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Original Paper

Aberrant endosperm formation caused by reduced production of major allergen proteins in a rice *flo2* mutant that confers low-protein accumulation in grains

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Abstract Rice *flo2* mutation produces grains showing a reduced amount of storage proteins. Using Nipponbare and the *flo2* mutant, we created rice transformants that showed defective production of major allergen proteins RA14 and RA33 (14–16 kDa and 33 kDa allergen proteins, respectively) by RNAi introduction. The knock-down transformant generated using Nipponbare showed greatly reduced accumulation of both allergen proteins, normal growth, and production of a sufficient amount of normal-shaped seeds. F_1 seeds were obtained by crossing between the transformants containing RNAi genes to RA14 and RA33, and showed decreased accumulation of both proteins. However, a peculiar phenotype was observed in the *flo2* transformants that lacked accumulation of RA14 or RA33. They showed significantly reduced fertility. A wrinkled grain feature was found on the transformant lacking accumulation of RA14. F_1 seeds obtained by crossing these transformants showed significantly lower fertility. F_2 seeds showed decreases in both allergen proteins but morphological abnormality with small and severely wrinkled features. These results indicated that it is hard to obtain any transformant lacking accumulation of these allergen proteins using the *flo2* mutant, whereas a knock-down transformant of both allergen protein genes was obtained when a wild-type Nipponbare was used as a host. These facts strongly suggest that RA14 and RA33 have some roles in rice seeds.

Key words: knock-down transformant, major allergen proteins, reduced storage protein content, RNAi genes, wrinkled seed feature.

Introduction

In terms of total world food production, rice (*Oryza sativa*) is an important grain crop, along with wheat and maize (Binod et al. 2010). Although rice is a major nutrient source, the prevalence of IgE-mediated rice allergy is approximately 10% in atopic subjects (Ogo et al. 2014). These patients may not consume cooked rice, causing the deterioration of quality of life and reduction in nutrition ingestion. To solve these problems, various strategies have been proposed.

Among many of these schemes, some processing technologies are actually performed to remove allergenic proteins, such as high hydrostatic pressure, enzymatic digestion, and alkaline hydrolysis of endosperm proteins (Kato et al. 2000; Watanabe et al. 1990a, 1990b). However, these methods cannot entirely remove allergen proteins, and, therefore, a molecular breeding strategy was designed to make a mutant lacking the genes for the allergen proteins. Transgenic rice plants, in which an RNA interference (RNAi) gene against a major allergen protein gene is introduced, have been generated to reduce the allergen proteins (Ogo et al. 2014; Tada et al. 2003).

The allergen proteins show little structural similarity. It is presumed that various proteins could become a novel allergen protein. Currently, α-globulin (26 kDa), β-glyoxalase I (33 kDa), and α-amylase/trypsin inhibitor (14–16 kDa) have been determined as major rice allergens based on studies of recognition by IgE from individuals with food allergy (Alvarez et al. 1995; Limas et al. 1990; Usui et al. 2001). Allergy patients

Abbreviations: PCR, polymerase chain reaction; RT-PCR, reverse-transcriptase mediated PCR; RNAi, RNA interference; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.
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desire the creation of rice grains in which amounts of various storage proteins and these allergen proteins are sufficiently reduced.

We have found that the rice *FLO2* gene regulates the quality of grains and the quantity of the storage substances in endosperm, and we reported reduced production of storage proteins such as glutelins, globulins, and prolamins, as well as some of the allergen proteins, in the rice *flo2* mutant endosperm (She et al. 2010). This suggests that the *flo2* mutant produces grains with low-protein content and that this mutant is a desired host to establish a rice line with allergen-free properties. In this study, we generated rice *flo2* transformants harboring RNAi against the genes for the 14–16 kDa and/or 33 kDa allergen proteins (termed RA14 and/or RA33, respectively), in which the amount of these proteins was decreased. We also evaluated the properties of these grains.

Materials and methods

Plant materials

Oryza sativa L. cv. Nipponbare (wild-type) and a *flo2* mutant were used for this work. They were germinated at 30°C in a dark chamber, and their seedlings were grown under continuous light (13 mol $m^{-2}s^{-1}$) for three days. Then, these plants were cultivated in a green house.

