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Evaluation of allergenic potential for rice seed protein components utilizing a rice proteome database and an allergen database in combination with IgE-binding of recombinant proteins

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Among 131 rice endosperm proteins previously identified by MS-based proteomics, most of the proteins showed low or almost no sequence similarity to known allergens in databases, whereas nine proteins did it significantly. The sequence of two proteins showed high overall identity with Hsp70-like hazel tree pollen allergen (Cor a 10) and barley α -amylase (Hor v 16), respectively, whereas the others showed low identity (28–58%) with lemon germin-like protein (Cit l 1), corn zein (Zea m 50 K), wheat chitinase-like xylanase inhibitor (Tri a XI), and kinase-like pollen allergen of Russian thistle (Sal k 1). Immuno-dot blot analysis showed that recombinant proteins for these rice seed homologs were positive in the IgE-binding, but not necessarily similarity dependent, from some allergic patients. These results suggest that utilization of proteome and sequence databases in combination with IgE-binding analysis was effective to screen and evaluate allergenic potential of rice seed protein components.

Key words: rice seed; food allergy; rice proteome; allergen database

Allergenic potential of a given protein antigen is the ability to give allergic sensitization to naïve individuals and also to elicit allergic symptoms in individuals who have already been sensitized with the given antigen or some other cross-reactive antigens. Although the biochemical and physicochemical properties of a given protein itself is considered to determine the allergenic potential of the protein,^{1,2)} underlining basis of the determination is still not fully understood. Evaluating allergenic potential of protein antigens is also an important practical subject for clinical investigation,

diagnosis, and therapy on a variety of allergic diseases. Moreover, as for genetically modified agricultural products, the allergen risk assessment of novel food proteins has attracted attention socially.³⁾

Cereals including rice are important plant-derived food and feed as major source of calories and, therefore, have been major targets of plant breeding and gene manipulation for development of novel crop cultivars with improved traits.⁴⁾ At the same time, on the other hand, cereals are known as major causative food of some allergic disorders,⁵⁾ which are especially serious for people who feed chiefly on cereals. Therefore, assessment of allergenic potential for cereal seed proteins has attracted attention of researchers and stakeholders related to development of novel crop cultivars. Interest of allergenic potential assessment is taken in not only the direct products of introduced exogenous genes but also a variety of endogenous proteins which may increase in relative content as a consequence of breeding or gene manipulation.⁶⁾

Sequence and structural similarities of a given protein to known allergens, which have already been characterized biochemically and immunologically, are useful to assess allergenic potential of the protein. Overall amino acid sequence similarity has often been adopted as an index of allergenic potential, according to the WHO/FAO scientific panel recommendation, that is, whether identity matches are above 35% over any 80 amino acid residue segment of the query protein compared to any known allergens.⁷⁾ Another sequence similarity is also taken into account, especially on immuno-cross reactivity, that short segments consisting of 6–8 amino acid residues of the protein exactly matches any known allergen sequences, simply because eight amino acid residues are approximate size/length for epitopes recognized by IgE antibody.⁸⁾ Identification

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Abbreviations: TRX, thioredoxin; LTP, lipid transfer protein; PBS, phosphate-buffered saline; HRP, horseradish peroxidase.

Table 1. Primer pairs for specific amplification of ORF for cDNAs encoding rice putative proteins. Recognition sites for restriction endonucleases are underlined.

Names	Sequence of sense primer	Sequence of anti-sense primer
PA1	5'- <u>GATATC</u> ATGTCCAAGGCCAGG-3'	5'- <u>GATATC</u> ATGTCCAAGGCCAGG-3'
PA2	5'- <u>GAATTC</u> ATGGATCGGGTTCGC-3'	5'- <u>AAGCTT</u> CTACAGCTCGTCATGCTC-3'
PA3	5'- <u>GATATC</u> ATGGCTTCTTCTCCTTT-3'	5'- <u>GATATC</u> ATGGCTTCTTCTCCTTT-3'
PA4	5'- <u>GATATC</u> ATGCCCCCGACG-3'	5'- <u>CTCGAG</u> CTATAGGTGCCGCC-3'
PA5	5'- <u>GATATC</u> ATGTCCAAGGCCAGG-3'	5'- <u>GATATC</u> ATGTCCAAGGCCAGG-3'
PA6	5'- <u>GAATTC</u> ATGGATCGGGTTCGC-3'	5'- <u>AAGCTT</u> CTACAGCTCGTCATGCTC-3'
PA7	5'- <u>GATATC</u> ATGAAGATCATTTTCTTCT-3'	5'- <u>AAGCTT</u> CTACGCCACTTCCAGGTC-3'
PA8	5'- <u>GATATC</u> ATGAAGATCATTTTCTGCTT-3'	5'- <u>CTCGAG</u> TTACAAGACACCGCCAAG-3'
PA9	5'- <u>GATATC</u> ATGAAGCTCGTCAGCATCTACCT-3'	5'- <u>AAGCTT</u> TATCTTCTTCCAAGAGGCTAGAG-3'

and sequencing of a variety of food and respiratory allergens have greatly been increasing, and a large amount of biochemical and immunological information is stored in several public databases (www.ift.ac.uk/protail/database.html, www.allergen.org/, www.allergenonline.org/, allergen.nih.gov/ADFS/index.jsp?pagen=top, <http://www.allergome.org/>). In the Allergome database, for example, more than 2,500 allergens excluding isoforms have been registered and more than 7,500 sequences are available at this moment.

