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Biochemical Safety Evaluation of Transgenic Rice Seeds Expressing T Cell Epitopes of Japanese Cedar Pollen Allergens

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Transgenic rice seeds, which express a hybrid peptide comprising seven predominant human T cell epitopes (7Crp) derived from Japanese cedar pollen allergens, have been shown to function as an effective edible vaccine for the control of pollen allergen-induced responses. In this study, we characterized biochemical properties of transgenic seeds expressing the 7Crp peptide. The levels of chemical compositions, such as carbohydrate, protein, lipid, amino acid, fatty acid, mineral, and vitamin, were substantially equivalent between transgenic 7Crp and its nontransgenic counterpart seeds. The contents of three major allergenic proteins in transgenic seeds were not enhanced by expression of the 7Crp peptide when compared with those of nontransgenic seeds. The 7Crp peptide expressed in seeds was susceptible to simulated gastric/intestinal fluids. *N*-Glycosylation was not observed in the 7Crp peptide sequence. These results indicate that transgenic 7Crp seeds are substantially equivalent to nontransgenic parental seeds except for the presence of the 7Crp peptide.

KEYWORDS: Food safety assessment; transgenic rice seed; edible vaccine; peptide-based immunotherapy; Japanese cedar pollinosis

INTRODUCTION

Transgenic plants have come to be recognized as viable and efficient bioreactors for the large-scale production of peptides and proteins (*1*, *2*). Compared to conventional microbial and mammalian cell culture systems, plant production systems offer the advantages of low production costs, easy control of production scale, and low risk of contamination by human pathogens (*1*, *2*). Seeds of cereal crops may further provide an ideal vehicle for production of recombinant proteins and a direct delivery system for them without the need for extraction and purification (*3*, *4*). There is also no need for cold storage of seed-based recombinant products because they are stable for more than a year even if stored at room temperature (*4*, *5*). Rice is an attractive candidate as a host for production of recombinant proteins because it is widely consumed as a staple food, has a high grain yield, is easy to transform, is self-pollinated, has an established production and processing system, and provides a high yield of recombinant products (*6*).

Immunotherapy using allergen-specific T cell epitope peptides is an effective and safe treatment for IgE-mediated allergic diseases, such as Japanese cedar pollinosis (*7*, *8*). Recently, mouse T cell epitopes derived from two major allergens (Cry j 1 and Cry j 2) in Japanese cedar pollen (*Cryptomeria japonica*) were expressed in transgenic rice seeds and fed to BALB/c mice in preclinical trials (*9*). The mice expressed immune tolerance by inhibiting allergen-specific IgE and $CD4^+$ T cell proliferative responses, cytokine production, and histamine release (*9*). Moreover, development of clinical symptoms such as sneezing was significantly suppressed (*9*). These results demonstrated the efficacy of an edible recombinant peptide using a rice seed-expression system for the induction of immune tolerance against the Japanese cedar pollen allergens.

In the next step toward development of specific immunotherapy for humans, a hybrid peptide comprising seven human T cell epitopes, designated 7Crp, was directly expressed in transgenic rice seeds (*10*). The 7Crp peptide accumulated in seeds of transgenic rice up to 4% of total seed protein (60 μg per grain) (*10*). The efficacy of this transgenic seed for inducing allergic tolerance was examined in B10.S mice, which recognize one of the seven human T cell epitopes of pollen allergens (Cry j 1 p211−225) (*11*). Feeding of 7Crp seeds significantly suppressed the levels of serum IgE and $CD4^+$ T cell proliferative responses (*10*). This result clearly indicates the potential utility of transgenic 7Crp seeds for control of pollen allergy in humans.

