

Generation of Transgenic Rice Lines with Reduced Contents of Multiple Potential Allergens Using a Null Mutant in Combination with an RNA Silencing Method

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Rice seed proteins are known to be a causative antigen in some patients with food allergy, especially cereal allergy, with clinical symptoms such as eczema and dermatitis. The α -amylase/trypsin inhibitors (14–16 kDa), α -globulin (26 kDa) and β -glyoxalase I (33 kDa) are regarded as major potential allergens of rice (*Oryza sativa* L.) seed based on specific recognition by serum IgE from allergy patients. In order to suppress the production of these major allergens in rice grains, a mutant in the 'Koshihikari' background lacking the 26 kDa allergen (GbN-1) was used as a host for RNA silencing. A binary vector harboring two RNA interference (RNAi) gene cassettes for suppression of 14–16 kDa and 33 kDa allergens driven by the 13 kDa and 10 kDa prolamin endosperm-specific promoters, respectively, was introduced into the GbN-1 genome by *Agrobacterium*-mediated transformation. In the most promising transgenic line, the content of the three potential allergens was remarkably reduced to a very faint level without a change in seed phenotype. IgE binding of 15 patients' sera to the transgenic rice seed mostly deficient in the three major allergens was on average only about 10% that of the control wild-type rice, suggesting that these three accounted for the great majority of rice seed causative allergens recognized by patients' IgE and that the sequential allergen deletion/reduction strategy works in the development of hypo-allergenic rice lines.

Keywords: Food allergy • IgE antibody • *Oryza sativa* L • RNAi • Transgenic rice.

Abbreviations: ALS, acetolactate synthase; BiP1, binding protein; CNX, calnexin; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; HRP, horseradish peroxidase; PB, protein body; PDIL, protein disulfide isomerase-like; RNAi, RNA interference; SSP, seed storage protein.

Introduction

Rice (*Oryza sativa* L.) is a major staple cereal food consumed by more than half of the world's population, especially in East and South Asian countries. The prevalence of IgE-mediated rice allergy is about 10% in atopic subjects (<http://www.food-allergens.de/symposium-3-1/rice/rice-allergens.htm>). It is unique that rice allergy is six times more prominent in adults than in children, since other food allergies affect 5–8% of children and 1–2% of adults (<http://www.food-allergens.de/symposium-3-1/rice/rice-allergens.htm>). Symptoms frequently associated with rice allergy are atopic dermatitis, eczema and asthma. Multiple rice seed proteins have been reported to be responsible for rice allergy (Urisu et al. 1991). Among them, α -amylase/trypsin inhibitor (14–16 kDa), α -globulin (26 kDa) and β -glyoxalase I (33 kDa) have been suggested to be major rice allergens based on recognition by IgE from patients with food allergy (Limas et al. 1990, Alvarez et al. 1995, Usui et al. 2001, Matsuda et al. 2006). The 14–16 kDa allergens constitute a multigene family sharing >80% identity at the nucleotide sequence level, whereas 26 kDa and 33 kDa allergens are encoded by single-copy genes. The 14–16 kDa and 26 kDa allergens are members of the prolamin superfamily having three or four intramolecular disulfide bonds (A, B and C domains) (Mills et al. 2004). These allergens strongly reacted with IgE antibody in the sera from many rice allergy patients and caused eczematous and atopic dermatitis (Urisu et al. 1991).

Since avoidance of food containing allergens is the primary therapeutic strategy for food allergy, removal of allergens from rice grain has been achieved by several processing technologies such as enzymatic digestion, alkaline hydrolysis or high hydrostatic pressure (Watanabe et al. 1990a, Watanabe et al. 1990b, Kato et al. 2000). Some of these processes have already been commercialized to produce low-allergen rice in Japan; however,

the taste quality of processed rice is reduced by these chemical, enzymatic or physical treatments, and the treatments are costly. Creation of a cost-effective means to produce hypo-allergenic rice with good taste for rice allergy patients was the main objective of our study. To meet this goal, we tried to create low-allergen (hypo-allergenic) transgenic rice lines suppressing the major seed allergen genes. 'Koshihikari' is the most popular cultivated elite variety in Japan due to its excellent taste, and is suitable as a host to generate hypo-allergenic transgenic rice.

In this study, we created transgenic rice suppressing three major rice allergens (33, 26 and 14–16 kDa) in mature seed. When the IgE binding capacity of these transgenic rice seeds was examined using human sera, it was significantly reduced to about 10% on average for serum samples from 15 patients with suspected food allergy.

Results and Discussion

Strategy for generation of hypo-allergenic rice

In order to generate hypo-allergenic rice with reduced amounts of allergens, one approach is to screen for a null allergen mutant. The 26 kDa and the 33 kDa allergens are encoded by single-copy genes in the rice genome. These gene products are accumulated in seeds, but their expression patterns are quite different. The former is specifically expressed in seeds (inner starchy endosperm) (Qu and Takaiwa, 2004), and the latter is constitutively expressed throughout the plant. In contrast, the 14–16 kDa allergen genes constitute a multigene family of at least nine homologous members in the rice genome (Supplementary Fig. S1) that are expressed in an endosperm-specific manner (inner starchy endosperm) (Wu et al. 1998).

Iida et al. (1998) reported that a 26 kDa allergen null mutant (GbN-1) was isolated by screening mutants with altered seed protein compositions from panicle mutants irradiated with γ -rays (Fig. 1a). Mutants of the 14–16 kDa allergens were also isolated from the same population of γ -ray-irradiated mutants (Nishio and Iida 1993). The quantity of the 14–16 kDa allergens decreased to about a half of that of the wild type, but complete elimination of the allergen was difficult because the proteins are encoded by a multigene family. Therefore, in order to shut-off completely the expression of all members of the 14–16 kDa allergen gene family, specific silencing by a double-stranded RNA interference (RNAi) approach under the control of an endosperm-specific promoter was more suitable than antisense technology since the antisense approach reduced the content of 14–16 kDa allergens to only 20% of that in the wild type (Tada et al. 1996).

Furthermore, given that allergen proteins may be required for the growth of plant vegetative tissues, acquiring complete knock-out mutants may not be possible. The 33 kDa allergen gene is required for normal rice growth. To examine the effect of suppressing the 33 kDa allergen on vegetative growth, the

33 kDa allergen gene was constitutively suppressed by the double-stranded RNAi construct under the control of the maize ubiquitin promoter. As shown in Supplementary Fig. S2, growth of some vegetative tissues was apparently affected by the constitutive knock-down of the 33 kDa allergen gene. Not only culm length, ear length and number of grains per head, but, most importantly, yield were significantly reduced as compared with the wild type. In order to reduce the accumulation level without having an effect on agronomic characters, the 33 kDa allergen should be suppressed by the seed-specific RNAi technique, because gene silencing by the RNAi gene cassette is restricted to the endosperm tissue under the control of an endosperm-specific promoter. Effective suppression of food allergens by specific RNAi constructs has been reported for several transgenic plants such as peanut allergy protein Ara h 2 (Dodo et al. 2008), tomato allergy proteins Lyc e 1 and Lyc e 3 (Le et al. 2006a, Le et al. 2006b), soybean allergen P34 (Herman et al. 2003) and apple allergen Mal d 1 (Gilissen et al. 2005).

Thus, GbN-1 in the 'Koshihikari' background was used as a host for transformation in our strategy to create hypo-allergenic transgenic rice with reduced levels of three major allergens. Suppression of the 14–16 kDa and 33 kDa allergens was achieved by an RNAi approach. We used a different promoter for 14–16 kDa and 33 kDa allergen RNAi constructs in order to avoid the deletion of DNA fragment by recombination when a binary vector harboring both the 14–16 kDa and 33 kDa allergen RNAi cassettes was constructed (Fig. 1d) and their genes were integrated into the rice genome.

Characterization of transgenic rice

Three kinds of binary vectors were constructed, two binary vectors harboring the 33 kDa allergen RNAi cassette or the 14–16 kDa allergen RNAi cassette, independently (Fig. 1b, c) and the third binary vector harboring both gene cassettes (Fig. 1d). The 33 kDa and the 14–16 kDa allergen RNAi cassettes were directed by rice 10 kDa and 13 kDa prolamin endosperm-specific promoters, respectively. These gene cassettes were introduced into the GbN-1 genome via *Agrobacterium*-mediated transformation.

