

Development of Rice-Seed-Based Oral Allergy Vaccines Containing Hypoallergenic Japanese Cedar Pollen Allergen Derivatives for Immunotherapy

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Supporting Information

ABSTRACT: Allergen-specific immunotherapy is the only available curative treatment for IgE-mediated allergen diseases. A safe hypoallergenic allergen derivative with high efficiency is required as a tolerogen to induce immune tolerance to the causative allergens. In this study, to generate a rice-based oral allergy vaccine for Japanese cedar (JC) pollinosis, the tertiary structures of major JC pollen allergens, Cry j 1 and Cry j 2, were more completely destroyed by shuffling than the previous ones without losing immunogenicity and then were specifically expressed in the endosperm of transgenic rice seed. They accumulated at high levels and were deposited in endoplasmic reticulum (ER) and ER-derived protein bodies. The low allergenicity of these deconstructed Cry j 1 and Cry j 2 allergens was evaluated by examining their binding activities to the specific IgE antibody and by the basophil degranulation test.

KEYWORDS: allergen, pollen allergy, oral vaccine, rice endosperm, Japanese cedar

INTRODUCTION

Japanese cedar (JC) pollinosis is the most prevalent IgE-mediated allergic disease in Japan and affects >30% of the Japanese population.¹ Allergen-specific immunotherapy offers a disease-specific treatment by modifying the allergen-specific immune response.^{2,3} Immune tolerance or desensitization to the causative allergen is induced by the subcutaneous injection or sublingual administration of crude allergen extracts as a result of clinical treatment.⁴ However, the repeated administration of an allergen over >3 years (3 to 5 years) is laborious and inconvenient, leading to low patient adherence. Furthermore, these treatments sometimes induce adverse side effects such as mild symptom or anaphylaxis, so that the establishment of an effective protocol regarding the prevention and treatment of allergic diseases, the safety of the antigen, and the reduction of cost has been desired.

Plants have many advantages as a production platform for biopharmaceuticals (biologics) over the conventional fermentation system using bacteria, yeast, and mammalian cells in terms of productivity, safety, cost-effectiveness, and scalability.^{5,6} In particular, cereal seed (endosperm) has been utilized as one of the ideal bioreactors for the production of pharmaceutical recombinant proteins based on the robust production yield, the presence of a specialized deposition compartment, and the high stability at ambient temperature.^{7–9} Moreover, extensive purification is not required for administration because many cereal seeds are generally

regarded to have safe status. These benefits are accounted for by the findings that recombinant proteins highly and stably accumulate in protein bodies (PBs) when specifically expressed in the endosperm as secretory protein. Furthermore, it is notable that even artificial recombinant proteins can be accumulated more stably in the endosperm than other tissues.¹⁰ We have generated various transgenic rice lines expressing vaccines,^{11–14} bioactive peptides,¹⁵ and cytokines¹⁶ in the endosperm. In particular, hybrid T-cell epitope peptides^{11,12} or hypoallergenic antigens^{13,14} derived from native allergen proteins have been produced in transgenic rice seeds to reduce the IgE binding activity and the allergenic activity of the allergen, leading to adverse effects. When allergy vaccines are produced in rice seeds and are orally administered in a formulation of seed powder or the PB fraction without purification processing, they are effectively delivered to gut-associated lymphoid tissues (GALTs) without severe degradation by digestive enzymes in gastrointestinal tract.^{17,18} Immune tolerance against the causative allergen has been observed to be effectively induced in model mice through the oral administration of rice-based allergen vaccines, resulting in the alleviation of clinical symptoms.^{11,13,14} This is attributed to the

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bioencapsulation through the double barriers of the cell wall and PB characteristics of the seed, providing an innovative delivery system to the GALT.^{18,19} It is now desired that the allergenicity of tolerogens be eliminated by the destruction of the conformational structure required for IgE binding without a loss of immunogenicity (T-cell epitopes) to allow a high dose administration without side effects.

We previously generated transgenic rice seed containing hypoallergenic Cry j 1 and Cry j 2 derivatives, in which three overlapped Cry j 1 fragments were expressed as fusion proteins with the major rice seed protein glutelins (GluA, GluB, and GluC), and the Cry j 2 was expressed as a deconstructed form.¹⁴ Because these four constructs were expressed under the control of the four different endosperm-specific promoters for the production of the entire sequences of the Cry j 1 and Cry j 2 allergens, it was difficult to always produce constant levels of antigens in transgenic rice seeds even after they were genetically fixed. In light of the clinical use of a rice-seed-based allergy vaccine in the near future, they have to be produced under regulatory guidelines that comply with the principles of the Good Manufacturing Practice (GMP). In that case, the production of the deconstructed Cry j 1 antigen by shuffling rather than the fusion of Cry j 1 fragments with the glutelins is better and is easy to control on the production level. In this study, we aimed to produce high amounts of shuffled Cry j 1 and Cry j 2 antigens with lower allergenicities in transgenic rice seed. Furthermore, the reduction of allergenic and inflammatory activities from the developed modified Cry j 1 and Cry j 2 antigens was examined by competitive immune binding analysis and the basophil degranulation test.

MATERIALS AND METHODS

Generation of Transgenic Rice. DNAs encoding S-Cryj1-1 and S-Cryj2-1 were synthesized by GenScript (Piscataway, NJ), which were optimized by employing codons that are preferentially used in genes encoding many major rice seed storage proteins and by avoiding AT-rich sequences, including potential mRNA destabilizing sequences, polyadenylation signals, and some rare codons.⁹ DNA-encoding S-Cryj1-2 and S-Cryj2-2 were generated by introducing mutagenesis at the glycosylation sites (five sites for Cry j 1 and one site for Cry j 2) (Figure S1). Furthermore, DNAs encoding S-Cryj2-3 and S-Cryj2-4 were generated by shuffling DNA sequences using a method with two stages of polymerase chain reaction (PCR) amplification, as shown in Figure S1. These deconstructed Cry j 1 and Cry j 2 derivatives were attached to an endoplasmic reticulum (ER) retention signal (KDEL) at their C-termini. They were ligated downstream of the endosperm-specific 2.3 kb *GluB-1* or 1.3 kb *GluB-4* promoter containing an individual signal peptide, which was followed by a 0.65 kb *GluB-1* or 0.7 kb *GluB-4* terminator. The resulting constructs were introduced into the binary destination vector pCSPmALSag7-GW using MultiSite Gateway LR Clonase II Plus enzyme mix (Invitrogen, Carlsbad, CA).²⁰ To reduce the levels of endogenous prolamins, prolamins-suppressing construct (Proless construct) using the RNA silencing inducible sequence (RSIS)²¹ was introduced into the destination binary vector pCSPmALSag7-GW together with the Cry j 1 and Cry j 2 expression cassettes. These binary vectors containing the expression cassettes were transferred to *Agrobacterium tumefaciens* strain EH105 by electroporation and then introduced into the rice genome (*Oryza sativa* cv. Kitaake) via *Agrobacterium*-mediated transformation.²² Transgenic rice were selected by resistance to Pyriminobac by using a mutated acetolactate synthase gene (*mALS*) under the control of the callus-specific promoter (CSP) derived from the rice genome and then were generated.²³ They were grown in the closed control greenhouse (28 °C, 16 h light/8 h dark cycle) with at least 24 independent transgenic plants per individual construct.

Antibodies. Recombinant proteins of full-length S-Cryj1-2 and S-Cryj2-4 were expressed using pET15b in *E. coli*, and the purified recombinant proteins were used to raise anti-S-Cryj1-2 and anti-S-Cryj2-4 polyclonal antibodies in rabbits (Scrum, Tokyo, Japan). Polyclonal antibodies against 16 kDa prolamins, 14 kDa Cys-rich prolamins (RM1 and RM9), 13 kDa Cys-poor prolamins (RM2 and RM4), 10 kDa prolamins, and rice chaperones of OsBiP1, OsBiP4/5, OsPDIL1-1, OsPDIL2-3, and calnexin (CNX) were previously prepared.^{24–26}

SDS-PAGE and Immunoblot Analysis. Transgenic rice grains were harvested and ground completely into a fine powder using A Multibead Shocker (Yasui Kikai, Tokyo, Japan). Total protein was extracted from the powder with 400 μ L of urea–SDS buffer (50 mM Tris–HCl pH 6.8, 8 M urea, 4% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol (2-MER), 20% glycerol) by shaking for 1 h at room temperature, as previously described.²⁷ The extracts were centrifuged, and the supernatants were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. After separation by 12% SDS-PAGE, the proteins were visualized by Coomassie Brilliant Blue (CBB)-R250 staining or were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Burlington, MA) for immunoblot analysis. The blots were reacted with antibodies in blocking buffer, after which they were incubated with an anti-rabbit IgG horseradish-peroxidase-conjugated secondary antibody; then, the signals were detected using the Clarity Western ECL detection kit (Bio-Rad). The band images were scanned into a computer, and the corresponding bands were quantified with NIH image software (U.S. National Institutes of Health).

Detection of Glycosylation. To determine whether N-linked glycans were present on S-Cryj1-1 and S-Cryj2-1 proteins accumulated in transgenic seeds, total seed proteins were digested with endoglycosidase H (Endo H) and peptide N-glycanase (PNGase). The reaction mixture was separated by SDS-PAGE and transferred to a PVDF membrane for immunoblot analysis. The signals were detected using anti-Cry j-1 or anti-Cryj-2 antibody.