A clinical study on rice allergy, asthma, and eczema induced by rice flour exposure or cooked rice ingestion was reported first in Japan.⁹⁾ Later, in some broad-scale clinical studies with patients allergic to food or cereals, a substantial proportion of the patients was reported to be positive for skin reaction or serum IgE-binding to rice seed protein extracts.^{10,11)} Moreover, a recent clinical study showed that even ingestion of cooked rice could induce immediate-type hypersensitive reactions, including anaphylactic reaction.^{12,13)} Potentially allergenic proteins in rice seeds have been identified and characterized based on the reactivity with patients' serum IgE¹⁴⁾. The 14–16-kDa and 33-kDa allergenic proteins were identified to be α -amylase inhibitors¹⁵⁾ and glyoxalase-1,¹⁶⁾ respectively. These proteins as well as 26-kDa globulin were suggested to be major allergenic components of rice seeds based on the result that IgE-binding to rice seed proteins was markedly reduced by suppressing the expression of these three proteins in transgenic rice plants.¹⁷⁾ On the other hand, allergenic potential of minor components of rice seed proteins remains to be investigated. Putative extensin and polygalacturonase proteins have recently been identified as potential allergens in rice pollen proteins and detected in a trace amount also in rice seed proteins.¹⁸⁾

In this study, we aimed at searching components with allergenic potential by sequence similarity-based screening of rice seed whole proteins, which had previously been identified using MS-based proteomic analysis. Allergenic potential of several rice seed protein components with sequence similarity to known allergens was further estimated by analyzing the IgE-binding to their recombinant proteins.

Materials and methods

Databases and structural analysis. Information on proteins expressed in rice seeds was obtained from the Rice Proteome Database (National Institute of Agrobiological Sciences, <http://cdna01.dna.affrc.go.jp/>

<http://cdna01.dna.affrc.go.jp/cDNA/>), while cDNA sequences were from the rice full-length cDNA database, KOME (Knowledge-based Oryza Molecular biological Encyclopedia, <http://cdna01.dna.affrc.go.jp/cDNA/>). Similarity blast search was done with an allergen database, Allergome (<http://www.allergome.org/>). Known allergens with sequence similarity to rice seed proteins were searched using NCBI blastp (2.2.18) algorithm. Sequence alignment was done using Cobalt, Constraint Based Protein Multiple Alignment Tool (NCBI). Molecular structure diagrams were drawn using the program PyMOL (W.L. DeLano The PyMOL Molecular Graphics System, DeLano Scientific, Palo Alto, CA, 2002).

Preparation of recombinant proteins. Rice full-length cDNA clones were provided by Rice Genome Resource Center (RGRC, AFFRC, Tsukuba, Japan). Using these cDNAs as a template and the primers with sequences corresponding to 5'- and 3'-ends listed in Table 1, the open reading frame cDNA excluding the signal sequence was amplified and introduced into pET32a expression vector (Novagen, Madison, WI) as described previously.¹⁸⁾ His-tagged thioredoxin (TRX) fusion proteins were expressed in *E. coli* BL21 (DE3) and affinity purified in accordance with manufacturer's instruction. His-tagged bacterial TRX alone and His-tagged TRX-rice lipid transfer protein (LTP, AK071260) were also prepared as a negative control and a known seed allergen, respectively.

SDS-PAGE. Proteins were separated by SDS-PAGE (12.5% acrylamide) according to Laemmli's method,¹⁹⁾ followed by Coomassie Brilliant Blue R-250 (CBB) staining.

Patients' sera. Human blood samples were obtained from patients with suspected food allergy under medical treatment for atopic dermatitis and/or bronchial asthma at the Fujita Health University Hospital, Department of Pediatrics. The study using the patients' serum specimens was approved by the ethics committee of Fujita Health University School of Medicine. The informed consents were obtained from each patient or their guardians. Representative sera from 26 patients with higher IgE-ELISA values for rice seed salt-soluble proteins were selected among serum specimens from 60 patients in descending order as test samples (IgE-positive sera), while 2 sera with the lower

Table 2. Some biochemical and structural properties of nine rice endosperm proteins with sequence similarity to known allergens and corresponding cDNA clones in the rice full-length cDNA database.

Rice proteins identified by MS analysis					Rice full-length cDNAs in KOME				Allergens with sequence similarity			
Spot	Expression level (spot intensity)	IEF pI	SDS-PAGE MM (kD)	Protein ID from MS	Protein number in this study	Deduced		Overall identity with known allergen	Allergen designation (Protein name)	Protein ID	organism (common name)	Tissue & root of exposure
						Accession for mRNA	MM (kD)					
2	2752	5.16	84.95	AB036788	1	AK070271	39	casein kinase II subunit alpha-2	Sal k 2.0101 (Major antigen-like protein)	Q8L5K9	Salsola kali (Russian thistle)	Pollen inhalation
17	9696	4.73	66.77	AF006825	2	AK119653	73	luminal binding protein 3 precursor	Cor a 10.0101 (Putative luminal binding protein)	Q9FSY7	<i>Corylus avellana</i> (Hazel)	Pollen inhalation
								63%	Cla h 4 (Heat shock 70 kDa protein)	P40918	<i>Cladosporium herbarum</i>	Whole body inhalation
								64%	Pen c 19.0101 (Heat shock 70 kDa protein)	Q92260	<i>Penicillium citrinum</i> (Penicillium)	whole body inhalation
25	30225	8.51	58.39	AF042489	3	AK062862	23	germin-like protein subfamily 1 member 11 precursor	Cit i 1.1 (Germin-like protein)	I6UVE3	<i>Citrus limon</i> (Lemon)	Fruit ingestion
								31%	Ara h 3 (11S Globulin)	Q647H2	<i>Arachis hypogaea</i> (Peanut)	Seed ingestion
								26%	Cor a 9.0101 (11S Globulin)	Q8W1C2	<i>Corylus avellana</i> (Hazelnut)	Seed ingestion
33	50882	8.59	56.35	M24287	4	AK073487	52	alpha-amylase isozyme 3D precursor	Hor v 16 (Alpha-amylase)	O04964	<i>Hordeum vulgare</i> (Barley)	Seed ingestion
								29%	Asp o 21 (Alpha-amylase)	B0FZ76	<i>Aspergillus oryzae</i> (Fungi)	whole body Ingestion, inhalation
34	12309	4.54	55.43	M23745	5	AK242325	17	prolamin PPROL 17 precursor	Zea m 50kD Zein (50kD gamma zein)	Q946W1	<i>Zea mays</i> (corn)	Seed ingestion
								46%	Tri a 36 (LMW-GS)	H6VLQ1	<i>Triticum aestivum</i> (Wheat)	Seed ingestion
								45%	Ses i 1 (2S albumin)	Q9AUD1	<i>Sesamum indicum</i> (Oriental Sesame)	Seed ingestion
								25%	Ber e 1 (LMW-GS)	P0C8Y8	<i>Bertholletia excelsa</i> (Brazil Nut)	Seed ingestion
43	13042	5.69	53.79	AB027426	6	AK058882	31	xylanase inhibitor protein 2 precursor	Tri a XI (Xylanase inhibitor XIP-III)	Q4W6G2	<i>Triticum aestivum</i> (Wheat)	Seed ingestion
								35%	Ziz m 1.0101 (Chitinase)	Q2VST0	<i>Ziziphus mauritiana</i> (Chinese-dat)	Fruit ingestion
								36%	Hev b 14 (Hevamine-A)	P23472	<i>Hevea brasiliensis</i> (Para rubber)	latex contact skin, inhalation
45	9144	6.05	52.91	X60374	7	AK101344	33	cell division control protein 2 homolog 2	Sal k 2.0101 (Major antigen-like protein)	Q8L5K9	<i>Salsola kali</i> (Russian thistle)	Pollen inhalation