When single gene cassettes of the 33 kDa allergen (transgenic line named 26/33) and the 14–16 kDa RNAi (transgenic line named 14-16/26) construct were introduced independently, the target genes were effectively suppressed in half of the transformants [50.0% (5/10) and 53.1% (17/32)] as shown in Fig. 2a and Table 1. As expected, the 14–16 kDa allergen RNAi cassette could suppress all of the 14–16 kDa allergen gene families. Furthermore, simultaneous introduction of the two RNAi cassettes (transgenic line named 14-16/26/33) also reduced the expression of the two target genes at a level similar to the single gene cassette [45.4% (10/22), Fig. 2a and Table 1], accumulation of both the 33 kDa and 14–16 kDa allergens in mature seed was decreased to faint levels (<2%). The most highly suppressed transgenic line was selected from the T₁ population for

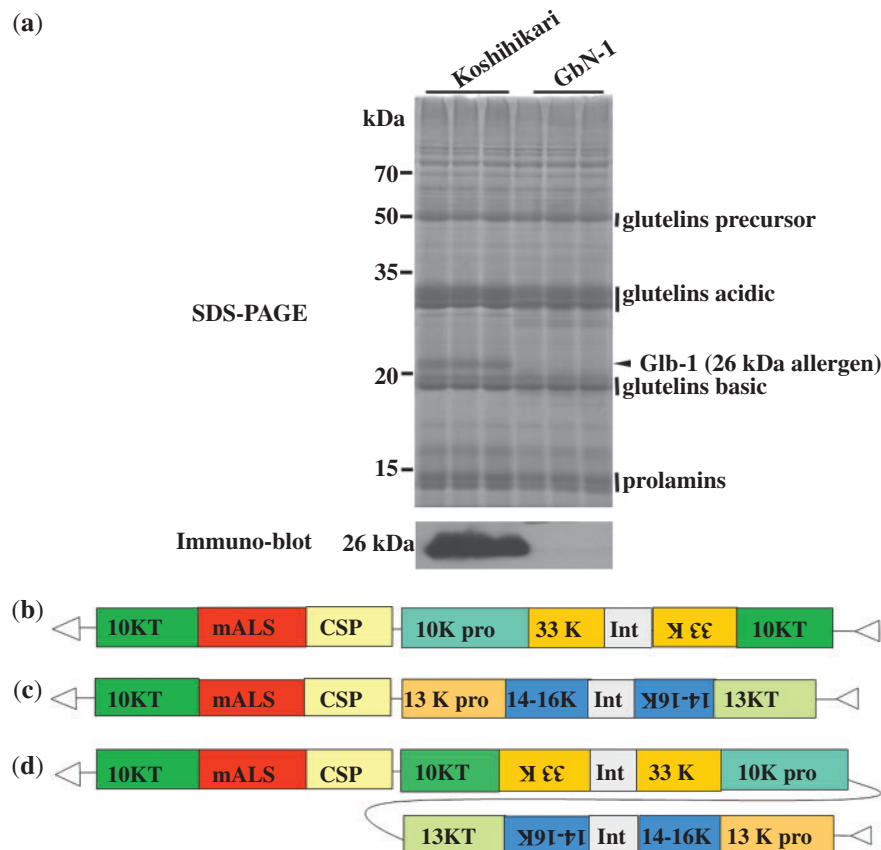


Fig. 1 Total seed protein of 'Koshihikari' and GbN-1, and a schematic representation of the binary vector construct. (a) The upper panel shows a Coomassie Brilliant Blue-stained SDS–polyacrylamide gel. The positions of seed storage proteins, glutelin precursor, glutelin acidic or basic subunit, globulin (Glb-1) and prolamin are indicated at the right of the panel. The lower panel shows an immunoblot analysis using anti-26 kDa allergen polyclonal antibody. (b) The 33 kDa allergen RNAi vector. (c) The 14–16 kDa allergen RNAi vector. (d) The 33 kDa and 14–16 kDa double RNAi vector. CSP, callus-specific promoter; mALS, mutated acetolactate synthase-coding gene; 10 K T, 10 kDa prolamin terminator. 10 K pro, 10 kDa prolamin promoter; 33 K, parts of 33 kDa allergen cDNA; 13 K pro, 13 kDa prolamin promoter; 13 K T, 13 kDa prolamin terminator; Int, rice *oryza* gene second intron. Upside-down characters indicate inverted sequence.

individual constructs and advanced to the next generation to obtain a homozygous line by self-crossing. We examined whether there was any effect on vegetative tissue using transgenic rice plants suppressing two allergens in an endosperm-specific manner (14-16/26/33) as a representative. It is important to note that 33 kDa allergen accumulated at almost the same level as non-transgenic rice in vegetative tissues, indicating that the expression level of the 33 kDa allergen was not affected by introduction of the endosperm-specific RNAi construct used here as well as 14–16 kDa allergens (Fig. 2b). Thus, a detrimental effect on plant growth was not observed in this selected 14-16/26/33 transgenic line, which was in contrast to transgenic rice constitutively suppressed 33 kDa allergen (Supplementary Fig. S2).

The seed morphology of transgenic rice was also similar to that of 'Koshihikari' and GbN-1 (Fig. 3a). On the other hand, seed length, width, thickness and the 1,000 kernel weight were slightly lower in transgenic seeds as compared with non-transgenic 'Koshihikari' and GbN-1 seeds (Table 2).

There were no significant differences in protein and amylose contents among 'Koshihikari', GbN-1 and transgenic rice (14-16/26/33) (Table 2). It should be noted that protein content (10–11% of total grain DW) of mature seed in 'Koshihikari', GbN-1 and transgenic rice was higher than the level (about 7%) in 'Koshihikari' grown in a paddy field (Table 2). One reason is that transgenic plants were grown on artificially controlled soil containing high nutrients such as nitrogen, phosphoric acid and potassium in a closed glasshouse. It is well known that cultivation under high nitrogen conditions enhances protein content in rice seed (Perez et al. 1996).

The gene copy number of the transgene integrated into the rice genome of the homozygous 14-16/26/33 transgenic line was examined by Southern blot analysis using the rice ALS (acetolactate synthase) coding region as a probe. When the probe was hybridized to genomic DNA digested with *Hind*III, two copies of the transgene were found to be introduced into the transgenic rice genome (Fig. 3b).

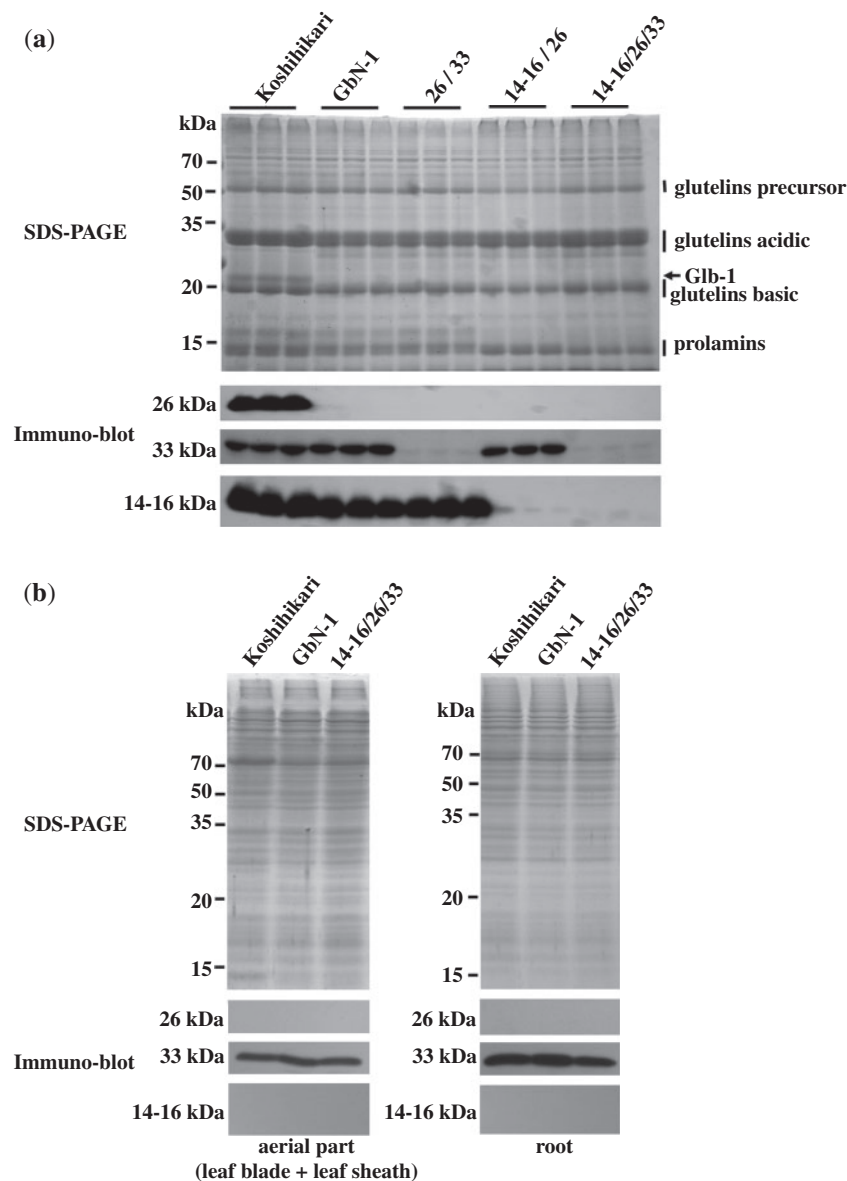


Fig. 2 Changes of major seed allergen protein levels in transgenic rice. (a) SDS-PAGE (upper panel) and immunoblot analyses (other panels) of 'Koshihikari', non-transgenic GbN-1, transgenic rice seed suppressing the 33 kDa allergen (26/33), transgenic rice seed suppressing the 14–16 kDa allergen (14-16/26) and transgenic rice seed suppressing both the 33 kDa and 14–16 kDa allergens (14-16/26/33). Three lanes for each sample correspond to three independent lines from 'Koshihikari', GbN-1 and three kinds of T_3 homozygous transgenic rice. (b) SDS-PAGE (upper panel) and immunoblot analyses (other panels) of vegetative tissues derived from GbN-1 and transgenic rice seed 14-16/26/33. Each allergen protein was examined by immunoblot analysis using anti-26 kDa, anti-33 kDa or anti-14–16 kDa allergen polyclonal antibodies.