RNA isolation, cDNA synthesis, RT-PCR, and construction of RNAi plasmids

Total RNA was prepared from rice cells as described previously (Imamura et al. 2007). The first-strand cDNA was synthesized from 1 g of total RNA using a ReverTra Ace cDNA synthesis kit (Toyobo, Osaka, Japan) with an oligo-dT (20) primer. RT-PCR was performed using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) under the following conditions: an initial denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s. Fragments for the RA14 and RA33 genes (acc. no. D11432 and no. AK066092, respectively) were amplified using the following primers: 5′-CAC CCT GCC GGG CGG TGG TGA GG-3′ and 5′-GTA GCA GAC ACC ACC TGT CC-3′ for RA14, and 5′-CAC CGG TTG TGC TGG AGT GGC CTA A-3′ and 5′-GAG TGA TCT TGC GCA CCA ACA ACT GG-3′ for RA33. The amplified fragment was introduced into pENTRTM/D-TOPO (Invitrogen) and transferred to the position following the CaMV-35S promoter regions in the pANDA vector (Miki and Shimamoto 2004) via an LR clonase (Invitrogen) reaction.

Plant transformation and selection of the transformant showing defective production of allergenic proteins

Rice was transformed by the Agrobacterium-mediated method (Hiei et al. 1994). Transformants were grown on half concentration of Murashige–Skoog (1/2-MS) plates (Murashige and Skoog 1962) supplemented with 50 mg Hygromycin B (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Hygromycin-resistant plants were selected and transferred onto soil for further cultivation. These plants were grown in a greenhouse. A seed obtained from these plants was divided into two parts, one of which including the embryo was used for germination to obtain the progeny plants, and the other part was subjected to protein blot analysis to determine the reduction in the amount of RA14 and RA33 in the endosperm (Figure 1). Among the seeds analyzed, the plants showing a reduced amount of allergenic proteins were grown to obtain the seeds of the next generation.

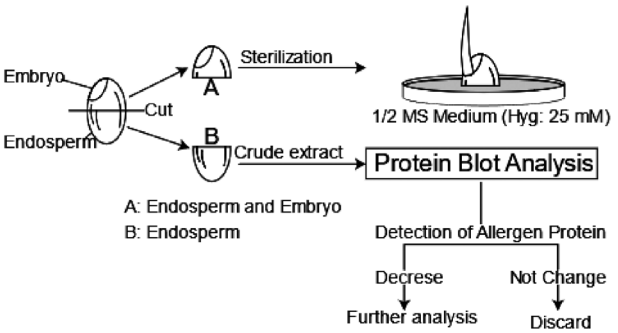

Figure 1. Schematic representation of the screening procedure for the rice seeds with reduced production of the allergen proteins. An unpolished brown rice grain was divided into two parts, a portion with embryo and endosperm, and a portion with endosperm. The former portion was used for germination test on the 1/2 MS medium supplemented with 25 mM hygromycin. The other portion was subjected to protein blot analysis.

Protein preparation, SDS-PAGE, and Protein blot analysis

Proteins were prepared from powdered seeds using the solution containing 1M NaCl as described previously (Morita and Yoshida 1986). One grain was used for preparation of total proteins, and amount of proteins was determined using a protein assay kit (Bio-Rad laboratories, Hercules, CA, USA). SDS-PAGE and protein blot analysis were performed according to Ausubel et al. (1987). RA14 and RA33 were detected by protein gel blot analysis using rabbit antisera raised against these proteins. Antisera against rice RA14 and RA33 were prepared using the corresponding recombinant proteins produced by an *E. coli* expression system. The antisera were affinity purified using a CNBr-activated sepharose 4B column.

Results

Generation of rice transformants harboring RNAi against the RA14 and RA33 genes

The RA14 genes consist of a multigene family with highly conserved nucleotide sequences (Adachi et al. 1993). To reduce amounts of RA14, an RNAi gene was created using a conserved region (Figure 2A, Supplementary Figure S1) and introduced into the rice *flo2* mutant and a wild-type rice cultivar, Nipponbare. Transformants

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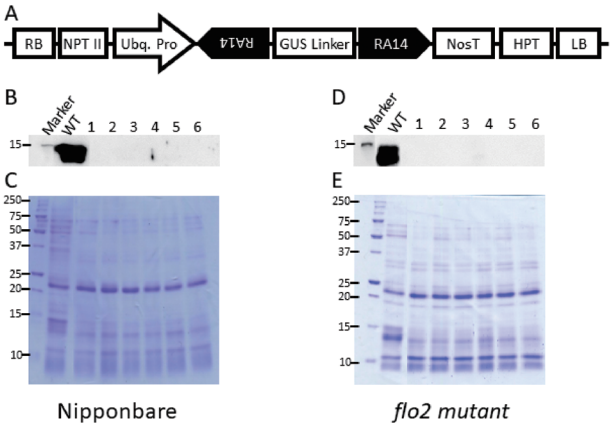

