

Oral immunotherapy against a pollen allergy using a seed-based peptide vaccine

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Summary

Peptide immunotherapy using dominant T-cell epitopes is safer and more effective than conventional immunotherapy for the treatment of immunoglobulin E (IgE)-mediated allergic diseases. When allergenic T-cell epitope peptides are expressed in the edible part of transgenic plants, successful mucosal immune tolerance to these allergens may be attainable by the consumption of these plants. In this study, we generated transgenic rice seed that accumulated high concentrations (about 60 µg per grain) of polypeptide consisting of seven dominant human T-cell epitopes derived from the Japanese cedar pollen allergens, Cry j 1 and Cry j 2, in the endosperm. Oral administration of these transgenic rice seeds to B10.S mice before or after they were immunized with Cry j 1 holoprotein reduced not only their T-cell proliferative response to Cry j 1, but also their serum IgE levels, proving the efficacy of oral immunotherapy for the treatment of pollinosis.

Keywords: edible vaccine, endosperm expression, immune tolerance, Japanese cedar pollen, pollinosis, T-cell epitope, transgenic rice.

Introduction

More than 30% of the population in developed countries is afflicted with immunoglobulin E (IgE)-mediated type I allergic diseases, such as seasonal and perennial rhinitis, asthma and atopic dermatitis. Although allergen-specific immunotherapy using intact native allergens has been employed successfully to treat these allergic diseases, this type of therapy has been associated with an increased risk of systemic anaphylaxis mediated by the cross-linking of allergen-specific IgE. To avoid side-effects such as this, peptide immunotherapy using dominant T-cell epitopes has been proposed as a treatment alternative. This is based on the observation that the administration of dominant T-cell epitopes in the absence of appropriate co-stimulatory signals induced either anergy, i.e. functional inactivation of T cells, T-cell deletion or active cellular suppression mediated by regulatory T cells, depending on the dose (Hoyne *et al.*, 1995; Van Neerven *et al.*, 1996; Wallner and Geffer, 1996; Weiner, 1997; Haselden *et al.*, 2000). Peptide immunotherapy has been performed by injection (Müller *et al.*, 1988; Briner *et al.*, 1993; Nicodemus *et al.*, 1997) and intranasal or oral administration (Hoyne *et al.*, 1993,

1996; Hirahara *et al.*, 1998; Yoshitomi *et al.*, 2002) in animal models and patients, and has resulted in a reduction in allergen-specific T-cell proliferation and IgE levels, and in changes in the pattern of cytokine release by T cells.

Japanese cedar (*Cryptomeria japonica*) pollinosis is one of the major allergic diseases in Japan, with nearly 23 million Japanese patients suffering from the condition from February to April each year. Two cedar pollen proteins, designated Cry j 1 and Cry j 2, have been characterized as major allergens based on the fact that patients with cedar pollinosis exhibit a high IgE titre (Hashimoto *et al.*, 1995) and T-cell reactivity (Sugiyama *et al.*, 1996) towards these allergens. The complementary DNA (cDNA) clones encoding Cry j 1 (Yasueda *et al.*, 1983; Sone *et al.*, 1994) and Cry j 2 (Komiyama *et al.*, 1994; Namba *et al.*, 1994) have been isolated and their sequences determined.

T-cell epitopes within the primary structure of the Cry j 1 and Cry j 2 allergens have recently been mapped using an *in vitro* peripheral blood mononuclear cell (PBMC) proliferation assay (Sone *et al.*, 1998; Saito *et al.*, 2000) with several of these found to be immunodominant. The sequences recognized by individual patients are highly variable due to

differences in their major histocompatibility complex (MHC) class II haplotypes or other genes encoded by the human leucocyte antigen (HLA) region of the genome, as well as their T-cell receptor. Use of hybrid peptides for immunotherapy, consisting of multiple predominant T-cell epitopes derived from the two major allergens, is a reasonable way to overcome genetic diversity that exists in response to epitopes. Five- and seven-linked epitope peptides derived from Cry j 1 and Cry j 2 were designed and produced in an *Escherichia coli* expression vector (Sone *et al.*, 1998; Hirahara *et al.*, 2001). It is noteworthy that the seven-linked epitope peptide (7Crp) was about 100-fold more effective at inducing T-cell proliferation than the mixture of the individual seven epitope peptides (Hirahara *et al.*, 2001). Furthermore, 92% of 48 volunteers who responded negatively with IgE antibody specific to cedar pollen allergen exhibited a positive T-cell response to these hybrid peptides (Hirahara *et al.*, 2001). Taken together, these results suggested that 7Crp was an ideal tolerogen for the induction of oral immune tolerance that could be used at a relatively low concentration and that would probably be effective in most patients.