Table 1 Target gene suppression efficiency in transgenic rice lines

Constructs (transgenic line name)	No. of transformants	No. of plants suppressed in the target genes
14–16 kDa RNAi (14-16/26)	10	5 (50.0%)
33 kDa RNAi (26/33)	32	17 (53.1%)
14–16 kDa 33 kDa double RNAi (14-16/26/33)	22	10 (45.4%)

14-16/26 means 14–16 kDa and 26 kDa allergen suppressed rice, 26/33 means 26 kDa and 33 kDa allergen suppressed rice and 14-16/26/33 means three major allergen suppressed rice.

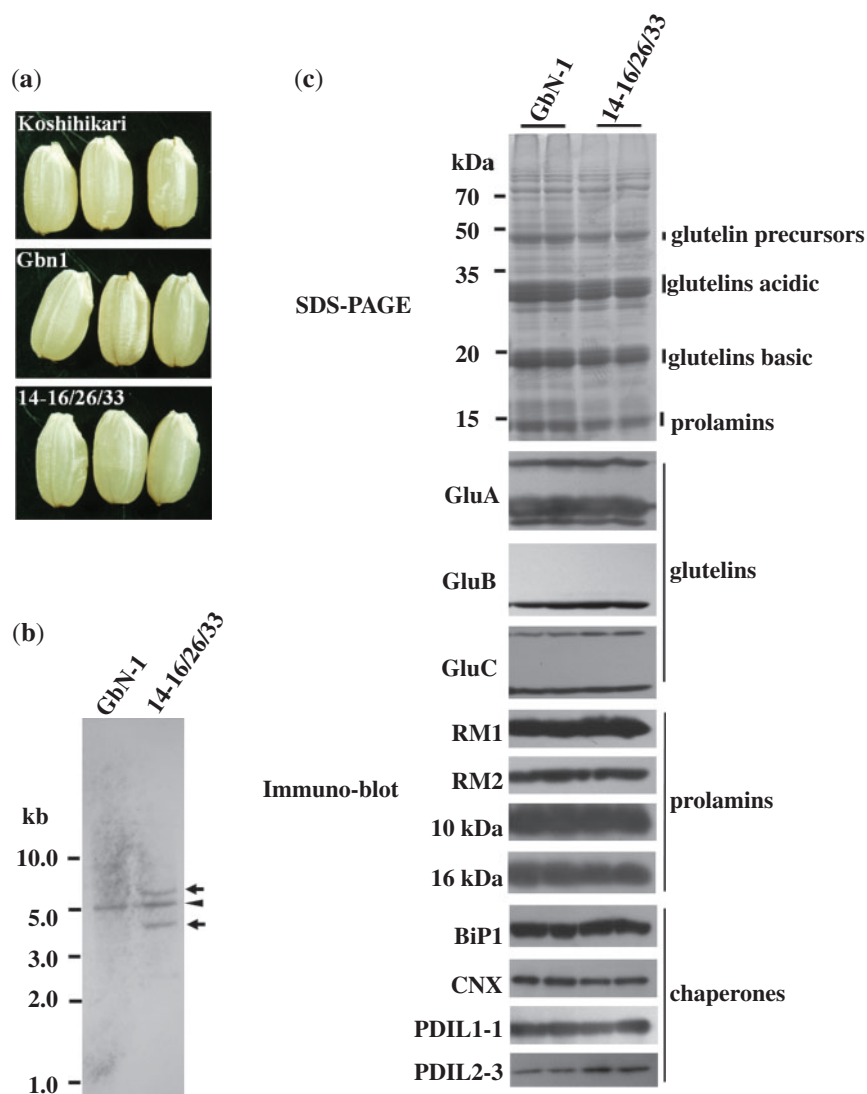


Fig. 3 Characterization of a transgenic rice line strongly suppressed in all three seed allergens (14-16/26/33). (a) Seed phenotype of 'Koshihikari', GbN-1 and 14-16/26/33. (b) Southern blot analysis using the mALS coding region as the probe. The arrowhead indicates an endogenous ALS signal, whereas the arrows indicate the mALS signal derived from the transgene. (c) SDS-PAGE and immunoblot analysis using polyclonal antibodies for SSPs [glutelins (GluA, GluB and GluC) and prolamins (RM1, RM2, 10 kDa prolamin and 16 kDa prolamin)] and protein folding-related chaperon proteins (BiP1, CNX, PDIL1-1 and PDIL2-3) in the ER.

Table 2 Seed features of Koshihikari, GbN-1 and transgenic (14-16/26/33) samples

Samples	Length (mm)	Width (mm)	Thickness (mm)	Thousand kernel weight (g)	Protein content (%)	Amylose content (%)
Koshihikari	5.00 ± 0.02	2.85 ± 0.03	1.91 ± 0.01	19.4	10.9 ± 0.95	16.7 ± 2.12
GbN-1	5.08 ± 0.02	2.92 ± 0.02	1.97 ± 0.01a	18.8	10.0 ± 0.33	15.6 ± 1.96
14-16/26/33	4.92 ± 0.06b	2.58 ± 0.01a,b	1.89 ± 0.003	18.1	10.3 ± 0.87	17.5 ± 0.57

'a' indicates $P < 0.05$ for the group of transgenic rice in comparison with the group of 'Koshihikari'. 'b' indicates $P < 0.05$ for the group of transgenic rice in comparison with the 'GbN-1' group. Length, width and thickness, $n = 20$; protein and amylose content $n = 3$

We investigated the effect of suppressing major allergens on the expression levels of seed storage proteins (SSPs) and chaperone proteins [binding protein (BiP1), calnexin (CNX), protein disulfide isomerase-like 1-1 and 2-3 (PDIL1-1 and PDIL2-3)]

(Fig. 3c). Although it was reported that a reduction of one or a few SSPs was compensated for by increases in other SSPs to maintain a relatively constant protein content (Kawakatsu et al. 2010), reduction of the 14–16 kDa and/or 33 kDa allergens

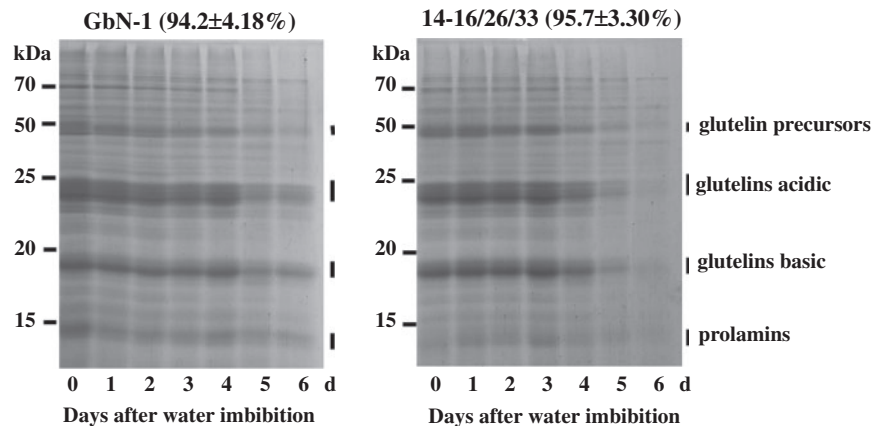


Fig. 4 Degradation of seed proteins during the seed germination stage. GbN-1 (left panel) and transgenic rice (14-16/26/33, right panel) seeds were investigated for up to 6 d after imbibition. The numbers in parentheses show the germination rate. There was no significant difference between GbN-1 and 14-16/26/33 in germination rate ($P > 0.05$).

did not cause a significant alternation in the accumulation pattern of other SSPs. This result may be explained by the normally low levels of these allergens (<5% by weight of total seed proteins) in rice seed proteins. Furthermore, differences in the regulatory mechanism for 14–16 kDa allergen gene expression compared with other seed proteins genes may be somewhat responsible for the lack of altered accumulation pattern for other SSPs, because 14–16 kDa allergen genes are controlled only by a RPBF Dof transcription factor (Kawakatsu et al. 2009).