Indirect Immunohistochemical Analysis by Confocal Laser Scanning Microscopy and Transmission Electron Microscopy. Developing seeds were harvested 13–18 days after flowering (DAF) from transgenic rice harboring the construct G (line 11) and nontransgenic rice and then cross-sectioned with a DTK-1000 microslicer (Dosaka EM) to ~ 150 μ m in thickness for indirect immunohistochemical analysis.²⁷ The immunohistochemical analysis by confocal laser scanning microscopy was performed as previously described.²⁷ The primary rabbit anti-Cryj1-2 and anti-Cryj2-4 antibodies were used at a 1:300 dilution. The specimens were observed with a confocal laser scanning microscope (Fv10i, Olympus, Tokyo, Japan) using Alexa-488-conjugated anti-rabbit IgG antibody and rhodamine to stain ER-derived protein bodies (PB-Is). The immunoelectron microscopy observation was performed as previously described.²⁸

Stepwise Extraction of Seed Proteins. Rice seed storage proteins were extracted from pulverized mature seeds using the previously described stepwise extraction method.²⁷ Extraction buffer containing 0.5 M NaCl and 10 mM Tris-HCl (pH 7.5) was used to extract albumin and globulin. The residues were then treated with 60% (v/v) *n*-propanol for the extraction of Cys-poor prolamins and further resuspended with 60% (v/v) *n*-propanol containing 10 mM dithiothreitol (DTT) for the extraction of Cys-rich prolamins. Individual residues of these steps were treated with 1% (v/v) lactic acid, and their extracts were applied to SDS-PAGE, which was followed by immunoblot analysis.

In Vitro Digestion of Transgenic Rice Seeds by Pepsin. Transgenic rice seeds were subjected to pepsin digestion, as previously described.²⁹ 5 mg of seed powder was added to 150 μ L of reaction buffer containing 0.01% (w/v) pepsin (Sigma, USA) and 30 mM NaCl (pH 1.2) and incubated at 37 °C. The reaction was terminated by neutralization with 100 μ L of 160 mM Na₂CO₃ at 0, 2, 5, 15, 30, 60, 120, and 180 min. After the addition of 400 μ L of urea–SDS buffer, the digested samples were analyzed by 12% SDS-PAGE, followed by immunoblot analysis.

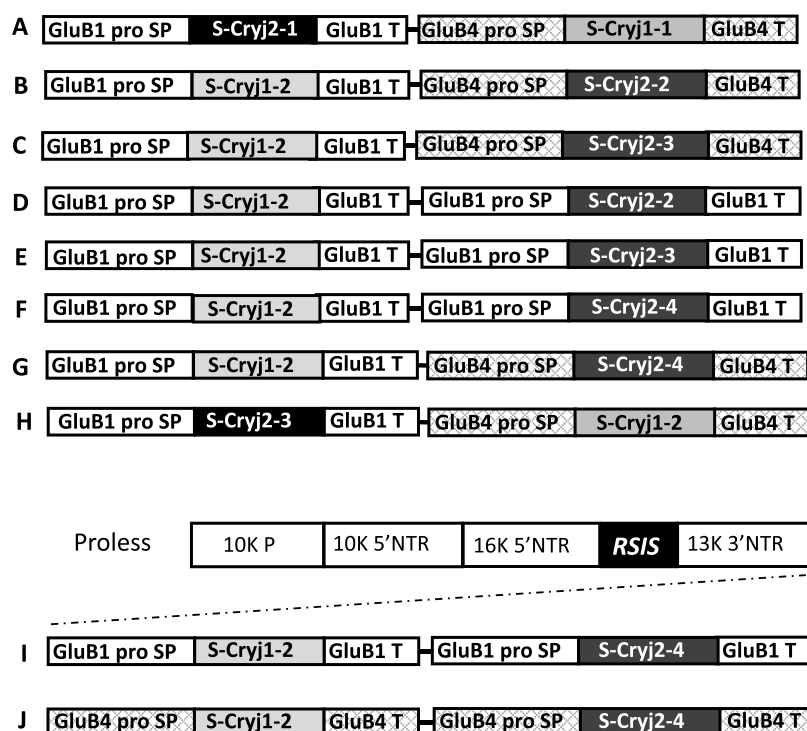


Figure 1. Schematic representation of the constructs used for the expression of the deconstructed Cry j 1 and Cry j 2 derivatives in transgenic rice seeds. They were expressed under the control of the endosperm-specific 2.3 kb *GluB-1* and 1.3 kb *GluB-4* glutelin promoters. GluB1 pro SP and GluB4 pro SP represent the 2.3 kb *GluB-1* promoter containing the signal peptides and the 1.3 kb *GluB-4* promoter containing the signal peptides, respectively, whereas GluB1 T and GluB4 T are 0.65 kb *GluB-1* and 0.7 kb *GluB-4* terminators. 10K P, 10 kDa prolamin 5' nontranslated region sequence; 10K 5'NTR, 10 kDa prolamin 5' nontranslated region sequence; 16K 5'NTR, 16 kDa prolamin 5' nontranslated region sequence; RSIS, RNA silencing inducing sequence; 13K 3'NTR, RM4 13 kDa 3' nontranslated region sequence.

Dot-Blot IgE-Binding Assay. Purified native Cry j 1 and Cry j 2 were purchased from Asahi Food & Healthcare (Tokyo, Japan). Recombinant Cryj1F, S-Cryj2, S-Cryj1-2, and S-Cryj2-4 were expressed by using pCold shock vector II (Takara, Tokyo, Japan) and *E. coli* strain BL21-RIL (Novagen, Darmstadt, Germany) according to the manufacturer's protocols. These recombinant proteins were purified with Ni Sepharose 6 Fast Flow resin (GE Healthcare). Cry j 1, Cry j 2, and recombinant proteins were dotted onto a protein nitrocellulose membrane (GE Healthcare) at 200, 50, 10, 5, 1, and 0 ng and incubated with sera from BALB/c mice immunized with JC pollen. IgE antibodies bound to Cry j 1, Cry j 2, and recombinant proteins were detected with antimouse IgE-biotin (Southern Biotech, Birmingham, AL) and streptavidin-HRP (R&D Systems).

Competitive IgE-Binding Assay. The binding ability of recombinant Cryj1F, S-Cryj2, S-Cryj1-2, and S-Cryj2-4 was determined by competitive IgE-binding ELISA.³⁰ An ELISA plate was coated with antimouse IgE antibody (Southern Biotech, Birmingham, AL). After washing and blocking of the plate, sera from BALB/c mice immunized with JC pollen mixed with varying amounts (0.0025–25 µg/mL) of Cry j 1, Cry j 2, and recombinant proteins were added to the wells. The plate was kept at room temperature for 2 h and then processed as a standard enzyme-linked immunosorbent assay (ELISA).³¹ The inhibition rate (%) was calculated by the formula $100 - [(OD_{450nm} \text{ of mouse serum mixed with Cryj1, Cryj2, and recombinant proteins}) / (OD_{450nm} \text{ of}) \times 100]$.

RBL-2H3 Basophil Degranulation Test. The basophil degranulation test was carried out according to the method of Naal et al.³² Rat basophilic leukemia RBL-2H3 cells were primed by incubating overnight with sera from BALB/c mice immunized with JC pollen and were stimulated with Cry j 1, Cry j 2, and recombinant proteins. The amount of β -hexosaminidase activity released by the RBL-2H3 cells was measured, and the total β -hexosaminidase release was determined by lysing the cells with 0.1% Triton X-100.

RESULTS

Accumulation of Destructed Full-Length Cry j 1 and Cry j 2 in Transgenic Rice Seed.

Cry j 1 is a basic glycoprotein with a molecular mass of about 41–45 kDa that is located in pollen cell walls and has pectate lyase activity.³³ The tertiary structure was reported to be highly responsible for recognition by the Cry-j-1-specific IgE.³⁴ Previously, we reported that production levels of the fragments derived from the native Cry j 1 or its full-length sequence were very low (<1 µg/grain) in transgenic rice seeds, even if the codon optimization of its sequence and the mutagenesis of the active sites involved in the enzymatic activity were achieved for their expression.³⁵ Then, to produce the whole Cry j 1 sequence at a higher level, three overlapped fragments covering the whole Cry j 1 molecule were expressed as fusion proteins with the major rice seed storage protein glutelins (GluA, GluB, and GluC).¹⁴ The accumulation levels of these three fusion products were improved but were highly variant (27–85 µg/grain).¹⁴ This may be accounted for by the fact that three Cry j 1 fragments fused to the different types of glutelins were expressed under the control of the different endosperm-specific promoters (glutelin *GluB-4*, 16 and 10 kDa prolamin promoters),^{14,36} even though three expression constructs were harbored in the same binary vector. In this study, the whole Cry j 1 sequence was shuffled based on information on the tertiary structure and characterized human T-cell epitopes.^{37–40} The deconstruction of Cry j 1 was achieved to alternate the tertiary structure without affecting the immunogenicity, resulting in the preservation of various T-cell epitope peptides containing major and minor ones, as shown in Figure

