Know. Food Facts*; FDA: Silver Spring, MD, 2013; <http://www.fda.gov/Food/ResourcesForYou/Consumers/ucm079311.htm>.
- (2) Kagan, R. S. Food allergy: An overview. *Environ. Health Perspect.* **2003**, *111*, 223–225.
- (3) World Allergy Organization (WAO). *WAO White Book on Allergy 2011–2012: Executive Summary*; WAO: Milwaukee, WI, 2011; [http://www.worldallergy.org/publications/wao\\_white\\_book.pdf](http://www.worldallergy.org/publications/wao_white_book.pdf).
- (4) Trcka, J.; Schad, S. G.; Scheurer, S.; Conti, A.; Vieths, S.; Gross, G.; Trautmann, A. Rice-induced anaphylaxis: IgE-mediated allergy against a 56-kDa glycoprotein. *Int. Arch. Allergy Immunol.* **2012**, *158*, 9–17.
- (5) Shibusaki, M.; Suzuki, S.; Nemoto, H.; Kuroume, T. Allergenicity and lymphocyte-stimulating property of rice protein. *J. Allergy Clin. Immunol.* **1979**, *64*, 259–265.
- (6) Arai, T.; Takaya, T.; Ito, Y.; Hayakawa, K.; Toshima, S.; Shibuya, C.; Nomura, M.; Yoshimi, N.; Shibayama, M.; Yasuda, Y. Bronchial asthma induced by rice. *Intern. Med.* **1998**, *37*, 98–101.
- (7) Nambu, M.; Shintaku, N.; Ohta, S. Rice allergy. *Pediatrics* **2006**, *117*, 2331–2332.
- (8) Besler, M.; Tanabe, S.; Urisu, A. Allergen data collection—Update: Rice (*Oryza sativa*). *Internet Symp. Food Allergens* **2001**, *3* (Suppl. 2), 1–17, <http://www.food-allergens.de/symposium-3-1/rice/rice-allergens.htm>.
- (9) Gonzalez-Mendiola, R.; Martin-Garcia, C.; Carnes, J.; Campos, J.; Fernandez-Caldas, E. Asthma induced by the inhalation of vapours during the process of boiling rice. *Allergy* **2003**, *58*, 1202–1203.
- (10) Fiocchi, A.; Bouygue, G. R.; Restani, P.; Gaiaschi, A.; Terracciano, L.; Martelli, A. Anaphylaxis to rice by inhalation. *J. Allergy Clin. Immunol.* **2003**, *111*, 193–195.
- (11) Lezaun, A.; Igea, J. M.; Quirce, S.; Cuevas, M.; Parra, F.; Alonso, M. D.; Martin, J. A.; Cano, M. S. Asthma and contact urticaria caused by rice in a housewife. *Allergy* **1994**, *49*, 92–95.
- (12) Kumar, R.; Srivastava, P.; Kumari, D.; Fakhr, H.; Sridhara, S.; Arora, N.; Gaur, S. N.; Singh, B. P. Rice (*Oryza sativa*) allergy in rhinitis and asthma patients: A clinic immunological study. *Immunobiology* **2007**, *212*, 141–147.
- (13) Monzon, S.; Lombardero, M.; Perez-Camo, I.; Saenz, D.; Lasanta, J. Allergic rhinoconjunctivitis after ingestion of boiled rice. *J. Invest. Allergol. Clin. Immunol.* **2008**, *18*, 487–488.
- (14) International Union of the Immunological Societies (IUIS). *IUIS Allergen Nomenclature Sub-Committee: Allergen Nomenclature*; IUIS: Vienna, Austria; <http://www.allergen.org/>.

- (15) Russell, S. D.; Bhalla, P. L.; Singh, M. B. Transcriptome-based examination of putative pollen allergens of rice (*Oryza sativa* ssp. *japonica*). *Mol. Plant* **2008**, *1*, 751–759.
- (16) Nakase, M.; Adachi, T.; Urisu, A.; Miyashita, T.; Alvarez, A. M.; Nagasaka, S.; Aoki, N.; Nakamura, R.; Matsuda, T. Rice (*Oryza sativa* L.)  $\alpha$ -amylase inhibitors of 14–16 kDa are potential allergens and products of a multigene family. *J. Agric. Food Chem.* **1996**, *44*, 2624–2628.
