Effect of an Antisense Sequence on Rice Allergen Genes Comprising a Multigene Family

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An antisense gene strategy was used to suppress the expression of rice allergen (RA) genes that comprise a multigene family. We produced transgenic rice plants harboring antisense genes that consisted of cDNA for a major RA driven by promoters of the RA, rice starch branching enzyme, rice prolamine and rice glutelin genes, which are specifically expressed in developing seeds. Among more than 100 transformants, the RA content in some transformants was reduced to no more than 20% of that in non-transformant. However, an allergen-free transformant was not obtained. Since the transcribed antisense mRNAs are not fully used for suppression of the RA genes in transformants, we examined here the possibility of a spatial or temporal difference in the expression of the introduced antisense and endogenous RA genes. However, we found no remarkable difference in the place or timing of their expression. We also examined the effect of antisense genes on each of the genes in the RA family in transformants by two-dimensional electrophoresis and immunoblotting. The results showed that the contents of the major RA and other RAs with nucleotide sequences that are highly homologous to the antisense sequence were markedly lowered, while the contents of other RAs with sequences showing relatively low homology to the major RA were hardly affected. These results suggest that the effect of the antisense gene on the RA gene family depends on the homology between the antisense sequence used and the target sequence.

Key Words: antisense RNA, rice allergen, multigene family.

Introduction

Antisense RNA is a naturally occurring mechanism for controlling gene expression in bacteria (Mizuno et al. 1984) and is used to control the expression of eukaryotic genes. In plants, antisense RNA has been shown to effectively inhibit the activity of chalcone synthase (van der Krol et al. 1988), polygalacturonase (Sheehy et al. 1988, Smith et al. 1988), granule-bound starch synthase (Visser et al. 1991, Shimada et al. 1993) and so on. However, there have been few reports on the suppressing effect of an antisense sequence on target genes that comprise a multigene family. An albumin of about 16 kDa has been identified as a rice major allergenic protein (RA) based on the results of a radioallergosorbent test (RAST) and immunoblotting analysis using sera from rice allergic patients (Matsuda et al. 1988, Urisu et al. 1991), and cDNAs for the major RA and its homologues have been isolated (Izumi et al. 1992, Adachi et al. 1993). We previously reported that the introduction of the antisense gene for the major RA into rice resulted in an 80% reduction in RA content in seed grains from transgenic rice (Tada et al. 1996). However, it is still uncertain whether such incomplete repression might be due to differences in the place or timing of the expression of the antisense and target genes or to the low degree of homology between their sequences.

Therefore, we investigated the spatial distribution and accumulation of RAs in seed grains from transformants and non-transformant by immunoblotting.

Materials and Methods

Plant materials and antisense RA gene constructs

Rice (Oryza sativa) varieties Kinuhikari and Nipponbare were used for transformation.

To produce hypo-allergenic rice, we constructed several antisense constructs using the major RA cDNA. Since allergenic proteins are produced in immature seeds, the antisense RA genes were expected to be expressed in this stage. Two plasmids, pRBA39 and pGRA11, each of which contains two antisense allergen gene units, were constructed as described previously (Tada et al. 1996). Plasmids pRA1, pBRA7, pGRA19 and pPRA13, which consist of the cDNA for the major RA driven by the 16 kDa RA promoter (Adachi et al. 1993), the rice branching enzyme I (BE) promoter (Kawasaki et al. 1993), the rice glutelin promoter (Nakase et al. 1996) and the rice prolamine promoter (Matsuda et al. unpublished), respectively, were the intermediate plasmids...
used for constructing pRBA39 and pGPA11. Construction of pHPT2, which contains a hygromycin phosphotransferase gene driven by the CaMV35S promoter, has also been described previously (Tada et al. 1996). The plasmids pGRA19 (glutelin promoter construction) and pPRA13 (prolamine promoter construction) were independently introduced into rice. To completely suppress RA gene expression, plasmids pRBA39 (RA and branching enzyme promoters construction) and pGPA11 (glutelin and prolamine promoter construction) were introduced simultaneously.

