

A 33-kDa Allergen from Rice (*Oryza sativa* L. *Japonica*)

cDNA CLONING, EXPRESSION, AND IDENTIFICATION AS A NOVEL GLYOXALASE I*

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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 allergenic 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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¹ 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.

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 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 as described previously (11). Briefly, proteins were extracted with 1 M NaCl by sonication and precipitated with ammonium sulfate (70% saturation). A part of the protein precipitated with ammonium sulfate was dialyzed against buffer A, (20 mM Tris-HCl, pH 8.6) and subjected to DEAE ion-exchange chromatography (DE52, Whatman International Ltd., Maidstone, United Kingdom). After being washed with buffer A, the column was subjected to a linear gradient of NaCl, from 0 to 0.5 M in buffer A. Protein peak fractions with UV absorbance at 280 nm were individually collected, and the reactivity to patients' IgE antibodies was determined for each fraction as described below. The fractions with reactivity to the IgE antibodies were dialyzed against buffer A and subjected to HPLC (Jasco, Tokyo, Japan) equipped with an ion-exchange column of DEAE-5PW (Tosoh, Tokyo, Japan), which had been equilibrated with buffer A. Proteins were eluted with a linear NaCl gradient from 0 to 0.25 M in buffer A.

cDNA Cloning and Sequencing—To obtain amino acid sequences for Glb33, its tryptic peptides were prepared as follows. The HPLC-purified Glb33 was digested with *N*- α -tosyl-L-phenylalanylchloromethyl ketone-treated trypsin at the enzyme/protein ratio (w/w) of 1/100 in 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM CaCl₂ for 6 h, and the tryptic peptides were applied to a reverse phase HPLC equipped with an ODS column (Biofine PO, Jasco, Tokyo, Japan). The peptides were separated by eluting with a linear gradient of acetonitrile from 0 to 50% in 0.1% trifluoroacetic acid. The isolated peptides were adsorbed on polyvinylidene difluoride membrane and subjected to a peptide sequencer (Applied Biosystems, model 476A, Foster City, CA).

For the cDNA cloning of Glb33 gene, degenerate sense oligonucleotide mixtures, 5'-AT(ATC)GG(GATC)AC(GATC)GA(A/G)GA(C/T)GT(GATC)TA(C/T)AT-3', synthesized according to the determined amino acid sequences for Glb33 were end-labeled with ³²P and used for the screening of a random-primed cDNA library of Agt11 (10, 12) derived from maturing rice seeds (*O. sativa* L. *Japonica* cv. Nipponbare). The cDNA inserts of positive phage clones were amplified by PCR and subcloned into a plasmid vector pUC118. For the extension of the 3'-end using the 3'-rapid amplification of cDNA ends technique, the first strand cDNA was synthesized by reverse transcriptase (SuperScript II, Life Technologies, Inc.) with an adapter-combined oligo(dT) primer, 5'-CAGAATTCAGCTGCAGGATCC(T)12-3', and poly(A)+RNA, which had been purified from maturing rice seeds with Oligotex dT-30 (TaKaRa, Osaka, Japan), as the template. The DNA was then amplified by PCR using a sense primer, 5'-GCCATGTTGGGCTATGCTGA-3' (nucleotide positions 616–635 of the sequence, GenBank™ accession number AB107042, registered in the data base), and an adapter primer as an antisense primer, 5'-CAGAATTCAGCTGCAGGATCC-3'. By using the PCR product as a template, a nested PCR was done with a sense primer, 5'-GGTGTACAGAATATACCAAGGG-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'-ATCCTCCACCTCGGCTCGA-3' (nucleotide positions 9–28), an antisense primer, 5'-CCTAGCAGACAACTTGGGCC-3' (nucleotide positions 921–940), and the first strand cDNA as a template as described above. Nucleotide sequence was determined by a DNA sequencer (Applied Biosystems, model 373) with the dye terminator cycle sequencing FS Ready reaction. Plasmid DNAs from at least four independent *Escherichia coli* clones were sequenced. Homologous sequences were identified by searching through the BLAST program of the National Center for Biotechnology Information; the obtained sequences were compared one to another by the computer-assisted method of Higgins and Sharp (15).