- (17) Limas, G. G.; Salinas, M.; Moneo, I.; Fischer, S.; Wittmann-Liebold, B.; Méndez, E. Purification and characterization of ten new rice NaCl-soluble proteins: Identification of four protein-synthesis inhibitors and two immunoglobulin-binding proteins. *Planta* **1990**, *181*, 1–9.
- (18) Usui, Y.; Nakase, M.; Hotta, H.; Urisu, A.; Aoki, N.; Kitajima, K.; Matsuda, T. A 33-kDa allergen from rice (*Oryza sativa* L. *japonica*). cDNA cloning, expression, and identification as a novel glyoxalase I. *J. Biol. Chem.* **2001**, *276*, 11376–11381.
- (19) Asero, R.; Amato, S.; Alfieri, B.; Folloni, S.; Mistrello, G. Rice: Another potential cause of food allergy in patients sensitized to lipid transfer protein. *Int. Arch. Allergy Immunol.* **2007**, *143*, 69–74.
- (20) Urisu, A.; Yamada, K.; Masuda, S.; Komada, H.; Wada, E.; Kondo, Y.; Horiba, F.; Tsuruta, M.; Yasaki, T.; Yamada, M.; Torii, S.; Nakamura, R.; et al. 16-kilodalton rice protein is one of the major allergens in rice grain extract and responsible for cross-allergenicity between cereal grains in the Poaceae family. *Int. Arch. Allergy Appl. Immunol.* **1991**, *96*, 244–252.
- (21) Jeon, Y. H.; Oh, S. J.; Yang, H. J.; Lee, S. Y.; Pyun, B. Y. Identification of major rice allergen and their clinical significance in children. *Korean J. Pediatr.* **2011**, *54*, 414–421.
- (22) Chen, M.-H.; Bergman, C. J. The influence of kernel maturity, milling degree and milling quality on rice bran phytochemical concentrations. *Cereal Chem.* **2005**, *82*, 4–8.
- (23) Krishnan, H. B.; Kim, W. S.; Jang, S.; Kerley, M. S. All three subunits of  $\beta$ -conglycinin are potential food allergens. *J. Agric. Food Chem.* **2009**, *57*, 938–943.
- (24) Krishnan, H. B.; Okita, T. W. Structural relationship among the rice glutelin polypeptides. *Plant Physiol.* **1986**, *81*, 748–753.
- (25) Krishnan, H. B.; Kerley, M. S.; Allee, G. L.; Jang, S.; Kim, W.-S.; Fu, C. J. Maize 27 kDa  $\gamma$ -zein is a potential allergen for early weaned pigs. *J. Agric. Food Chem.* **2010**, *58*, 7323–7328.
- (26) Krishnan, H. B.; Jang, S.; Kim, W.-S.; Kerley, M.; Oliver, M.; Trick, H. Biofortification of soybean meal: Immunological properties of the 27 kDa  $\gamma$ -zein. *J. Agric. Food Chem.* **2011**, *59*, 1223–1228.
- (27) Hauser, M.; Roulias, A.; Ferreira, F.; Egger, M. Panallergens and their impact on the allergic patient. *Allergy Asthma Clin. Immunol.* **2010**, *6*, 1–14.
- (28) de Leon, M. P.; Rolland, J. M.; O'Hehir, R. E. The peanut allergy epidemic: Allergen molecular characterisation and prospects for specific therapy. *Expert Rev. Mol. Med.* **2007**, *9*, 1–18.
- (29) Fu, C. J.; Jez, J. M.; Kerley, M. S.; Allee, G. L.; Krishnan, H. B. Identification, characterization, epitope mapping, and three dimensional modeling of the  $\alpha$ -subunit of  $\beta$ -conglycinin of soybean, a potential allergen for young pigs. *J. Agric. Food Chem.* **2007**, *55*, 4014–4020.
- (30) Yamagata, H.; Sugimoto, T.; Tanaka, K.; Kasai, Z. Biosynthesis of storage proteins in developing rice seeds. *Plant Physiol.* **1982**, *70*, 1094–1100.
- (31) Krishnan, H. B.; White, J. A. Morphometric analysis of rice seed protein bodies: Implication for a significant contribution of prolamin to the total protein content of rice endosperm. *Plant Physiol.* **1995**, *109*, 1491–1495.
- (32) Tian, Z.; Qian, Q.; Liu, Q.; Yan, M.; Liu, X.; Yan, C.; Liu, G.; Gao, Z.; Tang, S.; Zeng, D.; et al. Allelic diversities in rice starch biosynthesis lead to a diverse array of rice eating and cooking qualities. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 21760–21765.
