



Proteomic analysis of known and candidate rice allergens between non-transgenic and transgenic plants

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ABSTRACT

Salt-soluble proteins extracted from non-transgenic and transgenic rice were evaluated for the presence of known and potential allergens by proteomic techniques. The salt-soluble proteins were extracted, separated by 1D and 2D electrophoresis, and analyzed by Western blotting. 1D immunoblot analysis with patients' sera revealed few qualitative differences between the IgE-binding proteins of the non-transgenic and transgenic rice. 1D immunoblot with antigen-specific-animal sera revealed no qualitative or quantitative differences in two known allergens, RAG2 and glyoxalase I, between non-transgenic and transgenic rice. Multiple spots containing known and novel IgE-binding proteins were detected among the salt-soluble proteins of non-transgenic rice by 2D immunoblotting. Two globulin-like proteins, a 52 kDa protein and a 63 kDa protein, were identified as novel IgE-binding proteins that are candidates for rice allergens. These globulin-like proteins were homologous to Cupin superfamily allergens. Quantitative analysis of 19, 52, and 63 kDa globulins with protein-specific-animal sera showed no significant differences in the expression of these proteins between the transgenic rice and non-transgenic rice. These results indicate that none of the known or novel endogenous IgE-binding proteins detected in this study appear to be altered by genetic modification.

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1. Introduction

Rice is the staple food of over half of the world's population and contains carbohydrate and abundant protein as an energy source. On the other hand, the number of allergenic substances detected in various foods has been increasing in recent years, and cases of patients who are allergic to rice have also been reported. Manifestations of rice allergy include atopic dermatitis (Ikezawa et al., 1992; Lezaun et al., 1994; Uchio et al., 1998), eczema (Hoffman, 1975; Shibasaki et al., 1979), and food-protein-induced enterocolitis syndrome (Sicherer et al., 1998; Mehr et al., 2009).

Rice proteins are 80% acid- and alkali-soluble glutelins, 10% salt-soluble globulins, 5% water-soluble albumins, and 5% alcohol-soluble prolamins (Cagampang et al., 1966). Rice proteins account for 10% of the composition of rice grains by weight and are

mainly found in two different kinds of protein bodies, PB-I and PB-II. The digestion-resistant PB-I contains prolamins, while the easily digestible PB-II contains glutelins and globulins (Tanaka et al., 1980; Yamagata et al., 1982). PB-I mainly consists of 10, 13, and 16 kDa prolamin family proteins (Ogawa et al., 1987), and glutelin, which is a main component of PB-II, consists of two subunits, a 22–23 kDa protein and a 37–39 kDa protein (Yamagata et al., 1982).

It is known that allergenicity of rice is partly dependent on globulin and albumin fraction proteins (Shibasaki et al., 1979). Urisu et al. found that 22 proteins extracted from rice with 1 M NaCl exhibited binding activity with immunoglobulin E (IgE) in rice allergic patients' sera, and that a 16 kDa protein and a 33 kDa protein were the major allergens (Urisu et al., 1991). The 16 kDa allergen is a spheroidal protein that is homologous to α -amylase/trypsin-inhibitor family proteins and contains 10 cysteine residues (Matsuda et al., 1988; Izumi et al., 1992, 1999; Adachi et al., 1993). The 33 kDa allergen has been identified as a novel type of plant glyoxalase I (Usui et al., 2001). A 26 kDa protein, an α -globulin purified from salt-soluble proteins, has also been shown to be a rice allergen (Limas et al., 1990).

As a result of advances in molecular biology, crops that are adapted to poor conditions and that are abundant for nutrients

Abbreviations: 1D, one-dimensional; 2D, two-dimensional; HRP, horseradish peroxidase; IgE, immunoglobulin E; KSCN, potassium thiocyanate; MALDI-TOF MS/MS, matrix-assisted laser desorption/ionization-time of flight tandem mass spectrometry; PBS, phosphate-buffered saline (pH 7.2); PBS-T, PBS containing 0.05% Tween 20; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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are being developed by using transgenic techniques. The Codex Alimentarius Commission states that safety assessments of genetically modified (GM) foods need to include an investigation of tendencies to provoke allergy that might result from the gene insertion (ftp://ftp.fao.org/es/esn/food/guide_plants_en.pdf). Comprehensive analyses, such as by metabolomics and proteomics, may be useful tools for detecting unexpected effects in GM foods. Actually, the development of a comprehensive analysis procedure in recent years has made it possible to detect the allergenic components that had never been detected before (Batista et al., 2007; Nakamura et al., 2009, 2010a,b).

In this study we compared the qualitative and quantitative differences in known rice allergens between transgenic and non-transgenic rice by using transgenic rice with high-level tryptophan (Trp) accumulation (Tozawa et al., 2001; Wakasa et al., 2006). Trp is a key amino acid and is usually contained in small quantities in cereals. Anthranilate synthase (AS) catalyzes the first reaction in Trp biosynthesis. A mutant AS α -subunit (*OASA1*) gene from rice, *OASA1D*, that encodes a feedback-insensitive α -subunit of rice AS has been generated (Tozawa et al., 2001). Two homozygous lines, HW1 and HW5, have been obtained with stable overproduction of Trp in the seeds (Wakasa et al., 2006). Metabolic analysis revealed no significant changes in amount of metabolite other than Trp have been detected in these transgenic lines (Wakasa et al., 2006; Dubouzet et al., 2007). Furthermore, we performed comprehensive probing of IgE-binding proteins, which are candidates for allergens in the wild-type rice strain.