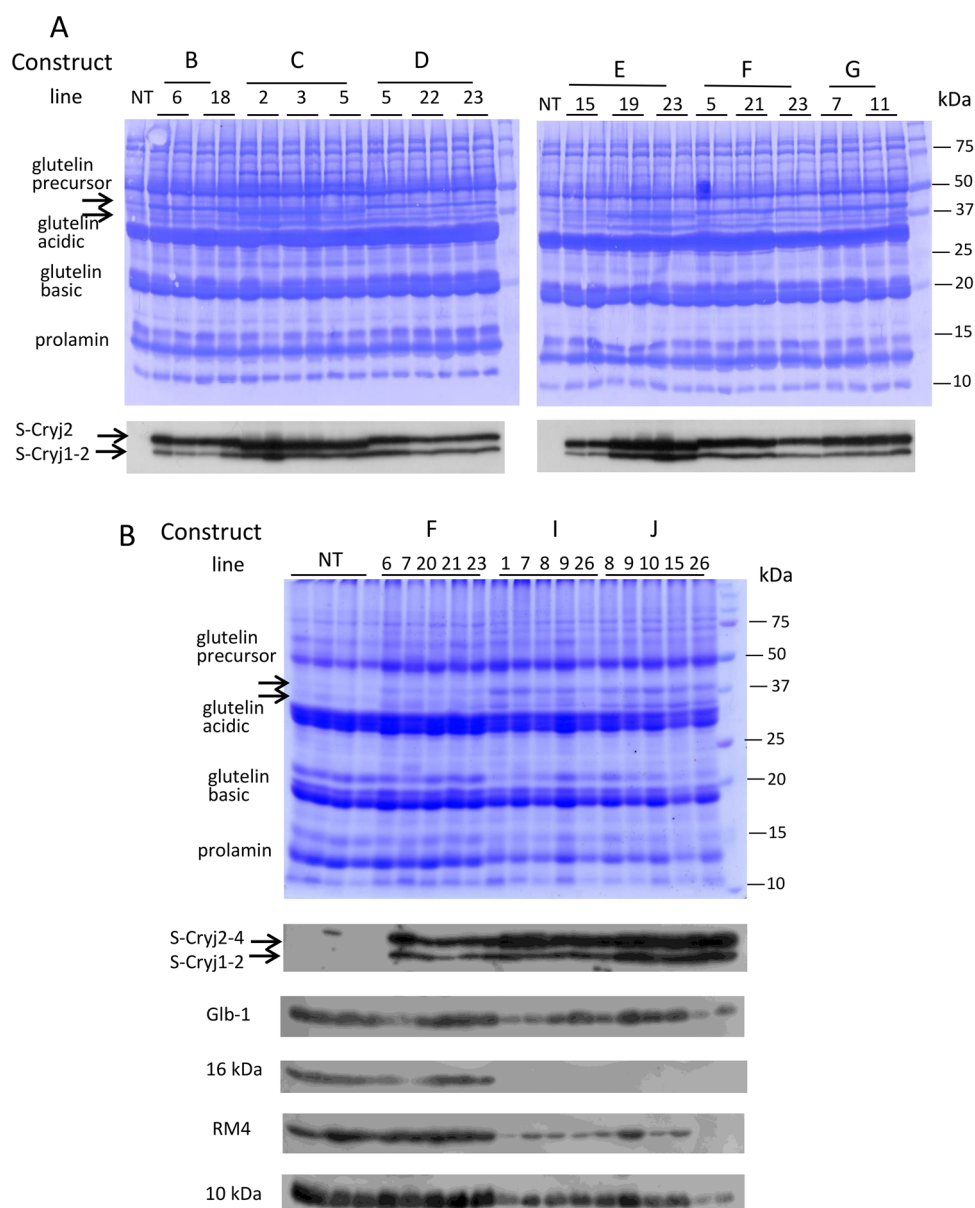


Figure 2. Analysis of the expression levels of deconstructed Cry j 1 and Cry j 2 derivatives in transgenic rice seeds. (A) Various deconstructed Cry j 1 and Cry j 2 were coexpressed under the control of the endosperm-specific glutelin *GluB-1* or *GluB-4* promoter. (B) Effect of the suppression of endogenous prolamins on the production of S-Cryj1-2 and S-Cryj2-4 in transgenic rice seeds. Their expression levels directed by the *GluB-1* promoter were compared with those in transgenic rice seeds that were expressed under the control of the *GluB-1* or *GluB-4* promoter along with the suppression of most prolamins by RNAi. Their expression levels were examined by electrophoresis on 12% SDS-PAGE and immunoblot analysis using anti-S-Cryj1-2 and anti-S-Cryj2-4 antibodies.

S1A. Four N-glycosylation sites (Asn-X-Ser/Thr motif) were also mutagenized by exchanging them with Asn-X-Ala because the presence of glycosylation is known to be involved in the T-cell response.⁴¹

The mature Cry j 2 protein has a molecular mass of 45 kDa and is located in pollen starch granules and has polymethylgalacturonase activity.⁴² To express the modified Cry j 2 with low allergenicity, the coding sequence was rearranged in the order of C-terminus to N-terminus. This original shuffled Cry j 2 accumulated at a low level ($\sim 10 \mu\text{g}/\text{grain}$), even when specifically expressed in the endosperm of the transgenic rice seed.⁴³ In this study, the original shuffled Cry j 2 was further destructed to reduce allergenicity and to increase the accumulation level, as shown in Figure S1B, in which the linear B-cell epitope sequence (KWVNGREI)⁴⁴ between

positions 124 and 131 was deleted and one N-glycosylation site was mutagenized.

Sequences encoding various deconstructed Cry j 1 and Cry j 2 derivatives were optimized using the rice seed storage proteins' preferred genetic codons, and the KDEL ER retention signal was attached at their C-termini to increase accumulation levels. Then, they were expressed under the control of the endosperm-specific glutelin 2.3 kb *GluB-1* or 1.3 kb *GluB-4* promoter containing its signal peptide sequence (Figure 1). Two expression cassettes were inserted into the same binary vector harboring the mALS selection marker using the MultiSite Gateway system. A binary vector containing two expression cassettes was introduced into the rice genome via *Agrobacterium*-mediated transformation.

Table 1. Accumulation Levels of Deconstructed Cry j 1 and Cry j 2 Derivatives in Transgenic Rice Seeds Containing Various Expression Constructs

construct	line number	accumulation levels ($\mu\text{g}/\text{grain}$)	
		shuffled Cry j 1	shuffled Cry j 2
A	12	20.2	70.4
	18	24	51.7
B	6	33.6	86.2
	18	37.2	82.7
C	2	66.9	100.2
	3	38.8	78.3
	5	47.4	74.6
D	5	51.5	65.6
	22	32.3	38.1
	23	24.2	53.1
E	15	29.7	59.2
	19	58.6	89.5
	23	87.4	85.7
F	5	40.7	67.2
	21	39.9	58.8
	23	33.8	50.2
G	7	35.7	64.1
	11	40.8	89.4
H	8	44.1	56
	15	45.1	60.9
I	1	95.2	120.3
	7	65.2	114.3
	9	53.5	94.3
J	9	104.7	132.7
	10	135.6	147.4
	15	140.3	130.7

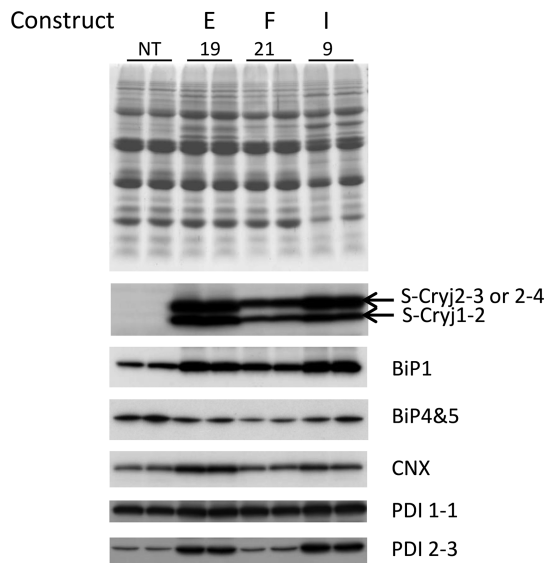


Figure 3. Expression of chaperone proteins in mature transgenic rice seeds. The effects on the expression of BiP1, BiP4&5, CNX, PDIL1-1, and PDIL2-3 by the production of S-Cryj-2 and S-Cryj2-3 or 2-4 in transgenic rice seeds containing the constructs E, F, and I were examined using specific antibodies. NT, nontransgenic rice.

Transgenic lines accumulating high levels of deconstructed Cry j 1 and Cry j 2 derivatives were screened by immunoblot analysis using anti-S-Cryj1-2 and anti-S-Cryj2-4 antibodies after the electrophoresis of total seed proteins from mature grains on 12% SDS-PAGE. Various deconstructed Cry j 1 and

Cry j 2 were detected as visible bands with molecular masses of 40 and 45 kDa when SDS-PAGE gels were stained with CBB after electrophoresis (Figure 2A and Figure S2). The accumulation levels of deconstructed Cry j 1 and Cry j 2 in matured seeds were presented for two or three representative lines (high expression lines) of the individual expression construct in Table 1. The accumulation levels were 24–87 $\mu\text{g}/\text{grain}$ and 38–100 $\mu\text{g}/\text{grain}$ for the deconstructed Cry j 1 and Cry j 2 derivatives, respectively. Regarding the expression of deconstructed Cry j 2 derivatives, the S-Cryj2-3 in the constructs C and F was most highly accumulated in the transgenic rice seed among the four constructs. Furthermore, it is notable that the shuffled Cry j 2 derivatives accumulated at higher levels than the shuffled Cry j 1 derivatives irrespective of the difference in the promoters used for their expression. These results suggest that the accumulation levels may be mainly determined by the physicochemical properties of the expressed product. Furthermore, it is interesting to note that the *GluB-4* promoter gave rise to slightly higher yields than the *GluB-1* promoter (construct B vs D, construct F vs G).

As shown in Figure S3, N-glycosylated bands observed in transgenic rice expressing S-Cryj1-1 or S-Cryj2-1, which were shifted by the treatment with Endo H or PNGase F, were not observed in those expressing S-Cryj1-2, S-Cryj2-2, S-Cryj2-3, or S-Cryj2-4, indicating that N-glycosylation was eliminated by the mutagenesis of N-glycosylation sites.

