

RNAi-mediated suppression of endogenous storage proteins leads to a change in localization of overexpressed cholera toxin B-subunit and the allergen protein RAG2 in rice seeds

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Abstract

Key message RNAi-mediated suppression of the endogenous storage proteins in MucoRice-CTB-RNAi seeds affects not only the levels of overexpressed CTB and RAG2 allergen, but also the localization of CTB and RAG2.

Abstract A purification-free rice-based oral cholera vaccine (MucoRice-CTB) was previously developed by our laboratories using a cholera toxin B-subunit (CTB) over-expression system. Recently, an advanced version of MucoRice-CTB was developed (MucoRice-CTB-RNAi) through the use of RNAi to suppress the production of the

endogenous storage proteins 13-kDa prolamin and glutelin, so as to increase CTB expression. The level of the α -amylase/trypsin inhibitor-like protein RAG2 (a major rice allergen) was reduced in MucoRice-CTB-RNAi seeds in comparison with wild-type (WT) rice. To investigate whether RNAi-mediated suppression of storage proteins affects the localization of overexpressed CTB and major rice allergens, we generated an RNAi line without CTB (MucoRice-RNAi) and investigated gene expression, and protein production and localization of two storage proteins, CTB, and five major allergens in MucoRice-CTB, MucoRice-CTB-RNAi, MucoRice-RNAi, and WT rice. In all lines, glyoxalase I was detected in the cytoplasm, and 52- and 63-kDa globulin-like proteins were found in the aleurone particles. In WT, RAG2 and 19-kDa globulin were localized mainly in protein bodies II (PB-II) of the endosperm cells. Knockdown of glutelin A led to a partial

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destruction of PB-II and was accompanied by RAG2 relocation to the plasma membrane/cell wall and cytoplasm. In MucoRice-CTB, CTB was localized in the cytoplasm and PB-II. In MucoRice-CTB-RNAi, CTB was produced at a level six times that in MucoRice-CTB and was localized, similar to RAG2, in the plasma membrane/cell wall and cytoplasm. Our findings indicate that the relocation of CTB in MucoRice-CTB-RNAi may contribute to down-regulation of RAG2.

Keywords Rice · Allergen · Localization · MucoRice · Mucosal vaccine

Abbreviations

BiP	Binding protein
CTB	Cholera toxin B-subunit
DAF	Days after flowering
IF	Immunofluorescence
immuno-EM	Immuno-electron microscopy
RNAi	RNA interference
PB-I	Protein body I
PB-II	Protein body II
PBS	Phosphate-buffered saline
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
T-DNA	Transfer DNA
WB	Western blotting
WT	Wild type
Ab	Antibody
mAb	Monoclonal antibody
BSA	Bovine serum albumin
NCS	Newborn calf serum
PSV	Protein storage vacuole
ER	Endoplasmic reticulum
RER	Rough endoplasmic reticulum
IL-10	Interleukin-10
PEG	Polyethylene glycol

Introduction

Seeds are an ideal site for the production of recombinant proteins such as vaccines and antibodies, because they are natural storage organs that accumulate proteins, starch and lipids, offering ample storage space (Nochi et al. 2007; Tokuhara et al. 2013). As a production system, rice (*Oryza sativa* L.) has several advantages over other crops, including its self-crossing ability, high yields, and ease of storage and processing. Rice transformation systems are established (Hiei et al. 1994), and the complete genome sequence is available (International Rice Genome Sequencing Project 2005). This genetic information can be easily used to create products by genetic manipulation. The

rice transgenic line MucoRice-CTB was developed to produce an oral cholera vaccine of the same name (Nochi et al. 2007, 2009; Yuki et al. 2009). MucoRice-CTB, as a non-purified vaccine, is stable at room temperature for more than 36 months, obviating the need for either a cold chain or needles (Tokuhara et al. 2010). These features provide a highly practical and cost-effective strategy for orally vaccinating large populations against mucosal infections.

In this rice line, the cholera toxin B-subunit (CTB) overexpression cassette is controlled by the endosperm-specific 10-kDa prolamin promoter (Kuroda et al. 2010). Recently, the MucoRice system has been advanced by the co-introduction of RNA interference (RNAi) sequences specific to 13-kDa prolamin and glutelin A (endogenous storage proteins) genes to increase the accumulation of CTB in seeds. MucoRice-CTB-RNAi expressed CTB (N4Q) with an Asn to Gln substitution at the glycosylation site. The entire amino acid sequence of the produced protein, the absence of N-glycosylation, and the absence of cross-linked complexes between CTB (N4Q) and other proteins have been confirmed by MS/MS analyses (Yuki et al. 2013). Rice starchy endosperm cells contain two types of protein storage organelles, called protein bodies I (PB-I) and II (PB-II), which differ in shape, density, and protein composition (Tanaka et al. 1980). Seed storage proteins localized in PB-I and PB-II/PSV (protein storage vacuole) are synthesized as secretory proteins in the rough endoplasmic reticulum (RER). PB-I proteins are formed in the RER lumen, whereas the PB-II/PSV proteins are transported into PB-II/PSV via Golgi or precursor-accumulating vesicles (Kawakatsu and Takaiwa 2010). PB-I organelles consist of prolamins, including 10-kDa RP10, 16-kDa RP16, and four 13-kDa isoforms (RM1, 2, 4, and 9), whereas PB-II organelles contain mainly 19-kDa globulin and glutelins, including glutelins A, B, C, and D (Katsube et al. 1999; Yamagata et al. 1982; Yamagata and Tanaka 1986).

Because MucoRice-CTB is used as polished rice flour without purification in clinical studies, the expression levels of allergenic proteins in rice seeds (termed “rice allergens” in this article) have to be estimated before their use in humans. Five major rice allergens (which can bind IgE) have been identified, namely RAG2 (a member of the α -amylase/trypsin inhibitor-like family), 33-kDa glyoxalase I (Usui et al. 2001), 19-kDa globulin, and 52- and 63-kDa globulin-like proteins (Satoh et al. 2011). The 19-kDa globulin is localized mainly in PB-II in WT rice (Krishnan et al. 1992). Glutelins are stored in the inner region of PB-II, whereas 19-kDa globulin is localized in the peripheral matrix surrounding the glutelins (Washida et al. 2012). There are few reports on the localization of RAG2, glyoxalase I, and the 52- and 63-kDa globulin-like

proteins (Wakasa et al. 2011a). Recently, five rice allergens (14–16-kDa) from the α -amylase/trypsin inhibitor-like family (including RAG2) were detected by proteomic approaches (Satoh et al. 2011; Teshima et al. 2012). The level of amino acid sequence identity among RAG2 and the two most closely related proteins is 72–95 % (Adachi et al. 1993). The 19-kDa globulin is encoded by a single-copy gene (Shorosh et al. 1992). The 52- and 63-kDa globulin-like proteins share 39 % amino acid sequence identity (Satoh et al. 2011). Proteomics has found a lower level of RAG2 in MucoRice-CTB-RNAi than in WT (Kurokawa et al. 2013).