On the other hand, it is known that endoplasmic reticulum (ER) chaperones are responsible for protein folding in the ER lumen at early stages of protein maturation, leading to the stable accumulation of endogenous SSPs. Furthermore, BiP1 and CNX act as sensing signals to assess whether the present state of secretory proteins in the ER lumen is normal or not. BiP1 and CNX levels are sensitive to perturbations in the initial protein folding and transport system in the ER lumen (Oono et al. 2010, Wakasa et al. 2011). As shown in **Fig. 3c**, there was little difference in the amounts of BiP1, CNX and PDIL1-1 that accumulated in non-transgenic GbN-1 and transgenic (14-16/26/33) rice seed, suggesting that the protein folding and transport system in the ER was not significantly perturbed in the transgenic rice seed cells. However, the PDIL2-3 level increased (approximately 2.2-fold) in transgenic rice compared with the levels in GbN-1. This result may be related to the finding that PDIL2-3 localizes in the ER lumen and the surface of type-I protein bodies (PB-I) (Onda et al. 2011), whereas the three allergen proteins localized in PB-II (**Supplementary Fig. S3**). On the other hand, PDIL1-1 also mainly accumulates in the ER lumen, but this protein facilitates folding of proglutelin which is finally trafficked into PB-II (Takemoto et al. 2002). Thus, reduction of the 14–16 kDa and 33 kDa allergens should influence PDIL2-3 accumulation in the ER. The PDIL2-3 level is likely to be more affected than PDIL1-1 by changes in the levels of accumulation other proteins in the ER lumen (Oono et al. 2010, Wakasa et al. 2011).

Next, we compared the germination rate and degradation pattern of seed proteins for 6 d after imbibition of seeds between GbN-1 and transgenic rice (14-16/26/33). The germination rate was almost the same for both of them ($94.2 \pm 4.18\%$ vs. $95.7 \pm 3.30\%$) (**Fig. 4**). Degradation of seed proteins in transgenic rice seed was slightly faster than in GbN-1 seed (**Fig. 4**). This result may be accounted for by the finding that 14–16 kDa allergens having a function of a protease inhibitor (α -amylase/trypsin inhibitor) related to the protein degradation process are highly reduced in transgenic rice seeds compared with GbN-1. In light of the finding that the 33 kDa allergen level was not changed in seedlings (**Fig. 2b**), seed-specific suppression of the three allergens would not be the key factor responsible for the germination rate or vigorous growth of seedling.

Immuno-dot-blot analysis of rice seed proteins recognized by IgE derived from allergy patients

To evaluate some other potential allergens remaining in the established transgenic lines with reduced contents of each or all of the three major allergens, total albumin–globulin proteins were extracted from rice seeds, and IgE binding to the extracted proteins was analyzed by immuno-dot-blot analysis. Immuno-dot-blot analysis was employed instead of enzyme-linked immunosorbent assay (ELISA) because of the higher protein adsorption capacity of nitrocellulose paper, which is suitable for rice seed crude extract containing various proteins.

'Koshihikari', GbN-1 and three transgenic rice lines (26/33, 14-16/26 and 14-16/26/33) were used in IgE binding tests using sera from allergy patients. Fifteen rice-positive and a control (rice-negative) sera were reacted with the albumin–globulin fraction containing 26 kDa, 33 kDa and 14–16 kDa allergens that had been extracted from transgenic and non-transgenic rice seeds. The IgE reactivity of these subjects to seed proteins from suppressed transgenic rice was examined by immuno-dot-blot analysis (**Fig. 5**). The 15 selected serum samples

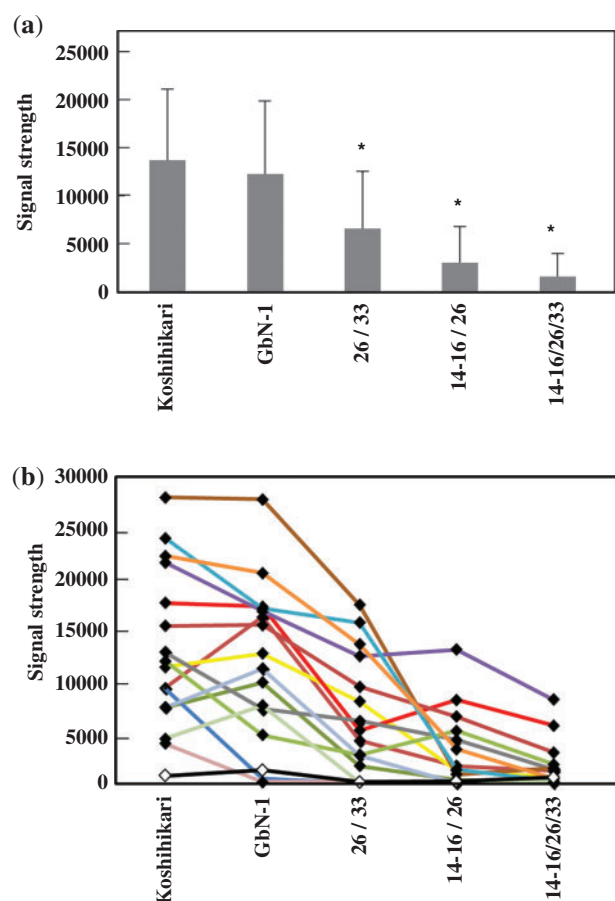


Fig. 5 IgE binding of serum samples from 15 patients to the albumin-globulin fraction of the transgenic rice seeds deficient in one, two and three allergenic proteins. The IgE binding to rice protein of the 15 serum samples was analyzed by chemiluminescence-based immunodot-blot analysis followed by quantification using Image-J. The intensity of IgE binding was represented as the mean $\pm$ SD binding to each of 'Koshihikari', GbN-1, 26 kDa and 33 kDa suppressed transgenic seed (26/33), 14–16 kDa and 26 kDa suppressed transgenic seed (14-16/26) and 14–16 kDa, 26 kDa and 33 kDa suppressed transgenic seed (14-16/26/33) proteins. (b) The intensity of IgE binding of the 15 serum samples to the five rice protein samples is represented individually as a closed diamond with a colored connecting line. The intensity of a negative control serum is shown as an open diamond with a black line for comparison. Asterisk indicates statistically significant reduction ($P < 0.001$) compared with the control rice, 'Koshihikari'. Differences in IgE binding between two rice samples were analyzed by paired *t*-test.

containing rice-specific IgE with considerable individual variability in the IgE concentration and/or the antigen binding affinity showed an IgE binding dot intensity 5–30 times higher than the intensity of the negative control serum binding to the albumin-globulin fraction from the control wild-type rice 'Koshihikari'. As shown in Fig. 5a, the mean value of IgE binding on the 15 serum samples gradually decreased with the stepwise deletion of the three major allergens from the rice seed protein. The mean value decreased to about 50 and 20%, respectively, of

the non-transgenic control when two allergens, the 33 kDa plus 26 kDa allergen and the 14–16 kDa plus 26 kDa allergen, were mostly deleted. The IgE binding intensity further decreased to about 10% of the control when all of the three allergens, 14–16 kDa, 26 kDa and 33 kDa, were mostly deleted. These results suggest that the allergenicity, at least on the IgE-dependent allergic reaction, of rice seed can be potentially reduced depending on the degree of suppression of the three major allergens. In other words, the three rice proteins, 14–16 kDa, 26 kDa and 33 kDa proteins, are estimated to account for the great majority of rice seed causative allergens recognized by IgE from patients' sera examined in the present study.

Individual variability among the 15 serum samples was observed in the IgE binding to the albumin-globulin fraction from not only the control non-transgenic rice 'Koshihikari' but also the mutant and transgenic lines (Fig. 5b). A few serum samples showed lower IgE binding to the GbN-1 proteins than that of the control 'Koshihikari', whereas the others showed comparable or higher binding than the control. The higher IgE binding to the GbN-1 seed proteins might be due to a technical reason. Removal of 26 kDa protein, which is abundant in the seed albumin-globulin fraction, might relatively increase the other protein components, including the allergens, solid-phased on a nitrocellulose membrane, resulting in an apparent increase in the IgE binding. It seems interesting to note that the IgE binding of some serum samples was markedly decreased by deletion of the 33 kDa protein plus the 26 kDa protein, whereas that of others was decreased by deletion of 14–16 kDa plus 26 kDa proteins. This result is consistent with weak correlation in patients' IgE binding to each purified allergen between 14–16 kDa and 33 kDa allergens, and suggests individual variability in the causative allergen(s) recognized by serum specific IgE. These transgenic rice lines mostly deficient in major allergen(s) may be useful in possible future application to clinical diagnosis of patients with cereal allergy for identification of the causative allergen(s). In the case of a few serum samples, the IgE binding was decreased at most to 30–40% of the control, suggesting that IgE of these serum samples had been raised against other unidentified proteins of rice seed.

Further studies on the allergenicity evaluation of the transgenic rice lines would be required based on analyses under more physiological conditions such as a cell-based assay using peripheral blood basophils from patients' fresh blood samples (Tokuda et al. 2009). Once the issue of safety evaluation of the transgenic rice lines is solved, the transgenic rice seed suppressing all or even two of the three major allergen proteins (14–16 kDa, 26 kDa and 33 kDa) may be utilized as a food tolerable to rice allergy patients whose serum IgE antibody predominantly recognizes these allergens. Furthermore, the transgenic rice approach has several advantages over conventional hypo-allergenic rice produced by processing of rice using enzymatic and physical treatment, because our hypo-allergenic rice was generated using the highly edible

quality 'Koshihikari' variety as host rice and the transgenic product is cost-effective.