**Transformation and DNA blot analysis**

Transformation and DNA blot analysis were performed according to Tada et al. (1990). The antisense genes were independently or simultaneously transferred into rice protoplasts concomitantly with pHPT2 by electroporation. Electroporated protoplasts were cultured and selected with hygromycin. Transgenic rice plants were regenerated from hygromycin-resistant calli. Rice genomic DNA was isolated and Southern blot analysis was performed using a gene-specific probe.

**Quantitation of RA content in rice grains by ELISA**

Total soluble proteins in phosphate-buffered saline (PBS) solution were prepared from rice grains (20 mg of rice grain in 100 µl of PBS) after physical crushing and centrifugation. The RA concentration in the resultant supernatant was quantified by ELISA. The wells of an ELISA plate were coated with 50 µl of anti RA-IgG (10 µg/ml) in coating solution (0.1 M Na2CO3, pH 9.6). After incubation at 4°C, the plate was washed three times with wash solution (10 mM Tris HCl, 0.05% Tween20, 0.02% NaN3, pH 8.0). The plate was then filled with blocking solution (1% BSA, 0.02% NaN3 in PBS) and incubated for 1 hr at room temperature. The solution was discarded and diluted samples and standard RA solutions were put into the wells. After 2 hr of incubation at room temperature, the plate was washed three times with wash solution and alkaline phosphatase-conjugated antibody was added. After 2 hr of incubation at room temperature, the plate was washed three times with wash solution. The plate was then filled with substrate solution, which was prepared by dissolving p-nitrophenyl phosphate at a concentration of 0.6 mg/ml in the substrate solution. After 30 min of incubation at room temperature, the enzyme reaction was stopped by adding 3N NaOH. The absorbance at 405 nm was then measured by an ELISA reader.

**SDS-PAGE, 2D-GE and immunoblotting**

Rice grains were milled using a small milling machine (Pearlest, Kett). PBS-soluble proteins were prepared from milled grains and milled powder. To examine the spatial distribution of RAs in seeds, rice seed grains were milled to 100, 90, 80, 70 or 60% and PBS-soluble proteins in the milled grains and milled powder were extracted and applied to SDS-PAGE as described by Tada et al. (1996). For analyzing the accumulation of RAs in developing seed grains, seeds were harvested at 5, 10, 15, 20, 25, 30 and 35 days after flowering (DAF) and PBS-soluble proteins were subjected to 2-dimensional gel electrophoresis and immunoblotting. For immunoblotting, separated proteins were electrically transferred to Clear Blot Membrane (ATTO) using Horize Blot (ATTO). For two-dimensional gel electrophoresis, PBS-soluble proteins were separated by isoelectric focusing prior to SDS-PAGE using Ampholine (Amersham Pharmacia Biotech) as a carrier, according to the supplier’s protocols.

**Sequence analysis**

The nucleotide and amino acid sequences were analyzed, including an estimation of the isoelectric point and molecular weight, using “GENETYX-WIN” software (SOFTWARE DEVELOPMENT).

**Results**

**RA contents in the transformants**

RA contents in the seed grains from transgenic rice plants were measured by ELISA (Fig. 1). Among transgenic lines co-transformed with pRBA39 and pGPA11, the transformant KRA17 showed the greatest reduction in RA content in selfed seeds (Tada et al. 1996). Southern blot analysis showed that KRA17 contains four intact antisense constructs (data not shown). The RA content in R1 and R4 seeds was measured to be 33% and 11% of that in Kinuhikari. The reason for the fluctuated RA content was unclear. The RA content in NRA37 and NRA85, which were Nipponbare-transformants with pRGA19 and pPRA13, respectively, were 11% and 14% of those in Kinuhikari. However, among more than 100 transformants, no RA-free plant was obtained.

