

Review

Rice Allergenic Protein and Molecular-genetic Approach for Hypoallergenic Rice

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Allergenic proteins with a molecular mass of about 14 to 16 kDa were isolated from a rice salt-soluble fraction based on the reactivity with IgE antibodies from patients allergic to rice. cDNA clones encoding these allergenic proteins were isolated from a cDNA library of maturing rice seeds, and the deduced amino acid sequences showed considerable similarity to wheat and barley α -amylase/trypsin inhibitors, which have recently been identified as major allergens associated with baker's asthma. An antisense RNA strategy was applied to repress the allergen gene expression in maturing rice seeds. Immunoblotting and ELISA analyses of the seeds using a monoclonal antibody to a 16-kDa allergen showed that allergen content of seeds from several transgenic rice plants was markedly lower than that of the seeds from parental wild type rice.

Key words: allergen; α -amylase inhibitor; antisense RNA; transgenic rice; hypoallergenic rice

Food allergy is an adverse reaction to food defined as the reaction in which an immunopathologic process can be demonstrated,¹⁾ and allergens can be defined as those substances that initiate and provoke the immunological reactions of allergy. In IgE-mediated food allergy, the allergens are usually-occurring proteins found in the food.²⁻⁵⁾ The most common allergenic foods tend to be commonly consumed food with comparatively high protein contents, especially food of animal or marine origin. However, not all proteins found in food can function as allergens. The major factors allotting the allergic sensitization to a particular food protein are the characteristics of the protein itself.⁶⁾

Furthermore, it is expected that there is heterogeneity in allergen recognition of IgE antibodies between individuals. Major allergens are those that elicit strong IgE responses from the majority of sensitive patients. Minor allergens, which play a role in the allergic reaction of only some of the patients, tend to elicit weaker IgE responses.⁷⁾ The factors that decide which proteins become the major allergens are unknown.

Rice is a grain produced and consumed in large quantities around the world and is a staple food for the Japanese, but there have been only a few reports on rice allergy. Some atopic patients show positive prick tests⁸⁾ and positive RAST values for rice grain allergens.⁹⁾ Recently, rice has been paid attention as a causative agent of atopic dermatitis,^{10,11)} but the clinical significance of this phenomenon has not been well established.

Rice grain contains proteins accounting for 8% of the dried endosperms, most of which are storage proteins accumulated in protein bodies. Most plant storage proteins in seed tissues are used as nitrogen, sulfur, and carbon sources during the post-germinative period of development. These proteins can be classified into four groups based on their solubility. Depending on the analytical method, genotype, and environmental conditions, rice seeds contain 5-10% alcohol soluble proteins (prolamines), 4-10% salt-soluble proteins (globulin and albumin), and 80-90% alkali soluble proteins (glutelin).¹²⁾ Identification of all the allergenic components in rice grain is necessary for the standardization of rice grain allergens and the study of rice allergy. Shibasaki *et al.*¹³⁾ first showed that a high degree

of allergenicity was found in a globulin fraction of rice seed proteins, but the exact properties of rice allergenic proteins had not been clarified.

1. Separation of Rice Allergenic Protein

To isolate the allergenic protein in rice grain, polished rice grain of the Japonica variety was extracted with 1 M NaCl solution and extracted proteins were fractionated by ion-exchange chromatography on a column of DEAE cellulose.¹⁴⁾ The reactivity with specific IgE antibody from patients allergic to rice was tested for each fraction. Since one fraction showed a positive reaction and high homogeneity, it was further purified by rechromatography on DEAE cellulose column and molecular sieve chromatography on Sephadex G-100. Finally we could isolate one purified allergenic protein from rice grain. The molecular weight of the purified allergenic protein was estimated to be about 16,000.

The reactivity of this allergenic protein was tested about 31 sera with positive RAST (Radio allerge sorbent test) values for rice grain extract.¹⁵⁾ As shown in Fig. 1, the sera examined all gave positive RAST values for the purified allergenic protein. Furthermore, there was a significant close correlation ($r=0.56$, $y=0.75x+25.5$, $p<0.01$) between RAST values for rice grain extract and

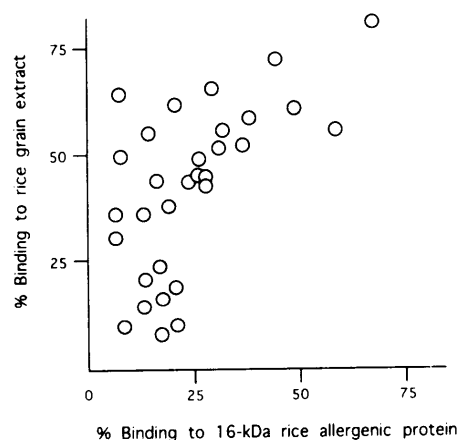


Fig. 1. Relationship between RAST Values for Rice Allergenic Protein and Rice Grain Extract.