Preclinical evaluation showed that oral administration of a major mouse T-cell epitope peptide (Hirahara *et al.*, 1998) or three-linked mouse T-cell epitope peptides (Yoshitomi *et al.*, 2002) induced immunological tolerance against Japanese cedar pollen allergen. These results suggested that, if 7Crp was present in high concentrations in the endosperm of rice seed, the use of this seed system in the form of an edible peptide vaccine might represent an efficacious new immunotherapeutic strategy.

The seed production system has tremendous advantages for the production of T-cell epitope peptide as a peptide vaccine: (i) it can be used as a direct oral delivery system without processing, such as extraction and purification; (ii) artificial T-cell epitope peptide can be highly and stably accumulated in seed; (iii) transgene products accumulated in seed are stable for years at ambient temperature; (iv) there is no need for a specific facility for the production of the transgene; (v) there is no contamination of animal virus and prion (safety); (vi) production can be easily scaled up. These advantages give rise to a very low cost production platform compared with other expression systems using transgenic animals, yeast and *E. coli*, as pointed out in the production of foreign genes, such as biopharmaceuticals, in transgenic crops (Giddings *et al.*, 2000; Hood and Woodard, 2002). In particular, production in rice seeds has many strong advantages, such as high expression levels, large biomass, low production costs and minimal risk of outcrossing, compared with other crops (Stoger *et al.*, 2002). Furthermore, rice is the

staple food in Japan, thus providing a better delivery system than other cereal seeds, such as maize or wheat. Vaccine accumulated in rice seeds can be expected to induce oral immune tolerance by daily diet through the mucosal immune system.

In this study, we generated transgenic rice plants that accumulated high concentrations ($60 \pm 2.5 \mu\text{g}$ per ~20 mg of grain) of 7Crp in the endosperm, which is the edible part of the seed. When these transgenic rice seeds were orally administered to mice before or after immunization against the whole Cry j 1 allergen, T-cell proliferation to the epitope peptide and Cry j 1-specific IgE levels were down-regulated compared with those in animals fed non-transgenic rice seeds. Our data are the first to support the notion that type I allergic diseases, such as pollinosis, may be alleviated by oral peptide vaccination therapy using rice seeds containing T-cell epitope peptides.

Results

Generation of transgenic rice seeds containing 7Crp

We first designed an artificial gene coding for the 96 amino acids of 7Crp using optimized codons preferentially employed for the translation of several rice seed storage protein genes. An improved recursive polymerase chain reaction (PCR) method was used to synthesize this gene. In order to express this 7Crp gene in the endosperm, the rice major seed storage protein glutelin 2.3-kb *GluB-1* promoter was used.

Three different targeting strategies were investigated to optimize the production of 7Crp in the rice endosperm, as described in Figure 1, with the three constructs differing in their coding regions. The glutelin *GluB-1* promoter, with or without its signal peptide (72 bp), was translationally or transcriptionally fused to the 7Crp gene with an endoplasmic reticulum (ER) retention signal (KDEL) at its C-terminus. The other was constructed by fusing the glutelin promoter with its signal peptide to the 7Crp gene without the KDEL signal. After cloning into the binary vector pGTV 35SHPT/GluB3', these constructs, designated as GluB-1pro sig/7Crp + KDEL, GluB-1pro/7Crp + KDEL and GluB-1pro sig/7Crp, respectively, were introduced into the rice genome by *Agrobacterium*-mediated transformation. Independent, stable transgenic rice plants (34–37) were generated for each of the three constructs.

