Reduction of 14–16 kDa allergenic proteins in transgenic rice plants by antisense gene

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Abstract An antisense gene strategy was applied to suppress the 14–16 kDa allergen gene expression in maturing rice seeds. Gene constructs producing antisense RNAs of the 16 kDa allergen under the control of some rice seed-specific promoters were introduced into rice by electroporation. Immunoblot and RNA blot analyses of the seeds from the transgenic rice plants using the allergen-specific monoclonal antibody and a sequence-specific antisense RNA probe demonstrated that the 14–16 kDa allergen proteins and their transcripts of the seeds from several transgenic lines were present in much lower in amounts than those of the seeds from parental wild-type rice. The high levels of reduction observed were stably inherited in at least three generations.

Key words: Rice allergen; Transgenic rice; Antisense RNA

1. Introduction

The ingestion or inhalation of cereal flours including rice has been reported to provoke allergic diseases such as asthma and eczema [1,2]. Several studies on allergic reaction to rice have indicated that patients' sera contain immunoglobulin E (IgE) reacting to rice proteins, and that soluble proteins, albumins and globulins have a high degree of allergenic activity [3,4]. The authors have isolated a rice seed protein of about 16 kDa with reactivity for IgE of several rice-allergic patients [5], and the 16 kDa protein was later identified as one of the major allergens in rice seed [6]. Monoclonal and polyclonal antibodies raised against the 16 kDa allergen were shown to cross-react with the 14–16 kDa allergens [7]. More than 10 homologous cDNA clones encoding the 14–16 kDa allergens have been obtained so far [8], indicating that the 14–16 kDa allergens are multigene products. DNA sequencing revealed that these cDNA clones all encoded proteins similar to wheat α-amylase inhibitor, barley trypsin inhibitor and the castor bean storage protein [9], which had been identified as major allergenic proteins associated with baker's asthma [10,11].

Antisense RNA with a complementary sequence of mRNA has been used experimentally to inhibit gene expression in bacteria, yeast, plant and animal cells [12], and the antisense strategy has also been reported to be practically applicable to transgenic crop plants, to which genes that produce antisense RNA are introduced to suppress the gene expression [13,14]. In the present study, the antisense genes for the 16 kDa allergen were introduced into rice by electroporation, and specific suppression of the 14–16 kDa allergen gene expression, resulting in the reduction of corresponding allergenic proteins in the mature seeds, was examined for regenerated transgenic rice plants and their progeny. The results show that antisense RNA markedly reduced the mRNA and protein contents of the 14–16 kDa allergens in the transgenic rice seeds.

2. Materials and methods

2.1. Plasmid construction

To express antisense allergen RNA in transgenic rice seeds, a 0.55 kb cDNa for 16 kDa allergen (RA17) [9] and the promoters of four rice-seed specific genes, the rice allergen gene (RAG1) [15], branching enzyme I gene (BE), prolamin gene and glutelin gene, were used. The 1.0 kb rice allergen promoter and the 0.8 kb glutelin promoter [16] were PCR-amplified by using the genomic clone, pRAG1 [15], and the rice genomic DNA prepared from rice leaves as a template, respectively. The 0.7 kb prolamin promoter and the 1.6 kb BE promoter were excised by *Eco*RI-*Sma*I digestion from a cloned prolamin gene, PG5a, [17] and by *Bam*HI-*Sma*I digestion from a cloned BE gene [18], respectively. The 0.67 kb *Bgl*II-*Sca*I fragment, containing the terminator region was prepared from a cloned rice waxy gene [19,20]. Two plasmids, pRBA39 and pGPA11, containing two antisense allergen genes were finally constructed (Fig. 1). The former plasmid contains a tandem repeat of two antisense RA17 genes with the GL or PR promoter, and the latter two inverted repeats of RA17 genes with the RA17 or BE promoter.