In this study, we investigated whether RNAi-mediated suppression of the endogenous storage proteins affects the localization of the overexpressed CTB and major rice allergens. To do so, we generated a transgenic line expressing RNAi without CTB (MucoRice-RNAi) and investigated the expression and localization of two storage proteins, CTB, and five major allergens in MucoRice-CTB, MucoRice-CTB-RNAi, MucoRice-RNAi, and WT rice. MucoRice-RNAi expresses RNAi against 13-kDa prolamin and glutelin A, but does not express CTB. Their mRNA and protein levels were estimated, and their localization was assessed by immunofluorescence (IF) and immunoelectron microscopy (immuno-EM). The expression levels and localization of three allergens (glyoxalase I and 52- and 63-kDa globulin-like proteins) were similar in the four rice lines. RNAi did not affect the expression of RAG2 and 19-kDa globulin, but led to a partial destruction of PB-II. Our data show that RNAi-mediated suppression of the endogenous storage proteins affects not only the levels of overexpressed CTB and RAG2, but also the localization of CTB and RAG2. In addition, these findings suggest that the low expression of RAG2 in MucoRice-CTB-RNAi can be attributed to the competition with CTB for the location site.

Materials and methods

Plant materials

The *japonica* rice cultivar Nipponbare (WT) was used. Two transgenic lines were established previously (MucoRice-CTB with or without RNAi; Yuki et al. 2013), and the MucoRice-RNAi line was developed as described below. Plants were grown in controlled growth chambers (Nochi et al. 2007). Developing seeds were collected 14 days after flowering (DAF).

Generation of MucoRice-RNAi transgenic line

The RNAi cassette pZH2Bik45G1B without the CTB overexpression cassette is shown in Fig. S2. The RNAi

sequence against 13-kDa prolamin was a 45-bp fragment of the respective gene (*RM1*: Os07g0206500; coding sequence positions 1–45). The RNAi sequence against glutelin genes was a 129-bp fragment (*GluA*: Os10g0400200; coding sequence positions 142–270) (Kuroda et al. 2010). Plants were transformed with the plasmids by using the *Agrobacterium*-mediated method (Nochi et al. 2007). Several independent transgenic lines were generated for MucoRice-RNAi, and glutelin A and 13-kDa prolamin expression levels in seeds were compared with those in WT rice. The line with the lowest levels of glutelin A and 13-kDa prolamin was selected and advanced to the T₄ generation by self-crossing to obtain homozygous lines.

Antibodies

The monoclonal antibody to glutelin A was raised in female BALB/c mice by a standard method. In brief, WT rice seeds were extracted with 1 M NaCl, and mice were immunized with this extract three times. Four days after the final immunization, lymphocytes were isolated from the spleen and fused with P3X63-AG8.653 myeloma cells in the presence of 50 % PEG 1500 (Roche). Established hybridomas were injected into ICR nude mice, and antibodies were purified from ascites by using protein G-Sepharose beads (GE Healthcare). Antibody specificity was confirmed by Western blotting (WB) (Supplementary Fig. S1). Polyclonal antibodies to 13-kDa prolamin and glutelin were raised in rabbits immunized with bands excised from an SDS-PAGE blot (Saito et al. 2012). Polyclonal antibody to rice binding protein (BiP) was raised in rabbits immunized with the recombinant N-terminal domain (residues 26–274) of BiP. The antibody specificity was confirmed by WB (Supplementary Fig. S1). Polyclonal antibody to CTB was raised in rabbits immunized with recombinant CTB (Yuki et al. 2001). Polyclonal antibodies to rice allergen proteins were raised in rabbits immunized with recombinant RAG2 (Os07g0214300, AK107328) and glyoxalase I (Os08g0191700, AK066092) (Satoh et al. 2011). Antibodies to 19-kDa (Os05g0499100, gi20159), 52-kDa (Os03g0793700, gi115455865), and 63-kDa (Os03g0663800, gi41469581) rice globulin allergens were raised in rabbits immunized with specific synthetic peptides corresponding to amino acid residues 23–36, 454–470, and 511–527, respectively (Satoh et al. 2011). All antibodies were purified by affinity chromatography on Protein A Sepharose (GE Healthcare). For IF analysis, anti-glutelin A and anti-13-kDa prolamin antibodies were conjugated with Cy3 or Cy5 by using the Cy3 mAb Labeling Kit or the Cy5 Ab Labeling Kit (GE Healthcare), respectively.

SDS-PAGE and Western blotting

Mature brown rice seeds from the WT and the three transgenic lines were harvested and pulverized separately

in a Multi-Beads Shocker (Yasui Kikai). Total protein was extracted from the fine powder with SDS buffer containing 5 % (w/v) SDS, 6 % (w/v) 2-mercaptoethanol, and 500 mM Tris-HCl (pH 6.8). Allergen proteins were also extracted with 1 M NaCl and analyzed in these and SDS buffer extracts. After separation by SDS-PAGE (15 % gels) (Laemmli 1970), proteins were stained with Coomassie Brilliant Blue or transferred onto PVDF membranes (Merck Millipore) for Western blotting. Proteins were detected with primary antibodies (0.5 µg/ml), followed by peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (1:20,000) (Jackson). The bands were visualized with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and detected in an ImageQuant LAS 4000 mini imager (GE Healthcare). For densitometric analysis of CTB, SDS-PAGE gels were stained by using Invitrogen LC6065 Simply Blue Safe Stain kit (Life Technologies), and spot intensity was measured on GS-800 calibrated densitometer (Bio-Rad Laboratories) by using the Bio-Rad Quantity One software (Yuki et al. 2013). Recombinant CTB was expressed and purified as described previously (Yuki et al. 2001).

Quantitative real-time PCR

Total RNA was extracted from developing seeds of WT and transgenic lines with an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Samples were treated with DNase (TaKaRa), and cDNA was synthesized from total RNA (0.5 µg) with the PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa). cDNA (20 µl) was diluted 1:50 with distilled water. Quantitative real-time PCR (qRT-PCR) was performed in triplicate in a total volume of 20 µl containing 0.5 µM each primer (Supplementary Table S1) and Fast SYBR Green Master Mix (Applied Biosystems) on a StepOnePlus Real-Time PCR System (Applied Biosystems) (95 °C for 20 s; 40 cycles of 95 °C for 3 s, 60 °C for 30 s; followed by 95 °C for 15 s and 60 °C for 1 min). The expression levels were normalized to 17S rRNA levels in WT. Differences between WT and transgenic lines were assessed by Student's *t* test, and *P* < 0.05 was considered to indicate statistical significance.