Higher Production of Shuffle Cry j 1 and Cry j 2 by

Suppression of Endogenous Prolamins.

Next, to examine whether the accumulation levels of the deconstructed Cry j 1 and Cry j 2 derivatives could be enhanced by the reduction of endogenous seed storage proteins, endogenous prolamins were specifically suppressed by the RNAi silencing inducing sequence (RSIS), which was linked to the 5' and 3' untranslated regions (UTRs) of 10, 16, and 13 kDa prolamins (Proless construct).¹⁶ When coinroduced with this Proless construct, the accumulation levels of endogenous 16, 10, and some 13 and 14 kDa prolamins (RM2 and RM4) were severely suppressed, as shown in Figure 2B and Figure S4. When compared with those directed by the 2.3 kb *GluB-1* promoter without a Proless construct (construct F), the production levels of S-Cryj1-2 and S-Cryj2-4, which were directed by the 2.3 kb *GluB-1* (construct I) or 1.3 kb *GluB-4* promoter (construct J), were significantly enhanced by the reduction of endogenous prolamins (Figure 2B). Production levels directed by the *GluB-4* promoter (construct J) were higher than those directed by the *GluB-1* promoter (construct I). That is, by the suppression of several endogenous prolamins by RNAi, S-Cryj1-2 and S-Cryj2-4 levels were enhanced 1.9- and 1.4-fold for the expression by the *GluB-1* promoter (construct I), whereas they were increased 4.2- and 1.85-fold for the expression by the *GluB-4* promoter (construct J). Furthermore, it is notable that high accumulation lines could be obtained more frequently from the generated transgenic rice lines than from the conventional expression constructs without the Proless construct.

Influence on Expression of Chaperon Proteins in

Mature Seed.

When the deconstructed Cry j 1 and Cry j 2 derivatives were highly produced as secretory proteins via the endomembrane system in the endosperm cells of transgenic rice seed, it was examined whether the unfolded protein response (ER stress) was induced in mature seeds. As shown in Figure 3, the expression levels of representative ER chaperone proteins such as binding protein 1 (BiP1) and folding enzymes

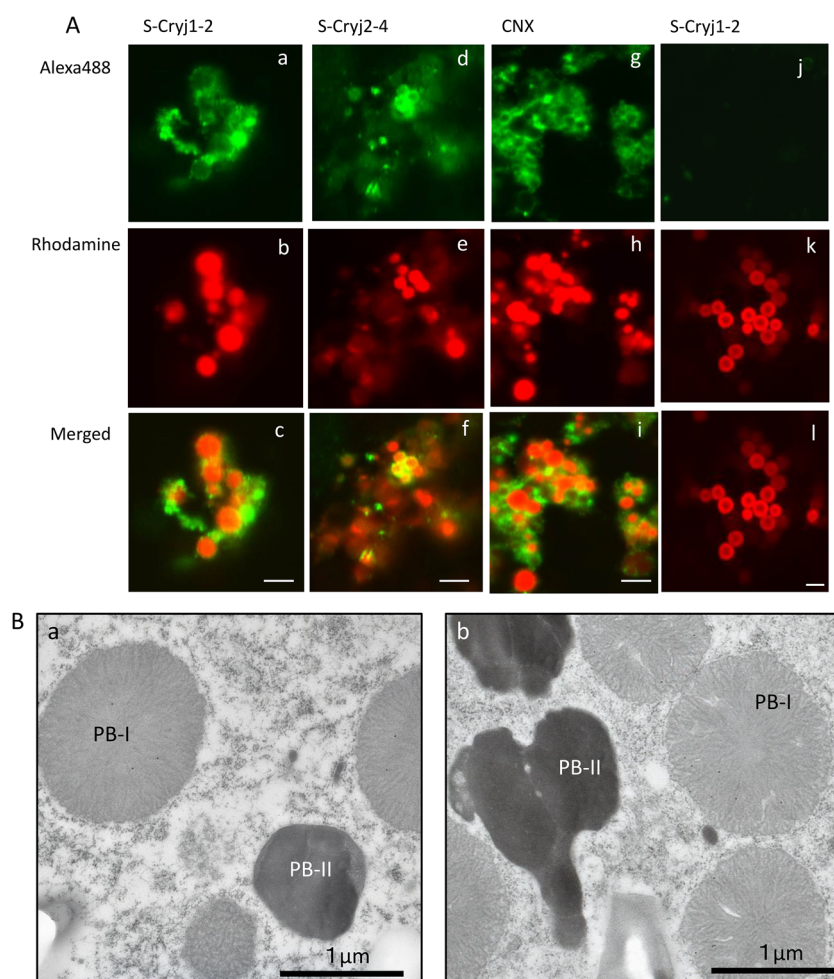


Figure 4. Intracellular localization of deconstructed Cry j 1 and Cry j 2 in developing endosperm cells. (A) Visualization of the intracellular localization of S-Cryj1-2 and S-Cryj2-4 was carried out by immunohistochemical analysis using anti-Cryj1-2 and anti-Cryj2-4 rabbit antibodies in maturing transgenic rice seeds (13–18 DAF) containing the construct G (a–i) and in nontransgenic rice seeds (j–l). Seed sections were stained with Rhodamine B to visualize PB-Is (red fluorescence in panels b, e, h, and k) and were reacted with antibodies against S-Cryj1-2 (a,j), S-Cryj2-4 (d), and CNX (g). Signals were detected using a second antibody with green fluorescence, Alexa488-conjugated second antirabbit IgG. bar = 1 μ m. (B) Immunoelectron microscopy analysis of S-Cryj1-2 (a) and S-Cryj2-4 (b) in transgenic rice endosperm cells containing construct I. Gold labeled particles are localized inside PB-I and ER lumen. PB-I, protein body I; PB-II, protein body II; bar = 1 μ m.

such as protein disulfide isomerase-like 2-3 (PDIL2-3) and CNX were significantly increased as compared with those of nontransgenic rice. This result indicated that high accumulation levels of the deconstructed Cry j 1 and Cry j 2 expressed as secretory proteins led to the upregulation of some chaperon proteins involved in refolding the highly produced artificial foreign proteins.

Intracellular Localization of Shuffle Cry j 1 and Cry j 2 Antigens in Endosperm Cells. The intracellular localization of deconstructed Cry j 1 and Cry j 2 in the endosperm cells of transgenic rice seed was investigated by confocal immunohistochemical and immunoelectron microscopy observation using anti-S-Cry1-2 and S-Cryj2-4 antibodies. Maturing endosperm cell of rice seed has two types of PBs that are different in morphology (shape and size) and electron density. One is the ER-derived protein body containing prolamins (16, 14, 13, and 10 kDa prolamins) called type I protein body (PB-I), and the other is the protein storage vacuole depositing glutelins and 26 kDa globulin (Glb-1) which is designated as type II PB (PB-II).⁴⁶ As shown in Figure 4A, shuffled Cry j 1 and Cry j 2 were

observed to be mainly deposited in ER lumen and at peripheral of rhodamine-stained ER-derived PB-Is with a size of ~ 1 μ m.

This localization pattern is characteristic of this transgenic rice because high amounts of these synthesized products are retained in ER lumen without sequestering to PB-Is or are not forming unique ER-derived PBs observed in various foreign recombinant proteins expressed in endosperm cell.¹⁸ Then, the detailed localization of these antigens was further investigated by immunoelectron microscopy observation. Immuno-gold signals were detected in the ER lumen in addition of PB-Is, which were mainly localized in the former (Figure 4B). This result agrees well with that observed by the confocal immunohistochemical analysis. The predominant localization in the ER lumen may be associated with the attachment of the KDEL ER retention signal at the C terminal ends and the physicochemical structures of these antigens.

Interactions between Modified Cry j 1 and Cry j 2 and Endogenous Cysteine-Rich Prolamins. A reducing agent has been demonstrated to be inevitable for the extraction of foreign antigens with the free Cys residue from rice seed.²⁹ We first examined whether there is an interaction between the

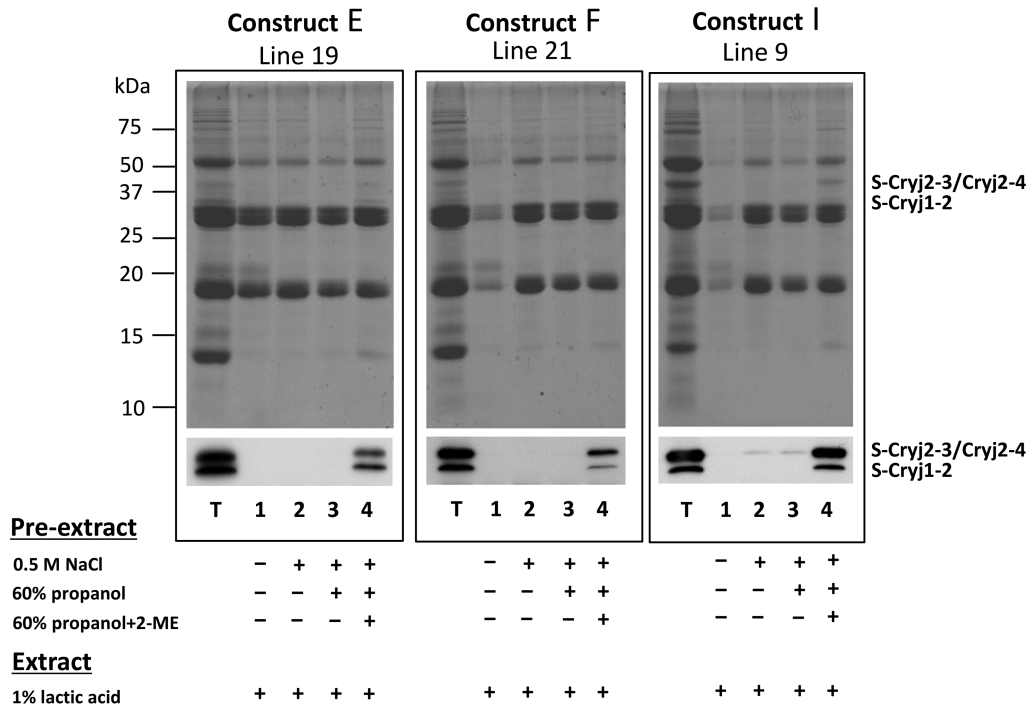


Figure 5. Stepwise extraction of deconstructed Cry j 1 and Cry j 2 derivatives from transgenic rice seed powder. SDS-PAGE and immunoblot analysis of deconstructed Cry j 1 and Cry j 2 derivatives extracted with 1% lactic acid after pre-extraction with different solvents of 0.5 M NaCl, 60% propanol, and 60% propanol with 10 mM DTT. The residues after the extraction with 0.5 M NaCl, 60% propanol, and 60% propanol with 10 mM DTT were also further extracted with 1% lactic acid. T, total seed protein; +, with pre-extraction, -, without pre-extraction.

