Cereal proteins are known to cause allergic reactions such as Baker's asthma and severe atopic dermatitis to certain populations. In rice allergy, proteins with molecular masses of 14–16, 26, 33, and 56 kDa have been demonstrated to be potentially allergenic. In this study, to identify and characterize the 33-kDa allergen, designated Glb33, this protein was first purified to homogeneity, and its cDNA clone was isolated. When expressed in Escherichia coli, the recombinant Glb33 was shown to be as reactive as the native Glb33 with mouse IgG and patients' IgE antibodies to Glb33. The Glb33 cDNA coded for a protein of 291 amino acids with two 120-amino acid residue repeats, and the amino acid sequence showed similarity to glyoxalase I from various organisms, including human, plant, yeast, and bacterium. As expected, both native Glb33 purified from rice seeds and the recombinant protein had glyoxalase I activity that catalyzes condensation of methylglyoxal and glutathione into S-lactoylglutathione. However, Glb33 had a higher sequence identity to the bacterial glyoxalase I rather than to known plant and yeast enzymes. Both the Glb33 transcript and the protein were detected not only in maturing seeds of rice but also in its stem and leaf. Taken all together, the rice allergen, Glb33, was identified to be a novel type of plant glyoxalase I that is expressed in various plant tissues, including maturing seeds.

Ingestion and inhalation of cereals and flours are known to be a cause of allergic disorders, such as asthma, eczema and dermatitis, and gluten-sensitive enteropathy (1). Among these cereal allergies, asthma, eczema, and dermatitis are thought to be caused mainly, or in part, by the IgE1-mediated type I allergic reaction against certain cereal proteins (1–3). Asthma caused by cereal is frequently found in workers handling cereal flours in European countries and considered to be an occupational disease known as “Baker's asthma.” Using the reactivity to IgE from the asthmatic and other cereal-sensitive patients as a gauge, the potent allergenic components in cereals was identified as the 15–20-kDa proteins of the plant α-amylase/trypsin inhibitor family (3, 4). Some other wheat proteins, glutenin and gliadin, have also been identified as IgE-reactive proteins (5).

Rice is a cereal produced and consumed in large quantity in South and East Asian countries. The association of rice seed proteins with allergic reaction was first reported in Japan for patients with histories of asthma induced by rice flour exposure and eczema exacerbated by rice ingestion (6). Several clinical studies on rice-induced allergy have been reported for asthma (7) and severe atopic dermatitis (8) in Japan. Rice seed proteins with molecular masses of about 14–16, 26, 33, and 56 kDa were shown to be reactive with IgE antibodies from patients with a suspected rice allergy (9). A group of homologous proteins of about 14–16 kDa recognized by IgE from the majority (90–95%) of the patients in rice seeds was isolated and characterized to be α-amylase inhibitors, which were immunologically cross-reactive proteins constituting a multigene family (10–12). Another allergen with a size of 26 kDa, recognized by patients’ IgE less frequently, was identified as the major seed storage protein, α-globulin (13). However, the other two proteins of about 33 and 56 kDa that exhibit IgE-reactivity have not yet been identified, though these proteins showed IgE-reactivity stronger than the 14–16-kDa allergens with only part of the patients allergic to cereals (9).

A trial to produce hypoallergenic rice was done by suppressing the allergic genes in transgenic rice plants (14). The expression of 14–16-kDa allergens in maturing seeds was demonstrated to be markedly reduced, without any biological side effect on the rice plants, to 10–20% of that of wild type by the introduction of specific antisense genes into rice plants. However, to develop hypoallergenic rice for therapeutic use, some other potent allergens should be decreased or eliminated by conventional breeding and/or genetic engineering.

Toward the hypoallergenic rice production and for a better understanding of IgE reactivity of rice seed proteins, it would be of interest and importance to know the structure of the major allergenic proteins and their expression as well as the real function of these proteins in rice plants. The aim of the present study is isolation of the protein and cDNA for the rice 33-kDa allergen and characterization of its expression and function, to decipher any biological roles of the rice 33-kDa allergen in rice plants. The cDNA and the deduced amino acid sequences indicated that the rice 33-kDa allergen consisted of two repeated sequences, each of which showed sequence simi-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB017042.

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The abbreviations used are: IgE, immunoglobulin class E; IgG, immunoglobulin class G; PBS, phosphate buffered saline; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; RAST, radio allergosorbent test; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; DAF, days after flowering.