63	14225	5.69	39.59	AF042201	8	AK242260	16	Prolamin precursor	45%	Zea m 50kD Zein (50kD gamma zein) Tri a 36 (LMW-GS)	Q946W1	Zea mays (corn)	Seed ingestion
107	30897	8.76	22.78	AF230515	9	AK068307	74	lectin-like receptor kinase 7	33%	Sal k 2.0101 (Major antigen-like protein)	H6VLQ1	Triticum aestivum (Wheat)	Seed ingestion
									33%		Q8L5K9	Salsola kali (Russian thistle)	Pollen inhalation

IgE-ELISA values were selected in ascending order and used as control samples (IgE-negative sera).

The total-IgE concentration of the serum specimens was determined by two-site ELISA using anti-human IgE monoclonal antibody (MCA IgE E-01, Yamasa Corporation, Tokyo) as a capture antibody, HRP-labeled anti-human IgE (Biosource International, Inc., Camarillo, CA) as a detection antibody, and human serum specimens with known total-IgE concentration determined using a test kit (ImmunoCAP, Thermo Scientific Inc., Uppsala, Sweden). ELISA plates (Nunc-Ummuno™, Roskilde, Denmark) were coated with the capture antibody (1 µg/mL in PBS), blocked and incubated with 100-fold diluted human serum specimens. After the incubation with the HRP-labeled detection antibody, HRP activity was measured as described previously.¹⁸⁾

Immuno-dot blotting assay for IgE-binding of recombinant proteins. Binding of human serum IgE to the recombinant proteins was analyzed using immune-dot blotting as described.¹⁸⁾ Briefly, sample proteins dissolved in 2 M urea/PBS was spotted on nitrocellulose membrane (1 µg/spot), incubated with human serum diluted by 100-fold, followed by HRP-labeled anti-human IgE (Biosource International, Inc.). HRP activity was detected using a chemiluminescent HRP substrate (Millipore, Billerica, MA). Using NIH Image-J program, the chemiluminescence dot images were standardized by the 'rolling ball' algorithm-based subtraction of background chemiluminescence, and the relative chemiluminescence intensity of each dot was quantified.

IgE-binding assay for heat-denatured recombinant proteins. Recombinant rice proteins dissolved in 2 M urea/PBS was further diluted with PBS to 5 µg/mL and heat denatured by boiling for 5 min and cooled immediately on ice. To evaluate possible contribution of disulfide bridges to protein heat stability, the recombinant proteins were diluted with 1% mercaptoethanol in 2 M urea/PBS and heat-treated similarly. IgE-binding to the native and heat-denatured proteins was assayed using ELISA as described previously.¹⁸⁾

Results

Search of rice seed proteins with sequence similarity to known allergens

In the database of rice endosperm proteome, 131 proteins with molecular mass of 14–86 kDa and pI of 4.4–8.8 identified by MS analysis as each spot in 2D (IEF/SDS)-PAGE have been registered with their ID in sequence databases. Using these registered sequences, the blastp similarity search was done for each deduced amino acid sequence of the rice seed proteins against the Allergome database of known allergens. Most of the proteins' sequence did not show significant similarity to that of any known allergens, but nine out of the 131 rice proteins were found to be homologous to one or more known allergens registered in the allergen database (Table 2). Then, full-length cDNA clones

A. Protein kinases homologous to an weed pollen allergen

1 AK070271 casein kinase II subunit alpha-2 (Protein No. 1)
 2 AK101344 cell division control protein 2 homolog 2 (Protein No. 7)
 3 AK068307 lectin-like receptor kinase 7 (Protein No. 9)
 4 Q8L5K9 Sal k 2 (Major antigen-like protein)

1 38 RKVGRGKYSEVFEGINVNNEKCIKILKPVKKKK-----IKREIKILQNLGGPNIVKLLDIVRDQHSKTPSLIFEYIN 112
 2 8 EKIGEGTYGVVYKGRHRTNETIALKKIRLBQDEGVPTAIRIEISLLEKEM-QHRNIVRLQDVVHKE--KCTYLDFEYLD 84
 3 367 CLLGVGGFGRVYKGVLPNSNVEIAVKRVSHDSQGG--VKEFVAEVVSLGRL-QHCNLRLLGYCRRKG--ELMLVVEYIS 441
 4 77 LKIGEGGYGPVYRAMLQHTP--VAIKVLRPNVSQGG--LKQFQOEIDVLGRM-RHPNMVLLVGACPEY----GCLVVEYIE 147

1 113 N---PTLTDPKVL--YTDYDI-RYYIYELLKALDYCHS---QGIMHRDVRPHNMIDHDLRLRLIDWGLAE-F -YH 178
 2 85 L---DLKHHMSSPDPKNNRIVKSFYQILRGIAVCHS---HRVILHRDLKQNLIDRRNTNSLKADFGARAF -GI 154
 3 442 NGSIDKYLHGQDNKPTLSWAQRFIKIDIASGLLYLHEECDKVIFHRDIKASNVLLDNEMNA-RLGDFGLARLY DHG 517
 4 148 NGSLEDRLFRKNSPPIPWKLRFKIAAEITATALLPLRDAKPEPMVHRDLKASNVLLDNGYIS-KIADVGLARLV[5]NET 228