	10	20	30	40	
RAP	DHHQVYSPGEQCRPGISYPTYSLPQCRTLVRRO	CVGRGASAADEQVW			
Wheat AI 28	SGPWSWCNPATGYKVSALTGCRAMVKLQ	CVGSQVPEA	..VL		
Barley TI	FGDSCAPGDALPHNPLRACRTYVVSQICHQGPRLTSDMK				
Castor Bean	PSQQGCRGQIQ	EQQNLRCQCEYIKQQVSGQGPR	RQERSL		
	50	60	70	80	90
RAP	QDCC RQLAAVDDGWCRCGALDHMLSGIYRELGATEAGHPMAEVFPGCR				
Wheat AI 28	RDCC QQLADINNEWCRGDLSSMLRAVYQELG	VREG	..KEVLPGCR		
Barley TI	RRCC DELSAIP	AYCRCEALRIIMQGVVTWQG	AFEG	AYFKDSPNCP	
Castor Bean	RGCC DHLKQM	SQCRCEGLRQAIQQQL	QQQNVFEAF		
	100	110	120	130	
RAP	RGDLERAAASL	..PAFCNVDPNGPG	..GVCY	..WLGYPRTFRTGH	
Wheat AI 28	KEVMKLTAAVS	..PEVCKVPIPNPSGDRAGVCYGDWCAYPDV			
Barley TI	RERQTSYAANLVTPQECNLGTH	..G	SA	YCPQLPGYG	
Castor Bean	RTAANL	..PSMC	GVSP	TQCRF	

Fig. 2. Alignment of the Deduced Amino Acid Sequence of Rice Allergic Protein with Sequences of α -Amylase/Trypsin Inhibitor Family Proteins, Namely, Wheat α -Amylase Inhibitor (AI 28), Barly Trypsin Inhibitor (TI), and Castor Bean Glutamin-rich Storage Protein.

The sequences are aligned for maximum similarity causing several gaps (shown as a dot) which may represent insertions/deletions. Numbering refers to the sequences for rice allergenic protein. Conserved cysteine residues are shown in boldface. The castor-bean protein consists of two polypeptide chains of 34 and 61 residues.

those for the purified allergenic protein. These indicate that this purified protein is the major allergenic protein in rice grain extract with response to IgE binding activity. The same conclusion was indicated by the close correlation in the histamine-releasing activities between the rice allergenic protein and whole extract of rice grain.

The allergenic protein was measured by using antibody raised against 16-kDa allergenic protein about 150 rice strains mainly provided by International Rice Germination Center of International Rice Research Institute and Philippine Rice Research Institute.¹⁶⁾ While all of the screened Japanese cultivars contain nearly the same amount of this protein, some cultivars and wild-type species from Asian countries either have a small amount of or no detectable 16-kDa allergenic protein. Rice grains of these cultivars might be useful for rice allergic patients in Japan as substitutes for Japonica rice, although no or low allergenicity should be confirmed by clinical tests such as RAST.

2. Properties of Rice Allergenic Protein

Since allergenic proteins obtained earlier often have a high heat stability, experiments have been done about the effects of heating against the antigenicity of rice allergenic protein.¹⁴⁾ Antigenicity was measured using both human sera of rice allergic patients and rabbit antisera against the rice allergenic protein. Almost the same results were obtained from both experiments; no less than 60% of the allergenicity was still remained even after heating at 100°C for 60 min. This high resistance to heat treatment may allow this protein to be a food allergen.

A cDNA clone encoding the rice allergenic protein was first isolated from cDNA libraries of maturing rice seeds by screening with a ³²P-labeled synthetic oligonucleotide mixture corresponding to the N-terminal amino acid sequence of 16-kDa allergen and its nucleotide sequence was analyzed.¹⁷⁾ The deduced amino acid sequence of rice allergenic protein showed a considerable similarity to the members of cereal α -amylase/trypsin inhibitor family. In Fig. 2, the deduced amino acid sequence of rice allergenic protein is compared with three members of the family. Among them, both wheat and barely α -amylase/trypsin

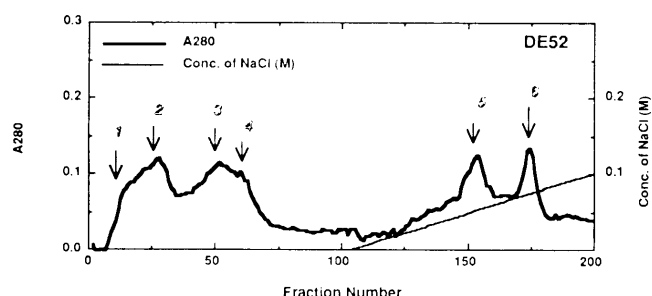


Fig. 3. Separation of Rice Salt-soluble Protein by DEAE Ion-exchange Chromatography.