Heterologous Expression of the Rice Glb33—The Glb33 cDNA inserted in pUC118 was amplified by PCR using a sense primer, 5'-GCTCGAAGCCATGGCAAGCGGT-3' (nucleotide positions 23–45), and an antisense primer, 5'-GAACCTGGGATATCTCTCATCT-3' (nucleotide positions 910–931), in which *Nco*I and *Eco*RV sites were incorporated, respectively. The PCR product was first cloned into pBlue-scriptKS(+), 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 instruction (Novagen). The purified protein was dialyzed against the cleavage buffer, 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM CaCl₂, and digested with enterokinase (Novagen), 2 units for 50 μ g 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, Nalgen Nunc International, Roskilde, Denmark) were coated with 50 μ l of purified Glb33 and rGlb33 at varied concentrations between 0.3 and 20 μ g/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 μ l of patient's serum 20 times diluted or 100 μ l of mouse antiserum 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., San Mateo, CA) diluted appropriately with 1% BSA in PBST. The peroxidase activity was determined with *o*-phenylenediamine as a substrate as described previously (11). Mouse antiserum was prepared by three-time intraperitoneal injections of the purified Glb33 (50 μ g of protein per mouse in 100 μ l 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 Rensink *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 centrifuged at 15,000 $\times g$ for 15 min. 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). After being blocked with 1% polyvinylpyrrolidone (*M_r* 40,000) in PBS or 3% BSA in PBS, the membrane was incubated with the mouse anti-Glb33 antiserum 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 μ g 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 Hybond-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 μ l) 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-

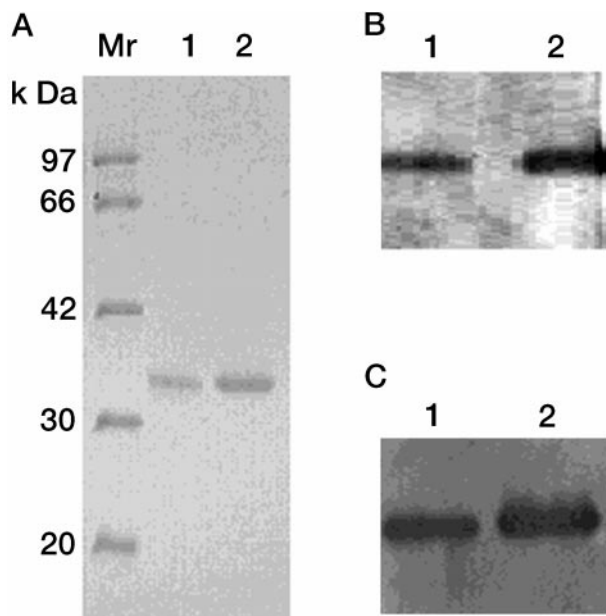


FIG. 1. Reactivity on immunoblotting of the purified native and recombinant Glb33 proteins with IgE of a patient's serum and IgG of a mouse antiserum raised against native Glb33. The native Glb33 purified from rice seed proteins (lane 1), the recombinant Glb33 expressed in *E. coli* (lane 2), and a molecular mass standard (M_r) were subjected to SDS-PAGE (12% acrylamide gel) under reducing condition and stained with Coomassie Brilliant Blue R-250 (A). The proteins were blotted onto polyvinylidene difluoride membrane and stained with IgE in a patient's serum (B) or IgG in the mouse antiserum raised against the native Glb33 (C) by the ECL method.

tachi, model U2001, Tokyo, Japan) and held at 25 °C. To the sample cuvette was added 10 μ l of the enzyme solution containing 4 μ g of protein and incubated at 25 °C for 1 min. Initial velocity of the reaction was determined by monitoring changes of absorbance of the reaction mixture at 240 nm. The UV absorbance was converted to mole quantity by using the molecular coefficient of 3,370 for *S*-lactoylglutathione (22). Yeast glyoxalase I used for comparison was from Sigma.