B. Germin-like protein homologous to a lemon allergen (Cit I 1)

1 AK062862 germin-like protein subfamily 1 member 11 precursor (Protein No. 3)
 2 I6UVE3 Cit I 1 (Germin-like protein)

1 79 NVTLVNVLQGLNLTGISTARLDFAPLGLNPPHTHPRATEIFVLEGTLYVGVFTS--NPDNRLLSKVLNKGDFVVFPEGLIHFOFNPNHK 169
 2 78 NETDANVEQIGLNTLTGISTPRIDYAPYQGRPHIHPRASEIFVLEGTLYVGVFTSDQLNNTLAKVLNKGDFVVFPEGLIHFOFNGKTN 169

C. Xylanase inhibitor homologous to an wheat allergen (Tri a XI)

1 AK058882 xylanase inhibitor protein 2 precursor (Protein No. 6)
 2 Q4W6G2 Tri a XI (Xylanase inhibitor XIP-III)

1 1 MK MKALLPVAAMLLLVSGQLAAPVTAD--GYVG[7]RHKEEGSLREACHTGRYNIIVVITFYNVFGYQGRY GL 75
 2 1 MA[10]LLALLSVVTALSLAAPGLAAGTGGVTVFWG RNKEEGSLREACHSGMYTMVTMSFLDVFGAK-GKY HL 79

1 76 DFGSHFVAIVGADIKHCQSKGVVLLSIGGGGGEYSLPSQSASDVADNLWNAYLGGRRGVPRPFQGVVDGIDFFIDQ 155
 2 80 DLSGHDLAIVGADIKHCQSKGVVLSLVGGYGTGYSLPSNRSALDLFDHLWNSYLGGSNIGVPRPFQGVLDGIDLFLFH 159

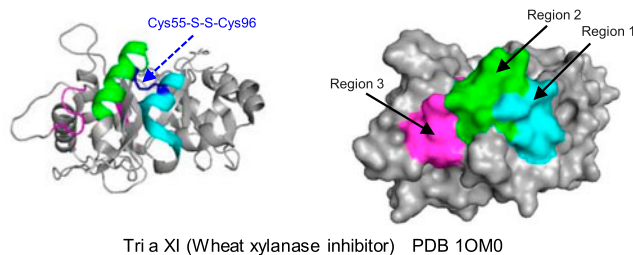


Fig. 1. Amino acid sequence alignment of putative rice proteins with sequence similarity to known allergens. (A) The sequences of three rice putative protein kinases (the rice proteins Nos. 1, 7, and 9 in Table 2) were aligned with that of a pollen allergen from Russian thistle (Sal k 2). Regions are boxed at which six contiguous residues match between the rice protein and Sal k 2. (B) The sequence of rice putative germin-like protein (the rice protein No. 3 in Table 2) was aligned with that of a fruit allergen (Cit I 1, germin-like protein) from lemon peel (flavado). Regions are boxed at which eight contiguous residues or more match between the two. (C) The sequence of rice putative xylanase inhibitor (the rice protein No. 6 in Table 2) was aligned with that of a food allergen (Tri a XI, xylanase inhibitor) from wheat seed. Regions are boxed at which nine contiguous residues or more match between the two. The crystal structure of wheat xylanase inhibitor (PDB: 1OM0) is shown as ribbon and surface models (Bottom). The matched contiguous residues in the three regions as well as the disulfide bridge between Cys55 and Cys96 are colored, respectively.

corresponding to the nine proteins were retrieved from the rice full-length cDNA database, KOME. The molecular masses of several of the nine proteins shown in the 2D-PAGE were larger than those estimated from the deduced amino acid sequence, probably due to incomplete dissociation of the proteins during the treatment with SDS-containing buffer before the secondary dimension SDS-PAGE (<http://cdna01.dna.affrc.go.jp/RPD/main.html>).

As summarized in Table 2, overall sequence identity of the rice proteins, except one (AK119653) described below, with known allergens was 70% or less, and four of them gave the identity of 50% or less. Three proteins (AK070271, AK101344, and AK068307), which had been identified as putative protein kinases, resembled a single allergen from pollen of Russian thistle (Sal k 2). Both of the two rice prolamin proteins (AK242325 and AK 242260) resembled prolamin allergens of corn (*Zea m* 50 kD Zein) and wheat (Tri a 36, LMW-glutenin). In particular, the putative rice luminal binding protein (Bip)/Hsp 70 (AK119653) showed high

similarity (~90% identity) to a hazel homolog known as a hazel pollen allergen (Cor a 10), and moderate similarity also to fungal aero allergens, Cla h 4 and Pen c 9, which are heat shock 70 kDa proteins of plant-pathogenic fungi, *Mycosphaerella tulasnei*. The other three (AK062862, AK073487, and AK058882) were similar to a lemon peel (flavado) germin-like allergen (Cit I 1), barley α -amylase (Hor v 16), and wheat xylanase inhibitor (Tri a XI), respectively. In summary, the seven known allergens with sequence similarity to the rice proteins included five food allergens of cereal seeds, two inhalant allergens of pollen from native grass and orchard, and one food/pollen allergen of citrus fruit.