The food safety of 7Crp seeds must be carefully examined prior to its commercial and clinical application. Regulatory standards based on "Substantial equivalent" have been used to determine the food safety of transgenic crops (*12*, *13*). A comparative assessment of the toxicology and nutrient composition is made between the newly developed transgenic food and its nontransformed host line (*12*, *13*). In this study, we compared the levels of nutrients and native rice allergenic proteins between parental nontransgenic seeds and transgenic 7Crp seeds. In addition, we analyzed the glycosylation and *in vitro* digestibility properties of the expressed 7Crp peptide.

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MATERIALS AND METHODS

Rice Plants. Transgenic rice line #10, which showed a high level of accumulation of the 7Crp peptide, was used in this study (*10*). Homozygous transgenic rice line #10 was selected by self-pollination and proceeded to T_6 generations in a glasshouse. T_7 plants were grown in an isolated field designed for field trials of transgenic plants at the National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, Japan. The field trials were conducted under the guidelines of the Ministry of Agriculture, Forestry and Fisheries of Japan. Nontransgenic control rice (*Oryza sativa* L., cv Kitaake) was used as host for transformation and cultivated at one part of the field site where transgenic rice line #10 was grown in the same year.

DNA Hybridization. Genomic DNA of rice plants was extracted from young leaves using a cetyltrimethylammonium bromide (CTAB) method as described previously (*14*). DNA (10 μg) was digested with *Sac* I and separated on 0.7% (w/v) agarose gels. The gels were then blotted to Hybond-N^+ membranes (GE Healthcare, Bio-Sciences Corp., Piscataway, NJ). A ^{32}P-labeled full-length 7Crp DNA was used as a probe. Hybridization in Rapid-hyb buffer (GE Healthcare, Bio-Sciences Corp., Piscataway, NJ) and posthybridization washes were performed at 65 °C. The *Sac* I restriction enzyme cuts once within the expression vector used for transformation, indicating that the number of bands represents the number of copies of the 7Crp gene introduced into the rice genome.

SDS-PAGE and Western Blots. Rice seeds were ground to a fine powder in a multibead shocker (Yasui Kikai, Osaka, Japan) (*14*). Total soluble seed protein extracted in an extraction solution (*14*) was evaluated by SDS-PAGE and Western blot analysis using a rabbit anti-7Crp antibody as described previously (*10*, *14*). Anti-glutelin A, anti-glutelin B, anti-glutelin C, and anti-13 kDa prolamin rabbit antibodies were produced against synthetic peptides: KRNPQAYRREVEEWSQ conserved in the glutelin A family (the middle region of the acidic subunit between positions 210 and 225 of the GluA-2, accession number X05664), QVQYSERQQTSSRW conserved in the glutelin B family (the C terminal region of the acidic subunit between positions 288 and 301 of the GluB-1, accession number X54314), SPRGFRGDQDSRHQ conserved in the glutelin C family (the N terminal region of the acidic subunit between positions 34 and 47 of the GluC-1, accession number AB016501), and YIAPRSIPTVGGVWY conserved in the 13 kDa prolamin (the C terminal region between positions 142 and 156 of the λRM1, accession number XM477075). Anti-10 kDa prolamin and anti-16 kDa prolamin rabbit antibodies were raised against *E. coli*-expressed, purified 10 kDa prolamin (between positions 25 and 125, accession number X15231) and 16 kDa prolamin (between positions 94 and 149, accession number D88210), respectively. Anti-α-amylase inhibitors, anti-α-globulin, and anti-glyoxalase I rabbit antibodies were kindly provided by Dr. T. Matsuda (Nagoya University, Nagoya, Japan).

Compositional Analysis of Rice Seeds. The chemical composition of unpolished nontransgenic and transgenic 7Crp seeds was examined by standard methods for analysis of food nutrients at Japan Food Research Laboratories (Tokyo, Japan) as described previously (*15*). All of the results reported in this study indicate the value of two replications of field-grown nontransgenic and transgenic 7Crp seeds.