2. Materials and methods

2.1. Rice extracts

Mature brown seeds of non-transgenic rice (*Oryza sativa* cv. Nipponbare) (WT) and *OASA1D* transgenic rice line HW5 (HW5) harvested from an isolated field were used in the experiments. Ten grains of brown rice were pulverized with a Multi Beads Shocker (Yasui Kikai Corp., Osaka, Japan) at 2500 rpm for 1 min. Salt-soluble proteins were extracted with 3 mL of 1 M NaCl with a Protease Inhibitor Cocktail (Sigma-Aldrich Corp., St. Louis, MO, USA) by shaking overnight at 4 °C with a Micro Tube Mixer (TAITEC Corp., Saitama, Japan). The solutions obtained from the seeds containing salt-soluble proteins of each variety were then centrifuged twice at 9300g for 10 min at 4 °C. The protein concentration of the supernatant was determined with a BCA Protein Assay Reagent Kit (Thermo Fisher Scientific Inc., MA, USA). The protein solutions were stored in aliquots at –80 °C until used.

The salt-soluble proteins of WT rice and HW5 rice (7.5 μ g/lane) were separated by SDS–PAGE on a 10–20% SDS–polyacrylamide gradient gel (DRC Co., Ltd., Tokyo, Japan) under reducing conditions. Silver staining (Pierce Silver Stain for Mass, Pierce Biotechnology, Rockford, USA) is used to detect proteins after electrophoretic separation on polyacrylamide gels. The stained gel was scanned with an Epson ES-7000H scanner (Epson, Nagano, Japan). The scanned images were processed with an Adobe Photoshop 8.0 and stored in TIFF image files (Adobe Systems, San Jose, CA, USA).

2.2. Patients' sera

Sera from 16 Japanese allergy allergic patients (#001 to #016) and 11 commercially available patient's sera/plasmas (#017 to #027, #024 and #025 are plasmas, PlasmaLab International, Everett, WA) whose rice-specific IgE ImmnoCAP score were positive (Table 1), and control sera from three healthy volunteers (SN-1 to -3) were used in this study. Informed consent was obtained from

all patients and volunteers. Our study was approved by the Ethical Review Committee of National Institute of Health Sciences.

2.3. Preparation of specific polyclonal antibodies

Rice full-length cDNAs encoding RAG2 (Os07g0214300, AK107328) and glyoxalase I (Os08g0191700, AK066092) were provided to us by the NIAS DNA Bank of the National Institute of Agrobiological Sciences (NIAS, Tsukuba, Japan) and were used to express recombinant proteins. Rabbit anti-RAG2 and anti-glyoxalase I polyclonal antibodies were produced by immunization to rabbits (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) with recombinant proteins expressed in *Escherichia coli* by transfection with the full-length cDNA. Rabbit anti-rice 19 kDa (Os05g0499100, gi 20159), 52 kDa (Os03g0793700, gi 115455865) and 63 kDa (Os03g0663800, gi 41469581) globulin antibodies were produced by immunizing rabbits (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) with synthetic peptides having rice globulin-like-protein-specific sequences (14 peptides [23–36 residues], 17 peptides [454–470 residues], 17 peptides [511–527 residues], respectively).

2.4. 1D Western blotting

2.4.1. SDS–PAGE and immunoblotting with patient's IgE

The salt-soluble proteins of WT rice and HW5 rice (10 μ g/lane) were separated by SDS–PAGE on a 5–20% SDS–polyacrylamide gradient gel (DRC Co., Ltd., Tokyo, Japan) under reducing conditions, and the separated proteins were transferred onto 0.22- μ m nitrocellulose membranes (S&S, Dassel, Germany). The membranes were blocked with 0.5% casein–phosphate-buffered saline (PBS) for 2 h at room temperature, and after cut them into ~4-mm strips, the strips were incubated for 1 h at room temperature with diluted sera from each of the 16 patients whose rice-specific IgE ImmnoCAP score was positive or from each of the three healthy volunteers (1:4 dilution with 0.1% casein–PBS) and then overnight at 4 °C. After washing with PBS containing 0.05% Tween 20 (PBS-T) three times, membrane strips were incubated with horseradish-peroxidase (HRP)-linked anti-human IgE (1:500 dilution with 0.1% casein–PBS; Nordic Immunological Laboratories, Tilburg, The Netherlands) for 90 min at room temperature. The bound IgE antibodies were detected by using Konica ImmunoStain HRP-1000 (Konica Minolta, Tokyo, Japan) according to the manufacturer's protocol. The membranes were scanned with an Epson ES-7000H scanner (Epson, Nagano, Japan). The scanned images were processed with an Adobe Photoshop 8.0 and stored in TIFF image files (Adobe Systems, San Jose, CA, USA).

2.4.2. SDS–PAGE and Western blotting of known and potential allergens with protein-specific animal antibodies

The salt-soluble rice proteins of WT rice and HW5 rice (7.5 μ g/lane) were separated by SDS–PAGE on a 15 or 10% SDS–polyacrylamide gel (DRC Co., Ltd.) and then transferred onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 0.5% casein–PBS for 2 h at room temperature and then incubated for 1 h at room temperature with each antibodies (anti-glyoxalase I antibody; 1:2500 dilution; others, 1:10,000 dilution with 0.1% casein–PBS) and then overnight at 4 °C. After washing with PBS-T three times, the membranes were incubated with HRP-linked anti-rabbit Ig (1:1000 dilution with 0.1% casein–PBS; Nordic Immunological Laboratories) for 1 h at room temperature. The bound secondary antibodies were detected by using Western Lighting Chemiluminescence Reagent Plus (NEL104, Perkin Elmer) according to the manufacturer's protocol. The signal intensities of the bands measured as optical densities were evaluated by using the Diana III luminescence imaging sys-

Table 1

Characterization of sera from patients with positive IgE binding to rice.