Immunofluorescence

Thin sections (500 nm) of developing seeds were incubated with avidin and biotin solutions (Vector Laboratories) (15 min each), blocked with 5 % donkey serum in PBS for 30 min at room temperature, and incubated with primary antibodies against CTB, RAG2, 19-kDa globulin, glyoxalase I, 52-kDa globulin, 63-kDa globulin (5 µg/ml), or Cy5-conjugated antibody against 13-kDa prolamin (1:30) in 1 % BSA-PBS for 1 h at room temperature. The

sections were washed with 0.05 % Tween-PBS, and incubated with biotin-SP-conjugated donkey anti-rabbit IgG (Jackson) (1:600) in 1 % BSA-PBS containing 10 % normal calf serum for 30 min. The sections were washed with 0.05 % Tween-PBS, incubated with Streptavidin-Alexa 488 (Molecular Probes) (1:1,000) and Cy3-conjugated mouse antibody against glutelin A (1:50) in 1 % BSA-PBS for 1 h, and washed with 0.05 % Tween-PBS. Images were captured with a confocal laser scanning microscope (Leica TCS SP2 AOBS).

Immuno-electron microscopy

Developing seeds were cut into 0.5-mm sections and fixed for 2 h in 4 % (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) at 4 °C. The sections were washed twice with the same buffer, and dehydrated with a series of ethanol concentrations (30, 50, 70, 80, 90 %, and twice 100 %, 10 min per change) at 4 °C. The sections were substituted at 4 °C with a series of solutions of LR White resin (London Resin Co., Ltd.) in ethanol (1:3, 1:1, and 3:1; 8–12 h per change), followed by 100 % LR White resin overnight. The LR White resin was polymerized at 50 °C for 48 h. Ultrathin sections (100–120 nm) were cut with a diamond knife on a Leica EM UC6 ultramicrotome (Leica) and mounted onto Formvar-coated nickel grids. The sections were treated with 10 % goat serum-PBS for 30 min at room temperature and incubated for 1 h at room temperature with primary antibodies against 13-kDa prolamin, glutelin (1:20,000), glutelin A, CTB, BiP, RAG2, glyoxalase I, and 19-, 52- and 63-kDa globulins (5 µg/ml), diluted in 1 % (w/v) BSA-PBS. The sections were washed with PBS six times for 5 min each and incubated with 18-nm gold-labeled goat (anti-mouse or anti-rabbit) IgG antibody (1:30) (Jackson) in 1 % (w/v) BSA-PBS for 1 h at room temperature. The sections were washed with PBS six times for 5 min each, and fixed in 1 % (v/v) glutaraldehyde-PBS for 5 min at room temperature, washed with distilled water, and stained with 2 % (w/v) uranyl acetate followed by Reynold's lead (5 min each). The sections were washed with distilled water and examined under a transmission electron microscope (model H-7500, Hitachi, Tokyo, Japan) at 80 kV.

Results

Expression levels of CTB, storage proteins, and major allergens in MucoRice lines and WT rice

We previously advanced MucoRice-CTB by introducing a combination cassette for RNAi-mediated suppression of endogenous storage proteins (13-kDa prolamin and glutelin

A) to increase the expression of CTB (MucoRice-CTB-RNAi; Yuki et al. 2013). Here, we established a transgenic line expressing RNAi against 13-kDa prolamin and glutelin A that does not produce CTB (MucoRice-RNAi, Supplementary Fig. S2). To characterize and compare these three MucoRice lines with WT rice, we analyzed the expression levels of BiP, glutelin precursor, glutelin A, 13-kDa prolamin, and CTB by WB. As expected, RNAi decreased the levels of 13-kDa prolamin, glutelin A, and glutelin precursor in MucoRice-RNAi and MucoRice-CTB-RNAi. In MucoRice-RNAi, and to a lesser extent in MucoRice-CTB-RNAi, the level of BiP (75-kDa) was higher than in WT. CTB was expressed in MucoRice-CTB-RNAi at a level six times that in MucoRice-CTB (Fig. 1A). The levels of five major rice allergens (RAG2, glyoxalase I, 19-kDa globulin, and 52- and 63-kDa globulin-like proteins) in 1 M NaCl (Fig. 1B) or SDS buffer (Supplementary Fig. S3) extracts prepared from MucoRice-RNAi and WT rice were not considerably different. CTB overexpression without RNAi (in MucoRice-CTB) slightly reduced the expression of RAG2, glyoxalase I, and 63-kDa globulin-like protein compared with WT. In 1 M NaCl extracts, the level of

RAG2 was the lowest, followed by that of 19-kDa globulin, in MucoRice-CTB-RNAi (Fig. 1B). In SDS buffer extracts, the level of RAG2 (but not that of 19-kDa globulin) was lower in MucoRice-CTB-RNAi than in WT. Thus, in line with our previous study, RAG2 is strongly down-regulated in MucoRice-CTB-RNAi; however, this effect was not observed in MucoRice-CTB or MucoRice-RNAi.

mRNA levels of storage proteins and major allergens in MucoRice lines and WT rice

The expression of 13-kDa prolamin and glutelin A transcripts was strongly suppressed in MucoRice-CTB-RNAi and in MucoRice-RNAi (Fig. 2). The transcripts of glutelins B and D (but not C) and of the 10- and 16-kDa prolamins were also suppressed (Fig. 2, Supplementary Fig. S4). Despite a strong suppression of RAG2 in MucoRice-CTB-RNAi (see above), only a limited reduction in their mRNA levels in MucoRice-CTB-RNAi and in MucoRice-RNAi was observed (Fig. 2). No silencing of mRNAs of glyoxalase I or of 52- and 63-kDa globulin-like proteins in MucoRice-CTB-RNAi or in MucoRice-RNAi was found