Figure 2. Protein blot analysis of the transformants harboring the RNAi gene against the RA14 gene. (A) Structure of the RNAi gene against the RA14 gene. The region between the left and right border regions is shown. RB: right border, LB: left border, Ubq Pro: ubiquitin promoter, RA14: the region for the trigger sequence used for the RNAi against the RA14 gene, GUS Linker: the region included in the RNAi gene derived from pANDA vector, NosT: terminator of nopaline synthase gene, HPT: hygromycin phosphotransferase gene. (B) Protein blot analysis of RA14 in the transformant grains, which were generated using Nipponbare. Panel (C) indicates the corresponding CBB staining image. (D) and (E) Protein blot analysis and corresponding CBB staining image of the transformants, which were generated using the rice *flo2* mutant. Numbers on the figure indicate the individual transformants. WT shows the nontransformant as a control. Equal amount of proteins was applied in a lane on each analysis. Sizes of protein markers are indicated on the left.

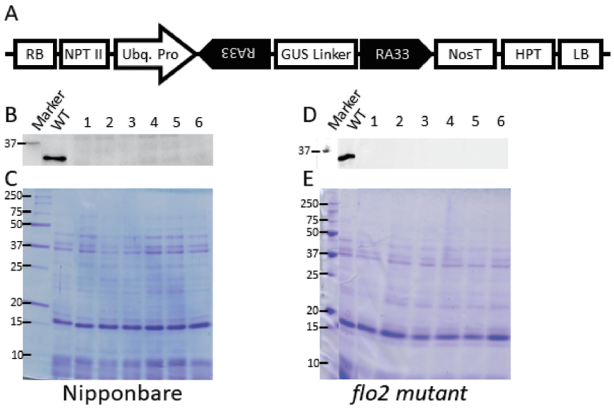

Figure 3. Protein blot analysis of the transformants harboring the RNAi gene against the RA33 gene. (A) Structure of the RNAi gene against the RA33 gene. The region between the left and right border regions is shown. RB: right border, LB: left border, Ubq Pro: ubiquitin promoter, RA33: the region for the trigger sequence used for the RNAi against the RA33 gene, GUS Linker: the region included in the RNAi gene derived from pANDA vector, NosT: terminator of nopaline synthase gene, HPT: hygromycin phosphotransferase gene. (B) Protein blot analysis of RA33 in the transformant grains, which were generated using Nipponbare. Panel (C) indicates the corresponding CBB staining image. (D) and (E) Protein blot analysis and corresponding CBB staining image of the transformants, which were generated using the rice *flo2* mutant. Numbers on the figure indicate the individual transformants. WT shows the nontransformant as a control. Sizes of protein markers are indicated on the left.

harboring the RNAi gene were selected, and their seeds were subjected to determination of the amount of RA14. Transformants showing reduced production of these allergen proteins were chosen as the low-allergen lines (Figure 2).

These transformants generated using the *flo2* mutant and the wild-type rice were grown normally and set normal shaped seeds. Among the seeds analyzed, the plants showing a reduced amount of RA14 were selected and grown to obtain the seeds for the next generation. The 12 randomly selected seeds all showed reduced amounts of allergen proteins and were considered homozygous transformant lines.

Next, RNAi against the RA33 gene was generated based on the rice RA33 gene (Figure 3A, Supplementary Figure S2). This gene was introduced into the *flo2* mutant and Nipponbare. Transformant lines whose seeds showed a lack of accumulation of RA33 were chosen by a similar method as transformants with reduced production of RA14 (Figure 3).

Phenotypes of the transformants that defectively accumulated RA14 or RA33

Phenotypes of these transformants generated using Nipponbare were analyzed. Seeds of these transformants harboring RNAi genes against RA14 and RA33 showed reduced amount of RA14 and RA33, respectively. These seeds had similar sizes based on weight, length, and thickness to those of the nontransformants (Figure 4A, Table 1). They grew similarly to the nontransformants and set a large number of mature seeds with sufficient fertility (Figure 4B). These results indicated that defective production of RA14 or RA33 caused no significant difference in the characteristics of their growth, fertility, and seed features.