Expression of 7Crp in rice seeds

Expression levels of the 7Crp gene in maturing seeds (15 days after flowering – 15 DAF) were first analysed by Northern

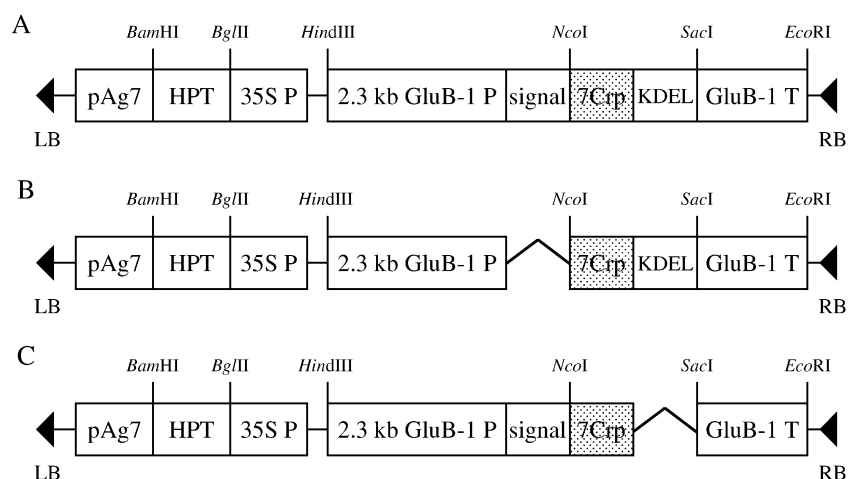


Figure 1 Schematic representation of the constructs used for the expression of 7Crp in transgenic rice seeds. GluB-1 pro sig/7Crp + KDEL (A), GluB-1 pro/7Crp + KDEL (B) and GluB-1 pro sig/7Crp (C) in the pGTV 35S HPT binary vector were used for 7Crp expression in transgenic rice plants. The 7Crp gene was under the control of the 2.3-kb glutelin *GluB-1* promoter. 2.3 kb GluB-1 P, 2.3-kb promoter region from the rice glutelin *GluB-1* gene; signal, signal peptide coding region from the *GluB-1* gene; 7Crp, a hybrid peptide comprising the seven predominant human T-cell epitopes derived from Japanese cedar pollen allergens; KDEL, tetrapeptide endoplasmic reticulum (ER) retention signal; GluB-1 T, 0.6-kb 3'-untranslated region from the *GluB-1* gene; 35S P, cauliflower mosaic virus 35S promoter; HPT, hygromycin phosphotransferase gene; pAg7, agropine synthase polyadenylation signal sequence; LB, left border; RB, right border.

blotting using the 7Crp coding region as probe. Accumulation levels of 7Crp in each mature dry grain were examined by Western blotting analysis using anti-7Crp antibody. When 7Crp was examined for the expression of the GluB-1 pro/7Crp + KDEL construct, all of the 35 independent lines had undetectable protein levels, even though high levels of transcripts were observed (data not shown). This result indicated that the glutelin signal sequence might be required for stable accumulation of 7Crp in rice seed. Thus, it was theorized that entry of the signal peptide into the ER lumen was required for its accumulation, as 7Crp expressed by this construct would be expected to remain in the cytoplasm or to exude outside the cell by bulk flow after translation. When the glutelin *GluB-1* signal sequence was introduced between the 2.3-kb *GluB-1* promoter and the 7Crp coding region (GluB-1 pro sig/7Crp + KDEL), high levels of 7Crp accumulated in many of the transgenic lines, as described in Figure 2A. It should be noted that there was little difference in the messenger RNA (mRNA) levels between transgenic lines from these two vectors, irrespective of the presence or absence of the glutelin signal sequence (data not shown).

As shown in Figure 3, artificial 7Crp was detectable as a visible band on Coomassie brilliant blue-stained sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in the highest expressing lines, a finding that was confirmed by Western blotting using an anti-7Crp antibody. The size of the band, estimated from electrophoresis, was consistent with that calculated from the amino acid sequence

[molecular weight (MW), 11 000 kDa]. Highly variable levels of 7Crp were observed not only amongst seeds from different transgenic lines generated from the same gene construct, but also amongst seeds derived from the independent line (Figure 2). The variation in 7Crp levels between lines may have been due to differences in the copy number and position of the transgene in the rice genome or to differences in the physiological conditions amongst the seeds. Similar results were observed when fungal laccase gene was expressed in transgenic maize plants (Hood *et al.*, 2003). In order to examine the role played by the KDEL ER retention signal in the accumulation of high levels of 7Crp, the KDEL-less construct (GluB-1 pro sig/7Crp) was introduced into rice. As shown in Figure 2B, omission of the KDEL retention signal resulted in a four- to sixfold reduction in 7Crp peptide levels compared with the levels obtained using the KDEL+ construct.