Six peaks used for further studies are indicated by arrows.

inhibitors were recently identified major allergens associated with baker's asthma.^{18,19)} The castor bean storage protein that had been identified as an allergen also had an amino acid sequence similar to those of this inhibitor family.²⁰⁾ This seems to show the proteins belonging to the α -amylase/trypsin inhibitor family may be potential prominent allergens in cereal and legume seeds. It is interesting that cross-reactivity with IgE antibodies among rice, wheat, corn, Japanese millet, and Italian millet in the Poaceae family was indicated by the RAST and RAST inhibition studies.^{21,22)} Further studies should be done about this immunological cross-reactivity among these cereal grain extracts.

Furthermore, the most prominent feature of the amino acid sequence of this family is the ten conserved cysteine residues that are all considered to be present as cystine in rice allergenic protein. The high heat-stability of the rice allergenic protein seems to depend on the compact structure caused by the presence of five cystine residues.

During these studies, we found that a monoclonal and polyclonal antibodies raised against the 16-kDa allergenic protein recognized several rice proteins with molecular masses of 14 to 16 kDa and isoelectric points between 6 and 8.²³⁾ Actually we could fractionated the salt-soluble fraction of rice grain into six components having allergenic activity by ion-exchange chromatography on a column of DEAE cellulose (Fig. 3).²⁴⁾ As shown in Fig. 4, the major components in two fractions (2 and 5) had similar molecular

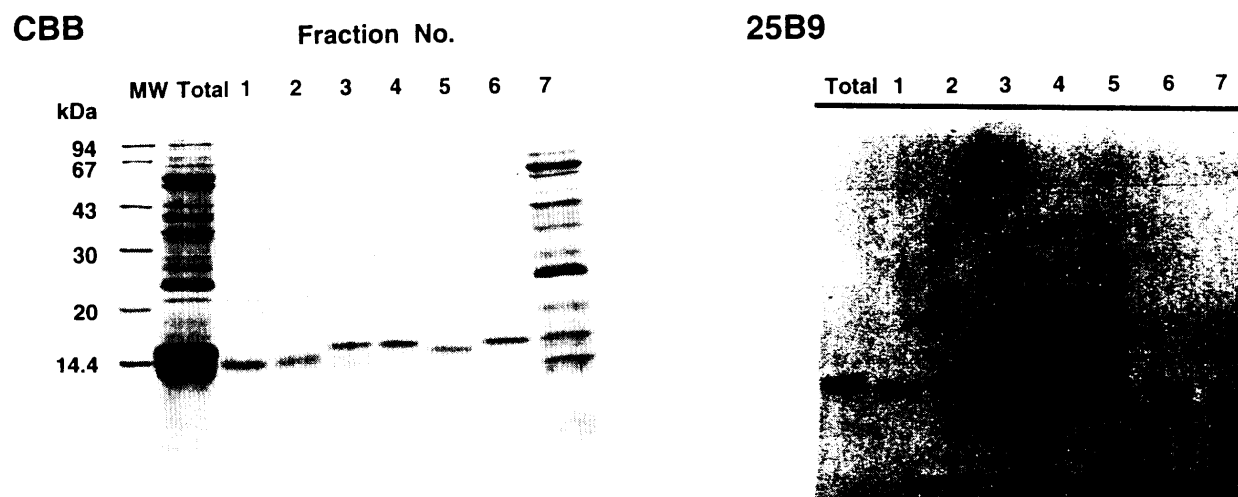


Fig. 4. SDS-PAGE and Immunoblotting of Isolated Rice Salt-soluble Proteins.

After SDS PAGE, gel sheets were stained with Coomassie brilliant blue (left) or immunoblotted with mAb 25B9 (right). In these figures, total means total salt-soluble proteins, and lane 7 shows proteins eluted with 0.5M NaCl.