The plasmid pHPT2 containing the hygromycin phosphotransferase (HPT) gene linked to the cauliflower mosaic virus (CaMV) 35S promoter was constructed as follows. The HPT coding region was prepared from plasmid pHph+1 (Böehringer Mannheim) digested with *Bam*HI. The HPT fragment was inserted into the *Bam*HI site of pSLG2, which contains the CaMV 35S promoter and β-glucuronidase gene [21]. The resulting plasmid was digested with *Sma*I and *Sac*I, blunt-ended by T4 DNA polymerase and self-ligated to eliminate the β-glucuronidase gene.

2.2. Protoplast culture and electroporation

The plasmids (pRBA39 and pGPA11) containing the antisense allergen genes, concomitantly with the plasmid (pHPT2) containing the HPT gene, were subjected to electroporation. Protoplasts were isolated from suspension cells of rice (*Oryza sativa* L. cv 'Kinuhikari'). The purified protoplasts were suspended at a population density of 2×10^6/ml in electroporation buffer, 20 μg of plasmid DNA was added, and electroporation was then performed as described in [22]. Electroporated protoplasts were cultured and selected with hygromycin [22]. Rice plants were independently regenerated from hygromycin-resistant calli, resulting in generation of transgenic rice plants containing the antisense RA17 gene.

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Abbreviations: CBB, Coomassie brilliant blue; PVDF, polyvinylidene difluoride; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PR, prolamin; GL, glutelin; BE, branching enzyme; CaMV, cauliflower mosaic virus; HPT, hygromycin phosphotransferase.

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2.3. DNA and RNA blot analyses

Plant DNA was extracted from leaves and purified by CsCl gradient centrifugation. The purified DNA was digested with restriction enzymes, separated by electrophoresis on 0.9% agarose gels and transferred onto nylon membranes. Hybridization was carried out using a non-radioactive, DIG-DNA labeling and detection kit (Böehringer Mannheim).

Total RNAs for RNA blotting were extracted from maturing rice seeds at 14 days after flowering using Extract-A-Plant RNA Isolation kit (Clontech). A *Bam*HI-*Kpn*I fragment containing the full-length 16 kDa allergen cDNa was cloned into pBluescriptII KS+ or pBluescriptII SK+ to produce transcription vectors pKSRA or pSKRA, respectively. Labeled single-stranded 16 kDa allergen sense or antisense RNA as a probe was synthesized in vitro using a DIG RNA-labeling kit, and the labeled probes were detected using a DIG Luminescence detection kit (Böehringer Mannheim).

2.4. SDS-PAGE and immunoblotting

Each of the individual dehulled grains was dipped in 200 μl of phosphate-buffered saline (PBS) at 4°C for 2 h, and crushed with forceps. Albumin and globulin proteins were then extracted by ultrasonication at 4°C for 10 min several times. The 14–16 kDa allergens can be extracted almost completely by this ultrasonic treatment (unpublished data). After centrifugation the supernatant containing about 0.2–0.5 mg/ml protein was used directly for SDS-PAGE [23] and immunoblotting [7,24]. 10 μl of the PBS extract was treated with SDS-PAGE sample buffer and applied to SDS-PAGE with 15% acrylamide gel. After electrophoresis, proteins were electrophoretically transferred onto polyvinylidene diffuoride (PVDF) membrane, then rice allergen was detected by immunostaining of the membrane with the monoclonal antibody, 25B9, specific for the 14–16 kDa allergens, and the peroxidase-labeled anti-mouse IgG [7].

2.5. Competitive ELISA

The allergen content of seeds from the transgenic rice plants was estimated by competitive ELISA [25] using the monoclonal antibody, 25B9 [7]. Isolated 16 kDa allergen was used as the standard. ELISA plates were coated with 50 μl of the isolated 16 kDa allergen (10 μg/ml in PBS), and blocked with 3% bovine serum albumin in PBS. 50 μl of an appropriately diluted monoclonal antibody (25B9) was mixed with 100 μl of definite concentrations of 16 kDa allergen solution or serially diluted rice seed extract, incubated overnight at 4°C, and then added to the wells of antigen-coated ELISA plates. The antibody bound to the antigen coated on the plate was detected by using peroxidase-labeled anti-mouse IgG. The allergen content was calculated from inhibition curves based on the standard inhibition curve of the isolated 16 kDa allergen.