Serum No.	Major diagnosis/symptoms	Rice-specific IgE levels (IU/ml)	Rice-specific IgE scores ^a	Age (yr)	Sex	Total IgE
001	Anaphylaxis	5.77	3	6	M	1270
002	Food allergy	16.6	3	3	F	6510
003	Bronchial asthma, food allergy, atopic dermatitis	3.01	2	1	M	15,000
004	Atopic dermatitis, food allergy, hay fever (cedar pollen)	1.14	2	1	M	185
005	Food allergy, asthma, atopic dermatitis, anaphylaxis	3.97	3	6	M	4040
006	Atopic dermatitis, food allergy	63.9	5	13	M	22,810
007	Atopic dermatitis, food allergy	N.D. ^b	4 ^c	4	F	14,230
008	Atopic dermatitis, food allergy, bronchial asthma	25.3	4	9	M	8381
009	Atopic dermatitis, food allergy	7.46	3	5	M	780
010	Food allergy, atopic dermatitis	N.D.	4 ^c	1	M	2545
011	Food allergy (wheat, milk, egg white), atopic dermatitis, bronchial asthma, anaphylaxis (milk)	N.D.	2 ^c	4	M	428
012	Atopic dermatitis, food allergy	26.2	4	1	M	9325
013	Food allergy, atopic dermatitis (shrimp, mayonnaise, tuna)	1.37	2	5	M	596
014	Atopic dermatitis, food allergy	1.23	2	1	F	978
015	Atopic dermatitis, asthma, food allergy	N.D.	4 ^c	1	M	4000
016	Atopic dermatitis, food allergy	N.D.	5 ^c	1	M	N.D.

^a The correlations between the ImmunoCAP® Scores and specific IgE concentrations were: (1) 0.35–0.69; (2) 0.70–3.4; (3) 3.5–17.4; (4) 17.5–45.9; (5) 50.0–99.9; (6) >100 IU/ml.

^b No data.

^c Scores tested using ImmunoCAP® (Phadia AB, Uppsala, Sweden).

tem (Raytest, Straubenhardt, Germany) and quantified with Scion Image software (NIH).

2.5. 2D PAGE and immunoblotting with patient's IgE

A 10 µg sample of rice protein was purified by using a 2-D Clean-Up Kit (Ettan Sample Preparation Kits & Reagents, GE Healthcare UK, Little Chalfont, UK) according to the manufacturer's protocol. Purified proteins were dissolved in 125 µl of Destreak Rehydration Solution (GE Healthcare UK) supplemented with 0.1% IPG buffer (pH 3–10, linear; GE Healthcare UK), and after applying them to an Immobiline Drystrip (pH 3–10, 7 cm long; GE Healthcare UK), they were rehydrated overnight at 20 °C. First-dimensional isoelectric focusing (IEF) and equilibration of the strips, and second-dimensional SDS-PAGE were performed as described previously (Nakamura et al., 2009). The separated proteins were transferred onto a PVDF membrane (Bio-Rad Laboratories) and labeled with Cy5 mono-reactive dye as described previously (Nakamura et al., 2009). After washing out the unbound Cy5, the membranes were immunostained with sera #016 and #012 (1:10 dilution) overnight at 4 °C. HRP development of the membrane was performed as described previously (Nakamura et al., 2009).

2.6. 2D PAGE and immunoblotting with protein-specific animal antibodies

For detection of 19, 52, and 63 kDa rice globulin proteins, the protein-blotted membranes labeled with Cy5 mono-reactive dye and washed out the unbound Cy5 described above were immunostained with each antibody (1:200,000 dilution with 0.1% casein-PBS) overnight at 4 °C. After washing with PBS-T three times, the membranes were incubated with HRP-linked anti-rabbit Ig (1:1000 dilution with 0.1% casein-PBS; Nordic Immunological Laboratories) for 1 h at room temperature. HRP development of the membrane was performed as described previously (Nakamura et al., 2009).

2.7. In-gel digestion and protein identification by MALDI-TOF-MS/MS

A 200 µg sample of WT rice protein was separated by 2D PAGE and stained with Pierce Silver Stain for Mass Spectrometry (Pierce

Biotechnology, Rockford, USA). The silver-stained protein spots corresponding to the protein spots that bound to patients' IgE were cut out from the gel and in-gel digested for protein identification by MALDI-TOF-MS/MS as described previously (Nakamura et al., 2010a,b).