masses of about 14–15.5 kDa, and the other three (3, 4, and 6) were about 16 kDa. All six proteins reacted strongly with the mAb 25B9 specific for 16-kDa rice allergen. All purified proteins inhibited human salivary α -amylase, but they inhibited neither bacterial α -amylase nor bovine trypsin. Radio allerge sorbent test (RAST), using IgE antibodies from patients allergic to rice, showed that the IgE from some patients reacted with all five proteins, but others specifically reacted with individual proteins (Fig. 5). These results suggest that the proteins responsible for rice allergy are major components with micro-heterogeneity and that human IgE can distinguish minor structural differences among the homologous proteins. The cDNA libraries were screened with a 370-bp ^{32} P-labeled *Sal*-*Eco*RI fragment of a formerly identified cDNA, RA17. More than 10 cDNA clones have been obtained.²⁵⁾ The cDNA sequencing showed that these cDNA clones all encoded homologous proteins with molecular weights of 14 to 16 kDa, which were classified into four subfamilies (Fig. 6). The N-terminal sequences of the isolated proteins suggested that proteins 3, 4, and 5 corresponded to the RA14 subfamily (Fig. 7). As summarized in the Table, the sequence identities among the seven cDNA clones sequenced were more than 80% for the nucleotide level and more than 70% for the amino acid level, suggesting that these allergens are encoded by a multigene family.

It is important to consider the localization of allergenic proteins in rice grain. For this purpose, experiment was done to study the difference of allergenic proteins content between bran and endosperms.¹⁴⁾ The amount of allergenic proteins in the endosperms was much larger than that of the bran, although total extractable protein from the endosperms was much smaller than that of the bran. A large portion of the storage proteins in the endosperms have been shown to be present in the granules called protein bodies.²⁶⁾ Since allergenic proteins can be easily extracted with a low concentration of NaCl, these proteins might not be present in the protein bodies. To ascertain this, the endosperm of maturing rice grains were homogenized with phosphate-buffered saline and fractionated by sucrose density gradient ultracentrifugation (unpublished data). All

the fractions obtained by this method were put on a SDS-polyacrylamide gel electrophoresis apparatus and examined after Western blotting using a monoclonal antibody raised against rice allergenic protein (Fig. 8). Allergenic proteins were only found in the low density fraction, but major storage proteins of rice grain were in the high density fraction. This seems to show that allergenic proteins are not present in protein bodies. Based on this finding, we constructed a novel method to remove the allergenic protein from rice grain: when ultrasonic treatment was done on the soaked rice, about 90% of the allergenic protein was removed without significant loss of other rice proteins. Although parts of the rice grain were crushed by this procedure, it might be useful for the production of hypoallergenic rice products.

3. Production of Hypoallergenic Rice

From the therapeutic view, it is essential to reduce the allergenic protein content of rice grain. Watanabe *et al.*²⁷⁾ established a novel method to produce hypoallergenic rice using a proteolytic enzyme in the presence of a surfactant at an alkaline pH. This process produced rice grains from which the major allergenic proteins were decomposed. Watanabe *et al.*²⁸⁾ further improved the color of these hypoallergenic rice grains by treatment with diluted hydrochloric acid and washing with water. The acid-treated rice grains were steamed at the surface layer to prevent breakage. Appearance of this hypoallergenic rice is almost the same as that of normal rice and its taste is good. Although this hypoallergenic rice is very useful as a substitute diet for rice allergic patients, its price is rather high owing to the use of expensive proteolytic enzyme. Production of hypoallergenic rice with a reasonable price is now expected.

Regulation of expression of specific genes by antisense RNA is a naturally occurring mechanisms in bacteria,^{29,30)} although gene regulation by this mechanism has not yet been observed in higher eukaryotes. However, the finding that antisense RNA can inhibit gene expression in natural systems led to the development of strategies to artificially regulate genes using antisense RNA. Expression of antisense