3. Results

Rice plants were transformed with the plasmids pRBA39 and pGPA11 to produce transgenic rice that express antisense RA17 gene in a seed- and stage-specific manner. About 2000 hygromycin-resistant colonies were transferred onto the plant regeneration medium. 120 plants were selected based on the results of a PCR analysis using specific primers for the antisense constructs and DNA blot analysis, in which an additional band with an expected size of the antisense gene was observed (data not shown). 11 lines among them were diploid with fertility. Several lines were selected by immunoblot screening of their seed allergen, and the second and third generations of progeny for these transgenic lines were obtained.

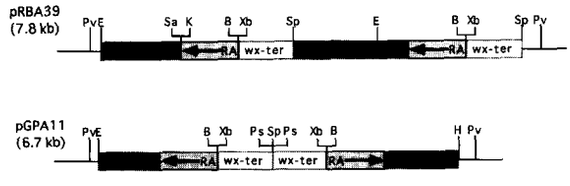

Fig. 1. Construction of plasmids for the antisense RA genes. The promoters (pro) of 16 kDa allergen, RA17, (RA), prolamin (PR) and glutelin (GL) and coding sequence for RA17 were cloned by PCR. The rice blanching enzyme (BE) gene [17] was kindly donated by Dr. Kawasaki (Mitsui Plant Biotechnology Research Institute). Two expression plasmids were constructed by insertion of these DNA fragments and the RA17 cDNA into pUWt containing the waxy gene terminator (wx-ter). Restriction enzyme sites: Pv, *Pvu*II; E, *Eco*RI; Sa; *Sac*I; K; *Kpn*I; B, *Bam*HI; Xb, *Xba*I; Sp, *Sph*I; Ps, *Pst*I; H, *Hin*dIII.

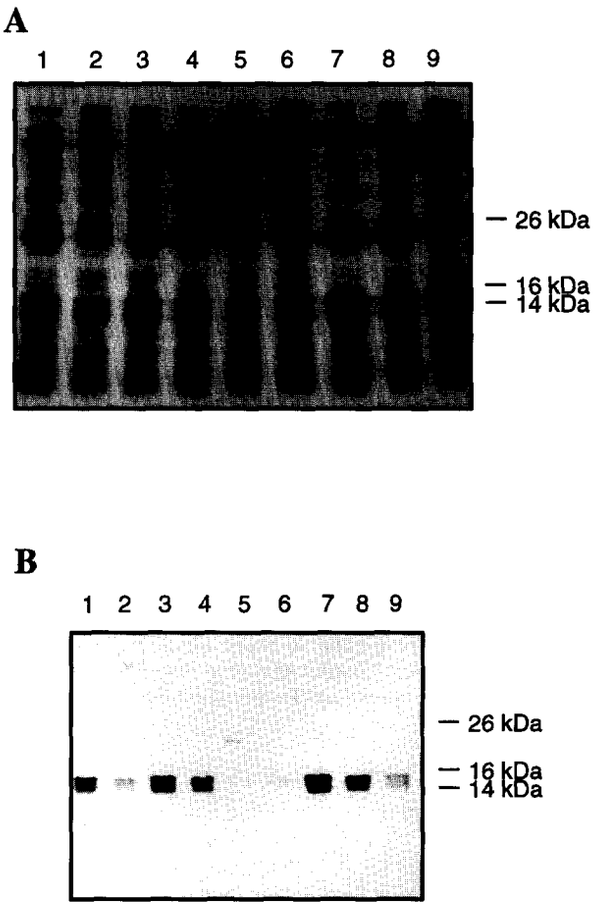

Fig. 2. SDS-PAGE and immunoblot analyses of selfed seeds from a transgenic rice plant. The PBS-extracted proteins of nine seeds (nos. 1–9) randomly collected from a transgenic rice plant were analyzed by SDS-PAGE followed by CBB staining (A), and by immunoblotting using the allergen-specific monoclonal antibody, 25B9 (B).