2.8. Affinity purification of 19, 52, and 63kDa rice globulin proteins

The 19, 52, and 63 kDa rice globulin proteins that are candidate rice allergens were purified from the salt-soluble WT rice proteins. For ammonium sulfate precipitation, 4 ml of rabbit anti-rice 19, 52, and 63 kDa globulin antibodies were diluted 1:1 with saturated ammonium sulfate and rotated for 1 h at 4 °C. After centrifugation at 9300g for 10 min at 4 °C, the 50% ammonium sulfate fraction of antibodies was collected, and the suspension buffer was replaced with PBS twice with Amicon Ultra-15 (Millipore Corp., Billerica, MA, USA). The antibody fraction was then redissolved in 4 ml of coupling buffer [0.1 M NaHCO₃ (pH 8.3), 0.5 M NaCl] as a ligand. A 250 mg amount of CNBr-activated Sepharose 4B (GE Healthcare UK) was suspended in 50 ml of 1 mM HCl, and the solution was allowed to stand at room temperature for 15 min. The CNBr-activated Sepharose 4B solution was replaced with 5 ml of coupling buffer twice, and the buffer was removed by centrifugation. Then the CNBr-activated Sepharose 4B was mixed with the ligands (anti-19, 52, and 63 kDa antibodies) and rotated overnight at 4 °C. After centrifugation, the ligand-coupled Sepharose 4B gels were washed with coupling buffer twice, and mixed with 20 ml of 0.1 M Tris-HCl (pH 8), and then allowed to stand for 2 h at room temperature. The ligand-coupled gels were washed with 20 ml of 0.1 M acetate buffer (pH 4) containing 0.5 M NaCl and then with 20 ml of 0.1 M Tris-HCl (pH 8) containing 0.5 M NaCl. After performing these washing operations three times, the ligand-coupled gels were washed with PBS twice.

Solvent of a 200 µg sample of the salt-soluble WT rice proteins was replaced with PBS twice with Microcon YM-10 (Millipore Corp.), and the protein solution was mixed with the ligand-coupled gels, and rotated overnight at 4 °C. After washing with PBS three times, bound proteins were eluted with an equal volume of 3 M KSCN/PBS by incubation for 15 min at 4 °C. The eluted fractions of rabbit anti-rice globulin antibody-bound proteins were immediately neutralized by mixing with two volumes of 1 M Tris-HCl (pH

8) and solvent was replaced with PBS twice. The concentrations of rabbit anti-rice globulin antibody-bound proteins were determined with a BCA Protein Assay Reagent Kit (Thermo Fisher Scientific Inc.).

2.9. Dot-blot immunoassay

Dot-blot immunoassays were performed by blotting 4 µg of the rabbit anti-rice globulin antibody-bound proteins onto nitrocellulose membranes. The membranes were then blocked with 0.5% casein–PBS for 1 h at room temperature and incubated with diluted sera #012 and #016 (1:10 dilution with 0.1% casein–PBS) overnight at 4 °C. After washing with PBS–T three times, the membranes were incubated with HRP-linked anti-human IgE (1:500 dilution with 0.1% casein–PBS; Nordic Immunological Laboratories) for 90 min at room temperature. The bound IgE antibodies were detected by using Konica ImmunoStain HRP-1000 (Konica Minolta) according to the manufacturer's protocol.

3. Results and discussion

3.1. IgE-immunoblot analysis of the salt-soluble proteins extracted from non-transgenic and transgenic rice

WT and HW5 rice seeds were used in experiments to analyze salt-soluble proteins in rice. Approximately 3 mg of salt-soluble proteins were obtained from 10 grains of WT and HW5 rice. A 10 µg sample each of the salt-soluble extracts from WT and HW5 rice was subjected to 1D SDS–PAGE, and the gels were detected by silver staining. The result indicated that there are few new band produced by the GM rice (Fig. 1).

The IgE-binding components of the salt-soluble proteins of WT and HW5 rice were also separated by 1D SDS–PAGE and compared by immunoblotting with sera from allergy patients whose rice-specific IgE ImmunoCAP score was positive (Table 1) and with control sera. Multiple IgE-binding bands were detected with allergic patients' sera, and no new bands produced by the HW5 rice could

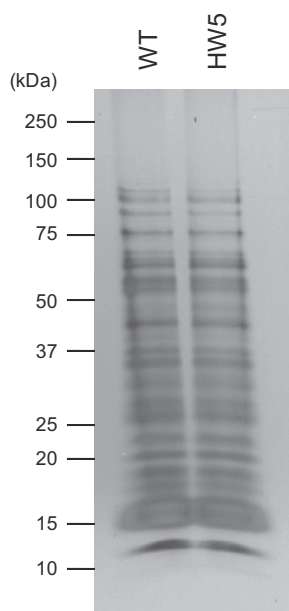


Fig. 1. 1D SDS–PAGE of salt-soluble proteins from WT and HW5 rice with silver staining. WT and HW5 indicate the non-transgenic rice line (Nipponbare) and the transgenic rice line HW5, respectively. Representative results of four similar experiments are shown.