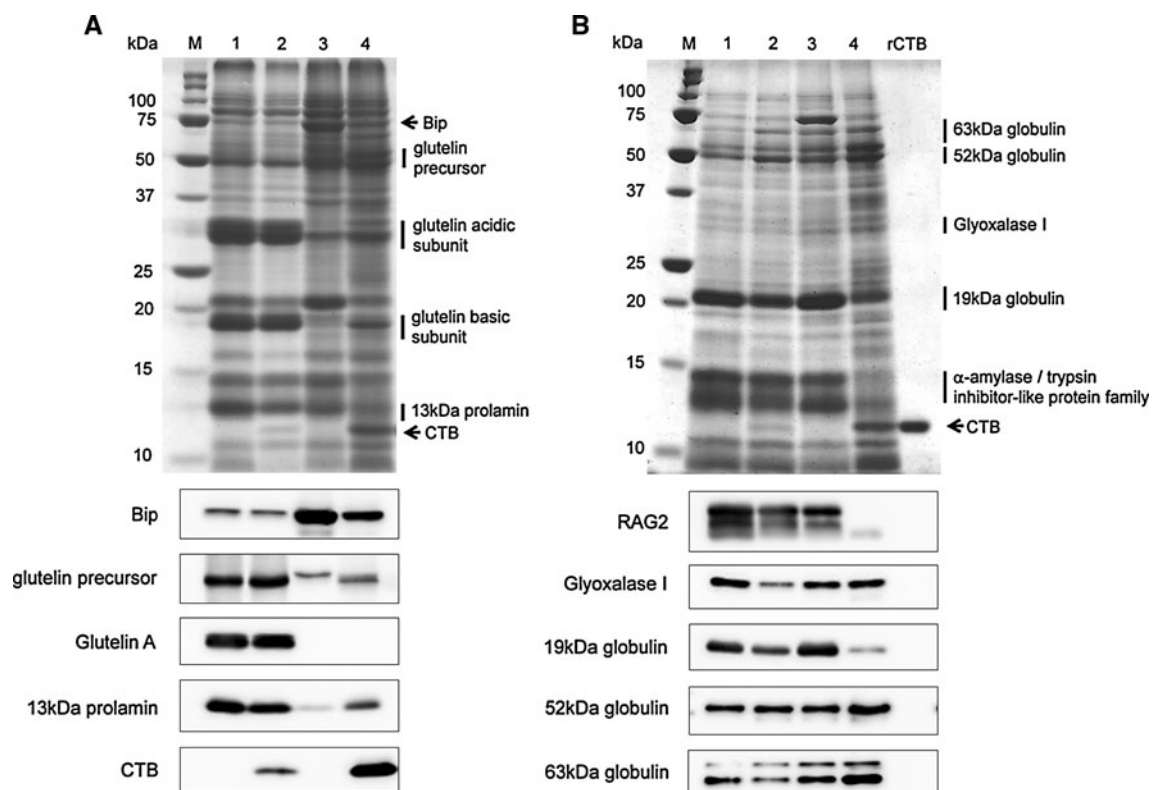
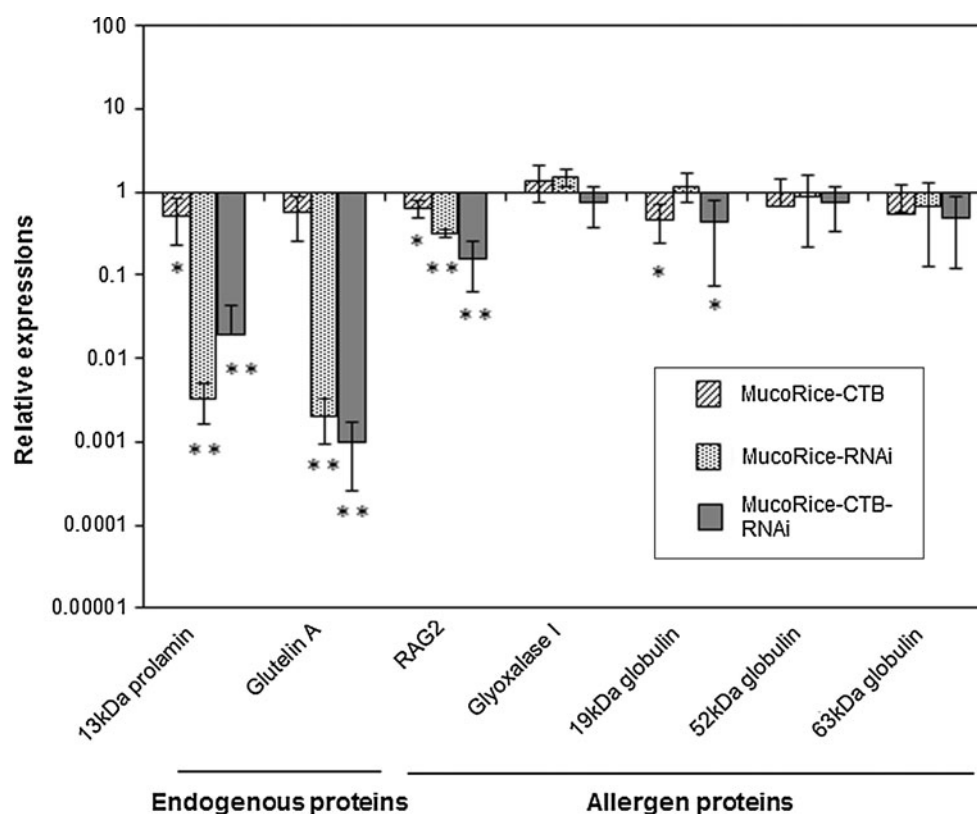


Fig. 1 SDS-PAGE and Western blot analysis of **A** total and **B** salt-soluble proteins from WT and MucoRice seeds. Proteins from WT (#1), MucoRice-CTB (#2), MucoRice-RNAi (#3), and MucoRice-CTB-RNAi (#4) seeds were extracted with SDS-sample buffer (**A**) or 1 M NaCl (**B**), separated by SDS-PAGE (upper panels), and Western blotted with indicated antibodies (panels in black frames). Expression

levels of endogenous storage proteins (glutelin precursor, glutelin A, and 13-kDa prolamin) were lower in MucoRice-CTB-RNAi and MucoRice-RNAi than in WT rice (**A**). The levels of major allergens (RAG2 and 19-kDa globulin) were lower in MucoRice-CTB-RNAi than in WT when extracted with 1 M NaCl. *M* molecular weight markers, *rCTB* recombinant CTB

Fig. 2 mRNA levels of endogenous storage and allergen proteins in MucoRice seeds. Expression levels were analyzed by qRT-PCR of RNA extracted at 14 DAF. Expression levels are normalized to 17S rRNA, and are represented relative to the expression levels in WT rice. Values are averages of three replicates. ** $P < 0.01$; * $P < 0.05$; MucoRice-CTB vs. WT



(Fig. 2). These results showed that the effect of RNAi on down-regulation of RAG2 was indirect.