Selfed seeds obtained from transgenic rice which had the expected size of the antisense gene were analyzed for allergenic protein content by immunoblotting using the specific monoclonal antibody. Total proteins extracted with PBS from several seeds were analyzed by SDS-PAGE. A representative result is shown in Fig. 2. No large differences were observed in the CBB-stained band intensity of major PBS-soluble proteins, albumins and globulins, among seeds from the transgenic and non-transgenic rice forms (Fig. 2A). On the other hand, the band intensity not only of the 16 kDa allergen but also of other 14–16 kDa allergens specifically stained with the monoclonal antibody was remarkably faint or below detectable level in several seeds of each transgenic clone, although the allergen content of a seed appeared to differ largely among seeds even from a transgenic rice plant (Fig.

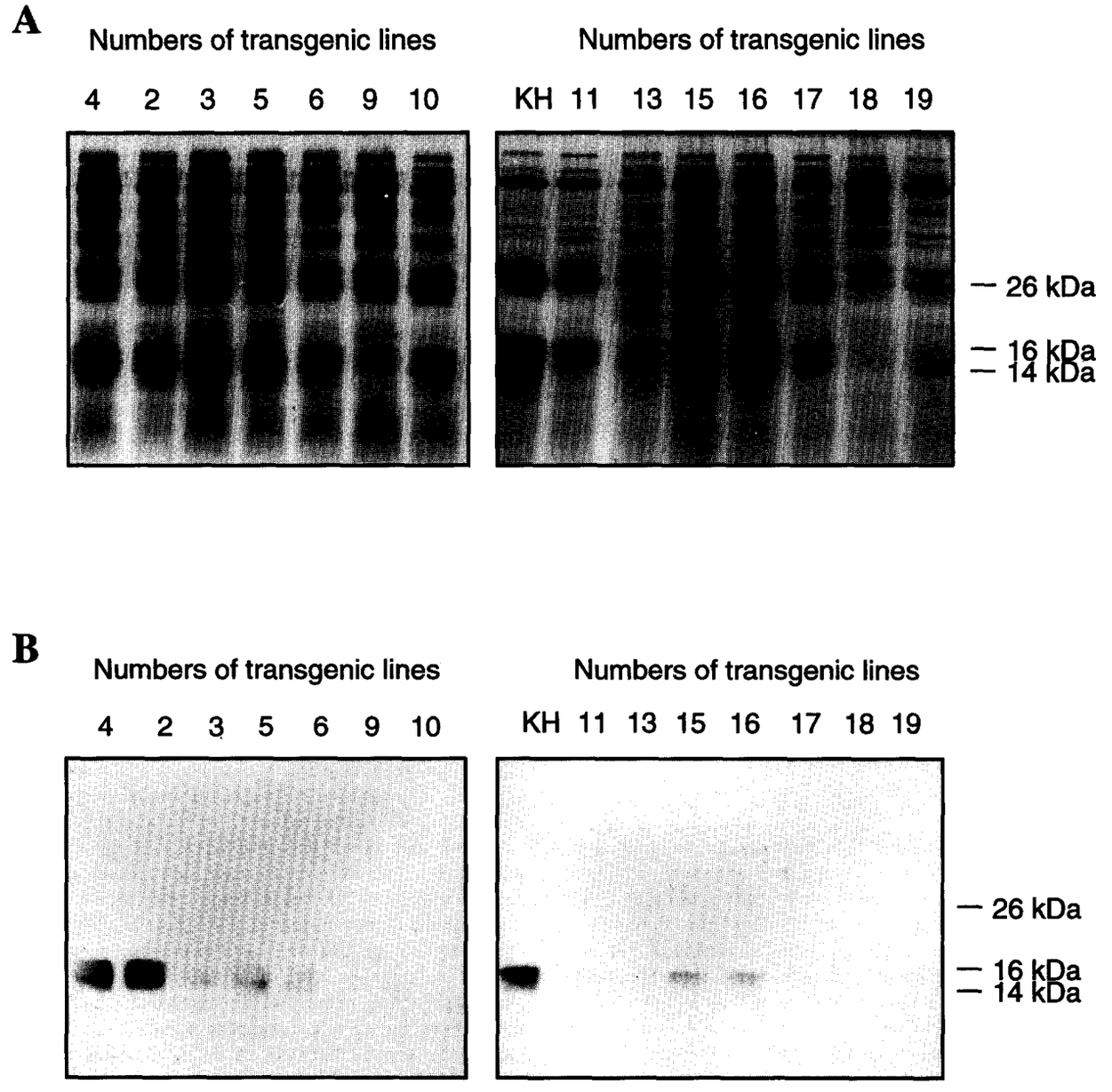

Fig. 3. SDS-PAGE and immunoblot analyses of selfed seeds from the progeny of transgenic rice plants. The PBS-extracted proteins of one seed randomly collected from 14 lines of progeny plants were analyzed by SDS-PAGE followed by CBB-staining (A), and by immunoblotting using the allergen-specific monoclonal antibody, 25B9 (B). Line No. 4 is the progeny of a transgenic plant in which no effective reduction of the allergen content had been observed. A seed of the parental non-transgenic rice, cv. Kinuhikari (KH) was also analyzed as a control.

2B). These differences should be due to heterozygosity in the transgene of the regenerated rice plants.