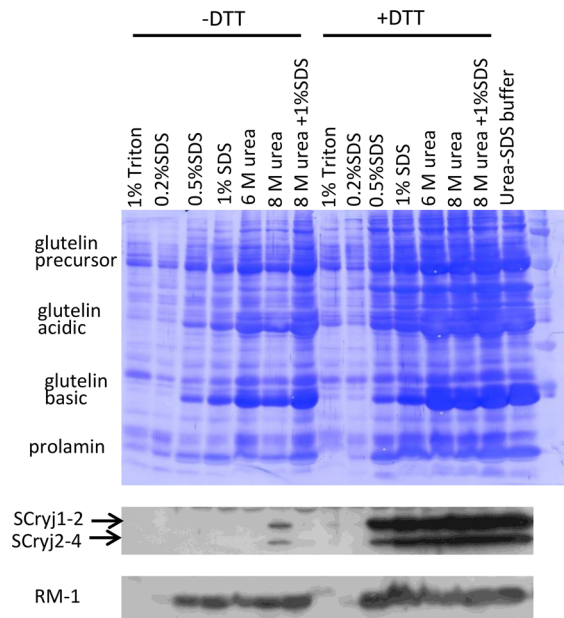


Figure 6. Extraction of S-Cryj1-2 and S-Cryj2-4 from transgenic rice seeds with various extraction buffers. Transgenic rice seed powder (construct G line 11) was extracted with various concentrations of SDS (0.2, 0.5, and 1%), 1% Triton-X, and 6 and 8 M urea in the saline buffer (50 mM Tris-HCl pH 7.5, 0.5 M NaCl) in the presence or absence of 10 mM DTT. Extracts were subjected to electrophoresis on 12% SDS-PAGE gel and then analyzed by immunoblotting using anti-His and anti-GluA antibodies.

deconstructed Cry j 1 or Cry j 2 and Cys-rich prolamins (16, 14, and 10 kDa prolamins). To investigate the deposition status of shuffled Cry j 1 and Cry j 2 produced in rice endosperm cells, seed proteins were extracted with various

buffers in a stepwise manner using 0.5 M NaCl, 60% propanol, and 60% propanol containing 10 mM DTT, which gave rise to the extraction of the globulin fraction, 13 kDa Cys-poor prolamins, and Cys-rich prolamins, respectively. As shown in Figure 5, shuffle Cry j 1 and Cry j 2 could be extracted when endogenous Cys-rich prolamins were removed with 60% propanol containing 10 mM DTT after the removal of globulin fraction and Cys-poor prolamins. These results suggest that shuffled Cry j 1 and Cry j 2 may interact with Cys-rich prolamins via intermolecular disulfide bonds.

Next, we examined what kinds of solubilizing conditions are required for the extraction of the shuffle Cry j 1 and Cry j 2 to elucidate their deposition mode in ER lumen and PB-Is. They were extracted with different concentrations of SDS (0.2, 0.5, and 1%) or 1% Triton X-100 or under denaturing conditions (6 and 8 M urea) in the presence or absence of 10 mM DTT. As shown in Figure 6, they could be solubilized with a higher concentration than 0.5% SDS or 6 M urea in the presence of a reducing agent, whereas the absence of a reducing agent resulted in no extraction with these detergents or denaturant, except for 8 M urea. It is notable that Cys-rich prolamins (RM1) could be extracted even with 0.5% SDS or 6 M urea without a reducing agent. Taken together, it was suggested that the shuffle Cry j 1 and Cry j 2 may aggregate via inter- and intramolecular disulfide bonds in ER lumens, resulting in the formation of insoluble aggregates.

In Vitro Digestion of Transgenic Rice with Gastric Digestive Enzymes. To compare the digestibility of antigens (shuffled Cry j 1 and Cry j 2) deposited in the ER lumen and PB-Is of transgenic rice and endogenous seed storage proteins (14 kDa Cys-rich RM1 prolamins and glutelin GluA) localized in the PB-Is and PB-IIs of nontransgenic and transgenic rice, respectively, seed powders were subjected to in vitro digestion

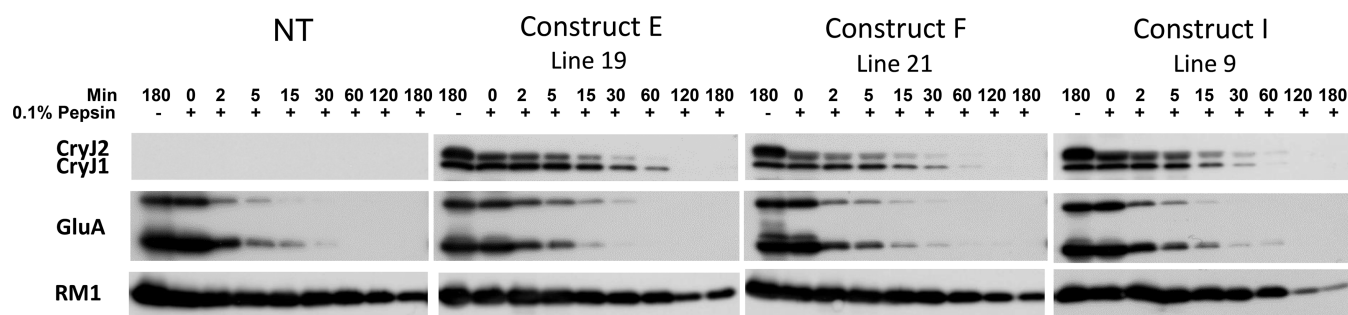


Figure 7. In vitro digestibility of seed protein in nontransgenic and transgenic rice. Panels indicate the digestibility by pepsin at 37 °C for up to 20 h. Proteins were detected by immunoblot analysis using anti-S-Cryj1-2, anti-S-Cryj2-4, and anti-GluA antibody and anti-13-kD prolamin RM1 antibody.

with pepsin. Shuffled Cry j 1 and Cry j 2 were more sensitive to proteolysis with pepsin than Cys-rich prolamin (RM1) in transgenic and nontransgenic rice (Figure 7). That is, these recombinant antigens were digested by treatment for ~60 min, whereas 14 kDa Cys-rich RM1 prolamin was resistant to pepsin digestion, even for 180 min. On the contrary, glutelin GluA deposited in PB-IIs of transgenic and nontransgenic rice was proteolyzed with pepsin within 30 min. These results indicated that shuffled Cry j 1 and Cry j 2 are slightly more highly resistant than glutelin GluA. Notably, there was little difference in stability for 14 kDa Cys-rich RM1 prolamin between transgenic and nontransgenic rice, although there was a ragged appearance and surface cracking in PB-IIs of transgenic rice. These results suggest that a higher sensitivity of the deconstructed Cry j 1 and Cry j 2 to pepsin than Cys-rich prolamin may be due to the difference in their localization or deposition mode.

Binding Activity of Deconstructed Cry j 1 and Cry j 2 to Their Specific IgE. It was examined whether the alternation of Cry j 1 or Cry j 2 structure by molecular shuffling has any effect on the binding activity to the specific IgE antibody. First, deconstructed Cry j 1 and Cry j 2 proteins (S-Cryj1-2 and S-Cryj2-4) expressed in *E. coli* were subjected to a binding assay to the specific IgE antibody. As shown in Figure 8A, S-Cryj1-2 and S-Cryj2-4 did not bind to their specific IgE antibody in a similar manner as the Cry j 1 F1 fragment (Cryj1F1) or the original shuffled Cry j 2 (S-Cryj2) previously reported,¹⁴ whereas native Cry j 1 and Cry j 2 bound to the specific IgE antibody depending on their concentrations. Furthermore, the binding activities of deconstructed Cry j 1 and Cry j 2 antigens were examined using inhibition ELISA. As shown in Figure 8B, the addition of native Cry j 1 or Cry j 2 (positive control inhibitors) inhibited the binding of biotinylated Cry j 1 or Cry j 2 (secondary antibody) to the allergen-specific IgE in the sera from BALB/c mice immunized with JC pollen, whereas the addition of bovine serum albumin (BSA) (negative control inhibitor) showed a lower inhibition rate (~10%). The inhibition rates of S-Cryj1-2 and S-Cryj2-4 were also low, indicating that the binding activity of S-Cryj1-2 and S-Cryj2-4 to the allergen-specific IgE was very weak, like Cry j1F1 and S-Cryj2. These results indicate that the binding activities of the S-Cryj1-2 and S-Cryj2-4 to their specific IgE are highly reduced or are abolished by the drastic alternation of the tertiary structure.