Localization of CTB and storage proteins analyzed by IF and immuno-EM

Glutelin A and 13-kDa prolamin were found in separate locations in WT and MucoRice-CTB (Fig. 3A). Glutelin A (but not 13-kDa prolamin) signals were much weaker in MucoRice-RNAi and MucoRice-CTB-RNAi (Fig. 3A). High-intensity CTB staining appeared mostly in the cytoplasm and cell walls in MucoRice-CTB-RNAi (Fig. 3Af), whereas low-intensity CTB staining (mostly outside of glutelin A-expressing PB-II) was found in MucoRice-CTB (Fig. 3Ae). The immuno-EM analysis revealed that PB-II formation was strongly disturbed (Fig. 3Bd, g, h, j, j'), and PB-I formed smaller rough-shaped structures (Fig. 3Bc, d, g, h, j, j') in MucoRice-RNAi and MucoRice-CTB-RNAi than in WT. Glutelin A-expressing PB-II formed electron-dense and irregularly shaped granules in WT (Fig. 3Be), whereas 13-kDa prolamin-expressing PB-I formed spherical structures with ring-shaped layers (Fig. 3Ba). In addition, immuno-EM analysis confirmed CTB localization in the cell wall/plasma membrane, PB-I/II, and PB-like structures in MucoRice-CTB-RNAi (Fig. 3Bj, j'), whereas CTB was localized in PB-II and cytoplasm in MucoRice-CTB (Fig. 3Bi). The immuno-EM analysis also confirmed

BiP localization at the periphery of PB-I in WT and all MucoRice, and also in PB-like structures in MucoRice-RNAi and MucoRice-CTB-RNAi (Supplementary Fig. S5). The results of immuno-EM analysis are summarized in Table 1. These findings showed that CTB location was altered when it was co-expressed with RNAi against glutelin A and 13-kDa prolamin.

Comparison of localization of rice allergens and glutelin A (IF analysis)

A similar approach was used to compare the localization of glutelin A with those of the five major allergens. RAG2 and 19-kDa globulin were localized mainly in glutelin A-expressing PB-II in WT and MucoRice-CTB (Fig. 4a, b, i, j). Although present in the endosperm cells, glyoxalase I was not localized in PB-II in WT and MucoRice-CTB (Fig. 4e, f); 52- and 63-kDa globulin-like proteins were found in cells other than the endosperm cells (Fig. 4m, n, q, r). Similar to the data shown in Fig. 3A, very weak glutelin A signals were found in MucoRice-RNAi and MucoRice-CTB-RNAi, i.e., the lines with the disturbed PB-II formation (Fig. 4). As expected, the signal intensity of glyoxalase I did not change (Fig. 4g, h), whereas RAG2 signal became weak in MucoRice-RNAi and MucoRice-CTB-RNAi (Fig. 4c, d), in line with the Western blot data (see

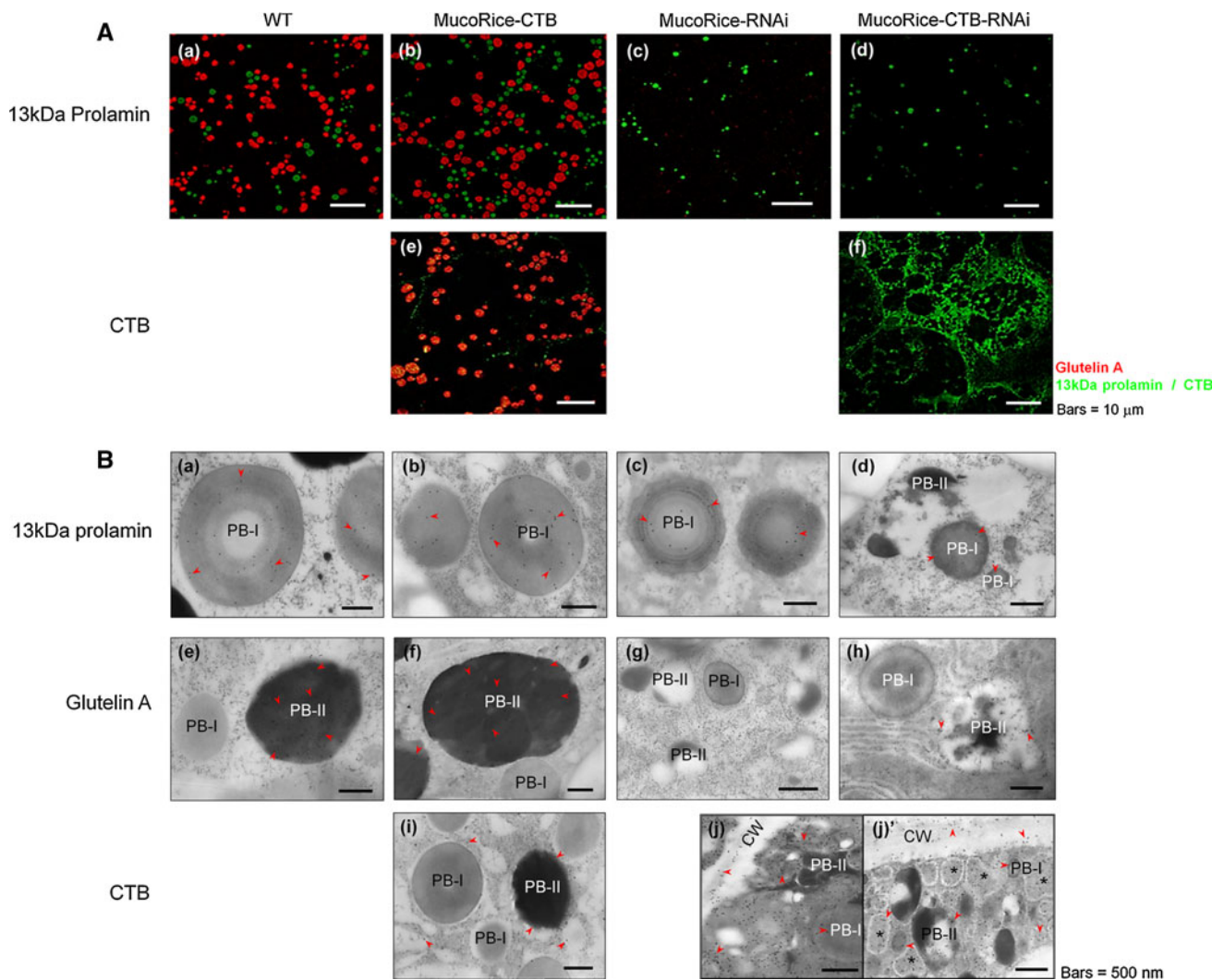


Fig. 3 **A** IF and **B** immuno-EM analyses of storage proteins and CTB in WT and MucoRice seeds. **A** Analysis carried out by double labeling with the mouse monoclonal antibody against glutelin A (red) and rabbit polyclonal antibodies against CTB or 13-kDa prolamin (green). **a** WT: glutelin A and 13-kDa prolamin; **b** MucoRice-CTB: glutelin A and 13-kDa prolamin; **c** MucoRice-RNAi: glutelin A and 13-kDa prolamin; **d** MucoRice-CTB-RNAi: glutelin A and 13-kDa prolamin; **e** MucoRice-CTB: glutelin A and CTB; **f** MucoRice-CTB-RNAi: glutelin A and CTB. Scale bars 10 μ m. **B** Sections of caryopses stained with mouse monoclonal antibody against glutelin A