Sequence comparison of rice proteins to known allergens

Sequences of three rice putative protein kinases, AK070271, AK10334, and AK068307 (protein Nos. 1, 7, and 9), were aligned with the corresponding

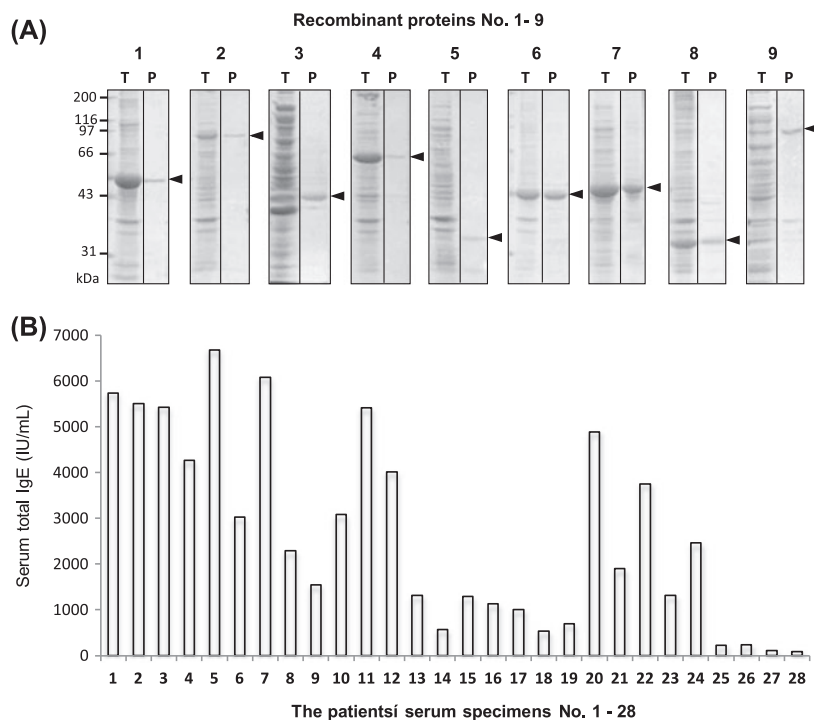


Fig. 2. Expression and affinity purification of recombinant rice proteins and total-IgE concentration of patients' serum specimens. (A) The putative rice seed proteins encoded by the nine full-length cDNA clones (GeneBank ID: AK070271, AK119653, AK062862, AK073487, AK242325, AK058882, AK101344, AK242260, and AK068307) were expressed as His-tagged TRX-fusion proteins by an *E. coli* expression system and purified using Ni-affinity chromatography. Total proteins of transformed *E. coli* lysate (T) and affinity-purified proteins (P) were analyzed by SDS-PAGE (12.5% acryl amide gel stained with CBB R-250). The protein number (1–9) in this study is shown above each strip of the PAGE gel. The bands of His-tagged TRX-fusion proteins are indicated with black arrowhead. (B) Total-IgE concentration of the patients' serum specimens (rice-positive, Nos. 1 to 26; rice-negative, Nos. 27 and 28) was determined by two-site ELISA using two kinds of anti-human IgE as described in Materials and Methods. The total-IgE concentration is expressed as international units (IU) per mL.

homologous allergen, a pollen allergen from Russian thistle (Sal k 2) (Fig. 1(A)), showing only limited matches of these rice proteins with Sal k 2. There was only one match of 6 contiguous residues with Sal k 2 in AK068307 or AK10334, but not in AK070271.

Putative rice prolamin 17, AK242325 (protein No. 5), which resembles another rice prolamin protein, AK242260 (protein No. 8), showed similarity in some parts of whole sequence to several plant food allergens, i.e., maize 50-kDa glutenin/zein (Zea m 50 kDa zein), a large number of wheat LMW-glutelin subunit isoallergens (Tri a-series), and 2S globulins from sesame (Ses I 1) and Brazil nut (Ber e 1) (Table 2). Sequence similarity between these rice prolamin proteins and the cereal seed allergens was found only in limited regions of whole sequences and many of the matched residues were Gln, an abundant amino acid residue of cereal prolamin proteins (data not shown). One segment with 12 contiguous amino acid residues, CQVM-QQCCQQL, of the rice p 17 (AK242325) was conserved in the sequence of wheat LMW-glutenin, Tri a 36, (10 matches, CHVMQRQCCQQL) and corn 50-kDa glutenin, Zea m 50 K, (9 matches, CQVLQQCCCHDL).

The rice putative germin-like protein 16, AK062862 (protein No. 3), was similar to several plant food allergens, including a lemon peel (flavedo) germin-like allergen (Cit l 1), a hazelnut allergen (Cor a 9), and a peanut allergen (Ara h 3), though the overall sequence identity was low (Table 2). The sequence of a putative rice germin-like protein was aligned with that of the

lemon allergen (Cit l 1) with the highest overall similarity (Fig. 1(B)). Three complete matches consisting of 8, 9, and 13 contiguous amino acid residues were present between AK062862 and Cit l 1, though overall similarity was 58%.

The sequence of a putative xylanase inhibitor, AK058882 (protein No. 6), was similar to wheat chitinase-like xylanase inhibitor (Tri a XI), a tropical fruit (Indian plum) cross-reacting allergen (Ziz m 1) and a rubber contact allergen, hevine-A (a chitin-binding protein, Hev b 14) (Table 2). The sequence of AK058882 was compared to that of Tri a XI (Fig. 1(C) top). Overall identity of the rice protein (AK058882) was 50% to the wheat allergen (Tri a XI). There were three complete matches consisting of 9, 9, and 14 contiguous amino acid residues apart each other. These three matched regions were mapped on the 3D diagrams (ribbon and surface models) of Tri a XI (Fig. 1(C) bottom). The regions 1 and 2 consisting of 9 and 14 matched residues formed two parallel-arranged α -helix structures connected with a disulfide bridge between Cys55 and Cys96 on the molecular surface. The region 3 with nine matched residues was located adjacent to the region 2 with α -helix. Thus, the three regions conserved between the rice putative xylanase inhibitor and wheat allergen, Tri a XI, were mapped adjacently on the surface of the Tri a XI molecule.