Relatively high accumulation of 7Crp was observed in lines 1, 10, 17 and 34 containing the GluB-1 pro sig/7Crp + KDEL construct, and in lines 17 and 19 containing the GluB-1 pro sig/7Crp construct. Each seed was divided in half, and one half seed was used to determine the expression levels, while the other was germinated to recover homozygous lines through self-pollination for further screening. The homozygous lines 1, 10, 17 and 34 accumulated 41 ± 2.4 , 62 ± 2.5 , 25 ± 1.3 and 34 ± 1.3 μg of 7Crp per grain on average, whereas the amounts in lines 4, 17 and 25, created with the KDEL-less construct, were 7 ± 0.4 , 16 ± 0.6 and 9 ± 0.3 μg per grain. These accumulation levels did not change over five generations

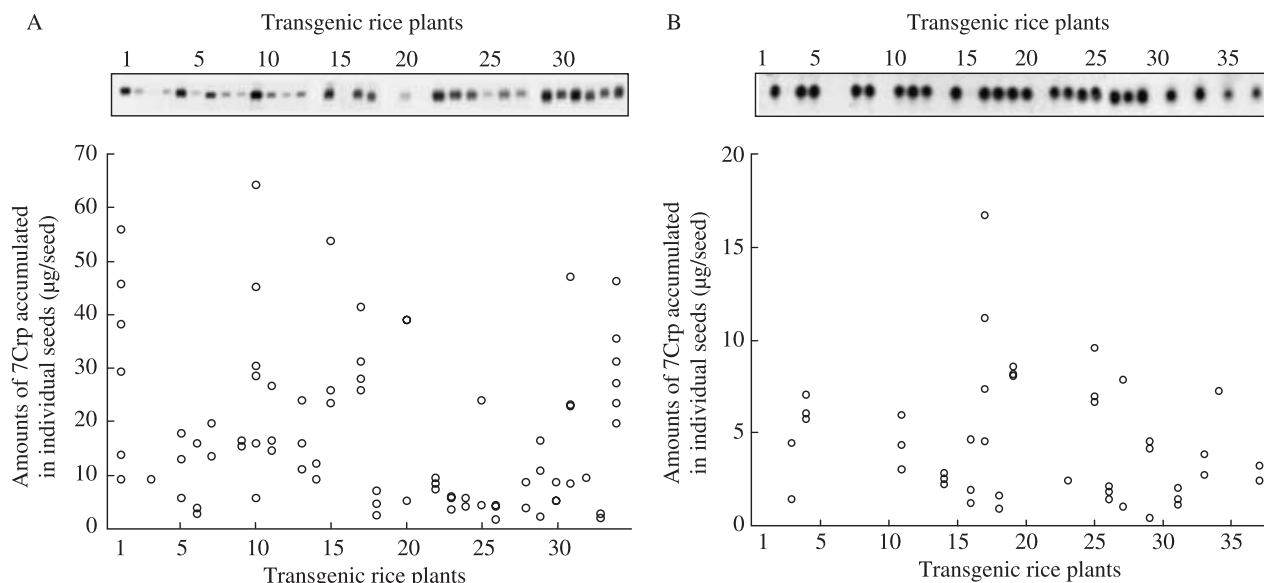


Figure 2 Expression of 7Cp in rice seeds transformed with (A) GluB-1pro sig/7Cp + KDEL and (B) GluB-1pro sig/7Cp. Northern blot analysis was carried out with total RNA extracted from immature seeds of transgenic plants at 15–20 days after flowering (DAF) using the 7Cp coding region as a probe. Amounts of 7Cp in mature seeds were examined by Western blotting analysis for individual transgenic rice lines in which 7Cp transcripts were detected. Each open circle represents the concentration of 7Cp in a seed (µg/seed) for an individual transgenic line.