***In Vitro* Digestion by Pepsin and Pancreatin.** Fine transgenic rice seed powder was dissolved in 100 mM sodium acetate (pH 1.7) and 30 mM sodium chloride with 0.32% (w/v) pepsin (Sigma, St. Louis, MO) or in 50 mM potassium dihydrogen phosphate (pH 7.5) with 1% (w/v) pancreatin (Nacalai Tesque, Kyoto, Japan) at a 7Crp peptide concentration of 170 ng/μL. Digestion reactions were incubated for 0, 2, 5, 15, 30, 60, and 120 min at 37 °C and terminated with addition of protein extraction solution (*14*). Reaction mixtures were subjected to Western blot analysis as described above.

Detection of *N*-Linked Glycan. In order to test for the existence of *N*-linked glycan chains on the 7Crp peptide accumulated in transgenic seeds, total seed protein extracted in a buffer containing 50 mM Tris-HCl (pH 7.5) and 2 M urea was digested with *N*-glycosidase F according to the manufacturer's protocol (Roche, Basel, Switzerland). The reaction mixture was then subjected to SDS-PAGE/Western blot analysis to examine apparent molecular mass. A shift to a lower molecular mass after digestion with *N*-glycosidase F indicates the presence of *N*-linked glycan on the peptide.

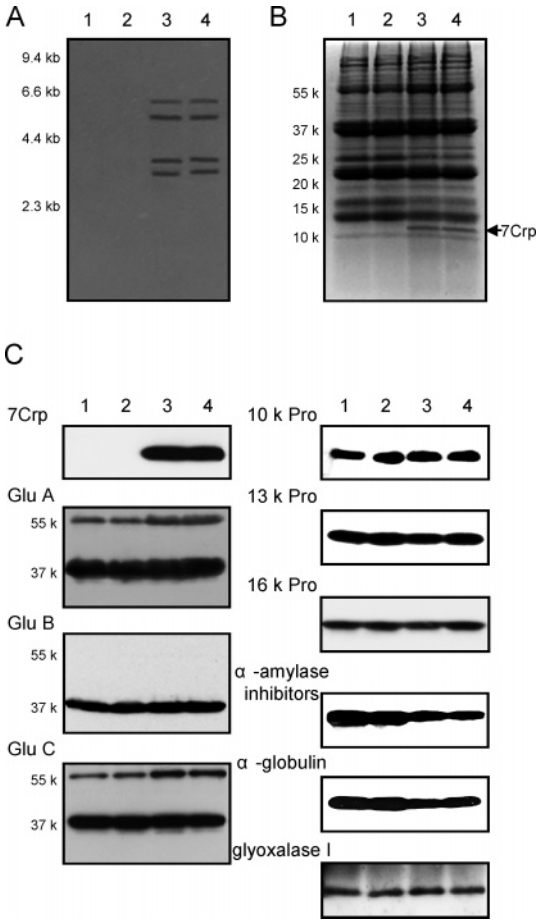

Figure 1. Characterization of nontransgenic and transgenic rice plants. (A–C) Lane 1, glasshouse-grown nontransgenic seed; lane 2, field-grown nontransgenic seed; lane 3, glasshouse-grown transgenic T_2 7Crp seed; lane 4, field-grown transgenic T_7 7Crp seed. (A) Southern blot analysis of rice genomic DNA prepared from young leaves and digested with *Sac* I. A ^{32}P-labeled full-length 7Crp DNA was used as a probe. (B) Coomassie brilliant blue (CBB) stained SDS-PAGE and (C) Western blot analysis of rice seed proteins. The same amount of protein (20 μg) was loaded in each lane. The membranes were probed with each of 10 primary antibodies.