The putative rice α -amylase (AK073487) showed high similarity (~70%) to barley seed food allergen, α -amylase (Hor v 16), and low similarity (below 30%)

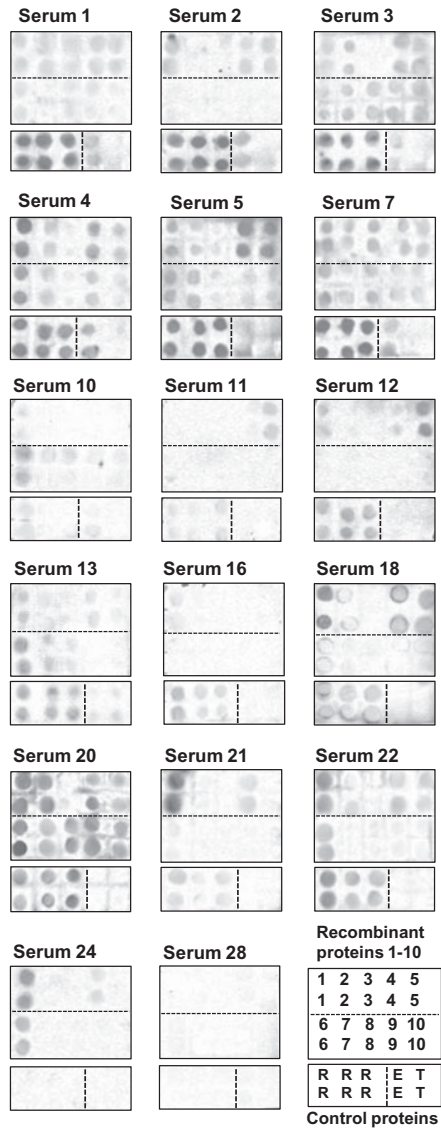


Fig. 3. IgE-binding to recombinant rice proteins with sequence similarity to known allergens.

Notes: The purified recombinant rice proteins [number 1–9 in Table 2 and LTP (No. 10)] were used as antigens for IgE immuno-dot blotting analysis in duplicate. Rice seed proteins from three lines (R), egg albumin (E), and the recombinant TRX (T) were also used for comparison. IgE-binding to the recombinant proteins was analyzed by immuno-dot blotting. Representative 16 blotting images and one image for a negative serum (No. 28) are shown with relatively characteristic IgE-binding profiles to the nine rice proteins. Background chemiluminescence of each blot was subtracted using Image-J based on the ‘rolling ball’ algorithm. The dot-blotting images of rice recombinant proteins (1–10) and control proteins (A–E), which are shown separately to reduce the image size, are from the blot analyzed simultaneously on a single membrane for each serum specimens.

to a fungal occupational aeroallergen (Asp o 21), α -amylase from *Aspergillus oryzae* (Table 2).

IgE-binding to the recombinant rice seed proteins with sequence similarity to known allergens

The nine rice proteins Nos. 1 to 9 (see Table 2) were prepared as His-tagged TRX-fusion recombinant proteins and affinity-purified. The SDS-PAGE of these protein preparation showed that the recombinant rice proteins were expressed as the TRX-fusion with molec-

ular mass expected from the size of each full-length cDNA (Fig. 2(A)). Although expression levels of the rice proteins No. 3 (germin-like protein), No. 5 (prolamin 17), and No. 9 (kinase-like protein) were relatively low as seen in the lanes of total cell lysate, the recombinant proteins for all of the nice rice proteins were prepared in amounts large enough to be used for the following IgE-binding immunoassay. The 28 serum specimens were characterized by measuring the total-IgE concentration using two-site ELISA (Fig. 2(B)). The total-IgE level differed remarkably from one serum to another, and the average of total-IgE with SD of the 26 rice-positive ones was 2861 ± 2034 . The residual 2 serum specimens selected as rice-negative ones showed very low total-IgE concentration.

Reactivity with patients’ IgE of the rice proteins was analyzed using immuno-dot blotting with TRX as a negative control as well as rice seed soluble proteins, LTP, and egg ovalbumin (OVA) for comparison. Representative blots are shown in Fig. 3. In many cases of the 26 serum specimens, markedly positive IgE-binding signals were detected for at least one of the nine recombinant proteins or more, though there was considerable individual variability among the serum specimens tested. Some serum specimens (e.g., Serum 7 and 20) showed IgE-binding most of the nine proteins, while the others only one (e.g., Serum 11 and 21) or a few (e.g., Serum 18 and 24). As a whole, serum specimens with strong IgE-binding to rice seed soluble proteins (positive controls) tended to show marked IgE-binding to the recombinant rice proteins with sequence similarity to known allergens. Exceptionally, a few specimens (e.g., serum 10 and 24) with weak IgE-binding to the rice seed showed strong IgE-binding to some recombinant proteins.

The staining intensity for the IgE-binding of each dot was semi-quantitated and plotted against each protein tested (Fig. 4(A)). IgE-binding signals of the serum specimens higher than those of the control TRX were detected frequently for the four rice proteins 1, 4, 5, and 6, that is, putative protein kinase (AK070271), α -amylase (AK073487), prolamin 17 (AK242325), and xylanase inhibitor (AK058882), respectively. Moreover, the mean value of the IgE-binding intensity of the four proteins tended to be higher than that of egg albumin but lower than that of rice seed proteins, though a large variation was observed from one serum to another. To analyze the IgE-binding to each rice protein on individual serum specimen in detail, the signal intensity of each spot was divided by that of TRX on each blot, and the values relative to TRX over 2.0 (regarded as positive) were compared among the nine rice proteins and the 26 serum specimens tested (Fig. 4(B)). The four rice proteins 1, 4, 5, and 6 (AK070271, AK073487, AK242325, and AK058882) gave the positive IgE-binding to 12 (43%) serum specimens or more out of 28. Twelve serum specimens gave strong positive signals (over 5.0) to at least one rice protein. Among them, a half of the serum specimens (serum 4, 7, 11, 12, 20, and 22) showed relatively high total-IgE levels, whereas some others (serum 13, 18, and 26) showed low levels of total IgE (see Fig. 2(B)).