**Spatial distribution of RAs**

In our previous study, antisense transcripts could be detected in the transformant even though the RA content was...
not completely suppressed, suggesting that not all of the antisense transcripts were fully utilized to suppress the expression of the target RA genes (Tada et al. 1996). To examine whether the existence of unused antisense mRNA is due to a spatial difference in the expression of the introduced antisense and endogenous RA genes, we analyzed the spatial distribution of RAs in seeds from R2 plant of KRA17 and Kinuhikari. PBS-soluble proteins in the milled grains and milled powder were separated by SDS-PAGE. CBB-stained gel (Fig. 2A and Fig. 2C) and immunoblotting (Fig. 2B and Fig. 2D) showed that the contents of PBS-soluble proteins were highest in the aleurone layer (Fig. 2C, milled powder of the 100-90% part), and lower in the central parts of grains (Fig. 2A and Fig. 2C) from both Kinuhikari and KRA17. However, RAs were not detected in the aleurone layer and the RA content was higher in the central part of the grain (Fig. 2B and Fig. 2D). The RA content of KRA17 was lower than that of Kinuhikari at all degrees of milling (Fig. 2B) and in all parts of the grain (Fig. 2D). Thus, there was no reduction of RAs in specific grain parts.

Effect of antisense genes on each RA

At the beginning of this research, the rice RA gene family was estimated to consist of a few genes by SDS-PAGE analysis. However, more than 10 partial or full-length cDNA clones hybridizing to the major RA (RA17) sequences have been obtained so far. DNA sequencing revealed that these cDNA clones encoded homologous proteins with molecular masses of 14 to 16 kDa (Table 1). The nucleotide sequence identity among the coding regions of the full-length cDNA clones sequenced so far was more than 79%, suggesting that these allergens are encoded by members of a multigene fam-

![Fig. 2. SDS-PAGE and immunoblotting of milled grains and milled powder from the transformant KRA17.](image-url)
ily (Adachi et al. 1993). Their estimated isoelectric points (pI) and molecular weights are shown in Table 1.

By two-dimensional gel electrophoresis (2D-GE) using isoelectric focusing (IEF) for the first dimension and SDS-PAGE for the second dimension, proteins of 14 to 16 kDa were separated into more than 10 spots (Fig. 3A). After immunoblot analysis, these proteins were detected as rice RAs (Fig. 3B). In this analysis, while the monoclonal antibody against the major RA (16 kDa) was used for immunoblotting, other RAs were also detected due to their affinity for the antibody.

Next, RAs in the transformants were detected by two-dimensional immunoblotting. The estimated position of each RA is shown in Fig. 4A for comparison. In the low-allergen transformant KRA17 (Fig. 4B), only a few faint signals were detected around pI 6.4, one of which was estimated to be the major RA (RA17), and no other spots could be detected around the RA. RA14 and an RA homologue (accession No. X62091), which are 83.4% and 84.8% homologous to the major RA (Table 1), are estimated to be included in these spots. On the other hand, other spots, especially spots with pI 8.3 and higher, were slightly weaker, and were estimated to include RA5 and an RA homologue (accession No. AF042200), which are 80.1% and 79.5% homologous to the major RA (Table 1). Similar results were obtained with the other transformants, NRA37 and NRA85 (Fig. 4C and Fig. 4D), which were transformed with the antisense construct driven by the glutelin or prolamine promoter. Thus, the accumulation of RAs of around pI 6.5, including the major RA, was reduced almost completely in transformants, while other RAs being less homologous to the major RA were less affected. These results suggest that the effect of antisense RNA strictly depends on the homology between the sequence used in the antisense gene and the target sequence.