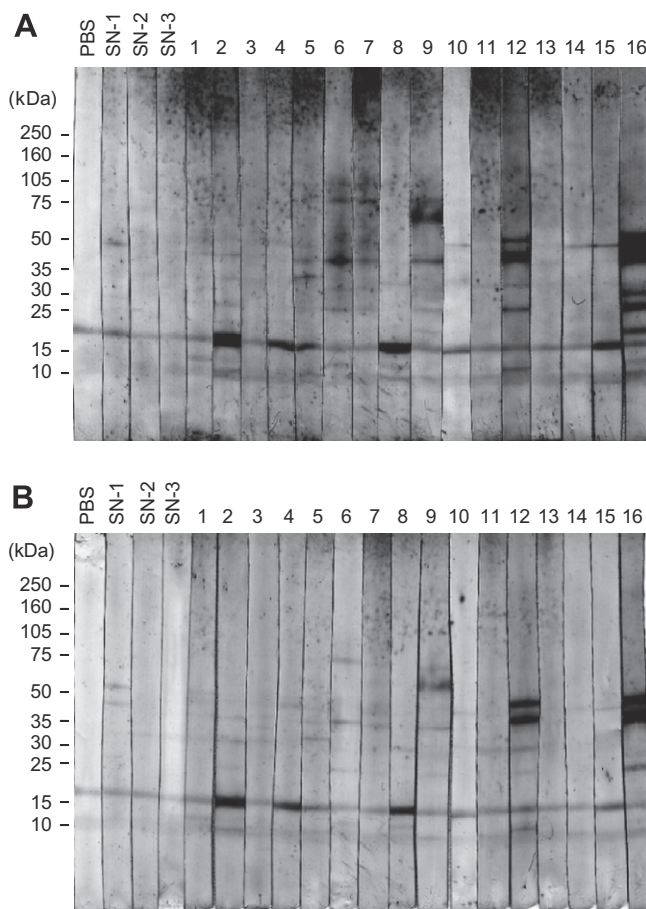


Fig. 2. IgE reactivity with salt-soluble proteins from WT and HW5 rice. (A) WT (B) HW5. PBS lanes, PBS as a negative control; lanes SN-1 to SN-3, sera from healthy volunteers; lanes 1 to 16, sera from patients whose rice-specific IgE immunoCAP score was positive. Representative results of four similar experiments are shown.

be detected (Fig. 2), indicating that there are few additional IgE-binding components in HW5 rice compared to WT rice. In addition, since slight IgE-binding to an approximately 19 kDa protein from both WT and HW5 rice was observed not only with allergic patients' sera but also with the control sera, the 19 kDa band seemed to have weak non-specific IgE-binding activity.

3.2. Comparison between expression of known allergens by non-transgenic and transgenic rice by analysis with protein-specific animal antibodies

Two proteins in rice, RAG2 and glyoxalase I, are known to be allergens (Adachi et al., 1993; Usui et al., 2001). In order to determine the qualitative and quantitative differences of these two allergens in non-transgenic and transgenic rice, we performed the 1D SDS–PAGE and Western blot analysis with rabbit anti-RAG2 and –glyoxalase I antibodies. The band corresponding to RAG2 could be observed at 16 kDa in the results for both non-transgenic and transgenic rice (Fig. 3A), and the densitometric detection of the bands and subsequent statistical analysis revealed no significant difference in expression of RAG2 between the non-transgenic and transgenic rice (Fig. 3B). Similar results were obtained for a 33 kDa band that corresponded to the glyoxalase I allergen (Fig. 3C and D). These results indicate that there are no obvious qualitative and quantitative differences in expression of two known allergens, RAG2 and glyoxalase I, between WT and HW5 rice.

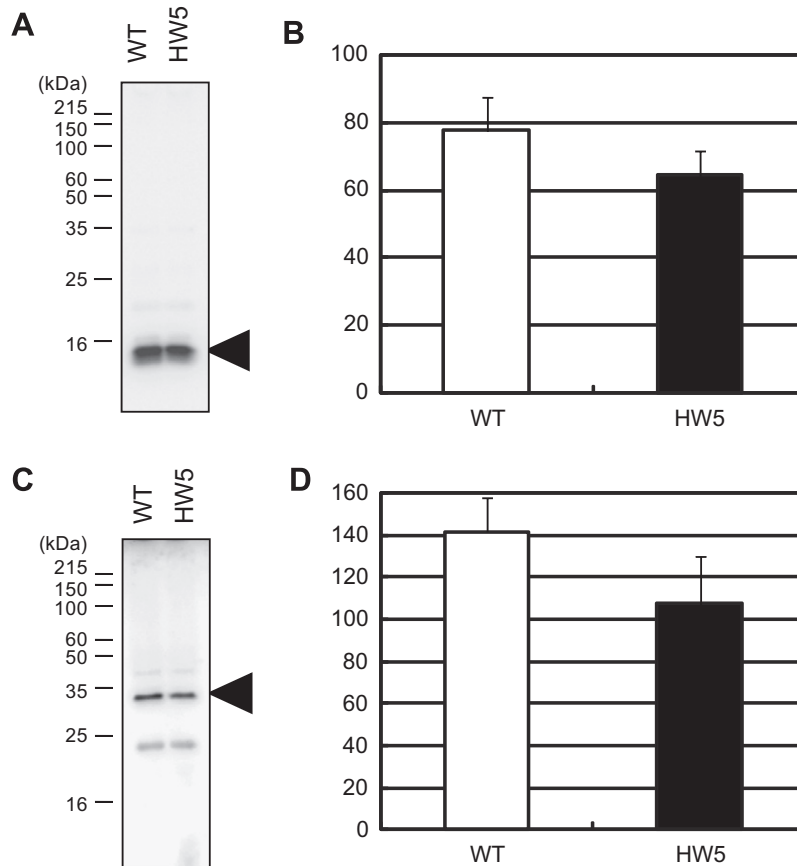


Fig. 3. Comparison of expression of the known allergens, RAG2 and glyoxalase I by WT rice and HW5 rice. (A and B) RAG2; (C and D) glyoxalase I; (A and C) SDS–PAGE and Western blots; (B and D) Densitometric analysis of specific antibodies. A representative result obtained from three experiments was shown. Values are means $\pm$ standard deviation (SD) of values obtained in three independent experiments, each performed in duplicate. The arrowhead indicates the band corresponding to the target allergen.