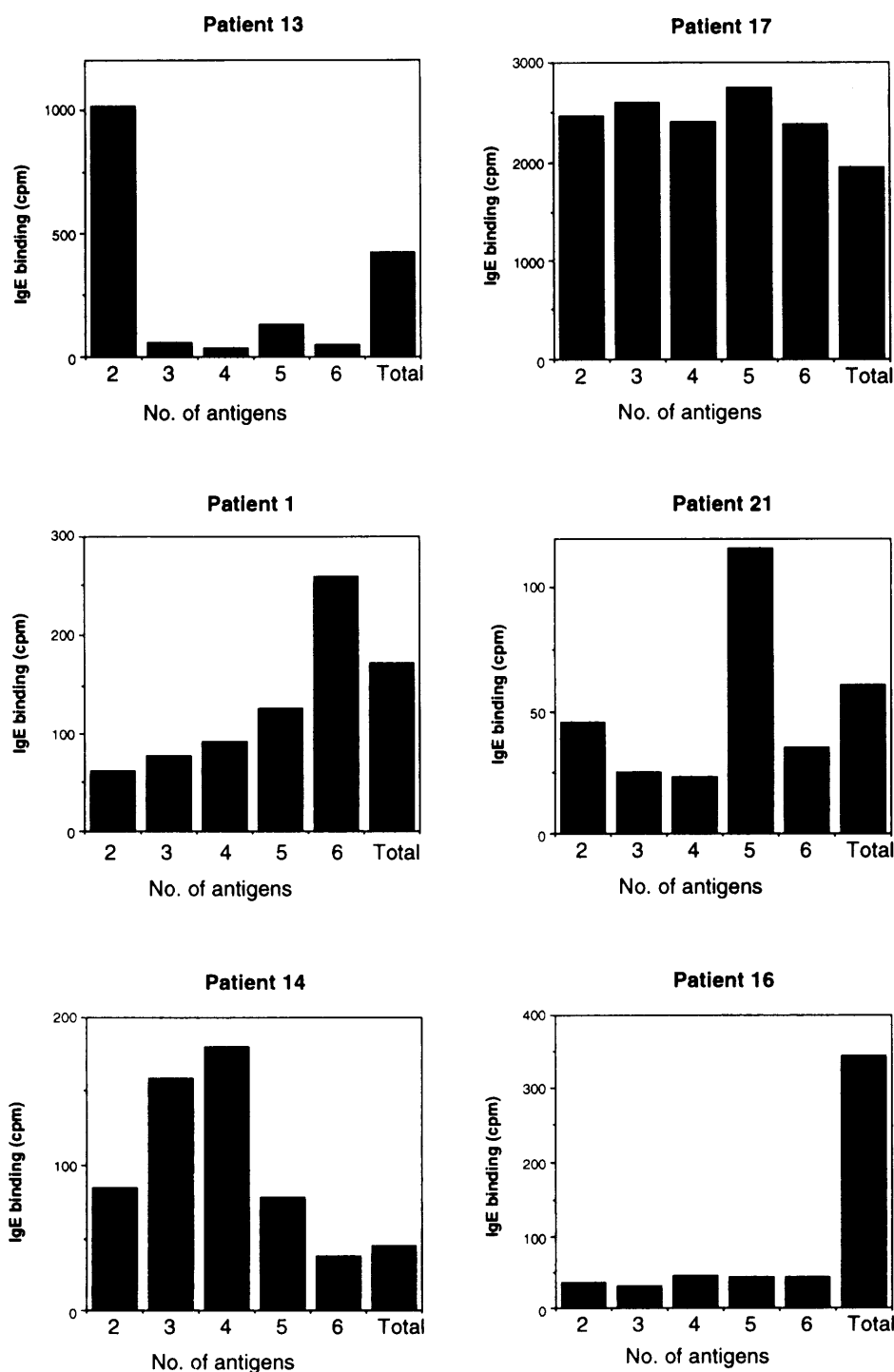


Fig. 5. RAST of Isolated Salt-soluble Proteins Using Sera from Patients Allergic to Rice.

Reaction patterns of several representatives are shown. Isolated proteins (2-6) and total salt-soluble proteins were used as antigens for RAST.

RNA to the rate-limiting enzyme in the biosynthetic pathway of ethylene inhibited the formation of ethylene and fruits ripening in tomato plants.³¹⁾ To reduce allergen contents in rice grain, this antisense RNA strategy was applied in collaboration with the Life Science Institute of Mitsui Toatsu Chemicals, Inc.

A part of the genomic sequence encoding rice allergenic protein in the antisense direction was constructed between the promoter of the rice allergenic protein gene³²⁾ and the rice waxy terminator. These antisense genes were introduced into rice protoplasts with the hygromycin phosphotransfer-

ase gene as the selection marker by an electroporation method³³⁾ and several fertile plants were regenerated. Introduction of the antisense genes into the transgenic rice plants was confirmed by Southern blot analysis using a cDNA of allergenic protein as a probe. Reduction of allergenic protein content in the seed of transgenic rice plants was measured by immunoblot analysis using a mouse monoclonal antibody raised against the rice allergenic protein. No large differences were observed in the amount of major seed proteins, glutelin and prolamin, between the transgenic and non-transgenic rices. On the other hand, the