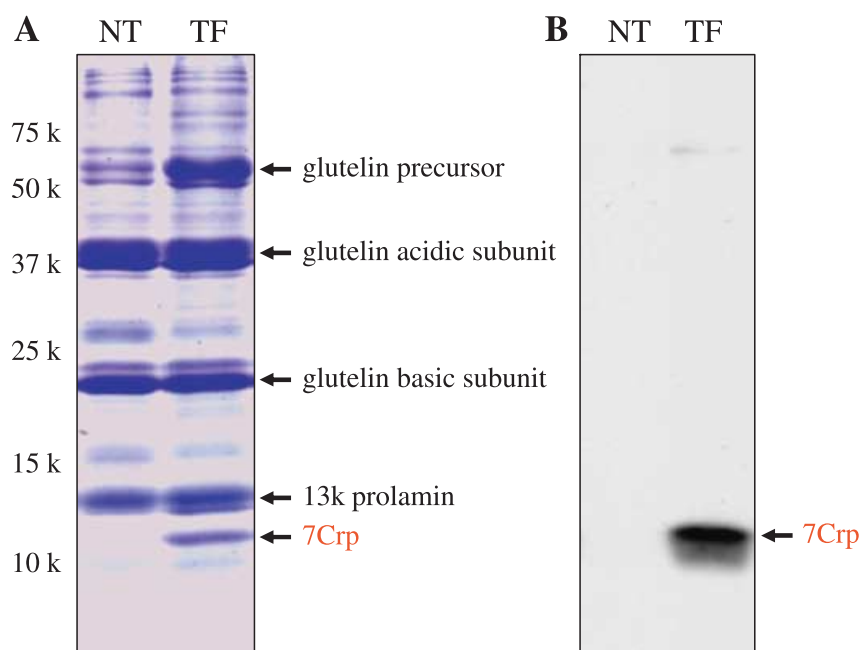


Figure 3 Accumulation of 7Cp in transgenic rice seeds. Total protein extracted from seed of non-transformed (NT) and transformed (TF, GluB-1pro sig/7Cp + KDEL, line 10) rice was subjected to 15% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). (A) Coomassie brilliant blue staining. (B) Immunodetection of 7Cp with anti-7Cp antibody.

from T1 to T5, suggesting that gene silencing did not occur. The amount of 7Cp corresponded to about 4% of the total seed protein in line 10, which showed the highest accumulation of the GluB-1 pro sig/7Cp + KDEL construct, whereas 1.5% of total seed protein accumulated in line 17 containing construct GluB-1pro sig/7Cp.

The gene copy number for GluB-1pro sig/7Cp + KDEL in the four high expression lines was examined by Southern

analysis (data not shown). The 7Cp gene in these transgenic rice lines was estimated to consist of 2–4 copies per haploid genome. The highest accumulation line, i.e. 10, had four copies of the 7Cp gene, whereas two copies of the gene were incorporated in the other lines. The four copies of the 7Cp transgene in line 10 were demonstrated to be located at multiple loci, which were not arranged in tandem. When the genomic DNA of line 10 was digested with rare cutter