3.3. 2D immunoblotting and identification of IgE-binding proteins in non-transgenic rice

Fig. 2 shows that the IgE in sera #016 and #012 bound to multiple salt-soluble proteins extracted from both WT and HW5 rice. In order to characterize the rice allergens and IgE-binding proteins in greater detail, we performed a 2D immunoblot analysis by using sera #016 and #012. As shown in Fig. 4, several IgE-binding proteins having molecular weights (kDa) of approximately 16, 19, 33, 50, and >50 were detected in both WT and HW5 rice, and few new spots produced by the HW5 rice could be detected compared to WT rice.

In order to characterize the IgE-binding proteins in WT rice, 11 IgE-binding proteins were selected and the spots corresponding to IgE-binding proteins were cut from silver-stained gels for protein identification by MALDI TOF-MS/MS (Fig. 4). The result revealed that the IgE-reacting spot 6 at 33 kDa was identified as a glyoxalase I (gi 16580747) encoded by gene locus Os08g0191700, which is previously reported as a rice allergen (Table 2). Database search showed that spot 9 has homology with allergenic protein (gi 218197), which is homologous to several allergenic proteins containing RAG2 (data not shown). On the other hand, spots 10 and 11 corresponded to the protein (gi 115471187) were encoded by gene locus Os07g0216700 (Table 2). The genome of the japonica rice cultivar Nipponbare has been completely sequenced (IRGSP, 2005), and then Rice Annotation Project Database (RAP-DB, <http://rapdb.dna.affrc.go.jp/>) has been created (Ohyanagi et al., 2006). The RAP-DB provides us the genome sequence, gene loci, and gene annotations in rice. A search of the RAP-DB revealed that

gene locus Os07g0216700 encode a putative bifunctional trypsin/ α -amylase inhibitor domain containing protein that is homologous with the rice seed allergenic proteins (RAs), including RAG2. This indicates that the spots 10 and 11 seem to belong to RA family proteins. In search of RAP-DB, RAG2 is encoded by the gene locus Os07g0214300. RAs are encoded by a multigene family that consists of at least 4 genes (Adachi et al., 1993). Because RAs are highly homologous with each other (Adachi et al., 1993), the rabbit anti-RAG2 antibody seems to detect RA family proteins widely. These results suggest that the IgE in sera #016 and #012 has binding activity with two known allergen family proteins in rice.

Spots 7 and 8 corresponded to a 19 kDa globulin precursor (gi 20159), which is encoded by gene locus Os05g0499100 (Table 2). The cDNA clone for the gene encoding the 19 kDa globulin, which is an α -globulin, has already been isolated (Shorrosh et al., 1992), and molecular weight of the 19 kDa globulin has been evaluated at 26 kDa by SDS–PAGE (Nakase et al., 1996). Genomic Southern blot analysis indicated that the 19 kDa globulin is encoded by a single-copy gene (Shorrosh et al., 1992). A BLAST search of rice genes in the RAP-DB revealed no genes that are highly homologous with the 19 kDa globulin gene, supporting the previous result. This suggests that spots 7 and 8 identified as the 19 kDa globulin are encoded by a single gene.

Spots 1, 4, and 5 were identified as a globulin-like protein (gi 115455865) encoded by gene locus Os03g0793700 and that has molecular weights around 52 kDa, whereas spots 2 and 3 corresponded to a putative globulin (gi 41469581) encoded by gene locus Os03g0663800 and that has molecular weights 63 kDa (Table 2). These two globulin-like proteins share 39% amino acid