A 33-kDa Allergen from Rice (Oryza sativa L. Japonica)
cDNA CLONING, EXPRESSION, AND IDENTIFICATION AS A NOVEL GLYOXALASE I*

Yumiko Usui, Masayuki Nakase, Hirotaka Hotta, Atsuo Urisu‡, Naohito Aoki, Ken Kitajima, and Tsukasa Matsuda§
larity to bacterial glyoxalase I enzymes and to plant enzymes, albeit with lower identity. In fact, the purified and recombinant 33-kDa proteins revealed not only reactivity with patients’ IgE antibodies but also catalytic activity characteristic of glyoxalase I. This is the first report on a novel type of a plant allergen possessing glyoxalase I activity.

**EXPERIMENTAL PROCEDURES**

**Isolation of the Rice Seed 33-kDa Protein (Glb33) with IgE Reactivity**—Rice seed proteins were extracted from dehulled grains of rice (*Oryza sativa L. japonica cv. Nipponbare*) and concentrated on a column (AppliBiosystems, model 476A, Foster City, CA). For the cDNA cloning of Glb33 gene, degenerate sense oligonucleotide mixtures, 5'–ATGATCGGGA/TCA/GCA/AG/AG/GAC/TG/GT/GAT/CAG/GTAT/GCT-3' (nucleotide positions 616–635), in which GT(G/A/T/C)TA(C/T)AT-3' (nucleotide positions 910–931), in which RV sites were incorporated, and the antisense primer as above, and the PCR product were amplified by PCR and subcloned into a plasmid vector pUC118. For the cDNA cloning of Glb33 gene, degenerate sense oligonucleotide mixtures, 5'–ATGATCGGGA/TCA/GCA/AG/AG/GAC/TG/GT/GAT/CAG/GTAT/GCT-3' (nucleotide positions 616–635) of the sequence, GenBankTM accession number AB107042, registered in the database), and an adapter primer as an antisense primer, 5'–CAGAATTCGTCGAGGATCCT-3' (nucleotide positions 921–940), 5'–GCTGGAGCTATATACCGAGG-3' (nucleotide positions 676–698), and the antisense adapter primer as above, and the PCR product was cloned into pUC118. Finally, to obtain a full-length cDNA for Glb33, PCR was done by using a sense primer, 5'–ATCTCCACATCTCGGCTGA-3' (nucleotide positions 921–940), and the first strand cDNA was synthesized by reverse transcriptase (SuperScriptII, Life Technologies, Inc.) according to the manufacturer's instructions. Total RNA (1 μg each) with specific primers for the full-length cDNA synthesis, the obtained sequences were compared one by another with the computer-aided method of Higgins and Sharp (15). Glb33 cDNA in pUC118 was amplified by PCR using a sense primer, 5'–GCTGGAGCTATATACCGAGG-3' (nucleotide positions 676–698), and an antisense primer, 5'–GAACTTGGGATATCTCTCAT-3' (nucleotide positions 910–931), in which NcoI and EcoRV sites were incorporated, respectively. The PCR product was then cloned into pBlueScriptKS(+), amplified, and then cloned again into pET-32a(+) (Novagen, Darmstadt, Germany) to construct a plasmid designated as pET-33k, for Glb33 expression. The Glb33/thioredoxin fusion protein was expressed in *E. coli* and purified by a stepwise elution with varied imidazole concentrations in accordance with manufacturer's instructions (Novagen). The purified protein was digested for the cleavage buffer, 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM EDTA, and treated with enterokinase (Novagen), 2 units for 50 μg of protein in 2 ml of the buffer. After analysis by SDS-PAGE, the enterokinase digest was subjected again to the column of His-bind resin to remove released thioredoxin as well as undigested fusion protein. The flow-through fraction containing recombinant Glb33 (rGlb33) was dialyzed against phosphate-buffered saline (PBS) and stored at −20 °C until further use.