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 to be GNAYAQVAIGTGVY and IASFLDPDGWK, and consequently, a degenerate oligonucleotide mixture for the sequence underlined was synthesized. By screening of the rice cDNA library with the degenerate oligonucleotide probe, three positive clones were obtained, and their DNA sequences were determined. Since the chemically determined peptide sequence was present in the deduced amino acid sequence of the cDNAs, but neither stop codon nor polyadenylation signal sequence was found in the DNA sequences, the 3'-end of the cDNAs were extended by PCR using the gene-specific primer. About 500-

base pair DNA fragments amplified by the PCR were cloned into plasmid and sequenced. Finally, RT-PCR was performed followed by DNA sequencing of four independent clones to obtain the precise sequence using a set of primers to amplify the open reading frame of the cDNA.

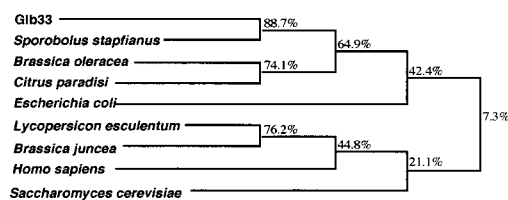
The cDNA contained the 873 base pairs of open reading frame (excluding the stop codon), which encoded a 291-amino acid protein with a theoretical molecular mass of about 32.6 kDa and pI of 5.4. At 73 and 115 base pairs downstream from the stop codon (TGA) in the 3'-untranslated region, two polyadenylation signals were found. The sequences of the two tryptic peptides from purified Glb33 can be localized in the deduced 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 GenBankTM 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 (*Lycopersicon 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 (GenBankTM 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 Ni^{2+} -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



B

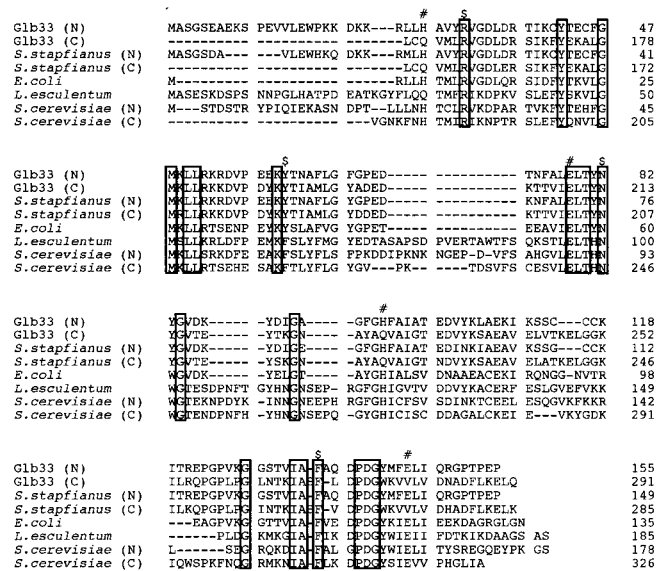


FIG. 2. Sequence similarity of Glb33 to glyoxalase I and related proteins. A, phylogenetic tree of glyoxalase I enzymes and related proteins. The amino acid sequences of rice Glb33 (*O. sativa* L.), the hypothetical proteins of resurrection grass (*S. stapfianus*, GenBankTM accession number Y10782) (23) and cabbage (*B. oleracea*, GenBankTM accession number Z74962) (23), grapefruit (*Citrus paradisi*, GenBankTM accession number Z97064) (24), and glyoxalase I enzymes of *E. coli* (27), tomato (*L. esculentum*) (26), Indian mustard (*B. juncea*) (29), human (*Homo sapiens*) (31), and yeast (*Saccharomyces cerevisiae*) (25) are subjected to phylogenetic analysis by the method of Higgins and Sharp (15). B, amino acid sequence alignment of rice Glb33, a hypothetical protein from *S. stapfianus* (resurrection grass), and glyoxalase I enzymes from *E. coli*, *L. esculentum* (tomato), and *S. cerevisiae* (yeast) as analyzed by the method of Higgins and Sharp (15). Insertions added to obtain maximum matching are shown by hyphens. The N-terminal (N) and C-terminal (C) halves of Glb33, resurrection grass, and yeast glyoxalase I enzymes are separately aligned. The amino acid residues conserved among the eight sequences are boxed, and putative binding sites for metals and glutathione are indicated by the symbols \$ and #, respectively, above the sequence.

antibodies; the rGlb33 and the nGlb33 showed comparable reactivities (Fig. 3, A and B).