The transmission of the antisense genes to the progeny was examined for several transgenic rice plants. DNA blot analysis of leaves of the second generation obtained from the selfed seeds of the first generation was performed to detect the antisense genes introduced. The results revealed that the introduced antisense gene was stably transmitted to the progeny (data not shown). Immunoblot analysis was also carried out for the estimation of 14–16 kDa allergens in the selfed seeds from the progeny plants. As shown in Fig. 3B, only slight staining, if any, was observed for the bands with the monoclonal antibody for the seed proteins of most second-generation lines, indicating that strong repression of allergen synthesis was induced by the antisense genes in the progeny plants. There were slight differences between the wild-type and transgenic rice species in the CBB-stained band intensity of some PBS-extracted proteins other than 14–16 kDa allergens (Fig. 3A).

The rice allergen content of seeds from the transgenic rice plants was also estimated by the competitive ELISA using the monoclonal antibody (data not shown). Isolated 16 kDa allergen was used as standard. The allergen content of non-transgenic rice (cv. Kinuhikari) was about 300 μg per seed (20 mg), whereas the transgenic rice species with antisense allergen genes possessed 60–70 μg per seed, suggesting that the 14–16 kDa allergen content in rice seeds were markedly reduced by the antisense RNA produced in transgenic rice plants.

The accumulation of sense and antisense allergen transcripts in the maturing seeds of transgenic and non-transgenic

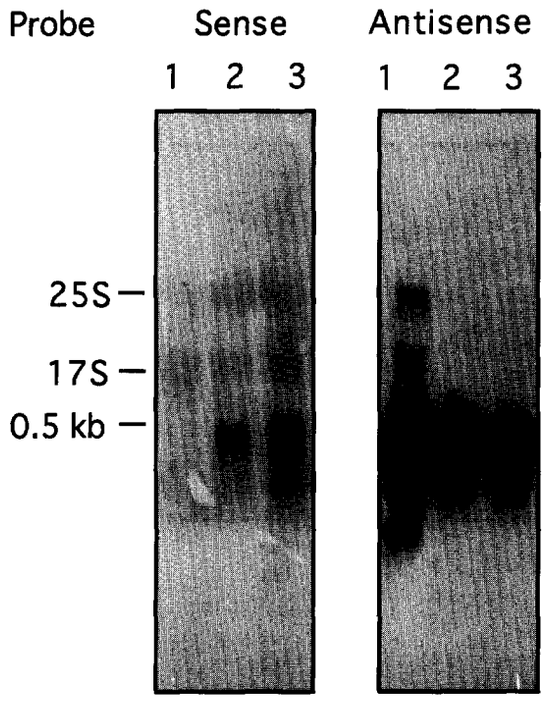

Fig. 4. RNA blot analysis for the expression of the sense and antisense allergen genes in transgenic rice plants. Each lane contains 10 μg of total RNA prepared from each of a mixture of five developing seeds (14 days after flowering) of the parental non-transgenic rice, cv. Kinuhikari (lane 1) and the transgenic lines (lanes 2,3). The sense and antisense sequence-specific probes were used for the RNA blot analysis.