Effect of heat-induce protein denaturation on IgE-binding was examined for rice α -amylase (AK073487)

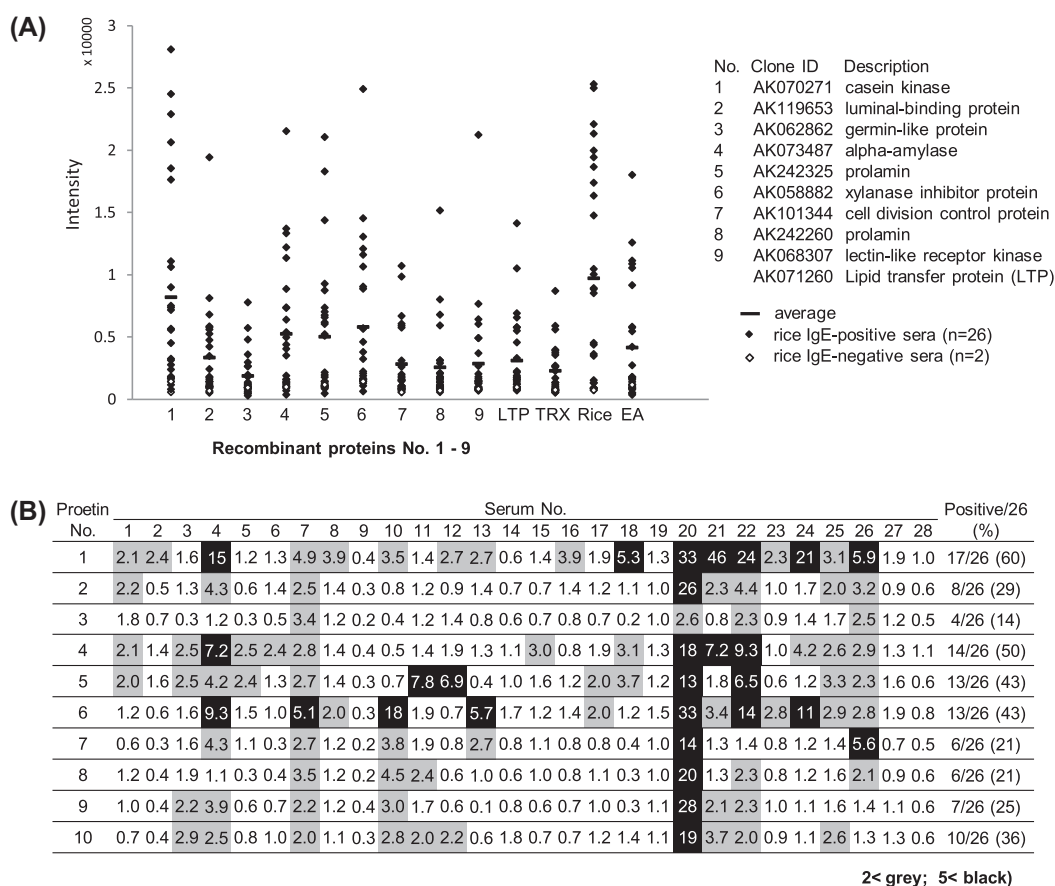


Fig. 4. Semi-quantitative analysis of IgE-binding to recombinant rice proteins as estimated by immuno-dot blotting. (A) Signal intensity of IgE-binding to each protein dot for the 28 serum specimens was semi-quantified using Image-J and plotted on the nine recombinant rice proteins and the control proteins, LTP, TRX, rice seed proteins, and egg albumin (EA). The mean value of the 26 positive sera is also shown as a horizontal bar. (B) TRX-rice protein fusion/TRX ratio in the IgE-binding intensity is compiled as a matrix of the nine rice recombinant proteins and the 28 serum specimens including 2 negative controls. The relative values above 2.0 and 5.0, which were regarded, respectively, as positive and strong positive in the IgE-binding, were highlighted with gray and black backgrounds, respectively.

and xylanase inhibitor (AK058882). The IgE-binding as determined using ELISA for three serum specimens available relatively in a large quantity is shown in Fig. 5, in which net IgE-binding to each rice protein was obtained by subtracting the IgE-ELISA value to TRX as the fusion partner. When the proteins were heat-treated in PBS, in all of the three representative serum specimens, the heat treatment decreased the IgE-binding by 25–50% to α -amylase, but almost not to xylanase inhibitor. By contrast, when they were heat-treated in the presence of 2-mercaptoethanol, IgE-binding was decreased by 25–35% also as to xylanase inhibitor.

Discussion

Rice proteins expressing in seed endosperm and resembling known allergens in the amino acid sequence were selected and the binding of recombinant proteins with IgE from patients' serum was analyzed to assess allergenic potential of these rice proteins. Although the IgE-binding was analyzed using a limited number of serum specimens in this study, all of the nine recombinant rice proteins showed positive IgE-binding with 4–17 of the 26 serum specimens (15–65%) as analyzed by immuno-dot blotting, suggesting that the similarity-

based search of unknown potential allergens using the NCBI blastp (2.2.18) algorithm was reasonable. However, IgE-binding was not necessarily dependent of or correlate with the sequence similarity. For example, the putative rice luminal binding protein (Bip)/Hsp 70 (AK119653) showed high similarity (~90% identity) to a hazel homolog known as a hazel pollen allergen (Cor a 10), but gave relatively low IgE-binding. It seems likely that high IgE-binding of the putative rice Bip/Hsp 70 is detected if the test samples are serum specimens from patients with pollen allergy. It is noteworthy that four (rice proteins 1, 4, 5, and 6) of the nine proteins were positive in the IgE-binding to 13 (50%) serum specimens or more and strongly positive to 3–7 of the 26 serum specimens. These results may indicate that proteins with sequence similarity to known allergens show IgE-binding with high frequency to serum specimens from patients with some sort of allergy. The rice seed protein components reactive with patients' IgE found in this study had not ever been found in previous studies on rice seed potential allergens, in which traditional procedures had been adopted, i.e., protein fractionation and isolation followed by IgE-binding assay for isolated proteins or IgE-immunoblotting after SDS-PAGE separation of rice proteins. Such unidentification of the potential allergens in these studies may be ascribed to the fact that the rice proteins with allergenic