In order to create hypo-allergenic rice, further work will be required. Because most patients with rice allergy are sensitized to multiple rice allergens, suppression of a number of allergenic proteins must be targeted at the same time. We are now trying to isolate and identify undescribed rice allergen proteins. In the near future, we will need to create a transgenic rice suppressing not only the three major allergen proteins but also the other rice allergen proteins to a level below the threshold for sensitization or hypersensitive response of many rice allergy patients.

Materials and Methods

Plant materials

A rice (*O. sativa* L.) seed storage protein mutant, named GbN-1 (cv. Koshihikari background), lacking 26 kDa allergen (Iida et al. 1998), was used as the host for transformation (Fig. 1a). Seeds grown on artificially controlled soil (0.5 g l⁻¹ nitrogen, 1.3 g l⁻¹ phosphoric acid and 0.73 g l⁻¹ potassium) in a closed glass-house were used for SDS-PAGE, immunoblotting and the other seed characterizations.

Production of transgenic rice

Binary vector construction for *Agrobacterium*-mediated transformation was performed by using the MultiSite Gateway LR reaction (Invitrogen) as described previously (Wakasa et al. 2006). Briefly, gene cassettes consisting of an endosperm-specific promoter [13 kDa prolamin (1.23 kb) or 10 kDa prolamin promoter (0.87 kb)], the inverted repeat structure of sense and antisense fragments from the full-length 14–16 kDa allergen (AK107328, 0.5 kb) or 33 kDa allergen (AK066092, 0.88 kb) cDNA coding regions separated by the rice *Oryzasin1* intron (0.99 kb) (Asakura et al. 1995, Kuroda et al. 2010), and a terminator [(13 kDa prolamin (0.18 kb) or 10 kDa prolamin terminator (0.16 kb)] were inserted into the Gateway entry clones pKS221 MCS and pKS 4-1 MCS, respectively. These gene cassettes were introduced into pCSP mALS GW in the case of single gene cassette integration and into pCSP mALS 24 GW in the case of double gene cassette integration using MultiSite Gateway LR Clonase II Plus Enzyme Mix (Invitrogen) (Fig. 1b–d). CSP mALS is a selectable marker gene consisting of a mutated acetolactate synthase gene (mALS) under the control of a callus-specific promoter (CSP). All of these DNA elements were derived from the rice genome. Furthermore, selection was restricted to the callus stage only; thus, this selectable marker can be used for marker-free-like transgenic rice production, a method that should be more acceptable for commercial cultivation.

Three binary vector plasmids were introduced into the GbN-1 genome via *Agrobacterium*-mediated transformation as described previously (Wakasa et al. 2007).

DNA extraction and Southern blot analysis

Genomic DNA was extracted from 1.0 g of young leaves by the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980). A 5 µg aliquot of DNA was digested with *Hind*III, fractionated by electrophoresis on a 0.8% agarose gel and transferred to a Hybond-N+ nylon membrane (Amersham Pharmacia Biotech). Southern hybridization and signal detection were carried out using the Gene Images AlkPhos Direct Labeling and Detection System (GE Healthcare). A probe was prepared from purified PCR products of the full-length ALS coding region. Both endogenous ALS and exogenous mALS DNA fragments can be detected by this probe.

Total protein extraction and immunoblot analysis

Mature seeds from the transgenic rice lines were harvested. Each grain was separately ground into a fine powder with a Multi-beads Shocker (YASUI KIKAI). For total protein extraction, 500 µl of extraction buffer [50 mM Tris-HCl (pH 6.8), 8 M urea, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol, 0.1% Bromophenol Blue] was added to the seed powder and vortexed for >1 h at room temperature. The mixture was centrifuged at 12,000×g for 20 min at room temperature, and the crude soluble protein sample was decanted into a new tube. A 2 µl aliquot of total protein was subjected to immunoblot analysis using specific polyclonal antibodies after electrophoresis on 12% SDS-polyacrylamide gels as described by Yasuda et al. (2005). Signals were detected by an ECL detection system (GE Healthcare).

Seedlings (6 d after water absorption) were separated into the aerial parts and roots. Total proteins were extracted from these samples by 200 µl of extraction buffer. A 5 µl aliquot of total protein was subjected to SDS-PAGE and immunoblot analysis.

Protein and amylose contents of rice seed

Seed protein content was measured by an RC DC protein assay kit (Bio-Rad) as described in the manufacturer's protocol. Amylose content was measured using an amylose/amylopectin assay kit (Megazyme) as described in the manufacturer's protocol.

Confocal immunohistochemical analysis

Immature 'Koshihikari' seeds were collected at 15 d after flowering and used for immunocytochemical microscopic analysis using the anti-14–16 kDa allergen, 26 kDa allergen, 33 kDa allergen and anti-OsTiP3 antibody. These antibodies were used as a primary antibody at a 1:300 dilution. Alexa488-conjugated goat anti-rabbit IgG (Invitrogen) was used at a 1:500 dilution as the secondary antibody. Rhodamine B was used to stain PB-I. The samples were observed with a confocal laser scanning microscope (FLUOVEIW; Olympus).

Protein degradation analysis

Seeds derived from GbN-1 and transgenic rice (14-16/26/33) were put onto 0.8% agar (Wako) after sterilization treatment. Three seeds were sampled every day after plating on agar for up to 6 d. Total proteins were extracted from these seeds by using 1.5 ml of extraction buffer. A 2 μ l aliquot of total protein was subjected to electrophoresis on 12% SDS–polyacrylamide gels.

Extraction of the albumin–globulin fraction

Milled rice seeds of the wild type and two transgenic lines were mixed with 0.5 M NaCl/10 mM Tris–HCl, pH 6.8 (100 mg in 1 ml), kept at 4°C overnight and then ultrasonicated on ice. The milled rice suspension was centrifuged at 15,000 $\times$ g for 20 min. The supernatant was used for the IgE binding assay as the albumin–globulin fraction.

Patients' serum

The study using patients' serum IgE was performed with the approval of the Ethics Committee of Fujita Health University School of Medicine. Informed consent was obtained from the patients or guardians of infants or child subjects. From among the sera of patients with suspected food allergy under medical treatment for atopic dermatitis and/or bronchial asthma at the University Hospital, 15 sera with IgE-positive ELISA values for rice seed proteins, and a serum with negative values for the rice albumin–globulin proteins were selected and used for the following experiments.

Immuno-dot-blot analysis of rice seed proteins recognized by human IgE

IgE binding to rice seed proteins was analyzed by dot-blotting, in which a smaller volume of patients' sera can be used and a larger amount of allergen proteins can be attached to a nitrocellulose membrane compared with a polystyrene ELISA plate. A 1 μ l aliquot of each albumin–globulin fraction, equivalent to 100 μ g of milled rice seeds, was spotted on a nitrocellulose membrane, dried in air and blocked with gelatin solution [0.15 M NaCl, 5 mM EDTA, 50 mM Tris–HCl, pH 7.5, 0.05% Triton X-100, 0.25% gelatin (NETG)]. After being washed, the membrane was incubated with human serum diluted 100-fold with NETG, followed by incubation with a 1:10,000 dilution of horseradish peroxidase (HRP)-labeled anti-human IgE (Biosource International, Inc.). HRP activity was detected using the Immobilon Western chemiluminescent HRP substrate (Millipore) with a light capture system (AE-6962, ATTO). The relative chemiluminescence intensity of each spot was quantified using NIH Image-J.

Supplementary data

Supplementary data are available at PCP online.