- (33) Isshiki, M.; Morino, K.; Nakajima, M.; Okagaki, R. J.; Wessler, S. R.; Izawa, T.; Shimamoto, K. A naturally occurring functional allele of the rice waxy locus has a GT to TT mutation at the 5' splice site of the first intron. *Plant J.* **1998**, *15*, 133–138.
- (34) Hirano, H. Y.; Eiguchi, M.; Sano, Y. A single base change altered the regulation of the *Waxy* gene at the posttranscriptional level during the domestication of rice. *Mol. Biol. Evol.* **1998**, *15*, 978–987.
- (35) Hirano, H. Y.; Sano, Y. Molecular characterization of the *Waxy* locus of rice (*Oryza sativa*). *Plant Cell Physiol.* **1991**, *32*, 989–997.
- (36) Wang, Z. Y.; Zheng, F. Q.; Shen, G. Z.; Gao, J. P.; Snustad, D. P.; Li, M. G.; Zhang, J. L.; Hong, M. M. The amylose content in rice endosperm is related to the posttranscriptional regulation of the *Waxy* gene. *Plant J.* **1995**, *7*, 613–622.
- (37) Mikami, I.; Aikawa, M.; Hirano, H.-Y.; Sano, Y. Altered tissue-specific expression at the *Wx* gene of the opaque mutants in rice. *Euphytica* **1999**, *105*, 91–97.
- (38) Cheng, J.; Khan, M. A.; Qiu, W.-M.; Li, J.; Zhou, H.; et al. Diversification of genes encoding granule-bound starch synthase in monocots and dicots is marked by multiple genome-wide duplication events. *PLoS One* **2012**, *7* (1), No. e30088.
- (39) Dian, W.; Jiang, H.; Chen, Q.; Liu, F.; Wu, P. Cloning and characterization of the granule-bound starch synthase II gene in rice: Gene expression is regulated by the nitrogen level, sugar and circadian rhythm. *Planta* **2003**, *218*, 261–268.
- (40) Takumi, K.; Udaka, J.; Kimoto, M.; Koga, T.; Tsuji, H. Structural and immunochemical homologies between foxtail millet glutelin 60 kDa and starch granule-bound starch synthase proteins from rice, barley, corn and wheat grains. *J. Nutr. Sci. Vitaminol.* **2000**, *46*, 109–112.
- (41) Momma, M.; Fujimoto, Z. Interdomain disulfide bridge in the rice granule bound starch synthase I catalytic domain as elucidated by X-ray structure analysis. *Biosci., Biotechnol., Biochem.* **2012**, *76*, 1591–1595.
- (42) Watanabe, M.; Miyakawa, J.; Ikezawa, Z.; Suzuki, Y.; Hirano, T.; Yoshizawa, T.; et al. Production of hypoallergenic rice by enzymatic decomposition of constituent proteins. *J. Food Sci.* **1990**, *55*, 781–783.
- (43) Kato, T.; Katayama, E.; Matsubara, S.; Yuko Omi, Y.; Matsuda, T. Release of allergenic proteins from rice grains induced by high hydrostatic pressure. *J. Agric. Food Chem.* **2000**, *48*, 3124–3129.
- (44) Nakamura, R.; Matsuda, T. Rice allergenic protein and molecular-genetic approach for hypoallergenic rice. *Biosci., Biotechnol., Biochem.* **1996**, *60*, 1215–1221.
- (45) Wakasa, Y.; Hirano, K.; Urisu, A.; Matsuda, T.; Takaiwa, F. Generation of transgenic rice lines with reduced contents of multiple potential allergens using a null mutant in combination with an RNA silencing method. *Plant Cell Physiol.* **2011**, *52*, 2190–2199.
- (46) Iida, S.; Miyahara, K.; Nishio, T. Rice mutant lines lacking  $\alpha$ -globulin. *Breed. Sci.* **1998**, *48*, 45–49.
- (47) Nishio, T.; Iida, S. Mutants having a low content of 16-kDa allergenic protein in rice (*Oryza sativa* L.). *Theor. Appl. Genet.* **1993**, *86*, 317–321.
- (48) Mason-Gamer, R.; Weil, C. F.; Kellogg, E. Granule-bound starch synthase: Structure, function, and phylogenetic utility. *Mol. Biol. Evol.* **1998**, *15*, 1658–1673.