		10	20	30	40	50	
RA14	1	MASNKVVFS	LLLIIVSVLA	AT-TRMADHH	KDQVVYSLGE	RCQPQMGYPM	50
RA14B	1	MASNKVVFS	LLLIIVSVLA	AT-GPMADHH	KDQVVYSLGE	RCQPQMGYPM	50
RA14C	1	GTR-----	LLLIIVSVLA	AT-RRMADHH	KDQVVYSLGE	RCQPQMGYPM	50
RA14d	1	-----	-----	-----	-----	-----	50
RA14e	1	R-----	-----	-----	-----	-----	50
RA14f	1	-----	-----	-----	-----	-----	50
RA14g	1	-----	-----	-----	-----	-----	50
RA5	1	MASNKVVFS	LLLAUVSVLA	ATATMAEYHH	QDQVVYTRAR	-CQPQMGYPM	50
RA5B	1	MASNKVVFS	LLLAUVSVLA	ATATMAEYHH	QDQVVYTPAP	LCQPQMGYPM	50
RA16	1	MASNKVVIPA	LLVVVSVLA	ATTIMAD-HH	QDQVVYTPGQ	LCQPQGIYPT	50
RA17	1	MASNKVVFS	LLLVVLSVLA	AAMATMADHH	Q---VYSPGE	QCRPGISYPT	50
		60	70	80	90	100	
RA14	51	YSLPRCRAVV	KRQCVGTRSP	GA-VDEQLAQ	DCCRQLAAVD	DSWCRCALN	100
RA14B	51	YSLPRCRAVV	KRQCVATAHP	AARGNEQLRQ	DCCRQLAAVD	DSWCRCALN	100
RA14C	51	YSLPRCRAVV	KRQCVGHGAP	GGA-VDEQLRQ	DCCRQLAAVD	DSWCRCALN	100
RA14d	51	-----RHEV	KRQCVATTHP	AAPGTEQLRQ	DCCRQLAAVD	DSWCRCALN	100
RA14e	51	-----VV	KRQCVATRRT	GGA-DEQLRQ	DCCRQLAAVD	DSWCRCALN	100
RA14f	51	--FGT-R-FR	QRQCVGPRRT	RRRWTOQLRQ	DCCRQLAAVD	DSWCRCALN	100
RA14g	51	-----CRALA	K-QC-----P	GGA-VDEQVRQ	DCCRQLAAVD	DSWCRCALN	100
RA5	51	YSLPRCRAVV	KRQCVGTRSP	SAAAQVRR	DCCRQLAAVD	DSWCRCALN	100
RA5B	51	YSLPRCRAVV	KRQCVGTRSP	TAAAQVRR	DCCRQLAAVD	DSWCRCALN	100
RA16	51	YSLPRCRAVV	KRQCVGTRSP	TL--DEQVR	GCCRQLAGID	SSWCRCALN	100
RA17	51	YSLPRCRAVV	KRQCVGTRSP	ASAQVRR	DCCRQLAAVD	DSWCRCALN	100
		110	120	130	140	150	
RA14	101	HMVGGIYREL	GATDVGHFMA	EVFPGCRRGD	LERAAASLPA	FCNVDPNGT	150
RA14B	101	HMVGGIYREL	GATDVGHFMA	EVFPGCRRGD	LERAAASLPA	FCNVDPNGT	150
RA14C	101	HMVGGIYREL	GATDVGHFMA	EVFPGCRRGD	LERAAASLPA	FCNVDPNGT	150
RA14d	101	HMVGGIYREL	GATDVGHFMA	EVFPGCRRGD	LERAAASLPA	FCNVDPNGT	150
RA14e	101	HMVGGIYREL	GATDVGHFMA	EVFPGCRRGD	LERAAASLPA	FCNVDPNGT	150
RA14f	101	HMVGGIYREL	GATDVGHFMA	EVFPGCRRGD	LERAAASLPA	FCNVDPNGT	150
RA14g	101	HMVGGIYREL	GATDVGHFMA	EVFPGCRRGD	LERAAASLPA	FCNVDPNGT	150
RA5	101	HMLGGIYREL	GAPDVGHFMS	EVFPGCRRGD	LERAAASLPA	FCNVDPNGT	150
RA5B	101	HMLGGIYREL	GAPDVGHFMS	EVFPGCRRGD	LERAAASLPA	FCNVDPNGT	150
RA16	101	HMLGGIYREL	GAPDVGHFMS	EVFPGCRRGD	LERAAASLPA	FCNVDPNGT	150
RA17	101	HMLGGIYREL	GAPDVGHFMS	EVFPGCRRGD	LERAAASLPA	FCNVDPNGT	150
		160	170	180	190	200	
RA14	151	GGVCYWLGY-	PRTPTIGH..				200
RA14B	151	GGVCYWLGY-	PRTPTIGH..				200
RA14C	151	GGVCYWLGYT	PRTPTIGH..				200
RA14d	151	GGVCYWLGY-	PRTPTIGH..				200
RA14e	151	GGVCYWLGY-	PRTPTIGH..				200
RA14f	151	GGVCYWLGY-	PRTPTIGH..				200
RA14g	151	GGVCYWLGY-	PRTPTIGH..				200
RA5	151	GGVCYWLGY-	PRTPTIGH..				200
RA5B	151	GGVCYWLGY-	PRTPTIGH..				200
RA16	151	GGVCYWLGY-	PRTPTIGH..				200
RA17	151	GGVCYWLGY-	PRTPTIGH..				200

Fig. 6. Comparison of Deduced Amino Acid Sequences among Rice Allergenic Protein cDNA Clones. Identical amino acids are highlighted. Dashes are introduced for maximum alignment.