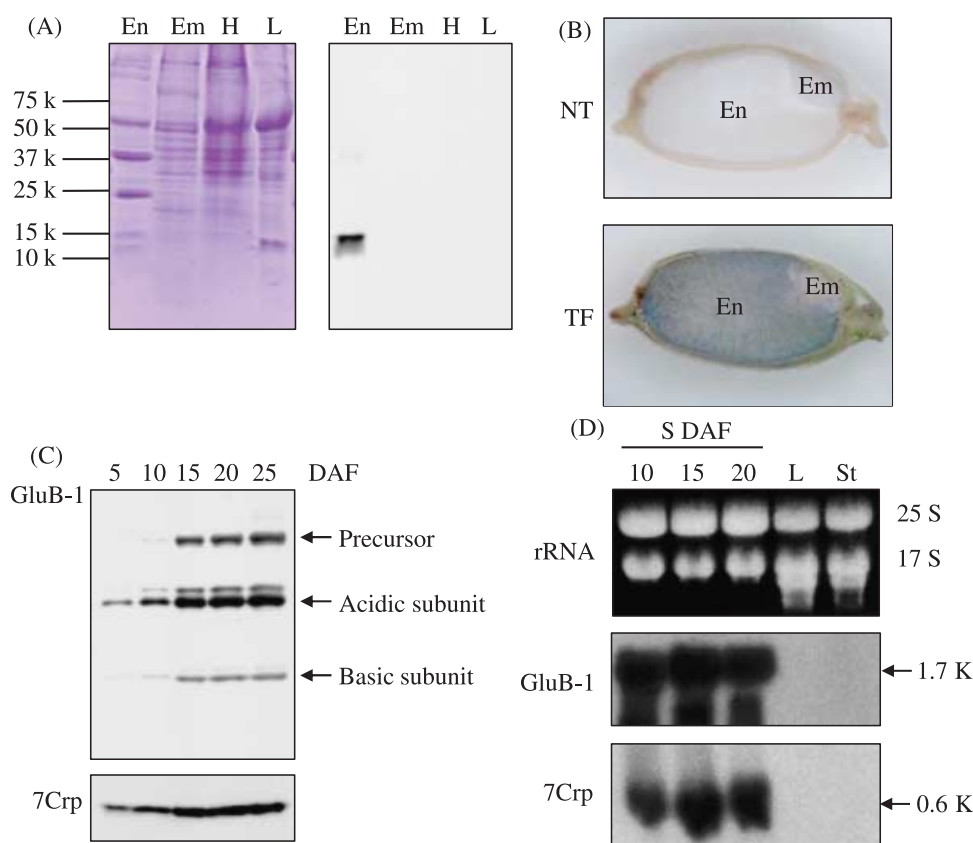


Figure 4 Specific expression of 7Crp in transgenic rice seeds containing GluB-1pro sig/7Crp + KDEL. (A) Analysis of site of accumulation of 7Crp in rice plants. Developing seeds were divided into endosperm, embryo and hull. Total protein extracted from each tissue and from leaves was analysed by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Em, embryo; En, endosperm; H, hull; L, leaf. (B) *In situ* immunolocalization of 7Crp in seeds. Developing rice seeds from non-transformants (NT) and transformants (TF) were cut in half vertically, and analysed by *in situ* hybridization using anti-7Crp antibody. For visualization of signals derived from 7Crp, anti-rabbit IgG antibody labelled with alkaline phosphatase was used as the secondary antibody. Em, embryo; En, endosperm. (C) Western analysis of glutelin (GluB family) and 7Crp accumulation in developing seeds. Total seed proteins extracted at 5, 10, 15, 20 and 25 days after flowering (DAF) were separated by 15% SDS-PAGE, and then immunoblotted with anti-GluB-1 glutelin or anti-7Crp antibody. (D) Accumulation of transcriptional products of glutelin (GluB-1) and 7Crp during seed development. Total RNAs were extracted from seeds (at 10, 15 and 20 DAF), leaves and stems. Coding regions of GluB-1 and 7Crp were used as probes. En, endosperm; L, leaf; S, seed; St, stem.

restriction enzyme *NotI*, hybridizing bands were reduced from four to two bands, suggesting that two individual transgenes were closely linked (data not shown). High accumulation may partially be accounted for by multigene insertion.

Tissue specificity of 7Crp

In order to examine whether 7Crp specifically accumulated in endosperm under the control of the glutelin *GluB-1* promoter, total protein extracted from endosperm, embryo, hull and leaf was subjected to Western blot analysis. As shown in Figure 4A, 7Crp was not detectable in tissues other than the endosperm. This tissue specificity was further confirmed by *in situ* staining of transgenic seeds, as shown in Figure 4B. Immunostaining of 7Crp was only observed in the endosperm of transgenic rice seeds, and not in any other tissues.

Levels of 7Crp were examined during seed maturation from 5 to 25 DAF, and were found to gradually increase from 5 DAF, reaching a plateau level at 15 DAF (Figure 4C).

This spatial and temporal expression pattern of the 7Crp gene was confirmed by Northern analysis. As shown in Figure 4D, the 7Crp gene was found to be specifically expressed in maturing seed, but not in the leaf or stem. The mRNA levels of 7Crp reached their peak at 15 DAF, after which they declined as the seed matured. These expression patterns were similar to those seen for glutelin genes that were used as controls because the promoter was derived from them. Taken together, these results indicated that the expression of the 7Crp gene and the accumulation of 7Crp protein were under the strict control of the glutelin *GluB-1* promoter.