RESULTS AND DISCUSSION

Detection of the 7Crp Gene and 7Crp Peptide in Transgenic Rice Plants. In this study, generations T_0-T_6 of the transgenic rice line #10 expressing the 7Crp peptide in seeds were grown in a glasshouse and generation T_7 was grown in an isolated field. Genomic DNA from generations T_2 and T_7 were examined by Southern blot analysis. The 7Crp hybridization patterns of the T_2 and T_7 generations were identical in size and intensity (**Figure 1A**), indicating that the 7Crp genes were stably inherited by the field-grown T_7 rice plants. Expression of the 11.2 kDa 7Crp peptide as a proportion of total seed protein was also the same in T_2 and T_7 plants, based on Western blots (**Figure 1B**, **1C**).

Seed Storage and Allergenic Proteins. There was as increase in the relative concentration of proteins with a molecular mass of around 55 kDa in 7Crp seeds but a decrease in ca. 25 and 15 kDa proteins (**Figure 1B**). To characterize these differences, we examined the expression levels of several storage proteins using specific anti-sera. The results showed that there were no

Table 1. Compositional Analysis of Rice Seeds[a]

content	nontransgenic seeds		transgenic 7Crp seeds	
moisture	10.6	10.6	10.3	10.1
protein	7.1	7.0	7.1	7.1
lipid	3.7	3.8	3.3	3.6
ash	1.9	1.9	1.9	1.9
carbohydrate	76.7	76.7	77.4	77.3
fiber	3.4	3.2	3.2	3.4

[a] Numbers represent the mean (g/100 g seed) of two independent measurements for nontransgenic and transgenic 7Crp seeds.

apparent differences in the levels of the mature acidic subunits of the glutelin A (Glu A), glutelin B (Glu B), or glutelin C (Glu C) subfamilies (around 38 kDa), whereas the precursors of Glu A and Glu C (around 55 kDa) were increased in transgenic 7Crp seeds (**Figure 1C**). The precursor of Glu B was not detected in our experiments. The levels of 10 kDa prolamin (10 k Pro), 13 kDa prolamin (13 k Pro), and 16 kDa prolamin (16 k Pro) were comparable to those of nontransgenic seeds (**Figure 1C**).

The α-amylase inhibitors (14−16 kDa), α-globulin (26 kDa), and glyoxalase I (33 kDa) have been identified as major rice grain allergens by several clinical investigations (*16*−*18*). The amount of glyoxalase I in 7Crp seeds was comparable with nontransgenic rice seeds, whereas the levels of α-amylase inhibitors and α-globulin were slightly lower in 7Crp seeds (**Figure 1C**). It is not known how 7Crp peptide affects the levels of other proteins and potentially toxic products in seeds but may be due to competition for transport or storage or there may be indirect effects on gene regulation, processing, or turnover. The effects of these minor alterations in seed storage proteins and allergens could have some impact on allergenicity and should be further examined.

Nutritional Comparison of Nontransgenic and Transgenic 7Crp Seeds. Quantitative comparison of nutritional properties (i.e., nutrient composition) has been proposed as a standard for food safety assessment (*13*). We evaluated whether the chemical composition of field-grown 7Crp seeds are equivalent to nontransgenic seeds. Moisture content, protein, lipid, ash, carbohydrate, and fiber of seeds are shown in **Table 1**. There were no apparent differences in the composition of nontransgenic and transgenic 7Crp seeds. The content of carbohydrate in 7Crp seeds was slightly higher than that of nontransgenic seeds; however, it is important to note that the difference is within the natural variability observed in conventional rice seeds (*19*). The contents of amino acids, fatty acids, minerals, and vitamins are shown in **Table 2**. There were small differences in the contents of several amino acids, fatty acids, sodium, calcium, and niacin. The differences in amino acids and fatty acids are within the natural distribution in rice seeds (*19*, *20*). Concerning the content of sodium and calcium, assays of glasshouse-grown seeds showed that there were no differences in the levels of calcium (8.7 mg/100 g nontransgenic seed; 8.1 mg/100 g 7Crp seed) and sodium (2.3 mg/100 g nontransgenic seed; 2.1 mg/100 g 7Crp seed). These results suggest that the differences in nutritional contents of field-grown seeds may be affected by field conditions.