**Measurement of Reactivity to Patients’ IgE Antibodies and Mouse Antiserum by ELISA**—Reactivity of rGlb33 to mouse anti-Glb33 antibodies and human IgE antibodies was also measured by ELISA (16). ELISA plates (Nunc-Immuno™ plate, Nalgene Nunc International, Roskilde, Denmark) were coated with 50 μl of purified Glb33 and rGlb33 at varied concentrations between 0.3 and 20 μg/ml in PBS at 4 °C for 16 h and blocked with 1% BSA in PBS at 37 °C for 30 min. After being washed with PBS containing 0.05% Tween 20 (PBST), the plates were incubated with 50 μl of patient’s serum 20 times diluted or 100 μl of mouse antiseraum 5,000 times diluted, with 1% BSA in PBST at 4 °C for 16 h. The plates were finally incubated with peroxidase-labeled anti-human IgE (BIOSOURCE International, Inc., Camarillo, CA), or peroxidase-labeled anti-mouse IgG (E. Y. laboratories Inc., CA) diluted appropriately with 1% BSA in PBST. The peroxidase activity was determined with o-phenylenediamine as a substrate as described previously (11). Mouse antiseraum was prepared by three time intraperitoneal injections of the purified Glb33 (50 μg of protein per mouse in 100 μl of emulsion with Freund’s complete or incomplete adjuvant) to female ddY mice (Japan SLC, Inc., Hamamatsu, Japan). The human sera were collected from patients whose consents were obtained. From among patients under medical treatment for atopic dermatitis and/or bronchial asthma at the University Hospital, 36 sera with positive RAST values for rice seed proteins, and 12 sera with negative RAST values for the rice proteins were selected and used for the following experiments.

**SDS-PAGE and Western Blot Analysis**—Proteins were extracted from each tissue of rice plants according to the methods of Renzikh et al. (17) and De Rocher et al. (18). Briefly, each tissue sample was ground to fine pieces in a blender and suspended in 10 ml/g PBS containing 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. After being kept at 4 °C with gentle shaking for 14 h and treated with ultrasonication for 1 min on ice, the tissue sample was passed through a piece of gauze and then homogenized with a fine pestle. The supernatant was dialyzed against distilled water and then freeze-dried.

The crude extracts of each tissue of rice plant and purified Glb33 proteins were separated by SDS-PAGE (12% acrylamide) according to Laemmli’s method (19); proteins in the gel were stained with Coomassie Brilliant Blue R-250. Proteins separated by SDS-PAGE were electrophoretically blotted onto a polyvinylidene difluoride membrane (20). Antiserum 5,000 times diluted with 1% BSA in PBST or 3% BSA in PBS, the membrane was incubated with the mouse anti-Glb33 antiseraum 500–1,000 times diluted with 1% BSA in PBS or with the patient’s serum 8 times diluted with 1% BSA in PBS, at 20 °C for 3 h. The membrane was washed with PBST and incubated with peroxidase-labeled anti-mouse IgG (E. Y. laboratories Inc.) or peroxidase-labeled anti-human IgE (BIOSOURCE International, Inc.) diluted appropriately with 1% BSA in PBS. Activity staining for peroxidase was done with either an enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech, Uppsala, Sweden) or 4-chloro-1-naphthol.

**RT-PCR Analysis**—Total RNA was isolated from seed, stem, leaf, and seedling tissues of rice by a phenol-SDS extraction method (12). RT-PCR was performed using SuperScript one-step RT-PCR system (Life Technologies, Inc.) according to the manufacturer’s instructions. Total RNA (1 μg each) with specific primers for the full-length cDNA described above were used for RT-PCR reaction at 25 cycles. Aliquots of PCR product were separated on a 1.0% agarose gel, blotted to Hy bond-N+ nylon membranes (Amersham Pharmacia Biotech); hybridization and detection with the digoxigenin-labeled Glb33 cDNA were performed using DIG DNA labeling and detection kit (Roche Molecular Biochemicals, Mannheim, Germany) according to manufacturer’s instructions.

**Assay for Glyoxalase I Activity**—Glyoxalase I was measured following a previously described method (21) with slight modification. Briefly, the substrate solution (100 μl) containing 0–20 mM methylglyoxal and 5 mM glutathione in 20 mM Tris-HCl, pH 7.0, was put in both sample and reference cuvettes set at a double-beamed spectrophotometer (Hi-
RESULTS

Isolation of Glb33 and Its Reactivity with IgE—Salt-soluble proteins extracted from rice were fractionated by an anion-exchange chromatography of DEAE-Sepharose, and fractions with a peak of UV absorbance were individually collected and subjected to SDS-PAGE and IgE binding assay. The fraction, which showed positive reaction to patients’ IgE and contained the 33-kDa protein, was subjected to a further purification by HPLC. Purity of the preparation was assessed by SDS-PAGE followed by Coomassie Brilliant Blue R-250 staining (see Fig. 1A). The purified 33-kDa protein, referred to as Glb33, was dialyzed against PBS and kept at –20 °C until use.