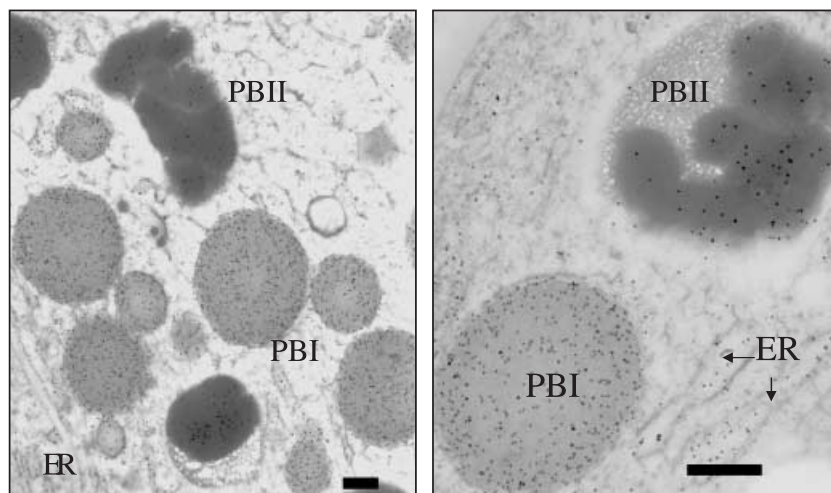


Figure 5 Immunolocalization of 7Crp in rice endosperm cells. The 15-nm gold particles reveal that 7Crp is localized in two types of protein body (PB) in transgenic rice seed (line 10) in which the GluB-1pro sig/7Crp + KDEL construct was introduced. ER, endoplasmic reticulum; PBI, protein body I; PBII, protein body II. Bar = 0.5 μ m.

Intracellular localization of 7Crp in endosperm cells

The subcellular localization of 7Crp in the endosperm cells was examined by immunocytochemical electron microscopy using a high-pressure freezing and freeze-substitution fixation method and antibodies against 7Crp. Immunogold labelling showed that 7Crp accumulated not only in the prolamin-containing, round, protein body I (PBI) with a diameter of 1–2 μ m, but also in the dark, irregularly shaped, protein body II (PBII) that had a diameter of 2–3 μ m and contained glutelin (Figure 5). Larger amounts of immunogold were observed in PBI. It should be noted that the amounts of 7Crp located in the ER lumen were very low compared with those observed in the protein bodies in spite of the presence of the KDEL ER retention signal (Figure 5B). When intracellular localization of the KDEL-less 7Crp was observed, the removal of the KDEL retention signal did not change the deposition pattern of 7Crp, even in the presence of the KDEL signal, although it did reduce its accumulation to less than about 25% (data not shown).

Tolerogenic potential of rice seeds containing 7Crp

We first investigated whether 7Crp synthesized in rice endosperm had the potential to induce specific T-cell activation. When crude proteins, extracted in 0.125 M NaHCO_3 , from seeds of the highest 7Crp-expressing line (GluB-1pro sig/7Crp + KDEL, 10) and from non-transgenic rice were examined for their ability to activate a specific SCR-1 T-cell line recognizing the Cry j 1 211–225 epitope in the presence of antigen-presenting cells (APCs), it was found that the crude extract including 7Crp induced proliferation to the

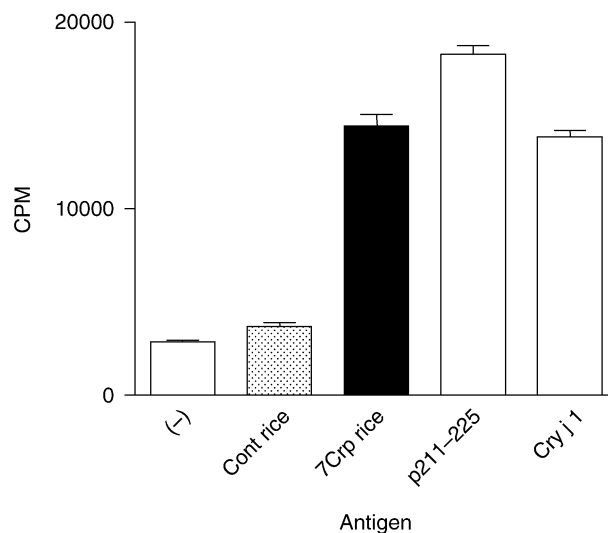
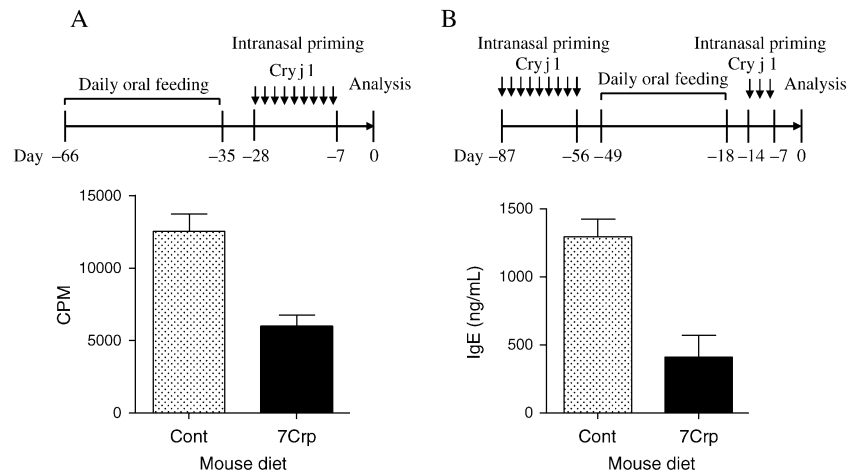