Furthermore, the low binding activity of S-Cryj1-2 and S-Cryj2-4 was further ascertained by the basophil degranulation test (Figure 8C). Native Cry j 1 and Cry j 2 bound and cross-linked to the specific IgE on the surface of basophil, leading to

degranulation of basophil, whereas shuffled Cry j 1 and Cry j 2 (S-Cryj1-2 and S-Cryj2-4) failed to bind to specific IgE and had little effect on basophil degranulation. Taken together, it is concluded that the reduction of IgE binding activity can be caused by the destruction of the tertiary structure

DISCUSSION

Allergen-specific immunotherapy is the only curative treatment for IgE-mediated allergy diseases with a long-lasting effect. The crude allergen extract has been administrated as a tolerogen of clinical treatment through subcutaneous and sublingual routes to induce the immunological tolerance to the causative allergens.² Because the native allergen has IgE binding activity, severe side effects such as anaphylaxis shock are sometimes induced by binding and cross-linking to IgE on the surface of mast cell and basophil cells.

To enhance the safety and efficacy of tolerogen, hypoallergenic allergenic derivatives with reduced allergenicity have to be produced at a higher level. For the treatment of JC pollinosis by allergen-specific immunotherapy using a rice-based oral allergy vaccine, we previously generated several types of transgenic rice containing 7 or 12 linked major T-cell epitopes derived from the Cry j 1 and Cry j 2 or destructed Cry j 1 and Cry j 2 (overlapped Cry j 1 fragments and shuffled Cry j 2) covering their whole sequences.^{15,12,14} The hybrid T-cell epitope peptide is very safe due to the very low allergenicity (very low IgE reactive capacity), but it does not cover all T-cell epitopes based on the difference in genetic background (variety of major histocompatibility complex (MHC) class II haplotypes). On the contrary, the deconstructed Cry j 1 and Cry j 2 allergens preserve immunogenicity and reduce allergenicity due to the alternation of the tertiary structure, leading to the treatment of a broad range of patients. In this study, we have generated new transgenic rice that accumulated high amounts of the hypoallergenic Cry j 1 and Cry j 2 derivatives by molecular shuffling. Furthermore, the production levels of these hypoallergenic Cry j 1 and Cry j 2 derivatives were further enhanced by the suppression of endogenous prolamins using RSIS. A similar enhancing effect on the production of foreign recombinant proteins by the suppression of endogenous seed storage proteins has been observed in the production of several cytokines such as human IL-10, mouse IL-4, mouse IL-18, or TGF- β in transgenic rice seeds.^{21,27,45,46}

The efficacy of a rice-based allergy vaccine containing the deconstructed Cry j 1 and Cry j 2 has already been examined by oral administration to model mice.^{14,47,48} When the transgenic rice seeds were fed to model mice and then

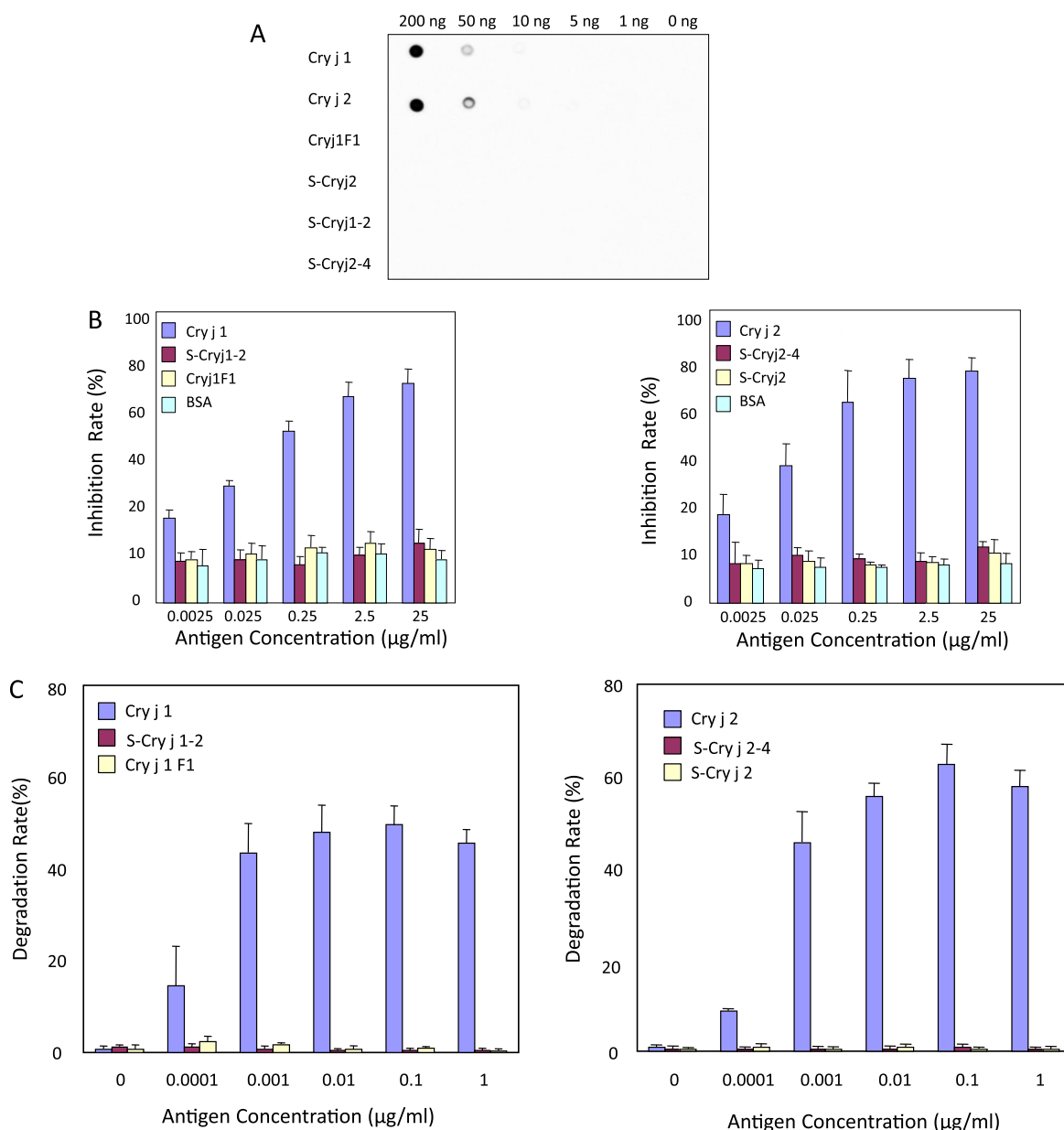


Figure 8. IgE binding activities of destroyed S-Cryj1-2 and S-Cryj2-4. (A) Dot-blot IgE binding assay of recombinant Cryj1F, S-Cryj2, S-Cryj1-2, and S-Cryj2-4. Native Cryj1, Cryj2, and recombinant Cryj1F, S-Cryj2, S-Cryj1-2, and S-Cryj2-4 were dotted onto a Protran nitrocellulose membrane at 200, 50, 10, 5, 1, and 0 ng and incubated with sera from BALB/c mice immunized with Japanese cedar pollen. IgE antibody bound to Cryj1, Cryj2, and recombinant proteins was detected with antimouse IgE-biotin and streptavidin–HRP, and then visualized by autoradiography. (B) Competitive IgE-binding assay of recombinant Cryj1F, S-Cryj2, S-Cryj1-2, and S-Cryj2-4. The IgE reactivity of recombinant Cryj1F, S-Cryj2, S-Cryj1-2, and S-Cryj2-4 was examined by competitive IgE-binding ELISA, as described in the manuscript. The inhibition rate (%) was calculated by the formula $100 - [(OD_{450nm} \text{ of mouse serum mixed with Cryj1, Cryj2, and recombinant proteins}) / (OD_{450nm} \text{ of}) \times 100]$. (C) RBL-2H3 basophil degranulation test of recombinant Cryj1F, S-Cryj2, S-Cryj1-2, and S-Cryj2-4. Rat basophilic leukemia RBL-2H3 cells were primed by incubating overnight with sera from BALB/c mice immunized with Japanese cedar pollen. Then, the cells were stimulated with Cryj1, Cryj2, and recombinant Cryj1F, S-Cryj2, S-Cryj1-2, and S-Cryj2-4. The amount of β -hexosaminidase activity released by the RBL-2H3 cells was measured, and the total β -hexosaminidase release was determined by lysing the cells with 0.1% Triton X-100.

challenged with a crude JC pollen allergen, allergen specific CD4⁺ T-cell proliferation (allergen-specific T-cell activation) was markedly suppressed compared with that of mice fed nontransgenic rice. Furthermore, the production levels of specific IgE and IgG, several Th2 type cytokines such as IL-4, IL5, and IL-13, and histamine were significantly down-regulated. Regarding clinical symptoms, the sneezing frequency and the infiltration of inflammatory cells such as eosinophils and neutrophils in the nasal lavage and the conjunctiva of eye

were also suppressed. It is notable that the induction of blocking IgG4 or IL-10 secreted from the regulatory T cell was not observed with the oral administration of transgenic rice. These results indicated that transgenic rice seed containing the modified whole Cry j 1 and Cry j 2 sequences can induce an immune tolerance to the Cry j 1 and Cry j 2 pollen allergens through oral administration. However, it is interesting to note that the immune tolerance mechanism induced by the oral administration of transgenic rice may be different from that

induced by the subcutaneous or sublingual injection of allergen extracts (natural allergen extract-based vaccine). Immune tolerance by the oral administration of transgenic rice has been suggested to be induced via anergy or the deletion of antigen-specific T cells. This difference has been suggested to be related to the dose concentration, administration frequency, administration root, or antigen formulation.⁴⁹