and rabbit polyclonal antibodies against 13-kDa prolamin or CTB, and then with 18-nm gold-conjugated goat anti-mouse IgG and rabbit IgG. **a** WT: 13-kDa prolamin; **b** MucoRice-CTB: 13-kDa prolamin; **c** MucoRice-RNAi: 13-kDa prolamin; **d** MucoRice-CTB-RNAi: 13-kDa prolamin; **e** WT: glutelin A; **f** MucoRice-CTB: glutelin A; **g** MucoRice-RNAi: glutelin A; **h** MucoRice-CTB-RNAi: glutelin A; **i** MucoRice-CTB: CTB; **j** and **j'** MucoRice-CTB-RNAi: CTB. PB-I, protein body I; PB-II, protein body II; CW cell wall. Arrowheads show each antigen. Asterisks, PB-like structures. Scale bars 500 nm

Fig. 1B). The location of 52- and 63-kDa globulin-like proteins was similar in all lines (Fig. 4m–t).

Localization of the major rice allergens (immuno-EM analysis)

Glyoxalase I was present in the cytoplasm, whereas the 52- and 63-kDa globulin-like proteins were found in the aleurone layer in all lines (Fig. 5). RAG2 and 19-kDa globulin were localized mainly in PB-II (and also in PB-I) in WT and MucoRice-CTB (Fig. 5a, b', i, j), whereas 19-kDa

globulin was also found in the cytoplasm (Fig. 5i, j). In both lines expressing RNAi (MucoRice-RNAi and MucoRice-CTB-RNAi), RAG2 was also found in the cell wall/plasma membrane in addition to PB-I and II (Fig. 5c, c', d), whereas 19-kDa globulin was present in both the cytoplasm and PB-II in all lines (Fig. 5i–l). The results are summarized in Table 1. The location of the 19-kDa globulin overlapped partially with that of CTB in MucoRice-CTB-RNAi (Figs. 3Bj, j', 5l). The RAG2 localization coincided with that of CTB in MucoRice-CTB-RNAi (Figs. 3Bj, j', 5d). These results show that the main localization of RAG2

Table 1 Location of storage proteins, allergens, and CTB in WT and MucorRice lines as determined by immuno-electron microscopic analysis

Location:	WT			MucorRice-CTB			MucorRice-RNAi			MucorRice-CTB-RNAi		
	Aleurone layer	Endosperm		Aleurone layer	Endosperm		Aleurone layer	Endosperm		Aleurone layer	Endosperm	
Protein												
13-kDa prolamin	ND	PB-I		ND	PB-I		ND	PB-I		ND	PB-I	
Glutelin A	ND	PB-II		ND	PB-II		ND	PB-II		ND	PB-II	
CTB	ND	ND		ND	PB-II/Cytoplasm +		ND	ND		ND	Plasma membrane/cell wall ++ PB-like structure +++ PB-I/PB-II +	
BIP	ND	PB-I +		ND	PB-I +		ND	PB-like structure +++ PB-I +		ND	PB-like structure ++ PB-I +	
RAG2	ND	PB-I + PB-II +++++		ND	PB-II +++++ Plasma membrane/cell wall +		ND	PB-I/PB-II + Plasma membrane/cell wall +++		ND	PB-II + Plasma membrane/cell wall +	
Glyoxalase I	ND	Cytoplasm +		ND	Cytoplasm +		ND	Cytoplasm +		ND	Cytoplasm +	
19-kDa globulin	ND	PB-I + PB-II +++++ Cytoplasm ++		ND	PB-I + PB-II +++++ Cytoplasm ++		+	PB-I/PB-II + Cytoplasm +++		ND	PB-I/PB-II + Cytoplasm ++	
52-kDa globulin	Aleurone particle (globoid) ++	ND		Aleurone particle (globoid) +	ND		Aleurone particle (globoid) ++	ND		Aleurone particle (globoid) +++	ND	
63-kDa globulin	Aleurone particle (globoid) +++	ND		Aleurone particle (globoid) ++	ND		Aleurone particle (globoid) +++	ND		Aleurone particle (globoid) +++	ND	