Next, we analyzed the phenotype of the transformants of the *flo2* mutant harboring the RNAi genes against RA14. They grew normally and showed similar features as those of the nontransformant plants during the vegetative stage. However, seeds of these *flo2* transformants showed a peculiar wrinkled feature (Figure 4C). The values of seed length, width, and thickness were similar to those of the nontransformant, but seed weight showed significantly smaller values (Table 2). This phenotype was observed on most of these transformants. Fertility of these transformants significantly decreased, and an average fertility of 55.7% was observed in the transformants (Figure 4D).

The *flo2* transformants with reduced accumulation of RA33 also grew normally, as did the nontransformant. The transformed seeds showed similar features to those of the nontransformant (Figure 4C, Table 2). The transformants lacking accumulation of RA33 resulted in significantly lower fertility that was 26.1% on average, whereas the fertility of the nontransformant (*flo2* mutant) was 79.2% (Figure 4D). These results suggest that suppression of RA14 and RA33 production may lead

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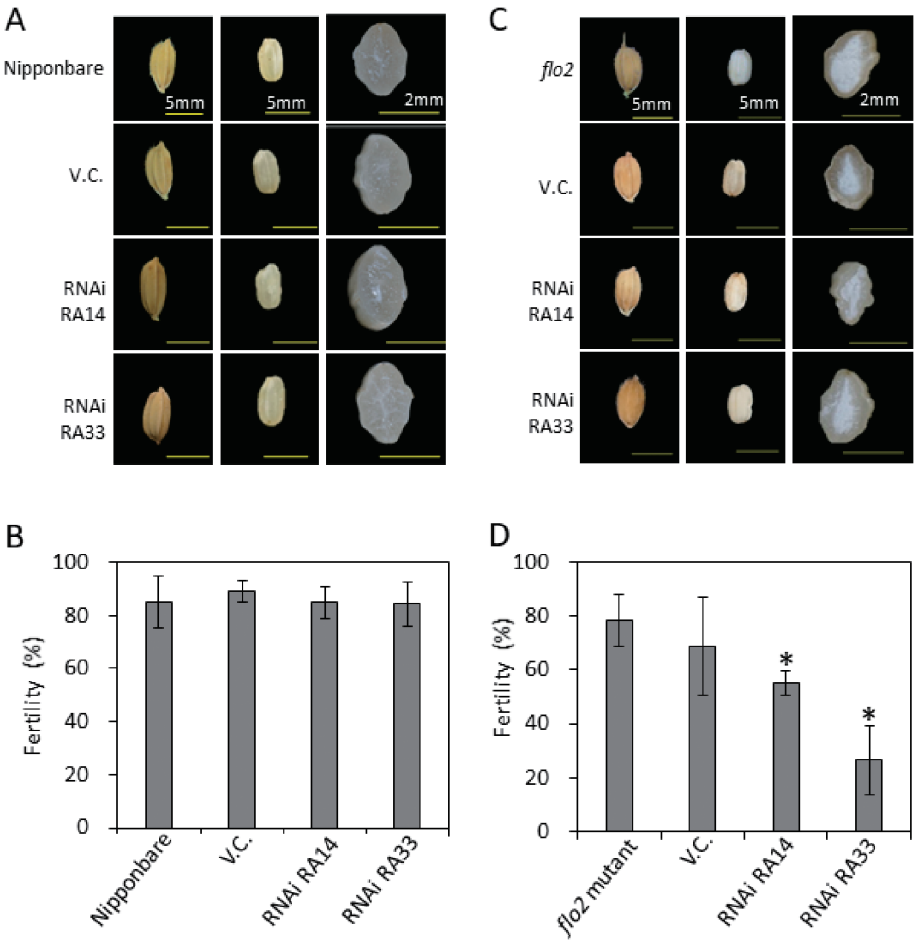

Figure 4. Phenotype of the transformants of Nipponbare lacking accumulation of RA14 or RA33. (A) and (C) Morphology of the grains from the representative transformants generated using Nipponbare and *flo2* mutants, respectively. Left panels indicate the feature of rice seeds. Middle and right panels show the feature of brown rice and their cross sections, respectively. Nipponbare and *flo2* indicate the features of the host plant seeds, respectively. V.C., RNAi RA14, and RNAi RA33 indicate the transformants harboring the vector plasmid and RNAi genes against the RA14 and RA33 genes, respectively. (B) and (D) Fertility of the transformants. Nipponbare and *flo2* mutant: nontransformants. V.C., RNAi RA14, and RNAi RA33: transformants harboring the vector plasmid and RNAi genes against RA14 and RA33, respectively. Error bars represent the means±SD (n=4). A mean that differs significantly is indicated by an asterisk (p<0.05).