Cloning and Sequence Analysis of a cDNA Encoding the Glb33—Because no N-terminal amino acid was recovered from the purified protein on the peptide sequence analysis, the Glb33 preparation was digested with trypsin to generate internal peptides. The N-terminal amino acid sequences of two peptides isolated from the tryptic digest of Glb33 were determined. The N-terminal amino acid sequence and start after a lysine residue, according to the tryptic proteolysis consensus site. From these sequence data and results of the expression experiments described below, this cDNA clone was concluded to encode Glb33.

The deduced amino acid sequence of Glb33 revealed neither hydrophobic region, found in typical signal sequences, at the 5′-end of the coding region nor potential N-glycosylation site. Neither signal sequence nor other hydrophobic region throughout the sequence suggests that Glb33 is a cytoplasmic protein. The Glb33 was shown to have two repeat homologous sequences, each containing several conserved regions.

By using the BLAST program, three hypothetical proteins (the GenBank™ accession numbers Z74962, Y10782, and Z97064 for Brassica oleracea (cabbage) (23), Sporobolus stapfianus (resurrection grass) (23), and Citrus X. paradisi (grapefruit) (24), respectively) with unassigned function were found to be highly homologous (about 90% identity) to Glb33. Furthermore, more than 20 sequences of glyoxalase I enzymes from plants, mammals, yeast, and bacteria were also retrieved from the protein data bases, though the sequence identities were low (30–50% identity). A phylogenetic tree of these homologous sequences is shown in Fig. 2A. Among these glyoxalase I enzymes, the overall structure, consisting of two repeat homologous sequences as was the case of Glb33, was found only in the yeast glyoxalase I sequence (25). Such a repeat sequence was also found in the plant hypothetical proteins described above, although it is yet unknown whether or not these hypothetical proteins exhibit glyoxalase I activity. The N- and C-terminal halves, named N- and C-halves, respectively, of these proteins with the repeat sequence were individually compared with one another and to some other glyoxalase I enzymes. An alignment of these sequences, including tomato (Lycoopersicon esculentum) (26) and E. coli (27) glyoxalase I enzymes, is shown in Fig. 2B. The N- and C-halves of Glb33 highly resemble their corresponding regions in the resurrection grass (GenBank™ accession number Y10782) in which all putative binding sites for metals and glutathione were conserved. Both the N- and C-halves of Glb33 were similar to glyoxalase I of E. coli rather than that of tomato in terms of the position of sequence deletions or insertions as well as sequence identity. The sequence identities of Glb33 with several homologous proteins and enzymes are summarized in Table I.

Immunological Reactivity of Native and Recombinant Glb33 Proteins—The recombinant Glb33 (rGlb33) was first expressed as a fusion protein with thioredoxin and His-tag, enzymatically cut apart from thioredoxin and His-tag, and then purified by the Ni2+-affinity chromatography. The purified rGlb33 is shown to have a molecular mass of 33 kDa, which is identical to that of native Glb33 (nGlb33), by SDS-PAGE analysis (Fig. 1A). Immunoblot analyses using the mouse anti-nGlb33 serum and pooled patients’ sera revealed that rGlb33 reacted well with both the Glb33-specific mouse IgG and human IgE (Fig. 1, B and C). Immunological similarity of rGlb33 to the native one was further confirmed by ELISA using the mouse and human...
A 33-kDa Rice Allergen with Glyoxalase I Activity

**Table I**

| Glyb33:C | Glyb33:N* |
|----------|-----------|
| S. stapfianus: N* | 41 | 46 |
| S. stapfianus: C* | 38 | 91 |
| Escherichia coli | 46 | 44 |
| L. esculentum | 33 | 31 |
| Saccharomyces cerevisiae:N* | 38 | 29 |
| Saccharomyces cerevisiae:C* | 30 | 34 |
| Homo sapiens | 35 | 31 |

* The N-terminal (N) and C-terminal (C) halves of Glyb33, S. stapfianus (resurrection grass), and S. cerevisiae (yeast) are compared separately.

**Fig. 3.** Reactivity on ELISA of the purified native and recombinant Glyb33 proteins with IgE antibodies of patients' sera and IgG of a mouse Glyb33 antiserum. ELISA plates were coated at varying concentrations with the native Glyb33 purified from rice seed proteins (closed symbols) and the recombinant Glyb33 expressed in E. coli (open symbols) and incubated with the sera of two RAST-positive (circles and squares) and one RAST-negative (triangles) patients (A) and the mouse anti-Glyb33 serum (B). The human IgE and mouse IgG, which bound to the plates, were detected with the peroxidase-labeled secondary antibodies, respectively. The antibody binding is represented as the absorbance at 492 nm (A492) on the ELISA assay.