ND not detected

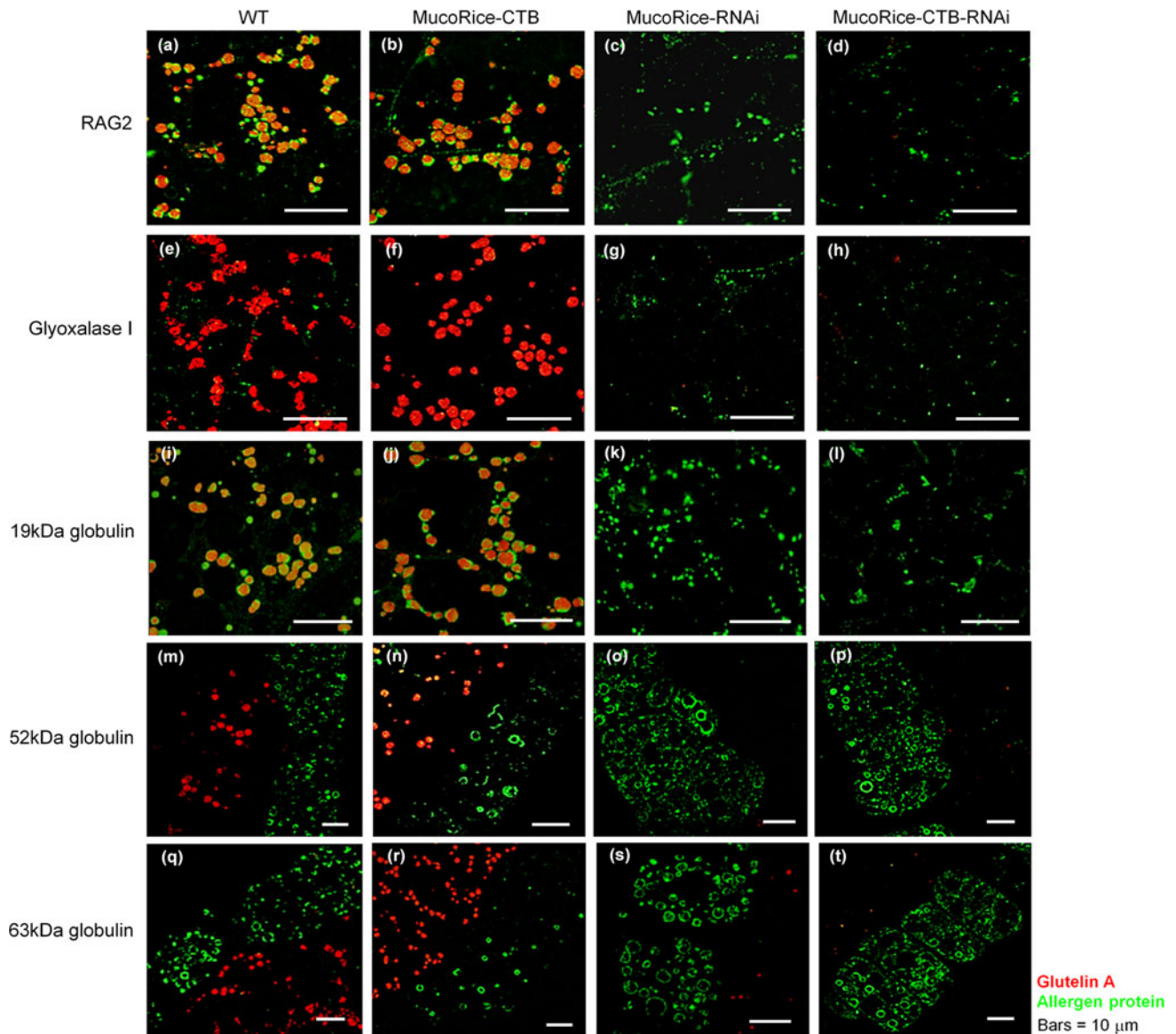


Fig. 4 IF analysis of rice allergens in WT and MucoRice seeds. The analysis was carried as in Fig. 3A with the mouse monoclonal antibody against glutelin A (red) and rabbit polyclonal antibodies (green) against allergens specified on the left side of the panels. Scale bars 10 μ m

in PB-II is altered when CTB is overexpressed together with RNAi-mediated suppression of endogenous storage proteins.

Discussion

Earlier, we confirmed that our rice-based vaccine, MucoRice, is stable for 3 years at room temperature as rice seeds or in a non-purified form, and we plan to use a polished rice powder preparation of MucoRice without purification in a clinical study to maintain the cold-chain-free property of MucoRice (Tokuhara et al. 2010; Yuki et al. 2013). The estimated dose of MucoRice powder (about 2 g) in the

clinical study is much lower than the usual consumption of cooked rice. In general, although rice is widely consumed, rice allergies are rare (Trcka et al. 2012). However, there have been some reports of immediate hypersensitivity reactions after rice ingestion, which lead to rhinoconjunctivitis, bronchial asthma, and atopic dermatitis, although the severity of allergic reactions to rice is low (Ikezawa et al. 1992; Kumar et al. 2007; Monzon et al. 2008). Although MucoRice is not a genetically modified (GM) food, but a non-purified form of oral vaccine, we should follow the safety assessment of GM foods proposed by the Codex Alimentarius Commission, which recommends an investigation of the potential of gene insertion to increase the content of allergen proteins.

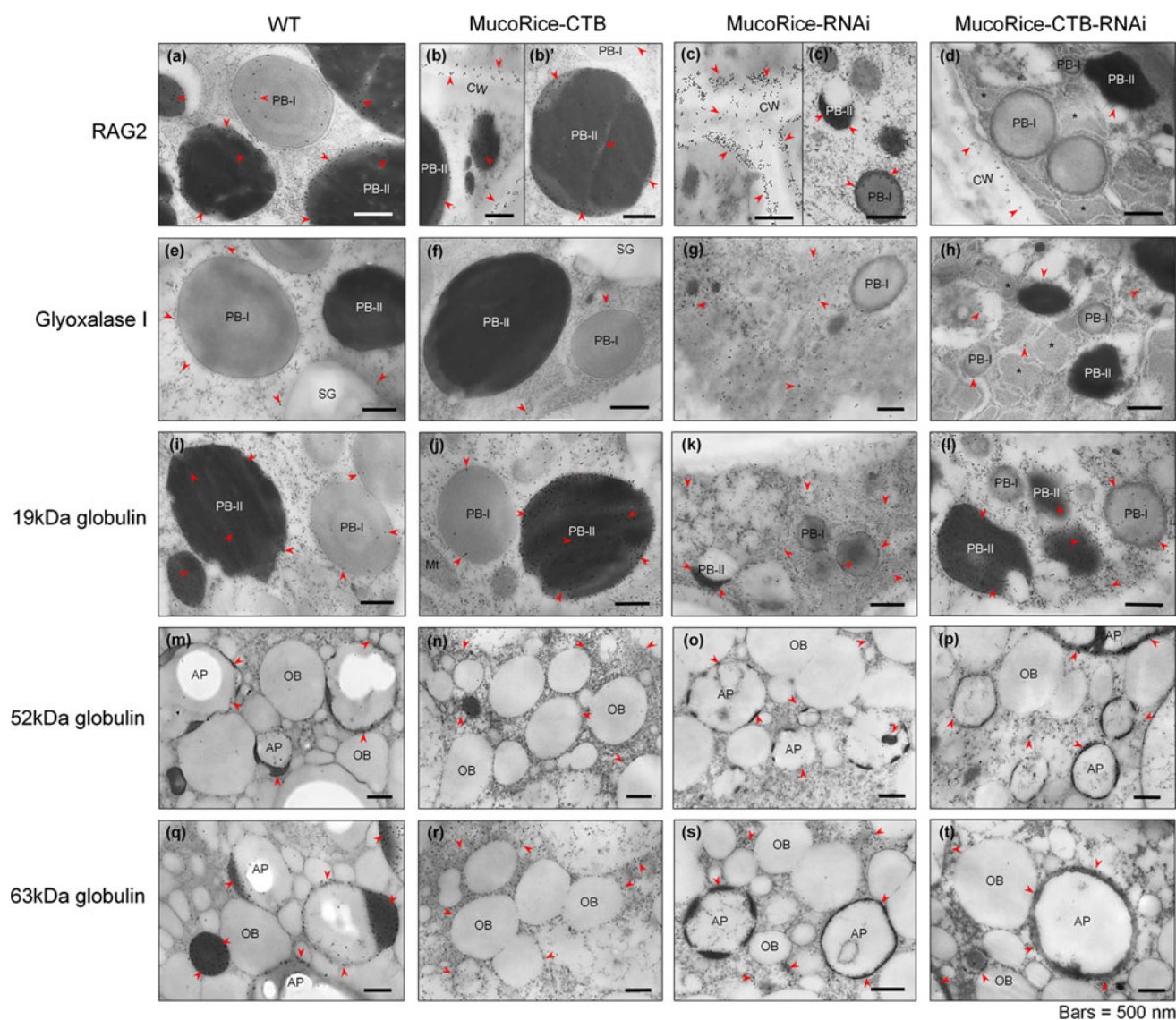


Fig. 5 Immuno-EM analysis of rice allergens in WT and MucoRice seeds. The analysis was carried out as in Fig. 3B with rabbit polyclonal antibodies against allergens specified on the left side of the

panels. CW cell wall, SG starch granule, Mt mitochondria, AP aleurone particle, OB oil body. Arrowheads show each antigen. Asterisks, PB-like structures. Scale bars 500 nm