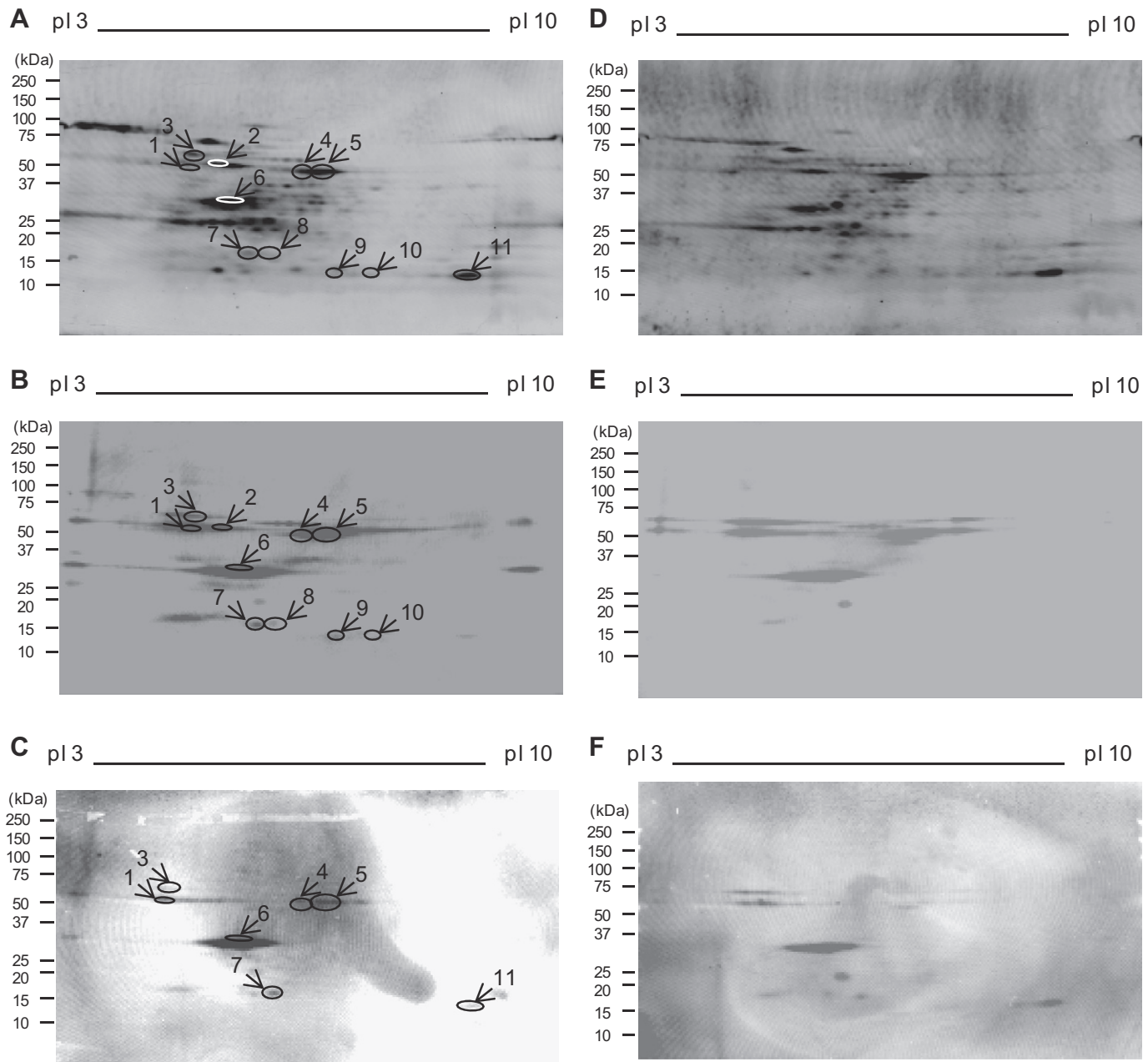


Fig. 4. 2D electrophoresis and immunoblot analysis of rice allergens. (A and D) whole rice proteins labeled with Cy5; (B and E) immunoblots probed with serum from patient #016 detected with ECL plus; (C and F) immunoblots probed with serum from patient #012 detected with ECL plus; (A–C) WT rice; (D–F) HW5 rice; circled and numbered spots indicate the protein spots used for the peptide identification by MALDI-TOF MS/MS.

Table 2
Results of protein identification of IgE-binding spots by MALDI-TOF MS/MS.

Spot No.	Annotation/locus ID	NCBI Accession No.	Theoretical M_w (Da)/pI	Mowse score	Sequence coverage (%)	Matched peptides
1	Os03g0793700 [<i>Oryza sativa</i> (japonica cultivar-group)]	gi 115455865	52,436/6.78	82	28	17
2	Putative globulin (with alternative splicing) [<i>Oryza sativa</i> (japonica cultivar-group)]	gi 41469581	63,845/8.35	151	8	5
3	Putative globulin (with alternative splicing) [<i>Oryza sativa</i> (japonica cultivar-group)]	gi 41469581	63,845/8.35	202	9	5
4	Globulin-like protein [<i>Oryza sativa</i> (japonica cultivar-group)]	gi 34495244	52,376/6.78	520	18	9
5	Globulin-like protein [<i>Oryza sativa</i> (japonica cultivar-group)]	gi 34495244	52,376/6.78	523	22	8
6	Glyoxalase I [<i>Oryza sativa</i> japonica group]	gi 16580747	32,861/5.51	126	16	4
7	19 kDa globulin precursor [<i>Oryza sativa</i> (japonica cultivar-group)]	gi 20159	21,492/7.51	234	21	4
8	19 kDa globulin precursor [<i>Oryza sativa</i> (japonica cultivar-group)]	gi 20159	21,492/7.51	238	21	4
9	Allergenic protein [<i>Oryza sativa</i> japonica group]	gi 218197	17,677/8.74	87	7	1
10	Os07g0216700 [<i>Oryza sativa</i> (japonica cultivar-group)]	gi 115471187	17,036/7.48	151	20	4
11	Os07g0216700 [<i>Oryza sativa</i> (japonica cultivar-group)]	gi 115471187	17,036/7.48	209	26	5

sequence identity. A homology search with PSI-BLAST on NCBI revealed that the proteins encoded by Os03g0793700 and Os03g0663800 are homologous to 7S globulin, vicilin, and several known allergens, i.e., Len c 1.0101, Ara h 1, and Gly m Bd 28 K. This finding strongly suggests that the 52 and 63 kDa globulins might be rice allergens that belong to the Cupin super family. Additional molecular and immunological characterizations might clarify whether they are.

3.4. Immunological reactivity of rice 19, 52, and 63kDa globulin proteins

To examine these rice globulins, we then generated the polyclonal antibodies specific to 19, 52, and 63 kDa globulins. When subjected to 2D and 1D Western blot analysis with rice salt-soluble proteins, each of the anti-rice 19 and 52 kDa globulin antibodies mainly recognized a single rice salt-soluble protein, whereas the anti-rice 63 kDa globulin antibody recognized several proteins (Fig. 5 and Supplementary Figs. 1–3). A search of the NCBI Database indicated the possible existence of alternative splicing forms of 63 kDa globulin. Additionally, the deduced amino acid sequence of 63 kDa globulin includes one potential *N*-glycosylation site (data