Accumulation of RAs in developing seed grains
To examine whether the incomplete reduction of RA content in the transformants is due to a temporal difference in the expression of the introduced antisense and endogenous RA genes, we analyzed the accumulation of RAs in developing seed grains from transgenic and non-transgenic rice. Developing seeds were harvested at 5, 10, 15, 20, 25, 30 and 35 days after flowering (DAF) and PBS-soluble proteins were subjected to 2D-GE and immunoblotting (Fig. 5).

### Table 1: Homology of RA homologues to the major RA at the DNA level, length of their amino acid sequence and their estimated isoelectric points and molecular weights

<table>
<thead>
<tr>
<th>Allergen</th>
<th>RA17</th>
<th>RA14</th>
<th>RA14b</th>
<th>RA14c (partial)</th>
<th>RA14d (partial)</th>
<th>RA14e (partial)</th>
<th>RA14f (partial)</th>
<th>RA5</th>
<th>RA5b</th>
<th>RA16</th>
<th>AF042200</th>
<th>X62091</th>
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<tbody>
<tr>
<td>Homology to RA17 cDNA (%)</td>
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<td>83.4</td>
<td>82.9</td>
<td>82.1</td>
<td>86.3</td>
<td>86.7</td>
<td>88.1</td>
<td>80.1</td>
<td>80.2</td>
<td>80.2</td>
<td>79.5</td>
<td>84.8</td>
</tr>
<tr>
<td>Length of AA (mature AA) (mature AA)</td>
<td>167</td>
<td>165</td>
<td>166</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Isoelectric Point</td>
<td>6.45</td>
<td>7.00</td>
<td>7.94</td>
<td>7.44</td>
<td>—</td>
<td>—</td>
<td>8.28</td>
<td>7.72</td>
<td>7.72</td>
<td>9.31</td>
<td>7.44</td>
<td></td>
</tr>
<tr>
<td>Molecular Weight (kDa)</td>
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<td>15.2</td>
<td>15.4</td>
<td>15.3</td>
<td>—</td>
<td>—</td>
<td>14.4</td>
<td>14.6</td>
<td>14.6</td>
<td>14.4</td>
<td>14.4</td>
<td>15.4</td>
</tr>
</tbody>
</table>

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Fig. 3. Two-dimensional separation of PBS-soluble protein from grains of Kinuhikari and immunoblot analysis.
A: CBB-stained gel, B: Immunoblotting using anti RA-IgG. Lane 1D, PBS-soluble protein was directly subjected to SDS-PAGE.
Antisense suppression of rice allergen genes

At 10 DAF, the amount and type of RAs in Kinuhikari and the transformant KRA17 were almost identical. At 15 DAF, the contents of RAs of pI 5.6 to pI 6.5, including the major RA, were increased in Kinuhikari, but were low in KRA17. The content of these RAs increased until 25 DAF in Kinuhikari, but remained low in KRA17. The contents of the other RAs were also slightly lower in KRA17 than in Kinuhikari at 15 to 35 DAF, but this difference was less than that for the major RA at all stages. Thus, the accumulation of some RAs was severely inhibited, while that of others was only slightly suppressed in seed grains from transformants at 10 to 35 DAF.

Discussion

We produced transgenic rice plants harboring antisense genes consisting of a cDNA for a major RA driven by promoters of the RA, rice starch branching enzyme, rice prolamine and rice glutelin genes. They were expected to be specifically expressed in developing seeds and to reduce the RA content in the seeds of transgenic rice. However, regardless of the combined introduction of several antisense constructs into rice, allergen-free rice was not obtained. Furthermore, antisense transcripts could be detected in the transformant even though the RA content was not completely suppressed, suggesting that not all of the antisense transcripts were fully utilized to suppress the expression of the target RA genes (Tada et al. 1996). To clarify why the antisense genes did not completely suppress endogenous gene expression, we examined the possibility of a spatial or temporal difference in the expression of the antisense and endogenous RA genes. Immunoblotting analysis of PBS-soluble proteins in the milled grains and milled powder from transformants revealed no specific grain part with reduction of RAs (Fig. 2). These results suggest that the detection of unused antisense RA RNAs was not due to a spatial difference in the expression of the sense and antisense genes in the grains. Then we

Fig. 4. Two-dimensional separation and immunoblotting of PBS-soluble protein from grains of Kinuhikari, Nipponbare and transformants.