	1	10
Peaks 1, 2	H ₂ N-DAH P G Q V V Y T P Q L X A A G-	
Peaks 3~5	H ₂ N-DH H K D Q V V Y S L G E R X Q P G M G-	
Peak 6	H ₂ N-DH H -- Q - V Y S P G E Q R P G I S -	

Fig. 7. N-Terminal Amino Acid Sequences of Isolated Salt-soluble Proteins.

Unidentified amino acid residues are shown by X. Identical amino acid residues (at least three of five proteins) are boxed.

amount of 16-kDa allergenic protein detected by the monoclonal antibody was significantly lower in several transgenic clones. The degree of the reduction of allergenic protein, however, fluctuated between seeds to seeds even in the single transformant.

Since this phenomenon seems to be dependent on the low expression of antisense RNA in transgenic rice seeds, some improvements were done in making antisense allergen genes using some other gene promoters of rice-seed specific proteins. Two kinds of antisense allergen genes were finally constructed; one contains a tandem repeat of two antisense 16-kDa allergen genes with the glutelin or prolamin promoter, and the other one two inverted repeats of 16-kDa

Table Percentage Identities of RA cDNA-Clones at the Nucleotide (DNA) and Deduced Amino Acid (AA) Levels of the Mature Protein-encoding Region

cDNA	Level	RA14B	RA14C	RA5	RA5B	RA16	RA17
RA14	DNA	96.7	97.1	85.9	81.0	75.6	83.6
	AA	91.4	95.0	78.3	72.7	68.7	77.1
RA14B	DNA	—	97.3	81.2	81.3	75.4	83.0
	AA	—	94.3	76.5	73.6	69.5	76.4
RA14C	DNA	—	—	81.1	79.8	76.6	84.3
	AA	—	—	75.5	76.6	70.9	77.1
RA5	DNA	—	—	—	95.1	82.1	82.1
	AA	—	—	—	93.3	71.8	72.3
RA5B	DNA	—	—	—	—	83.0	77.9
	AA	—	—	—	—	77.6	69.3
RA16	DNA	—	—	—	—	—	73.5
	AA	—	—	—	—	—	67.7

allergen genes with the prolamin or starch blanching enzyme promoter (Fig. 9). By this improvement the degree of the reduction of allergenic protein increased greatly. This tendency of the reduction of allergenic protein was further

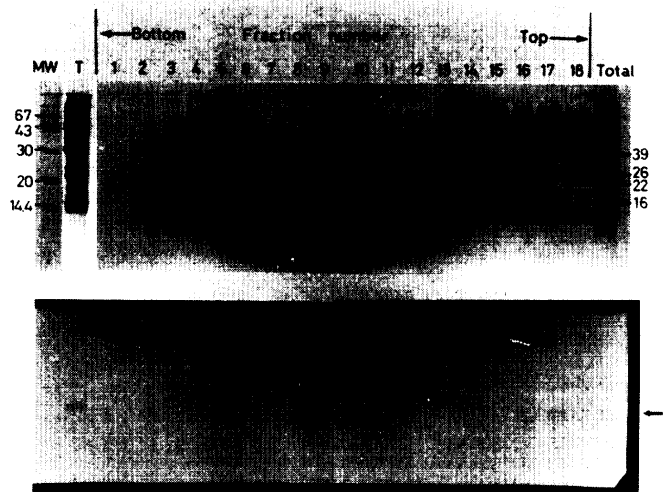


Fig. 8. SDS-PAGE and Immunoblotting Analyses of Rice Allergenic Protein in the Endosperms Fractions Separated by Sucrose Density Gradient Ultracentrifugation. Arrow shows the position of rice allergenic protein.

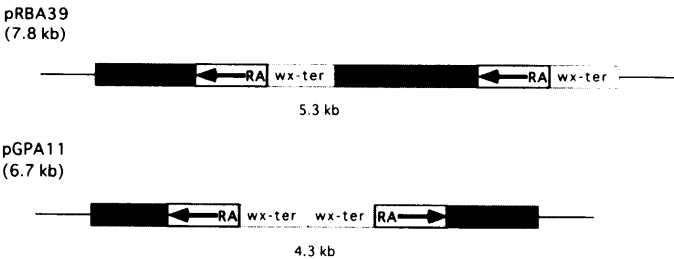


Fig. 9. Construction of Plasmids for the Antisense Rice Allergen Genes. RA-pro, rice allergen promoter; RA, rice allergen; wx-ter, waxy terminator; BE-pro, starch blanching enzyme promoter; PR-pro, prolamin promoter; GL-pro, glutelin promoter.