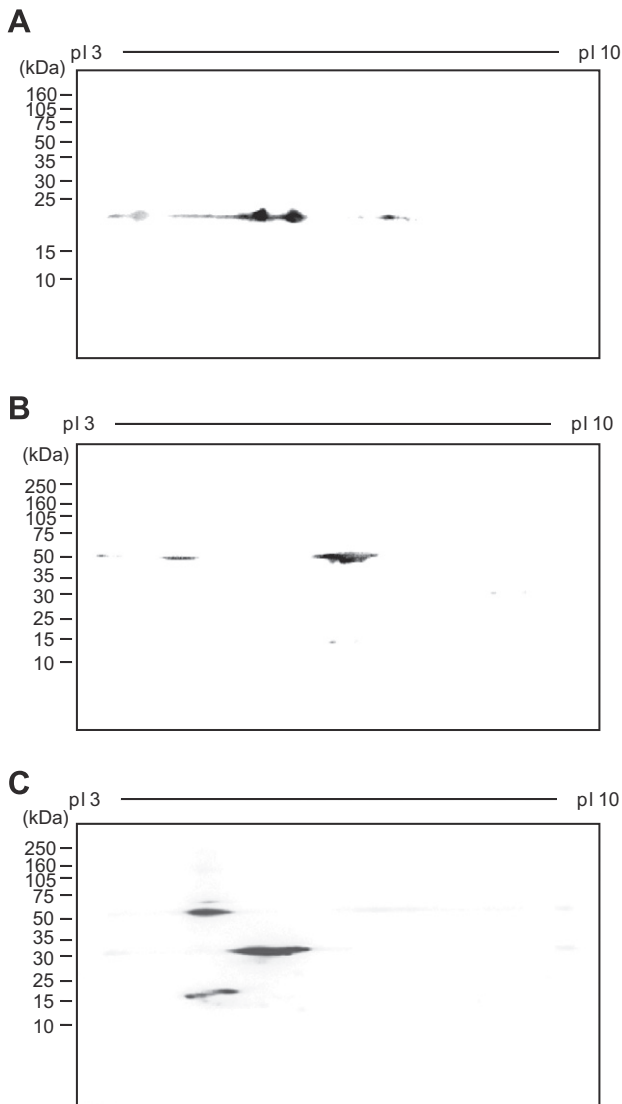


Fig. 5. 2D electrophoresis and immunoblot analyses of rice allergens with anti-rice globulin specific antibodies. (A) anti-19 kDa globulin antibody; (B) anti-52 kDa globulin antibody; (C) anti-63 kDa globulin antibody.

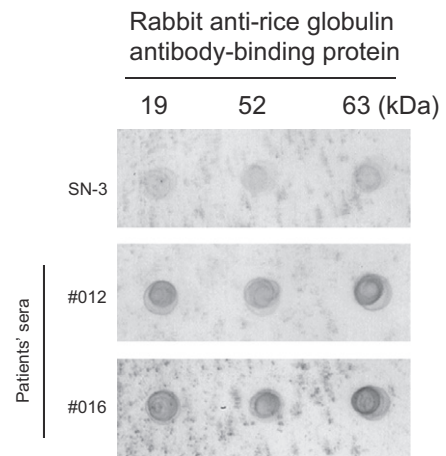


Fig. 6. Dot-blot immunoassay of 19, 52, and 63 kDa globulin proteins with sera #012 and #016 from patients whose rice-specific IgE immunoCAP score was positive.

not shown). These observations may be involved with the binding of anti-rice 63 kDa globulin antibody. To confirm the IgE-binding activity of the rice globulins, we affinity purified the 19, 52, and 63 kDa proteins from salt-soluble WT rice proteins with rabbit anti-rice 19, 52, and 63 kDa globulin antibodies. Dot-blot immunoassays with patients' sera revealed that the anti-rice 19, 52, and 63 kDa-antibody binding proteins had IgE-binding activity (Fig. 6). This suggests that the affinity-purified components are IgE-binding proteins that are present in rice. We then confirmed the qualitative and quantitative differences of these three proteins in non-transgenic and transgenic rice by using 1D Western blot analysis with rabbit anti-globulin antibodies. The results showed no significant increase in any of the three proteins in the HW rice in comparison with WT rice (Supplementary Figs. 1–3).

Proteomic studies of rice under different growth stages, different growth conditions, different level of phytohormones, and different biotic and abiotic stresses have been reported (Lin et al., 2005; Agrawal et al., 2006; Dai et al., 2007). More recently, we have reported on proteomic phenotyping of natural variants of rice (Teshima et al., 2010), and the genome of rice cultivar Koshihikari, which is one of the most popular staple rice cultivars in Japan, has been completely sequenced (Yamamoto et al., 2010). These studies will help us to further evaluate the quality and quantity of allergens in rice grown in different environments and may make it possible to identify a cultivar that contains lower levels of allergens.

In conclusion, we detected and compared candidate allergens among the salt-soluble proteins of non-transgenic and transgenic rice by a combination of proteomic techniques and serological methods. No clear qualitative or quantitative differences were found for two known allergen family proteins (RAG 2 and glyoxalase 1) between non-transgenic and transgenic rice. We also identified three other IgE-binding proteins, a 19, 52, and 63 kDa globulin-like protein, and the latter two were identified as IgE-binding proteins for the first time. Quantitative analysis of the 19, 52, and 63 kDa globulins with protein-specific-animal sera revealed no significant differences in expression of these proteins between the transgenic and non-transgenic rice. These results indicate that none of the known or novel endogenous IgE-binding proteins in this study appear to have been altered by genetic modification.

Conflicts of interest

None of the authors have any conflicts of interest in regard to this paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yrtph.2011.01.008.

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