Basic parts (pI 5-9) of the IEF-gel of Kinuhikari/Nipponbare and transformants were applied to the same SDS-gel. Immunoblot analysis was performed using anti RA-IgG.

A: Estimated positions of rice allergenic proteins on two-dimensional immunoblotting, B: Kinuhikari and KRA17, C: Nipponbare and NRA37, D: Nipponbare and NRA85.

Fig. 5. Accumulation of RAs in developing seed grains from Kinuhikari and KRA17.

Developing seeds were harvested at 5, 10, 15, 20, 25, 30 and 35 days after flowering (DAF) and PBS-soluble proteins were extracted and subjected to 2-dimensional gel electrophoresis and immunoblotting using anti RA-IgG. Basic parts (pI 5-9) of IEF-gel of Kinuhikari and KRA17 were applied to the same SDS-gel.
also analyzed the accumulation of RAs in developing seed grains from transgenic and non-transgenic rice by 2D-GE and immunoblotting. The results showed no evidence for stage-specific reduction of RAs (Fig. 5), suggesting that there is no remarkable difference in the temporal expression of the sense and antisense genes.

We also examined the effects of antisense genes on each RA in transformants by 2D-GE and immunoblotting analysis, since the RA genes comprise a multigene family. These transformants had markedly lower contents of the amount of the major RA and other RAs with nucleotide sequences highly homologous to the antisense sequences, but unaltered contents of RAs with sequences showing relatively low homology to the major RA (Table 1 and Fig. 4). These results suggest that the effect of the antisense gene on the RA gene family strongly depends on the homology between the antisense sequence used and the target sequence. Therefore, it seems that the antisense strategy is not suitable for completely suppressing the expression of all of the genes in this multigene family and produce RA-free rice.

It is still unclear whether such hypo-allergenic rice could be tolerated by patients allergic to rice, since even a small amount of residual allergens might elicit an allergic reaction. A preliminary examination showed that the sera from six rice-allergic patients reacted with rice protein from KRA17 equally as well as that from normal rice (Kinuhikari) (data not shown). Other strategies should be used to completely suppress the expression of all of the genes in this multigene family and produce RA-free rice.

The simultaneous down-regulation of the three catalase genes in tobacco by a sense cotton catalase gene has been reported, and specific catalase isozyme activity by antisense cassettes (Chamnongpol et al. 1996). This result suggests that a co-suppression strategy may be more suitable for the coordinated suppression of a multigene family. Ribozyme is an alternative tool for suppressing specific gene expression (Uhlenbeck 1987, Haseloff and Gerlach 1988), but this has not yet been successfully used for plant gene regulation. Plant gene expression has been successfully suppressed using the RNA interference (RNAi) method (Waterhouse et al. 1998), which has been reported to be superior to the antisense method (Smith et al. 2000). The expression of two self-complementary RNA constructs designed to initiate RNAi of *iabM* and *ipt* could confer resistance to crown gall resistance in transgenic *Arabidopsis thaliana* and *Lycopersicon esculentum* plants, the genes of which showed ~90% DNA sequence identity to studied *A. tumefaciens* strains (Escobar et al. 2001). The effect of RNAi-mediated gene silencing on the expression of a multigene family should be examined.

Furthermore, 77% of allergic patients could be allergic to some other component of rice seed protein (Urisu et al. 1991) even if the RA genes could be completely suppressed. Suppression of the regulator genes that control the expression of the genes for all rice allergenic proteins, if possible, seems to be a reasonable approach for producing hypo-allergenic rice.

**Acknowledgments**

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**Literature Cited**