With regard to the safety of administered tolerogens, we previously reported that the fragmented Cry j 1 and original shuffle Cry j 2 exhibited low IgE binding activity and low basophil degranulation activity to the specific mouse IgE.¹⁴ The low allergenicity of these modified allergens has recently been further evaluated using human-specific IgE samples taken from JC patients. When examined by the basophil activation test using blood samples from 29 patients with JC pollinosis, the binding and cross-linking activities of basophil-bound IgE from patients were shown to be very low compared with the deconstructed Cry j 1 and Cry j 2 produced in transgenic rice seeds.⁵⁰ These results indicated that the allergenicity can be significantly reduced by the conformational alternation of the Cry j 1 and Cry j 2 allergens through fragmentation or shuffling. In this study, both the Cry j 1 and Cry j 2 antigens were more completely destructed by shuffling than the previous ones (Figure S1). We demonstrated that they almost lost their allergenic activities because their binding activities to the specific IgE antibodies and basophil degranulation activities could not be detected, even by the presence of extensive high doses of these modified antigens. Therefore, the highly reduced allergenic activities of these modified antigens make it possible to allow high dose of oral administration of transgenic rice seed, resulting in the enhancement of efficiency and safety.

Deconstructed Cry j 1 and Cry j 2 derivatives were shown to be deposited in ER lumen and ER-derived PBs (PB-Is). Stepwise extraction from transgenic seed proteins showed that they interacted with Cys-rich prolamins. Furthermore, it is notable that the addition of a reducing agent to the extraction buffer containing the detergent (SDS) or denaturant (urea) is essential for their extraction from the mature seed, indicating that they are localized in the ER lumen and ER-derived PBs as insoluble aggregates via inter- or intra- disulfide bonds. These findings are related to their being slightly more highly resistant to the digestive enzyme (pepsin) than glutelins deposited in PB-IIIs. Therefore, they are expected to be efficiently delivered to the GALT without degradation in the gastrointestinal tract after oral administration. In light of the ER-derived PBs being taken up through the GALT,^{18,19} highly produced antigens deposited in ER-derived PB and ER are expected to be delivered to the GALT without degradation in the gastrointestinal tract.

In the previous study, we expressed the overlapped three fragments covering the whole Cry j 1 sequence by fusing with the rice glutelins (GluA, GluB, and GluC) to enhance the accumulation level. They were directed by three endosperm-specific promoters (GluB-4 and 16 and 10 kDa prolamins) different in promoter strength and temporal- and spatial-specific manner in transgenic rice seed.⁴⁴ As a result, these Cry j 1 fragments were produced at different levels. For the pharmaceutical application of a rice-based allergy vaccine, stability and constancy of yield levels for the expressed recombinant proteins are always required according to the guidance of the GMP-compliant manufacture. Therefore, it is important to note that the whole Cry j 1 and Cry j 2 sequences

could be expressed at high levels as the deconstructed hypoallergenic Cry j 1 and Cry j 2 derivatives using two expression constructs in place of the previous four constructs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b05421.

Figure S1. Schematic representation of various deconstructed Cry j 1 and Cry j 2 structures. Figure S2. Analysis of expression levels of destructed Cry j 1 and Cry j 2 derivatives in transgenic rice seeds containing construct A and construct H. Figure S3. Detection of glycosylation of various deconstructed Cry j 1 and Cry j 2 derivatives. Figure S4. Expression levels of S-Cryj1-2 and S-Cryj2-4 and various prolamins in mature transgenic rice seeds containing Proless construct (PDF)

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Author Contributions

F.T. designed the experiments. F.T., L.Y., H.T., and N.M. performed the experiments and analyzed the data. F.T. wrote this manuscript. Y.W. K.O. and C.H. critically revised the draft. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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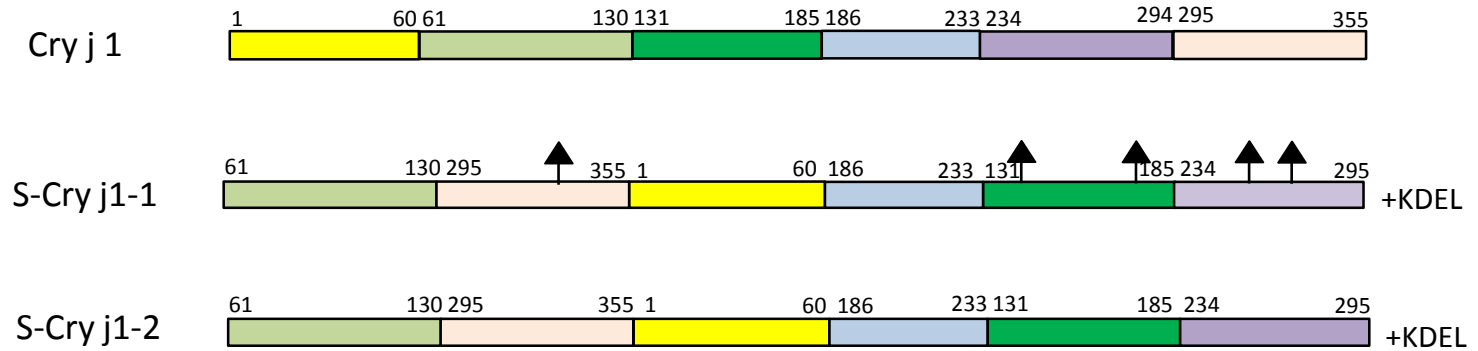
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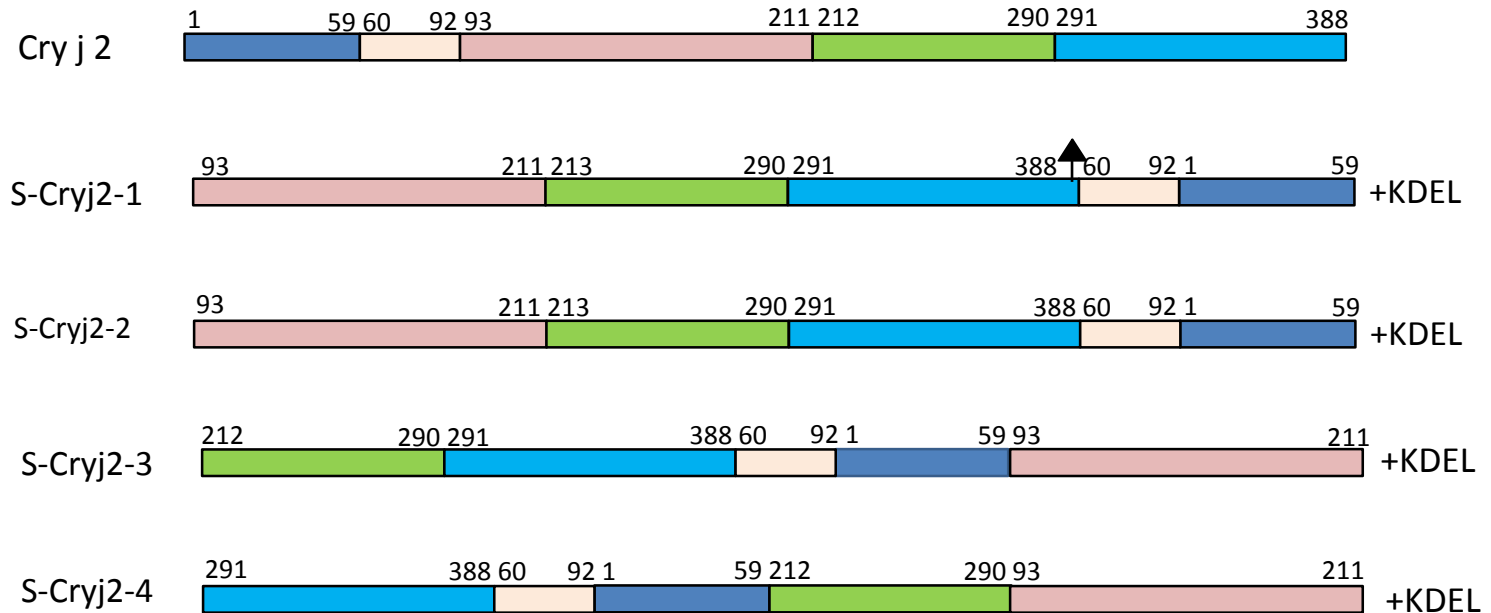


S-Cry j 1-2

M **GATRDRPLWII**FSGNMNIKLKMPMYIAGYKTFDGRGAQVYIGNGGPC**VFIKRVS****NVIHGLYLYGCSTSV**WQSTQDV
FYNGAYFVSS**SGKYEGGNIYTKKEAFNV**ENG**NAAP**QLTKNAGVLTCSLSKRC**DNPIDSCWRGDSNWAQ****NRMKLADCAV**
GFGSSTMGGKGGDLYTVNSDDDPVNPAPGTLRYTISNNLFFNHHKVMMLGHDDAYSDDK**SMKVTVAFNQFGPNCG**
QRMPRALGNVLINEAFGVEPVHPQDGDALTRLATNIWIDHNSFS**NSADGLVDVTLTSTGV****RYGLVHVANNNYDPWT**
IYAIGGSSNPAILSEGNSFTAPNEAYKKQVTIRIGCKTSSSCSNWV**KDEL**