**Fig. 4.** Glyoxalase I activity of Glyb33. A, the glyoxalase I activity was measured with the methylglyoxal concentration of 20 mM as described under "Experimental Procedures." Different doses: 0 (+), 0.1 μM (closed circles), and 0.2 μM (closed squares) of the recombinant Glyb33 protein were added to the reaction mixture. B, glyoxalase I activity was measured for the native and recombinant Glyb33 proteins (closed and open circles, respectively) and for the yeast enzyme (open squares). Methylglyoxal concentration varied from 0 to 20 mM and that of glutathione was set at 5 mM. Activity was represented as the initial rate (V) of the recombinant Glyb33.

**Fig. 2.** Sequence similarity of Glyb33 to glyoxalase I and related proteins. A, phylogenetic tree of glyoxalase I enzymes and related proteins. The amino acid sequences of rice Glyb33 (O. sativa L.), the hypothetical protein of resurrection grass (S. stapfianus), and S. cerevisiae were calculated to be 7.6 and 11 mM, respectively, which were four to five times higher than the value (2.0 mM) calculated for the yeast enzyme by the Lineweaver-Burk plot. The Vmax value for Glyb33 per unit protein was estimated to be about one-sixth of that of the yeast glyoxalase I. The catalytic ability (Vmax/Km) of Glyb33 as glyoxalase I was calculated to be about 1/20 to 1/30 that of the yeast enzyme.

**Stage- and Tissue-specific Expression of Glyb33—**Because the sequence of Glyb33 was homologous to several glyoxalase I enzymes even at several restricted regions, glyoxalase I activity was assayed for both nGlyb33 and rGlyb33 proteins. Both Glyb33 proteins were found to catalyze the formation of S-lactoylglutathione from methylglyoxal and glutathione in a dose-dependent manner (Fig. 4A). The enzyme kinetics of Glyb33 against methylglyoxal appeared to be different from that of yeast glyoxalase I, as analyzed by the method of Higgins and Sharp (15). The Lineweaver-Burk plot analysis indicated that the Km value for Glyb33 per unit protein was estimated to be about one-sixth of that of the yeast glyoxalase I. The catalytic ability (Vmax/Km) of Glyb33 as glyoxalase I was calculated to be about 1/20 to 1/30 that of the yeast enzyme.

**Stage- and Tissue-specific Expression of Glyb33—**Expression in some tissues of rice plants and in seeds at different stages of maturation was analyzed by mRNA and protein levels by RT-PCR/DNA blot and Western blot analyses, respectively (Fig. 5). Total RNA and protein were prepared from rice seeds

Antibodies; the rGlyb33 and the nGlyb33 showed comparable reactivities (Fig. 3, A and B).
antigenic N-linked sugar chain as epitope recognizable by the patients’ IgE and mouse IgG.

Glyoxalase I is a ubiquitous enzyme widely found in various organisms from mammals to bacterium. The enzymes of mammals, plants, and bacterial species have been reported to possess approximate molecular masses of 18–19 kDa, and only an exceptional one is that from yeast with a molecular mass of about 37 kDa (about twice as large as the others). The sequence of Glb33 was similar to that of yeast glyoxalase I in terms of the overall structural organization: two repeat homologous sequences. However, the sequence of each monomeric unit of Glb33 was similar to bacterial glyoxalase I rather than the yeast and known plant enzymes, such as Indian mustard (Brassica juncea) (29) and tomato (L. esculentum) (26). These two plant glyoxalase I enzymes possess the same size of polypeptide (185 residues) and similar amino acid sequence (76% identity). In contrast, Glb33 is larger in size (291 residues) and shows lower similarities (30–35% identity). These structural properties suggested that Glb33 is not a rice homologue of the glyoxalase I enzymes isolated from Indian mustard and tomato. The saturation curves of Glb33 and yeast glyoxalase I for methylglyoxal suggest that the kinetic property of Glb33 is different from that of yeast glyoxalase I (Fig. 4). Furthermore, the anti-Glb33 antibody did not cross-react with yeast glyoxalase I. Therefore, Glb33 would not be a plant homologue of the yeast-type glyoxalase I either. As a consequence, we propose that Glb33 isolated from rice seeds could be a novel type of plant glyoxalase I.

In conclusion, the 33-kDa protein in the salt-soluble fractions of rice seed proteins is one of the rice allergens and a novel type of plant glyoxalase I. The results presented here raise the possibility of eliminating or suppressing Glb33 by molecular genetic means (30), unless the presence of this allergen is critically important in plant cells.

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