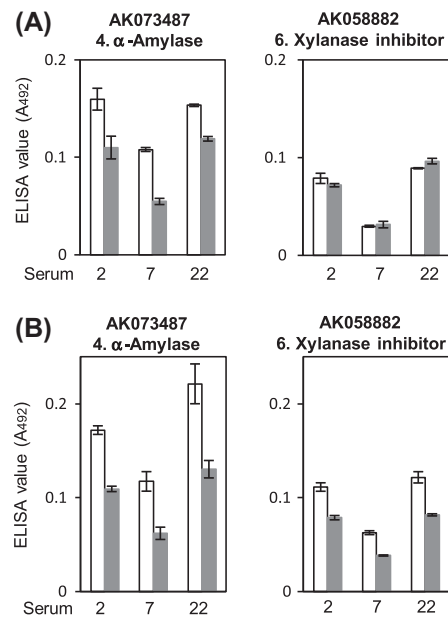


Fig. 5. Effect of heat-induced denaturation on the IgE-binding to putative rice α -amylase and xylanase inhibitor. The recombinant proteins of putative rice α -amylase (AK073487) and xylanase inhibitor (AK058882) were heat denatured by boiling in the absence (A) and presence (B) of 2-mercaptoethanol and then subjected to the quantitative IgE-binding analysis using ELISA on the three serum specimens, which were IgE-binding positive and available relatively in large amount. The ELISA values for native (white bars) and heat-denatured (gray bars) proteins are shown as mean $\pm$ SE of three determinations.

potential found in this study is too small in relative content in rice seed proteins to be purified and/or identified. Therefore, the potential allergens identified in this study are minor components and may not be relevant clinically. However, these potential allergens could be included in the list of rice proteins to be monitored in the safety assessment.

In the safety assessment of IgE immuno-cross reactivity of unknown proteins, short segments consisting of 6–8 amino acid residues have been considered from a viewpoint of sequence-dependent epitopes. Moreover, previous bioinformatic structural studies on known allergens suggested that conformation-dependent cross-reactive epitopes can be predicted and are worth considering for the allergenic potential estimation of novel proteins, when 3D structures of any allergens or related proteins are available.^{20,21} In this study, the contiguous residues matched in the sequence alignments between rice and wheat xylanase inhibitors were mapped on the 3D structure of the wheat one, showing that several separated segments of the matched residues sterically neighbor and correctively form a surface region in a folded protein molecule. This 3D mapping result suggests that the three matched contiguous residues form a surface area sterically similar to that of the wheat allergen, Tri a XI. Thus, allergenic potential of rice xylanase inhibitor may be higher than expected from the sequence similarity alone. Possible conformation-dependent epitope(s) of the putative rice putative xylanase inhibitor were further examined using heat-treated proteins. In contrast to rice α -amylase, rice putative xylanase inhibitor was heat stable in its IgE-binding ability. It remained to be analyzed further whether the

heat stability in IgE-binding was due to its conformational stability to heat or its sequence-dependent IgE-binding ability. Nevertheless, the 25–35% decrease in IgE-binding to putative xylanase inhibitor by the heat treatment in the presence of 2-mercaptoethanol suggested the presence of disulfide-dependent conformational epitopes in putative xylanase inhibitor and would be consistent with the above described assumption that allergenic potential of rice xylanase inhibitor is higher than expected from the sequence similarity alone.

A putative cAMP-dependent protein (Ser/Thr) kinase (AK070271) was one of the three rice protein kinases with sequence similarity to Sal k 2, which had been reported as a potential pollen allergen from *Salsola kali* (Russian thistle) based on *in vitro* IgE-binding.²² The sequence of Sal k 2 (Q8L5K9) had been registered in protein databases, but molecular properties of this pollen protein remained uncharacterized. Russian thistle is an annual weed in the genus of *Salsola*, growing often in arid region of the world. In several Middle East countries, pollen of this weed is known to be one of the main causes of respiratory allergic diseases,²³ whereas pollen allergy to the species of *Salsola* has not been reported so far in East-Asian countries including Japan. The expression level of the protein kinase (AK070271) as estimated by 2D-PAGE is not high in the rice endosperm (http://gene64.dna.affrc.go.jp/RPD/database_en.html), whereas the mRNA of AK070271 is expressed considerably in various tissues, including endosperm and anther, of rice plant (<http://ric.expro.dna.affrc.go.jp/>). Moreover, possible orthologs (over 95% sequence identity) are present widely in the Poaceae family plant, including cereals, millet, sorghum, and a model grass plant (*Brachypodium distachyon*). Therefore, it is likely that IgE reacting with the recombinant protein (AK070271) was produced originally against the possible ortholog antigens of seed and pollen from not only rice but also some other plant in the Poaceae family.

Rice α -amylase (AK073487) structurally resembled Hor v 16, presumed to be a barley homolog. The barley α -amylase, Hor v 16, was identified as a potential allergen recognized by patients' serum IgE with rather low frequency only in two large-scale survey researches using allergen microarray.^{24,25} Relative abundance of the α -amylase (AK073487) in rice endosperm (see Table 2), presence of several isozymes in rice, and presence of possible orthologs (over 70% sequence identity) in some other cereal and millet species suggest that the IgE in the serum specimens used in this study was produced against α -amylase by ingestion of rice and/or some other cereals and millet. Sensitization by inhalation of cereal flour could not be ruled out, as is the case with occupational allergy (asthma) to fungal α -amylase, Asp o 21.^{26–28}

In this study, allergenic potential of rice seed proteins was assessed and, consequently, several potential allergens in rice seeds were newly identified using a combination of a proteome database, sequence similarity search in known allergen databases, and IgE-binding assay for recombinant proteins of the selected candidates. This approach we took in this study will become more useful with enrichment of the databases, in both identification of potential allergens and

assessment of allergenic potential of plant and animal tissues for food and feed. The rice potential allergens newly identified in this study may be additional targets to be monitored in the safety assessment of novel rice varieties developed by molecular breeding or gene manipulation.

Author Contribution

K. Hirano carried out recombinant protein preparation, immunological assay, and data analysis and made manuscript draft. S. Hino generated expression plasmid constructs. K. Oshima and D. Nadano advised on recombinant protein preparation and plasmid construction, respectively. A. Urisu took blood samples and prepared serum. F. Takaiwa advised on research planning and rice databases. T. Matsuda directed the research and completed the manuscript with the other authors' contributions.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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