In our previous studies (Kurokawa et al. 2013; Yuki et al. 2013), we developed MucoRice-CTB-RNAi, which overexpresses CTB concurrently with RNAi-mediated suppression of two endogenous storage proteins, and showed (by using a proteomics approach) that not only was the expression of CTB up-regulated, but also the expression of RAG2 was down-regulated in MucoRice-CTB-RNAi compared with WT rice. Here, we examined whether RNAi-mediated suppression of the two storage proteins affected the expression level and localization of overexpressed CTB and five major rice allergens.

Because we used RNAi to suppress 13-kDa prolamin and glutelin A, first we confirmed the effect at the transcriptional level and investigated the effect on the expression of rice allergens. These RNAi sequences have

no similarity to RAG2 or 19-kDa globulin genes (Adachi et al. 1993; Shorosh et al. 1992), and there was only a weak suppression of the mRNA for each (although not of other allergens) in MucoRice-RNAi or MucoRice-CTB-RNAi. Thus, RAG2 down-regulation was not due to a direct effect of RNAi against the storage proteins. In this regard, others reported that decreased levels of individual storage proteins (13-kDa prolamin or glutelin B) are compensated by increased levels of almost all other storage proteins (Kawakatsu and Takaiwa 2010). Yet here, the simultaneous suppression of both 13-kDa prolamin and glutelin A did not produce this compensation effect.

Since rice allergens can be easily extracted with solutions containing ≤ 1 M NaCl (Nakamura and Matsuda 1996), they are unlikely to be located in PB-I, but may be

present in the PB-II/PSV, cytoplasm, plasma membrane/cell wall, or aleurone layer. In WT rice, 19-kDa globulin mainly surrounded glutelins of PB-II/PSV, which was consistent with previous reports (Krishnan et al. 1992; Washida et al. 2012). Although 19-kDa globulin localized similarly in WT and Mucorice-CTB-RNAi (Table 1), we found that the level of 19-kDa globulin was lower in 1 M NaCl extracts (but not SDS buffer extracts) in Mucorice-CTB-RNAi than in WT (Fig. 1B; Supplementary Fig. S3). Although RAG2 also mainly surrounded glutelins of PB-II/PSV, some was found in PB-I. Thus, localization of these allergens did not exactly coincide with that of glutelin A. There were no major differences in the expression levels of the allergens in Mucorice-RNAi or in WT, whereas the production of the storage proteins was strongly suppressed, accompanied by a partial destruction of PB-II and formation of smaller, rough-shaped PB-I. Although RAG2 was found at the plasma membrane/cell wall and in the remaining small PB-II, 19-kDa globulin was found mainly in the cytoplasm in Mucorice-RNAi. The localization of glyoxalase I in cytoplasm and of the 52- and 63-kDa globulin-like proteins in aleurone particles did not change in Mucorice-RNAi. These data show that RAG2, localized in PB-II/PSV in WT rice, is affected by the introduced RNAi. The mechanisms of this effect need further exploration.

We also examined the effect of the RNAi on recombinant CTB in Mucorice-CTB-RNAi. The CTB expression level dramatically increased in the presence of RNAi, and its localization changed from PB-II/PSV and the cytoplasm to the plasma membrane/cell wall, cytoplasm, the remaining small PB-I and PB-II, and PB-like structures. As CTB is produced as a secretory protein in endosperm cells and enters the ER, its subcellular destination may be affected by its interaction with seed storage proteins in the ER lumen. Suppression of storage proteins by RNAi led to a partial destruction of PB-II and formation of smaller PB-I as well as PB-like structures, which may result in unexpected CTB locations, such as the PB-like structures, plasma membrane, and cell wall in Mucorice-CTB-RNAi. Our results show that the RNAi changed not only the expression level, but also the location of CTB.

Some reports suggest that molecular chaperones affect PB morphology (Takemoto et al. 2002; Wakasa et al. 2011b). Thus, we also examined BiP localization (Supplementary Fig. S5). In WT, BiP was predominantly localized to the periphery of PB-I. In Mucorice-CTB and Mucorice-CTB-RNAi, BiP levels were increased, and BiP localized around smaller PB-I but also around PB-like structures. These results are consistent with observations of overexpressed IL-10 in rice (Yang et al. 2012).

Further, we examined the effect of CTB on the major rice allergens. Although their expression was only slightly reduced in Mucorice-CTB, the expression of RAG2 was strongly reduced in Mucorice-CTB-RNAi (which has much higher expression of CTB; see above). CTB almost did not alter the location of any of the allergens in Mucorice-CTB, but the location of RAG2 (but not of the other allergens) changed to the plasma membrane/cell wall and the remaining small PB-II in Mucorice-CTB-RNAi.

RAG2 down-regulation might be induced by competition with CTB at the transcriptional, translational, or transport/location levels in starchy endosperm cells. The RAG2 transcript level, as already discussed, was not affected. Because the locations of RAG2 and CTB overlap (Table 1), CTB overexpression may result in the location of RAG2 being taken over, leading to RAG2 down-regulation. The competition between CTB and 19-kDa globulin may be less pronounced, allowing the expression of the latter in the cytoplasm in Mucorice-CTB-RNAi. After being synthesized as a secretory protein in the RER, 19-kDa globulin is transported into PSV/PB-II via Golgi or precursor-accumulating vesicles (Takahashi et al. 2005). As RAG2 is also located in PB-II, it may be transported via the same pathway. Thus, when CTB is overexpressed in the presence of RNAi, it may compete with RAG2 for the trafficking machinery. The mechanisms of the possible competition need further exploration.

In conclusion, we investigated the expression levels and localization of five major rice allergens, the effects of suppression of endogenous storage proteins by RNAi, and the effects of overexpressed CTB. Although the RNAi specifically suppressed prolamin and glutelin genes, there was also an indirect effect on the expression of the rice allergens RAG2. Our data suggest that the dramatic overexpression of CTB in Mucorice-CTB-RNAi resulted in a competition with RAG2 expression and in down-regulation of the latter. These findings provide an important insight that would be valuable for assessing allergens in rice-based oral vaccines and in genetically modified rice.

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Conflict of interest No conflict exists.

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