Glyoxalase I Activity of Glb33—Because the sequence of Glb33 was homologous to several glyoxalase I enzymes even at several restricted regions, glyoxalase I activity was assayed for both nGlb33 and rGlb33 proteins. Both Glb33 proteins were found to catalyze the formation of *S*-lactoylglutathione from methylglyoxal and glutathione in a dose-dependent manner (Fig. 4A). However, the enzyme kinetics of Glb33 against methylglyoxal appeared to be different from that of yeast glyoxalase I analyzed for comparison (Fig. 4B). Based on the Hill's plot for these kinetic data, the K_m values on native and recombinant Glb33 proteins 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 V_{max} value for Glb33 per unit protein was estimated to be about one-sixth that of the yeast glyoxalase I. The cata-

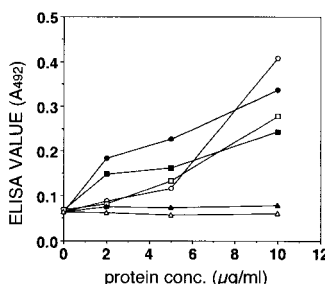
TABLE I

Amino acid sequence identity (%) of Glb33 with a theoretical protein from *S. stapfianus* (resurrection grass) and several glyoxalase I enzymes

	Glb33:N ^a	Glb33:C ^a
Glb33:C	41	
<i>Sporobolus stapfianus</i> :N ^a	87	46
<i>Sporobolus stapfianus</i> :C ^a	38	91
<i>Escherichia coli</i>	46	44
<i>Lycopersicon esculentum</i> (tomato)	33	31
<i>Saccharomyces cerevisiae</i> :N ^a	38	29
<i>Saccharomyces cerevisiae</i> :C ^a	30	34
<i>Homo sapiens</i>	35	31

^a The N-terminal (N) and C-terminal (C) halves of Glb33, *S. stapfianus* (resurrection grass), and *S. cerevisiae* (yeast) are compared separately.

A



B

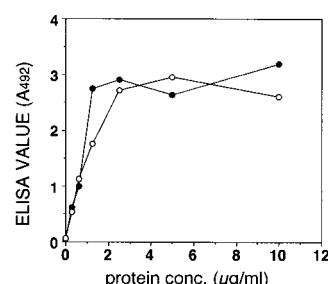
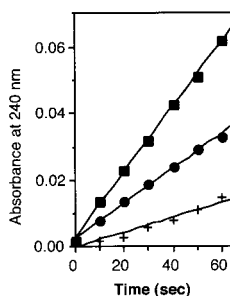


FIG. 3. Reactivity on ELISA of the purified native and recombinant Glb33 proteins with IgE antibodies of patients' sera and IgG of a mouse Glb33 antiserum. ELISA plates were coated at varying concentrations with the native Glb33 purified from rice seed proteins (closed symbols) and the recombinant Glb33 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-Glb33 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 (A_{492}) on the ELISA assay.

A



B

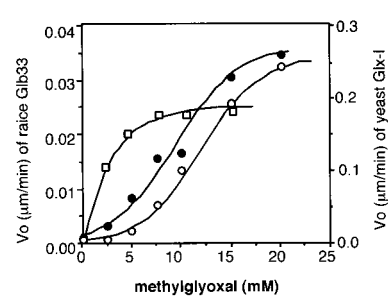


FIG. 4. Glyoxalase I activity of Glb33. A, the glyoxalase I activity was measured with the methylglyoxal concentration of 20 mM as described under "Experimental Procedures." Different doses: 0 (+), 0.1 μg (closed circles), and 0.2 μg (closed squares) of the recombinant Glb33 protein were added to the reaction mixture. B, glyoxalase I activity was measured for the native and recombinant Glb33 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_o) of *S*-lactoylglutathione production ($\mu\text{mol/min}$).

lytic ability (V_{max}/K_m) of Glb33 as glyoxalase I was calculated to be about $1/20$ to $1/30$ that of the yeast enzyme.