Digestibility of the 7Crp Peptide Expressed in Seeds. Protein stability in simulated gastric and intestinal fluids has been proposed as a standard criterion for protein allergenicity assessment (*21*). On the contrary, however, it has been reported that digestibility does not necessarily correlate with allergenicity (*22*, *23*). This conflicting theory suggests that a proteolytic digestion assay may not provide conclusive evidence either way.

Table 2. Comparison of Amino Acids, Fatty Acids, Minerals, and Vitamins in Rice Seeds[a]

	nontransgenic seeds		transgenic 7Crp seeds	
	amino acid			
arginine	0.58	0.58	0.59	0.60
lysine	0.30	0.30	0.32	0.33
histidine	0.20	0.20	0.21	0.21
phenylalanine	0.35	0.35	0.37	0.37
tyrosine	0.27	0.27	0.27	0.27
leucine	0.53	0.53	0.55	0.55
isoleucine	0.27	0.26	0.27	0.27
methionine	0.19	0.19	0.19	0.19
valine	0.39	0.38	0.40	0.40
alanine	0.40	0.40	0.41	0.42
glycine	0.35	0.35	0.36	0.37
proline	0.33	0.32	0.34	0.33
glutamic acid	1.16	1.13	1.18	1.17
serine	0.35	0.35	0.36	0.36
threonine	0.26	0.26	0.27	0.27
aspartic acid	0.65	0.65	0.68	0.69
tryptophan	0.11	0.10	0.11	0.10
cystine	0.18	0.18	0.18	0.18
	fatty acid			
myristic acid	0.024	0.024	0.025	0.025
palmitic acid	0.66	0.66	0.67	0.66
stearic acid	0.076	0.076	0.070	0.070
oleic acid	1.21	1.22	1.11	1.11
linoleic acid	1.10	1.11	1.13	1.12
linolenic acid	0.035	0.035	0.034	0.033
arachidic acid	0.021	0.021	0.018	0.018
eicosanoic acid	0.014	0.014	0.013	0.013
behenic acid	0.011	0.011	0.010	0.010
lignoceric acid	0.026	0.026	0.023	0.023
	mineral and vitamin			
sodium	2.5	1.7	1.7	1.6
iron	1.1	1.2	1.1	1.2
calcium	5.7	6.3	8.2	8.4
vitamin B1	0.50	0.54	0.48	0.51
vitamin B2	0.046	0.044	0.044	0.043
vitamin B6	0.48	0.50	0.50	0.53
vitamin E	2.6	2.5	2.4	2.4
niacin	7.6	7.2	8.3	7.9

[a] Numbers represent the mean (mg/100 g seed) of two independent measurements for nontransgenic and transgenic 7Crp seeds.

However, examination of digestibility might be ultimately useful for the safety assessment process (*13*, *24*). We examined the digestibility of the 7Crp peptide accumulated in seeds with pepsin and pancreatin. In this study, Glu A and the 7Crp peptide were completely digested within 15 min, whereas 13 k Pro was stable after digestion for 120 min with pepsin (**Figure 2A**). In the case of reaction with pancreatin, the 7Crp peptide in seeds was completely digested within 60 min, whereas GluA and 13 k Pro were detectable after digestion for 120 min (**Figure 2B**). These results indicate that the 7Crp peptide is rapidly degraded when compared with GluA and 13 k Pro.

***N*-Glycosylation of the 7Crp Peptide Expressed in Seeds.** Posttranslational modification of the 7Crp peptide by plant-specific glycosylation with β-(1,2)-xylose or α-(1,3)-fucose has the potential to provide new candidates as allergens (*25*, *26*). Even though there are no potential *N*-linked glycosylation sites (N−X−S/T) in the amino acid sequence of the 7Crp peptide, we examined whether seed-accumulated 7Crp peptide was *N*-glycosylated or not. After digestion with *N*-glycosidase F, the mobility of the *N*-glycoproteins Transferrin and Ribonuclease B shifted to a lower apparent molecular mass on SDS-PAGE, indicating release of *N*-glycan chains (**Figure 3A**). The 7Crp peptide did not change mobility with digestion, ruling out *N*-linked glycosylation of the 7Crp peptide (**Figure 3B**).