Table 1. Sizes of grains from the transformants using the wild-type Nipponbare plant.

Name	Length (mm)	Width (mm)	Thickness (mm)	Weight (mg)
Nontransformant	5.0±0.1	2.8±0.1	2.0±0.0	19.5±0.9
V.C.	5.0±0.2	2.8±0.1	1.8±0.2	16.3±1.9
RNAi RA14	4.9±0.1	2.8±0.2	1.8±0.1	16.7±1.3
RNAi RA33	5.1±0.2	2.8±0.2	1.9±0.1	18.4±1.4
RNAi RA14/RA33	5.0±0.2	2.7±0.2	1.9±0.1	18.1±1.6

Transformants containing RNAi genes for RA14 and/or RA33, which were created using the wild-type plant Nipponbare. "Nontransformant" and "V.C". indicate the control plants: untransformed Nipponbare and the transformant containing the vector plasmid, respectively. RNAi RA14, RNAi RA33, and RNAi RA14/RA33 indicate the transformants showing reduced accumulation of RA14, RA33, and both allergen proteins, respectively. Sizes of each grain are shown as the averaged values of 20 grains except for the transformant RNAi RNA14/RA33 in which 12 grains were used, with the standard errors.

Table 2. Sizes of grains from the transformants using the *flo2* mutant.

Name	Length (mm)	Width (mm)	Thickness (mm)	Weight (mg)
flo2 Nontransformant	4.5±0.3	2.6±0.1	1.8±0.1	15.2±1.3
flo2 V.C.	4.8±0.2	2.6±0.1	1.6±0.1	13.3±1.4
flo2 RNAi RA14	4.7±0.2	2.5±0.2	1.5±0.1	11.4±1.5*
flo2 RNAi RA33	5.0±0.2	2.8±0.2	1.6±0.1	14.8±1.9
flo2 RNAi RA14/33	4.4±0.2	2.4±0.2	1.6±0.2	11.5±1.2*

Transformants containing RNAi genes against RA14 and/or RA33, which were created using the rice *flo2* mutant. "*flo2* Nontransformant" and "*flo2* V.C". indicate the control plants: untransformed *flo2* mutants and the transformant containing the vector plasmid, respectively. RNAi RA14, RNAi RA33, and RNAi RA14/RA33 indicate the transformants showing reduced accumulation of RA14, RA33, and both allergen proteins, respectively. Sizes of each grain are shown as the averaged values of 20 grains except for the transformant RNAi RNA14/RA33 in which 12 grains were used, with the standard errors. An asterisk indicates a mean that differs significantly (p<0.05).

to decreased fertility when the *flo2* mutant was used as a host.

Phenotypes of the transformants harboring RNAi genes against both RA14 and RA33

To create a transformant whose seeds showed deficiencies in the accumulation of both RA14 and RA33,

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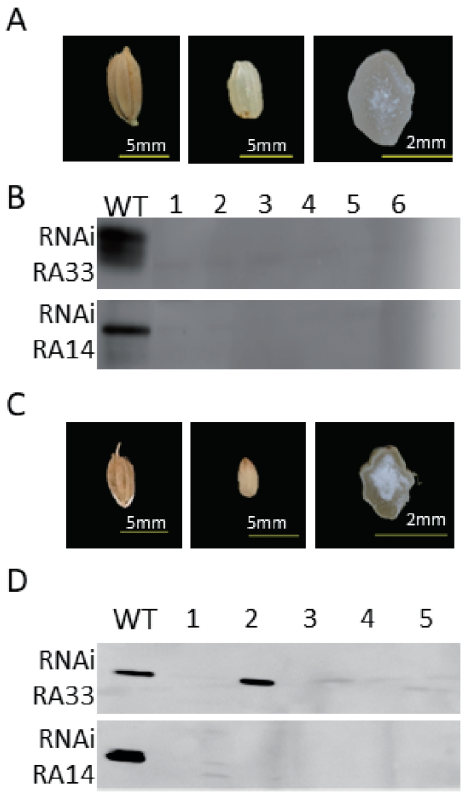