rice plants were estimated by RNA blotting analysis using the strand-specific probes (representative data are shown in Fig. 4). Total RNA was extracted from maturing seeds of one transgenic line and its progeny at 14 days after flowering. The antisense 14–16 kDa allergen gene transcripts were detected in seeds of the transgenic lines, but not in the parental non-transgenic rice. A large amount of sense 14–16 kDa allergen gene transcripts was detected in seeds of the non-transgenic rice, whereas the level of the sense gene transcripts was much lower in the transgenic lines with the antisense transgenes. The amount of sense gene transcript in the transgenic lines was about 20–30% of that of non-transgenic rice as measured with a photon counter (Argus-50, Hamamatsu Photonics), indicating that accumulation of the sense 14–16 kDa allergen gene transcripts was markedly, but not completely, repressed by the antisense transgene constructed with a cDNA clone RA17.

4. Discussion

The accumulation of 14–16 kDa allergenic proteins in matured rice seeds was markedly reduced by introducing the antisense gene for the 16 kDa allergen into rice plants, although the level of repression was rather lower than those of some other antisense gene systems reported previously on several transgenic plants [26–28]. The insufficient repression of the allergen gene expression might be due to the multigenicity of the 14–16 kDa allergen genes or due to the properties of gene promoters used for antisense gene expression as discussed below.

The 16 kDa allergenic protein was firstly isolated from rice seeds based on the reactivity with specific IgE antibodies from the sera of patients allergic to rice ingestion [5]. The subsequent screening of the immunologically cross-reacting proteins has demonstrated that most of the 14–16 kDa proteins in seed salt-soluble proteins are cross-reactive with the monoclonal antibody, 25B9 [7], and also reactive with the patients' serum IgE (unpublished data). The sequence data obtained so far on the cDNAs encoding the 14–16 kDa proteins showed that the 14–16 kDa allergen genes belong to a multigene family [8,15]. These results indicate that to produce 'hypo-allergenic rice' the expression of all genes encoding these homologous proteins must be suppressed. Since a 3'-end proximal region of the cDNAs is highly conserved among all the clones isolated [8], the antisense RNA method was applied to suppress their expression in maturing rice seeds in spite of such multigenicity of the allergen genes. The results obtained in the present study suggest that the antisense RNA derived from a representative cDNA clone, RA17 effectively represses the accumulation for the 14–16 kDa allergen gene transcripts, resulting in the marked but not complete reduction of allergenic protein content in the transgenic rice seeds.

Viral gene promoters such as the CaMV 35S promoter have often been used for the expression of foreign genes in transgenic plants. In the present study, however, the rice-derived gene promoters including the allergen gene itself were used for the antisense gene expression in transgenic rice plants, since rice grains harvested from transgenic rice plants containing only rice-derived genes are most suitable for transgenic food from a view point of public acceptance. The 5'-flanking region of RAG1 [15] was used at first as a promoter to transcribe the antisense gene, but the reduction of the allergenic proteins in transgenic rice plants was insufficient and unstable (unpublished data). Therefore, to increase the antisense gene expression in transgenic rice seeds, some other promoters of rice-seed specific genes, prolamin [17], glutelin [16] and starch branching enzyme [18] were used in combination with RA17 gene promoter. The level of expression of these genes was markedly decreased but still not complete even when using these promoters. Such incomplete repression might be explained by slight differences in the nucleotide sequence between the antisense RNA and some allergen gene transcripts and/or in the expression stages during seed maturation between the allergen genes and the other seed-specific genes used for the antisense gene promoters.

Thus, the rice allergen synthesis in maturing rice seeds could be suppressed by the antisense gene method, even though the allergenic proteins are products of a multigene family. However, it is still uncertain whether such 'hypo-allergenic rice' seeds obtained from the transgenic rice plants are tolerable to patients allergic to rice, because even a small amount of residual allergens could elicit an allergic reaction in patients. Furthermore, some patients have been reported to be allergic to some other components of rice seed proteins [6]. Further studies on the improvement of the antisense allergen genes and identification of the other allergenic proteins and their genes are in progress.

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