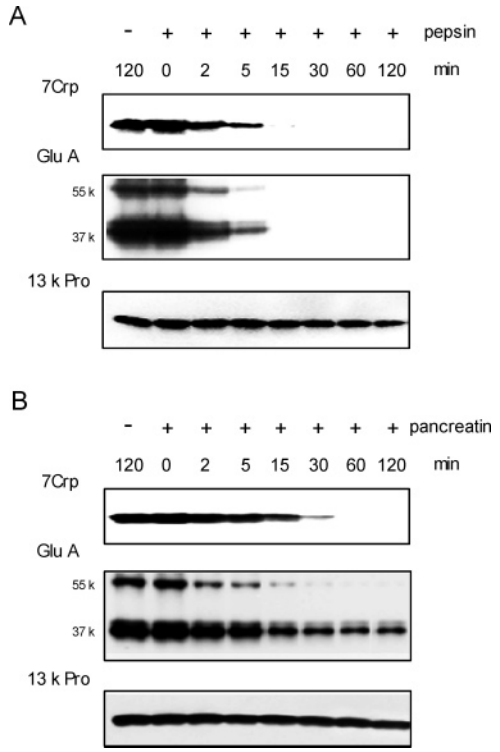

Figure 2. *In vitro* digestion of transgenic rice seeds expressing the 7Crp peptide. A solution of transgenic rice seeds containing 170 ng/μL of 7Crp peptide was mixed with 0.32% (w/v) of pepsin (A) or 1% (w/v) pancreatin (B). These reaction mixtures were incubated for up to 2 h at 37 °C and then examined by Western blot analysis.

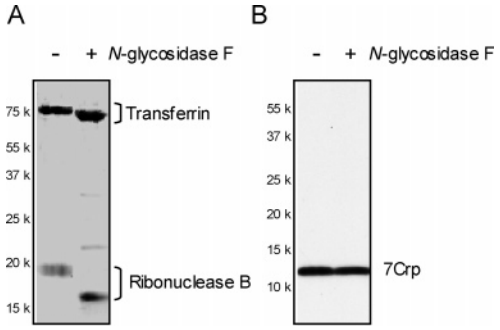

Figure 3. *N*-Glycosylation analysis of the 7Crp peptide accumulated in rice seeds. The *N*-glycosylated proteins Transferrin and Ribonuclease B and total protein of 7Crp seeds were digested with *N*-glycosidase F. Reaction mixtures were then subjected to SDS-PAGE (A) and Western blot analysis (B) to examine the apparent molecular mass for each sample. A shift to a lower molecular mass after digestion by *N*-glycosidase F indicates release of *N*-linked glycan.

These results indicate that no significant changes other than accumulation of the 7Crp peptide in the endosperm tissue have occurred in this transgenic rice and that 7Crp seeds are equivalent to nontransgenic seeds in their nutritional properties, within the variability of plant-to-plant variation. In addition, it may be reasonable to conclude that biochemical examination of 7Crp peptide and 7Crp seeds did not raise any concern about the safety of 7Crp seeds. For further characterization of 7Crp seeds, we are performing clinical, toxicological, and histopathological analyses in animal feeding studies, which are essential to evaluate the food safety of transgenic 7Crp seeds.

ABBREVIATIONS

7Crp, a hybrid peptide comprising seven predominant human T cell epitopes of Japanese cedar pollen allergens; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Glu, glutelin; Pro, prolamin; CBB, Coomassie brilliant blue; Safety, None.

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