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References

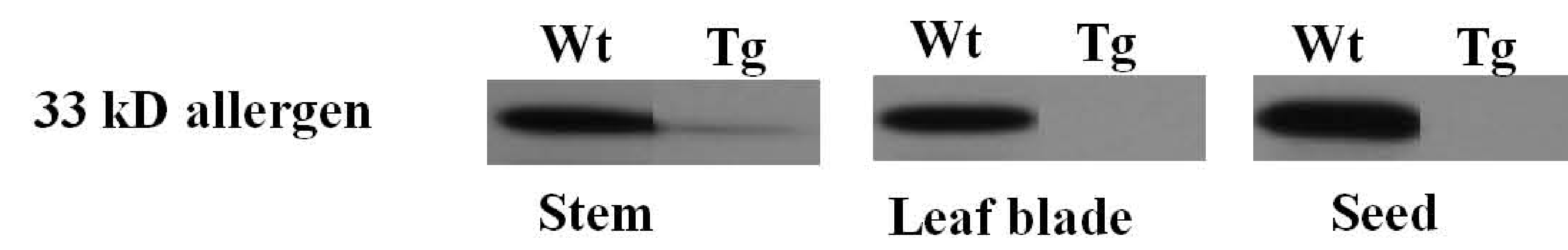
- Asakura, T., Watanabe, H., Abe, K. and Arai, S. (1995) Rice aspartic proteinase, oryzasin, expressed during seed ripening and germination, has a gene organization distinct from those of animal and microbial aspartic proteinases. *Eur. J. Biochem.* 232: 77–83.
- Alvarez, A.M., Adachi, T., Nakase, M., Aoki, N., Nakamura, R. and Matsuda, T. (1995) Classification of rice allergenic protein cDNAs belonging to the α -amylase/trypsin inhibitor gene family. *Biochim. Biophysica. Acta* 1251: 201–204.
- Dodo, H.W., Konan, K.N., Chen, F.C., Egnin, M. and Viquez, O.M. (2008) Alleviating peanut allergy using genetic engineering: the silencing of the immunodominant allergen Ara h 2 leads to its significant reduction and a decrease in peanut allergenicity. *Plant Biotechnol. J.* 61: 35–145.
- Gilissen, L.J., Bolhaar, S.T., Matos, C.I., Rouwendal, G.J., Boone, M.J., Krens, F.A. et al. (2005) Silencing the major apple allergen Mal d 1 by using the RNA interference approach. *J. Allergy Clin. Immunol.* 115: 364–369.
- Herman, E.M., Helm, R.M., Jung, R. and Kinney, A.J. (2003) Genetic modification removed an immunodominant allergen from soybean. *Plant Physiol.* 132: 36–43.
- Iida, S., Miyahara, K. and Nishio, T. (1998) Rice mutant lines lacking α -globulin. *Breed. Sci.* 48: 45–49.
- Kato, T., Katayama, E., Matsubara, S., Yuko Omi, Y. and Matsuda, T. (2000) Release of allergenic proteins from rice grains induced by high hydrostatic pressure. *J. Agric. Food Chem.* 48: 3124–3129.
- Kawakatsu, T., Yamamoto, M.P., Touno, S.M., Yasuda, H. and Takaiwa, F. (2009) Compensation and interaction between RISBZ1 and RPBF during grain filling in rice. *Plant J.* 59: 908–920.
- Kawakatsu, T., Hirose, S., Yasuda, H. and Takaiwa, F. (2010) Reducing rice seed storage protein accumulation leads to changes in nutrient quality and storage organelle formation. *Plant Physiol.* 154: 1842–1854.
- Kuroda, M., Kimizu, M. and Mikami, C. (2010) A simple set of plasmids for the production of transgenic plant. *Biosci. Biotechnol. Biochem.* 74: 2348–2351.
- Le, L.Q., Lorenz, Y., Schenrer, S., Fotisch, K., Enrique, E., Bartra, J. et al. (2006a) Design of tomato fruits with reduced allergenicity by dsRNA-mediated inhibition of ns-LTP (Lyc3) expression. *Plant Biotechnol. J.* 4: 231–242.
- Le, L.Q., Mahler, V., Lorenz, Y., Scheurer, S., Biemett, S., Vieths, S. et al. (2006b) Reduced allergenicity of tomato fruits harvested from Lyc e1-silenced transgenic tomato fruit. *J. Allergy Clin. Immunol.* 118: 1176–1183.

- Limas, G.G., Salinas, M., Moneo, M., Fischer, S., Wittmann-Liebold, B. and Mendez, E. (1990) Purification and characterization of ten new rice NaCl-soluble proteins: identification of four protein-synthesis inhibitors and two immunoglobulin binding proteins. *Planta* 181: 1–9.
- Matsuda, T., Nakasa, M., Alvarez, A.M., Izumi, H., Kato, T. and Tada, Y. (2006) Rice-seed allergenic protein and hypoallergenic rice. In *Nutraceutical Proteins and Peptides in Health and Disease*. Edited by Mine, Y. and Shahidi, F. pp. 493–511. Taylor & Francis Group, London.
- Mills, E.N., Jenkins, J.A., Alcocer, M.J. and Shewry, P.R. (2004) Structural, biological, and evolutionary relationships of plant food allergens sensitizing via the gastrointestinal tract. *Crit. Rev. Food Sci. Nutr.* 44: 379–407.
- Murray, M.G. and Thompson, W.F. (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* 8: 4321–4325.
- Nishio, T. and Iida, S. (1993) Mutants having a low content of 16-kDa allergenic protein in rice (*Oryza sativa* L.). *Theor. Appl. Genet.* 86: 317–321.
- Onda, Y., Nagamine, A., Sakurai, M., Kumamaru, T., Ogawa, M. and Kawagoe, Y. (2011) Distinct roles of protein disulfide isomerase and P5 sulfhydryl oxidoreductases in multiple pathways for oxidation of structurally diverse storage proteins in rice. *Plant Cell* 23: 210–223.
- Oono, Y., Wakasa, Y., Hirose, S., Yang, L., Sakuta, C. and Takaiwa, F. (2010) Analysis of ER stress in developing rice endosperm accumulating β -amyloid peptide. *Plant Biotechnol. J.* 8: 1–28.
- Perez, C.M., Juliano, B.O., Liboon, S.P., Alcantara, J.M. and Cassman, K.G. (1996) Effect of late nitrogen fertilizer application on head rice yield, protein content, and grain quality of rice. *Cereal Chem.* 73: 556–560.
- Qu, L.Q. and Takaiwa, F. (2004) Evaluation of tissue specificity and expression strength of rice seed component gene promoters in transgenic rice. *Plant Biotechnol. J.* 2: 113–125.
- Tada, Y., Nakase, M., Nakamura, R., Shimada, H., Takahashi, M., Fujiwara, T. et al. (1996) Reduction of 14–16 kDa allergenic protein in transgenic rice plants by antisense gene. *FEBS Lett.* 391: 341–345.
- Takemoto, Y., Coughlan, S.J., Okita, T.W., Satoh, H., Ogawa, M. and Kumamaru, T. (2002) The rice mutant esp2 greatly accumulates the glutelin precursor and deletes the protein disulfide isomerase. *Plant Physiol.* 128: 1212–1222.
- Tokuda, R., Nagao, M., Hiraguchi, Y., Hosoki, K., Matsuda, T., Kouno, K. et al. (2009) Antigen-induced expression of CD203c on basophils predicts IgE-mediated wheat allergy. *Allergol. Int.* 58: 193–199.
- Urisu, A., Yamada, K., Masuda, S., Komada, H., Wada, E., Kondo, Y. et al. (1991) 16-kilodalton rice protein is one of the major allergens in rice grain extract and responsible for cross-allergenicity between cereal grains in the Poaceae family. *Int. Arch. Allergy Appl. Immunol.* 96: 244–252.
- Usui, Y., Nakane, M., Hotta, H., Urisu, A., Aoki, N., Kitajima, K. et al. (2001) A 33-kDa allergen from rice (*Oryza sativa* L.). cDNA cloning, expression, and identification as a novel glyoxalase I. *J. Biol. Chem.* 276: 11376–11381.
- Wakasa, Y., Ozawa, K. and Takaiwa, F. (2007) *Agrobacterium*-mediated transformation of a low glutelin mutant of 'Koshihikari' rice variety using the mutated-acetolactate synthase gene derived from rice genome as a selectable marker. *Plant Cell Rep.* 26: 1567–1573.
- Wakasa, Y., Yasuda, H., Oono, Y., Kawakatsu, T., Hirose, S., Takahashi, H. et al. (2011) Expression of ER quality-control related genes in response to changes in BiP1 levels in developing rice endosperm. *Plant J.* 65: 675–689.
- Wakasa, Y., Yasuda, H. and Takaiwa, F. (2006) High accumulation of bioactive peptide in transgenic rice seeds by expression of introduced multiple genes. *Plant Biotechnol. J.* 4: 499–510.
- Watanabe, M., Miyakawa, J., Ikezawa, Z., Suzuki, Y., Hirano, T., Yoshizawa, T. et al. (1990a) Production of hypoallergenic rice by enzymatic decomposition of constituent proteins. *J. Food Sci.* 55: 781–783.
- Watanabe, M., Yoshizawa, T., Miyakawa, J., Ikezawa, Z., Abe, K., Yanagisawa, T. et al. (1990b) Quality improvement and evaluation of hypoallergenic rice grains. *J. Food Sci.* 55: 1105–1107.
- Wu, C.Y., Adachi, T., Hatano, T., Washida, H., Suzuki, A. and Takaiwa, F. (1998) Promoters of rice storage protein genes direct endosperm-specific gene expression in transgenic rice. *Plant Cell Physiol.* 39: 885–889.
- Yasuda, H., Tada, Y., Hayashi, Y., Jomori, T. and Takaiwa, F. (2005) Expression of the small peptide GLP-1 in transgenic plants. *Transgenic Res.* 14: 677–684.