Figure 5. Phenotype of the transformants of Nipponbare and the *flo2* mutant showing reduced accumulation of both RA14 and RA33. (A) Morphology of the grain from the representative transformants generated using Nipponbare, which showed reduced accumulation of both allergen proteins. Left, middle, and right panels show the feature of a rice seed, a brown rice, and its cross section, respectively. (B) Protein blot analysis of RA14 and RA33 in the grains of the Nipponbare transformants. Numbers on the figure indicate the individual F_3 grains. WT shows the nontransformant as a control. (C) Morphology of the grains from the representative transformants generated using the *flo2* mutant, which showed reduced accumulation of both allergen proteins. Left, middle, and right panels show panels indicating the feature of a rice seed, a brown rice, and its cross section, respectively. (D) Protein blot analysis of the RA33 and RA14 in the grains of the *flo2* transformants. Numbers on the figure indicate the individual F_3 grains. WT shows the nontransformant as a control.

we crossed the transformants containing the RNAi genes to these allergen protein genes. The F_1 seeds obtained were divided into two parts and subjected to analysis of the accumulation of RA14 and RA33. Among them, those showing production of RA14 and RA33 were selected and considered to possess the characteristics of reduced production of both proteins.

By crossing the transformants generated using Nipponbare, more than ten F_1 seeds were obtained. They grew normally and set a large number of F_2 seeds. Among them, we selected the seeds showing a lack of accumulation of both allergen proteins. These seeds had a normal shape (Figure 5A) and grew normally. Properties of low allergen protein accumulation were inherited in these progeny (Figure 5B), which led to the establishment of lines defective in these allergen proteins.

Next, we attempted to create an F_1 line crossed between the *flo2* transformants that showed a lack of accumulation of RA14 and RA33. In this case, we hardly obtained F_1 seeds, but some F_1 seeds were generated. Among them, we chose F_1 seeds lacking accumulation of both allergen proteins. These F_1 plants grew normally during the vegetative stage, but only a few F_2 seeds were obtained because the fertility of these plants was quite low. These grains showed a wrinkled seed phenotype (Figure 5C). The property of reduced accumulation of RA14 and RA33 was segregated in these progenies (Figure 5D). Their seed weights were observed as 11.5 ± 1.2 mg on average, which was significantly reduced from that of the host *flo2* mutant, whereas these seeds had similar grain sizes to those of the *flo2* mutant (Table 2). These results indicated that deficiency of RA14 and RA33 in the *flo2* mutant caused the appearance of a peculiar grain feature with a trait of reduced plant viability.

Discussion

In this study, we attempted to generate a rice grain lacking the production of major allergen proteins in a low-protein grain mutant. Grains of the rice *flo2* mutant are characterized as having reduced accumulation of many kinds of storage proteins as well as storage starch (She et al. 2010). We introduced RNAi against the genes for the major allergen proteins, RA14 and/or RA33, into the *flo2* mutant as a host.

Although we obtained the transformants harboring either of the RNAi genes, which reduced the accumulation of the corresponding allergen proteins, they resulted in peculiar seed features (Figures 3, 4). In particular, significantly low fertility was observed in these transformants (Figure 4). In addition, a peculiar and severe wrinkled seed feature occurred in the transformants showing decreased accumulation of both RA14 and RA33, whereas normal grain features were observed in the transformants derived from the wild-type Nipponbare (Figure 5).

A wrinkled seed phenotype has been observed in pea and rice mutants and is caused by mutation of a gene for starch biosynthesis (Bhattacharyya et al. 1990; Kubo et al. 2005). Maize mutant *fl3* also shows a similar phenotype. In this case, amounts of many tRNAs and 5S rRNA transcribed by RNAPIII are significantly reduced, resulting in inhibition of endosperm development and grain filling with storage substances (Li et al. 2017).

It has been reported that normal features of rice grains occurred in the transgenic Koshihikari with reduced accumulation of the three major allergen proteins (Ogo et al. 2014). Our results also revealed the formation of normal grain features along with the property of normal growth and sufficient fertility in the transformants

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with deficient production of RA14 and/or RA33 from Nipponbare (Figures 3, 5). These observations imply that suppression of RA14 and/or RA33 may exert little influence on grain formation when Nipponbare is used as a host.

However, obvious inferior traits occurred in the transformants when the low-protein grain mutant was used as a host. These transformants revealed significantly low fertility and a wrinkled seed feature (Figures 4, 5). These facts suggest that inhibition is induced in endosperm development and establishment of seed function when some species of major allergen proteins are removed in addition to the storage proteins from grains of the *flo2* mutant, which accumulated reduced amount of storage proteins. There might be many difficulties in generating an allergen-free rice grain. It is also suggested that these allergen proteins have some roles during seed formation.

Acknowledgements

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