Fig. S1a

B



S-Cryj2-4

MGSASACQNQRSQVQIQDVTYKNIRGTSATAAAIQLKCSDSMPCKDIKLSDSLKLTSGKIASCLNDNANGYF
 SGHVIPACKNLAPSNNLFFNGPCQPHFTFKVDGIIAAYQNPAWKNRR**RKVEHSRHD**AINIFNVEKYGAVGD
 GKHDCTEAFSTAWQAACKKPSAMLLVPGNKKFV**SNIVIEDLICGPGHG**ISIGSLGRENSRAEVSYVHNGAK
 FIDTQNGRLRIKTWQGGSGMASHIYENVEMINSENPIINQFYCTIWLQFAKLTGFTLMGKGVIDGQKGQWW
 ANDRDRPTAIKFDFSTGLIIQGLRLMNSPEFHLVFGNCEGVKIIIGISITAPRDSPTDGD**IFASKNFHLQKNTIGT**
 GDDCVAIGTGSKDEL

Figure S1. Schematic representation of various deconstructed Cry j 1 (A) and Cry j 2 structures (B). Amino acid sequences of the S-Cry j1-2 and S-Cryj2-4 were presented. Major T cell epitope peptides in the Cry j 1 and Cry j 2 were underlined. Black bold sequences are mutagenized N-glycosylation sites. Arrow represents N-glycosylation site

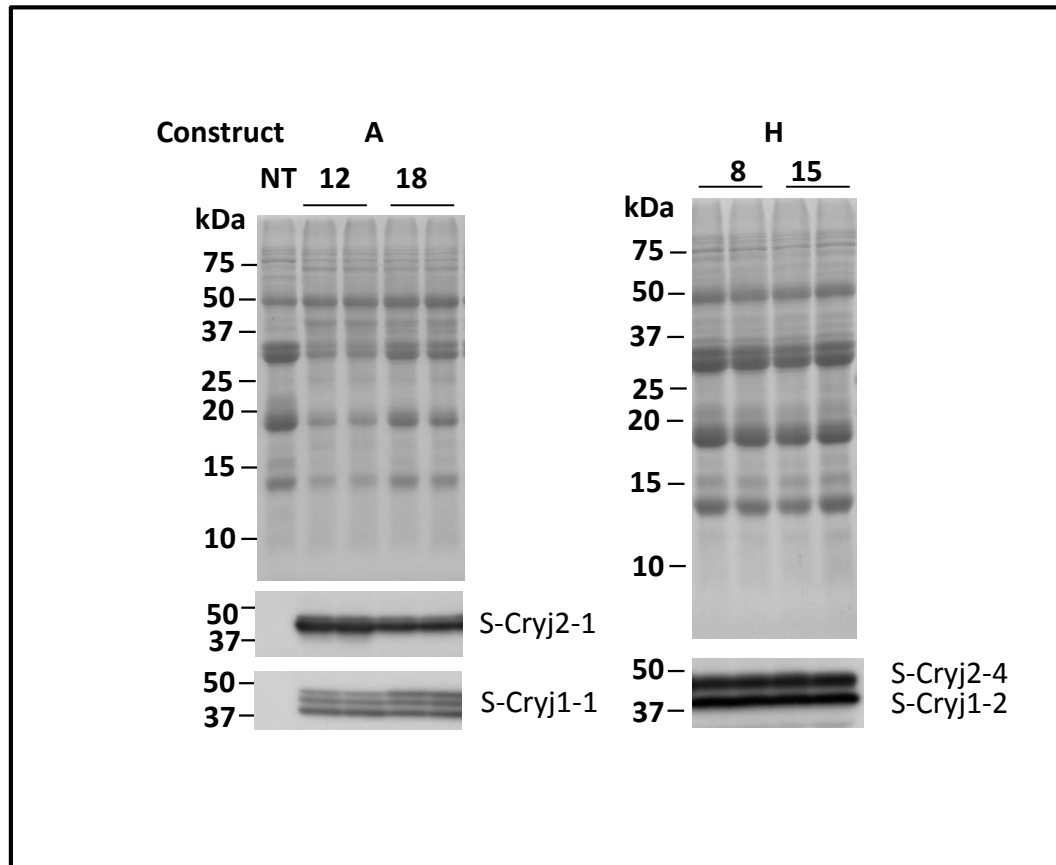


Figure S2. Analysis of expression levels of destructed Cry j 1 and Cry j 2 derivative in transgenic rice seeds containing construct A and construct H.

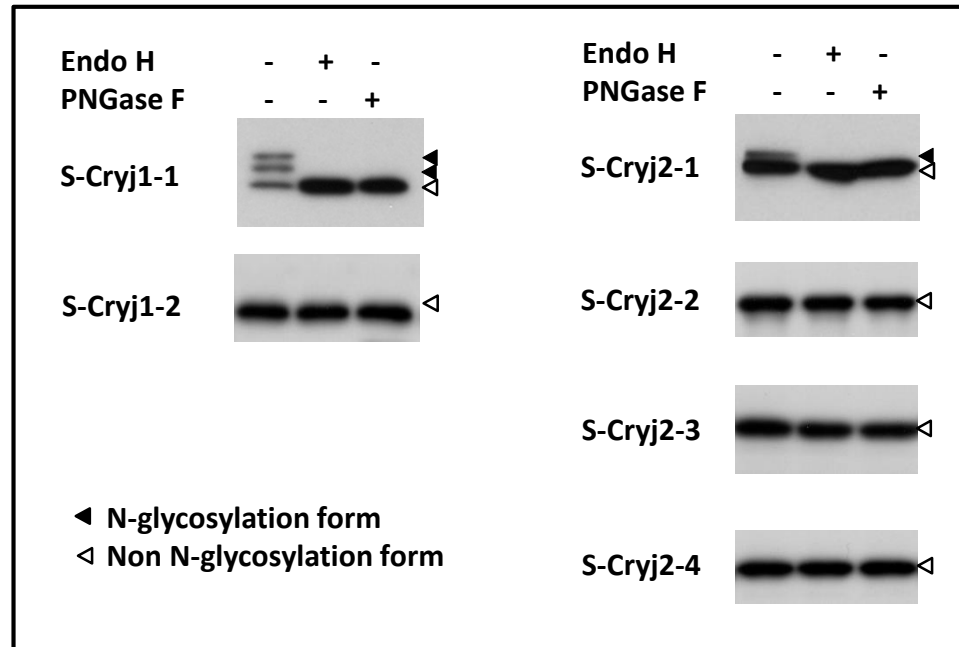


Figure S3. Detection of glycosylation of various deconstructed Cry j 1 and Cry j 2 derivatives. Total seed proteins were extracted from transgenic rice seeds and digested with Endo H and PNGase F. The digests were subjected to SDS-PAGE and analyzed by immunoblotting using anti-Cryj1-2 and anti-Cryj2-4 antibodies. A shift to a lower molecular mass after digestion with Endo H and PNGase, indicated the presence of N-linked glycans.

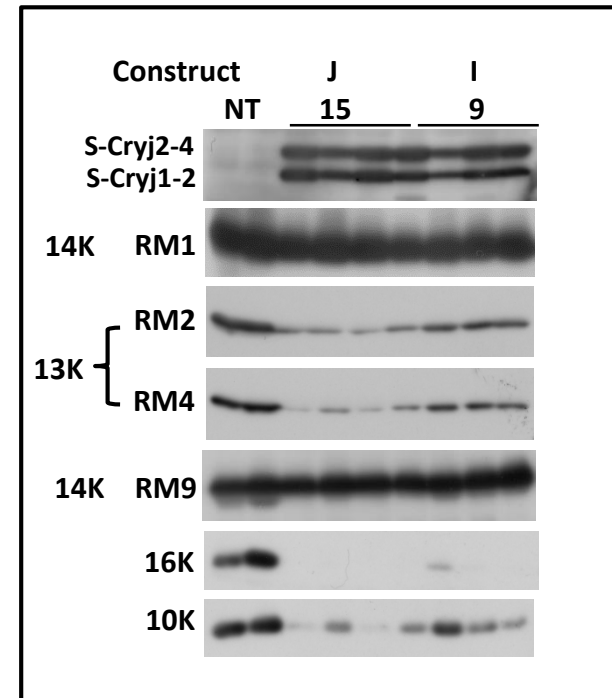
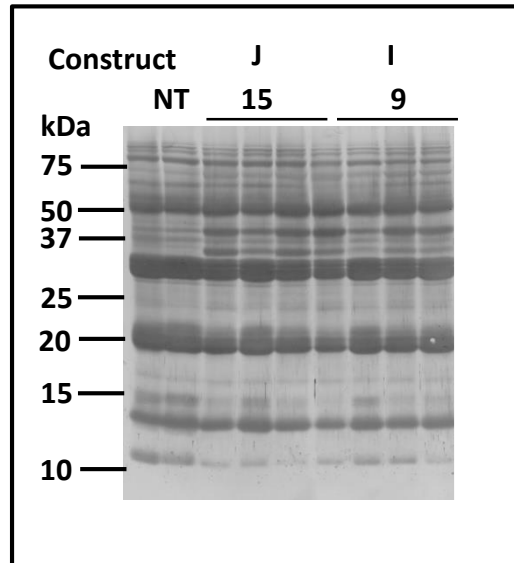


Figure S4. Expression levels of S-Cryj1-2 and S-Cryj2-4 and various prolamins in mature transgenic rice seeds containing Proless construct. Effect by suppression of prolamins on expression levels of S-Cryj1-2 and S-Cryj2-4, 16 kDa prolamin, Cys-rich 14 kDa prolamins (RM1, RM9), Cys-poor 13kDa prolamins (RM2, RM4) and 10 kDa prolamins were examined by immunoblotting using specific antibodies.