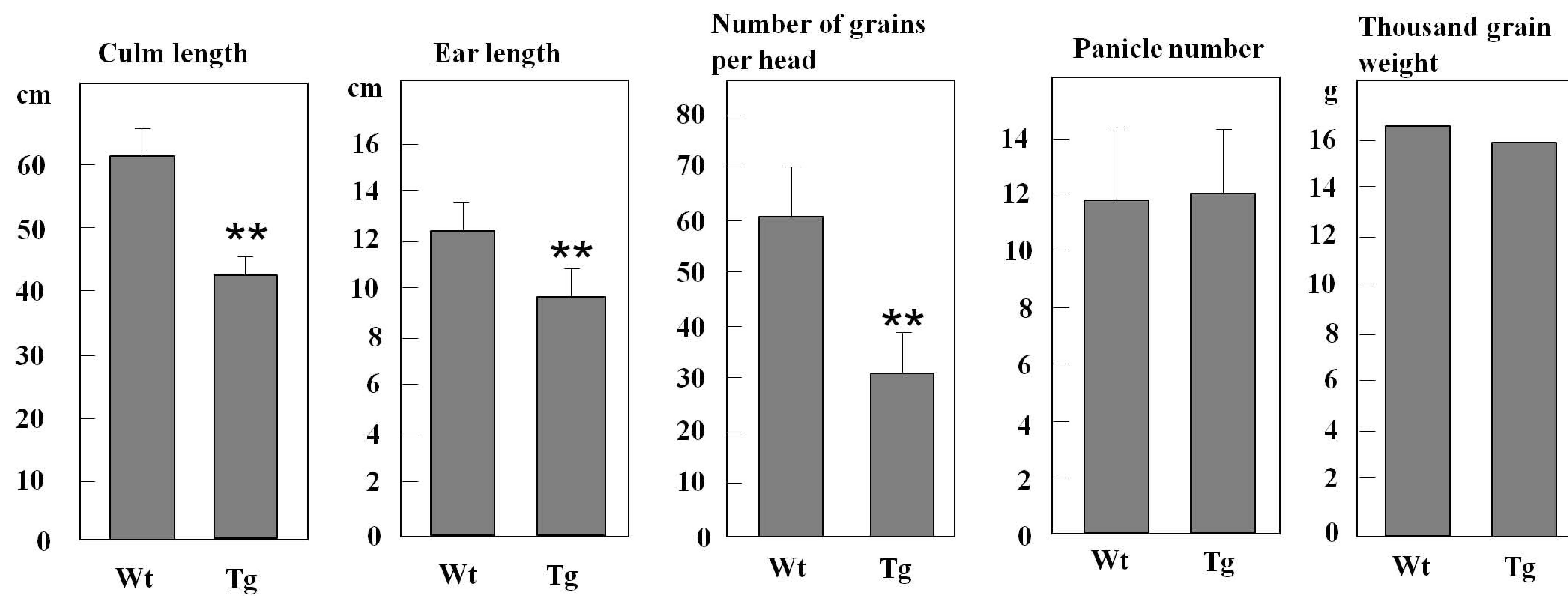
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D42121.gpt	115	AEVFR	GCRRGDI	ERAAASLP	AFCNVDIPNGV	GGVCYWLP	GTGY	----- 157
D42122.gpt	118	SEVFR	GCRRGDLERAAASLP	AFCNVDIPNGG	GGVCYWL	ARSGY	-----	160
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Wakasa et al. Fig. S1