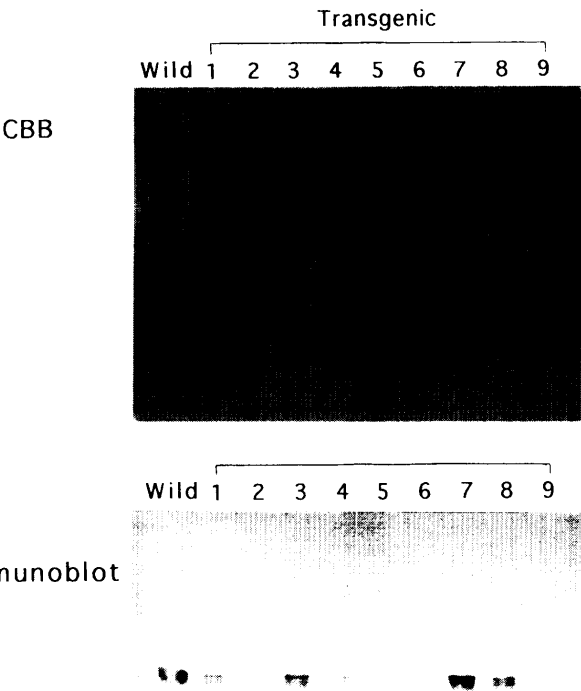


Fig. 10. SDS-PAGE and Immunoblotting of Rice Grains Obtained from Transgenic Rice Plants (The First Generation). Wild represents non-transgenic control.

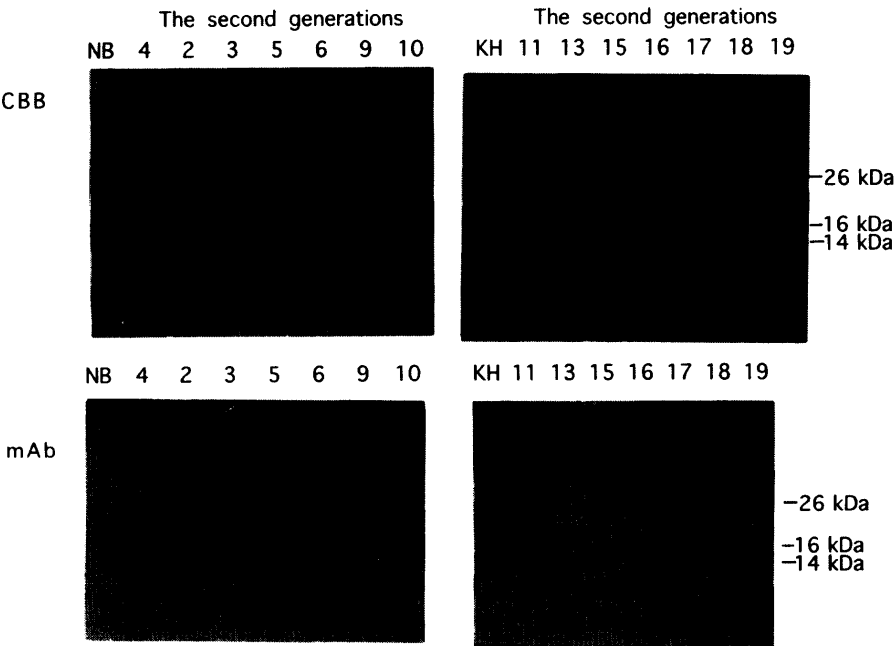


Fig. 11. SDS-PAGE and Immunoblotting of Rice Grains Obtained from Transgenic Rice Plants (The Second Generation). Both NB and KH represent non-transgenic control strains.

increased in the second generation of transgenic rice plants (Figs. 10 and 11).³⁴⁾

The rice allergen content of seeds from the transgenic rice plants were also estimated by the competitive ELISA (Enzyme-linked immunosorbent assay) using the monoclonal antibody. Isolated rice allergenic protein was used as a standard. The allergen content of non-transgenic rice (Kinuhikari) was about 300 micrograms per seed, while the transgenic rices with antisense allergen genes were 60–70 micrograms per seed, indicating that the rice allergen contents of the transgenic rice seeds were significantly lower than those of the non-transgenic control.

Thus, the rice allergen synthesis in maturing rice seeds could be repressed effectively by the antisense RNA method, even though the allergenic proteins are products of a multigene family. However, it is still uncertain whether such hypoallergenic rice seeds obtained from the transgenic rice plants are tolerable for the patients allergic to rice, because even a small amount of residual allergens might elicit an allergic reaction in the patients. Furthermore, some patients could be allergic to some other components of rice seed proteins.³⁵⁾ Further studies on improvement of the antisense genes and identification of the other allergenic components are in progress.

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