Stage- and Tissue-specific Expression of Glb33—Glb33 expression in some tissues of rice plants and in seeds at different stages of maturation was analyzed at 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

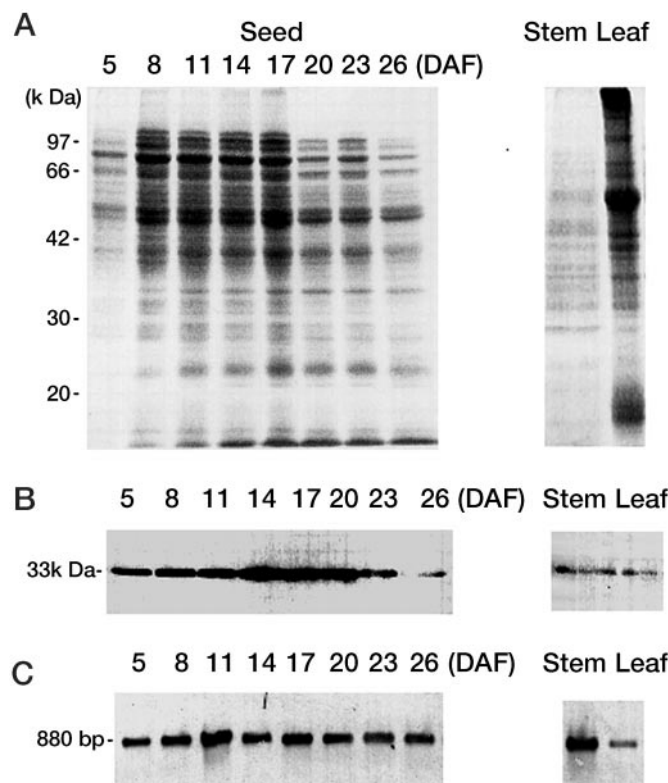


FIG. 5. Expression of Glb33 in various tissues of rice plant. SDS-PAGE (A) and Western blot analysis (B) were done for the total protein prepared from maturing seeds of 5, 8, 11, 14, 17, 20, 23, and 26 DAF, stem, and leaf. The protein amounts applied to each lane were one-fifth of total proteins extracted from one seed and total proteins extracted from 150 mg of stem and 15 mg of leaf. The gel was stained with Coomassie Brilliant Blue R-250, and Glb33 on the blotted membrane was detected with the mouse anti-Glb33 antibody. RT-PCR analysis (C) was also done for total RNA prepared from maturing seeds at 5, 8, 11, 14, 17, 20, 23, and 26 DAF, stem, and leaf. After being blotted on a nylon membrane the RT-PCR products were detected by hybridizing with the digoxigenin-labeled Glb33 cDNA.

at different stages of maturation (5, 8, 11, 14, 17, 20, 23, and 26 days after flowering (DAF)) and from other tissues. The RNA was used as a template for RT-PCR, and the protein was for SDS-PAGE/immunoblot analysis with the anti-Glb33 antibody. The Glb33 transcript was detected in seeds at all maturation stages tested and also in stem and leaf. At protein level, Glb33 was also detected in stem and leaf and in seeds harvested even at earlier stages of maturation (5–11 DAF).

DISCUSSION

In the present study, both of the isolated and recombinant Glb33 proteins revealed IgE reactivity to several patients' sera as examined by Western blotting and ELISA (Figs. 1 and 3), indicating that Glb33 and its cDNA are indeed for the rice 33-kDa allergen. Some *N*-linked carbohydrate chains containing xylose and fucose of plant and insect glycoproteins have been suggested to serve as an epitope recognized by a certain population of IgE antibodies from allergic patients, as well as IgG antibodies of rabbits immunized with a plant glycoprotein (28). However, it is unlikely that rice Glb33 has such carbohydrate epitopes of plant glycoproteins, because no potential *N*-linked glycosylation site was found in the Glb33 amino acid sequence, and the molecular mass (33 kDa) of Glb33 estimated by SDS-PAGE agreed well with the size of Glb33 polypeptide (32.4 kDa) calculated from the amino acid sequence. Furthermore, rGlb33 expressed in *E. coli* showed molecular mass and immunological reactivity almost identical with those of nGlb33 (Figs. 1 and 3). These results also suggest that Glb33 has no

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.² 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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