a

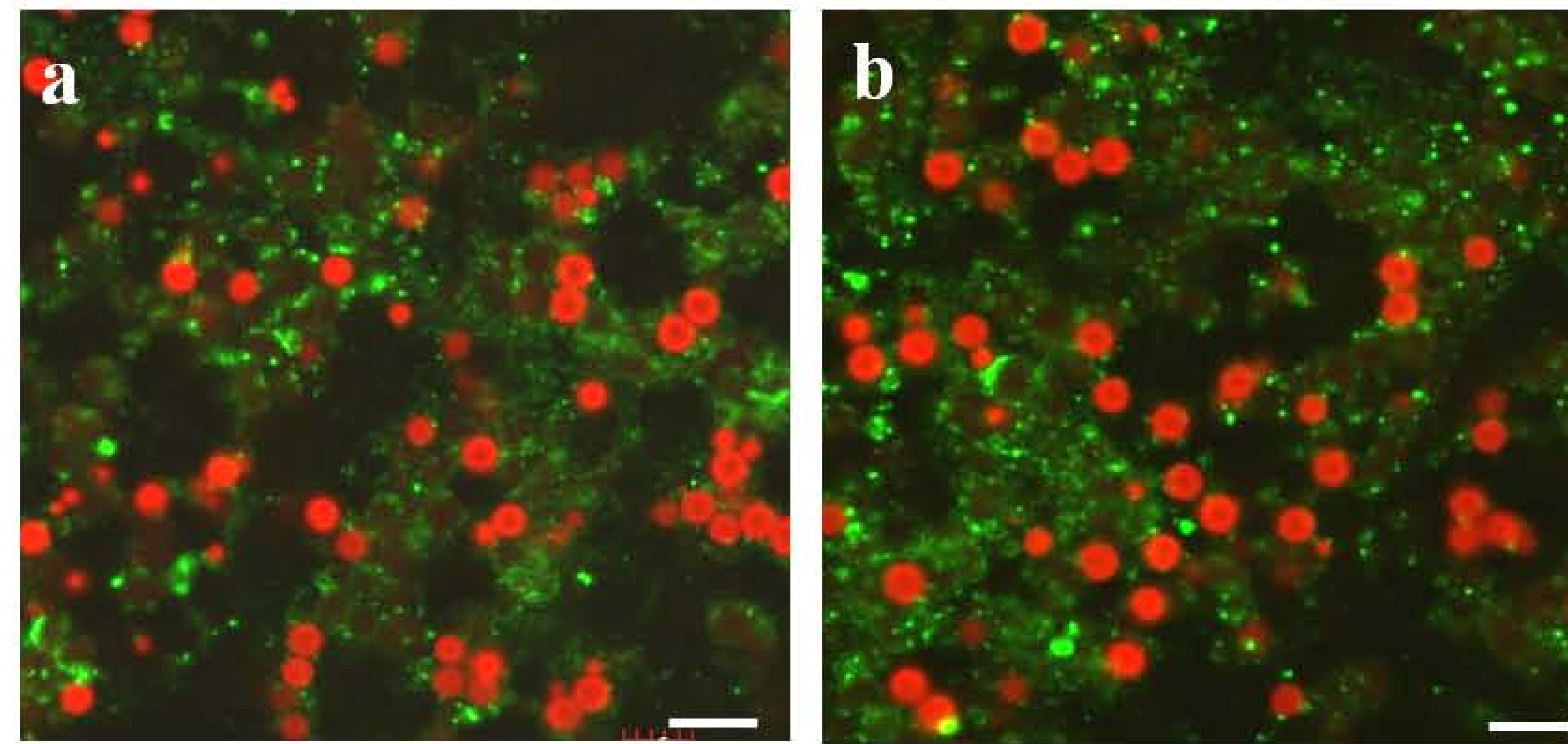


b

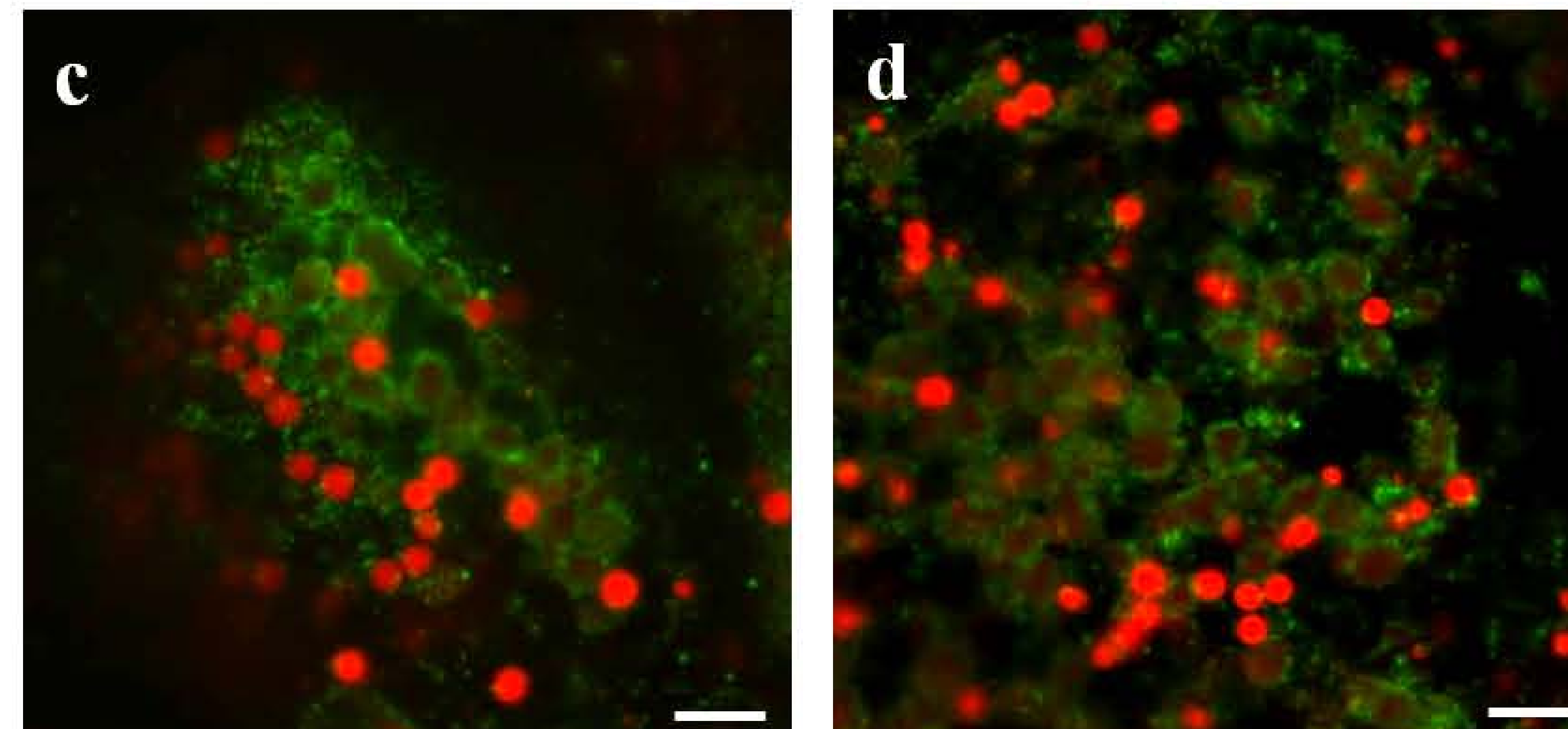


Wakasa et al. Fig. S2

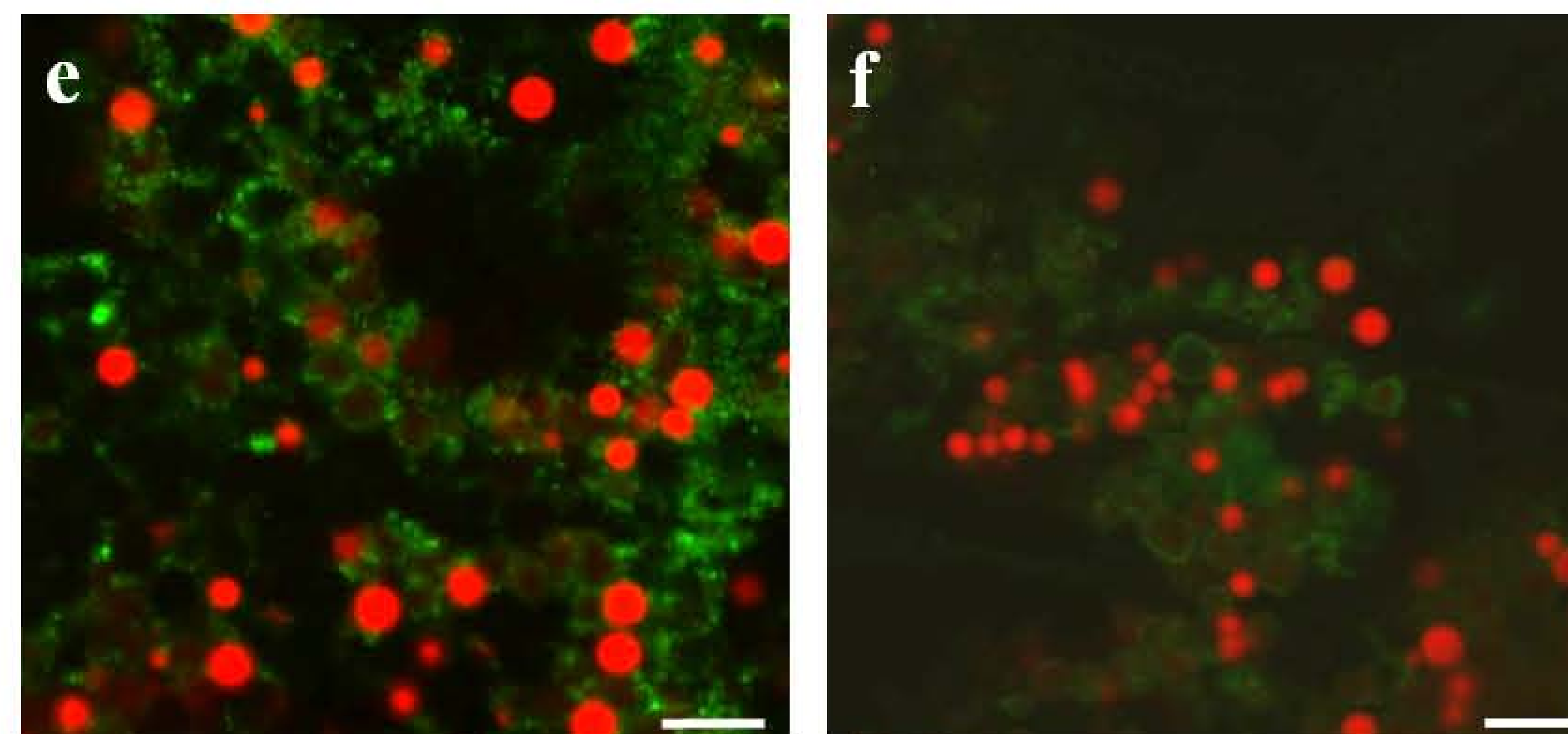
14-16 kD



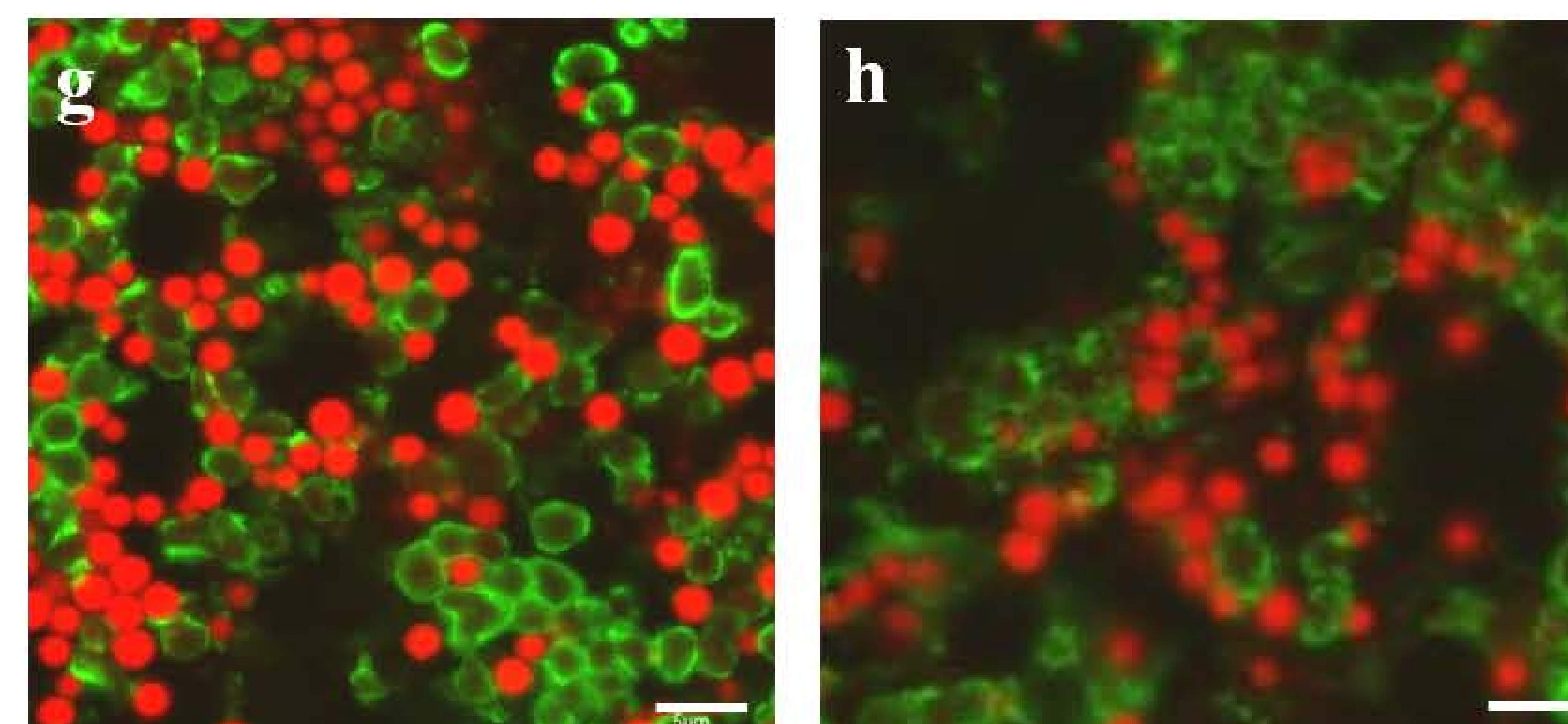
26 kD



33 kD



Os-TiP3



Bar 5 μ m

Supporting information

Figure S1. Multiple alignment of amino acid sequences for nine 14-16 kD allergen gene members in rice.

Figure S2. Accumulation of 33 kD allergen in wild type (cv. kitaake) and its transgenic rice tissues and corresponding plant phenotypes. a, Immuno-blot analysis of 33 kD allergen in stem, leaf blade and seed tissues in wild type (Wt) and transgenic (Tg) plants. b, Culm length, ear length, number of grains per head, panicle number and thousand kernel weights of wild type and a transgenic rice line. ** $p < 0.01$ for the group of transgenic rice in comparison with the group of non-transgenic rice.

Figure S3. Indirect immunohistochemical analysis of three allergen proteins (14-16 kD, 26 kD and 33 kD allergen) in 'Koshihikari'. Green and red signals indicate the localization of each allergen protein and protein body type-I (PB-I). a and b, 14-16 kD allergen; c and d, 26 kD allergen; e and f, 33 kD allergen. g and h show the localization of PB-II marker protein (Os-TiP3).