Figure 6 Antigenicity of 7Crp accumulated in transgenic rice seed. Protein extracts from transgenic (GluB-1pro sig/7Crp + KDEL, line 10) and non-transgenic wild-type (Cont rice) rice seeds were analysed for proliferative response to the Cry j 1 p211–230-specific T-cell line, SCR1. (–), no addition of protein extract.

same, or slightly lesser, extent as the whole Cry j 1 antigen and the Cry j 1 211–225 epitope peptide (Figure 6). This was in remarkable contrast with the low levels of T-cell proliferation obtained by the seed protein extracts of non-transgenic rice (control) or in the absence of seed extract (–). These results suggested that transgenic rice seeds that accumulated 7Crp were effective at inducing a T-cell proliferative response, and that even the embedded seven-linked epitope peptide could function as the active epitope. They further suggested that the individual epitopes in 7Crp (composed of seven major human T-cell epitopes) were correctly processed and

Figure 7 Tolerogenicity of transgenic rice seeds containing 7Crp. Mice were immunized by oral administration of transgenic rice seeds containing 7Crp. Oral feeding and intranasal challenge with Cry j 1 antigen are indicated. (A) Proliferative response of spleen cells from mice fed transgenic or non-transgenic wild-type rice seeds before being primed intranasally with Cry j 1. Data are expressed as the mean $\pm$ SD. (B) Serum IgE levels of mice fed transgenic or non-transgenic wild-type rice seeds after being primed intranasally with Cry j 1. Data are expressed as the mean $\pm$ SD.



presented by APCs in a similar manner to that seen for separate individual T-cell epitopes.

We went on to investigate whether the oral administration of rice seeds containing 7Crp could induce immunological T-cell tolerance against the whole Cry j 1 antigen. We first examined the protective effects of oral feeding on the immunological response to Cry j 1. Fine powder (200 mg) of transgenic rice seeds containing 560 μ g 7Crp and of control, non-transgenic rice seeds was orally administered to B10.S mice once a day for 32 days (Figure 7A). Mice were then intranasally immunized with the whole Cry j 1 allergen adsorbed to alum to elicit IgE-mediated allergic responses. T-cell proliferative responses against the whole Cry j 1 were reduced to about 50% of the control (non-transgenic rice seeds), as shown in Figure 7A. IgE levels, on the other hand, were below the level of detection. We then examined the potential therapeutic effect of oral feeding of rice seeds containing 560 μ g 7Crp on the immune response to Cry j 1. When B10.S mice were immunized intranasally with the whole Cry j 1 allergen before and after they were fed seeds containing 7Crp, their serum IgE levels fell to about 30% of those found in control mice fed non-transgenic rice seeds (Figure 7B), suggesting that the consumption of rice seeds containing 7Crp was an effective way to induce mucosal immunological tolerance to this antigen.

Discussion

The initiation and perpetuation of allergic reactions are strongly influenced by allergen-specific T-cell responsiveness. The regulation of such T-cell responses has been suggested as a potentially effective therapeutic approach for the treatment of allergies. Peptide immunotherapy using dominant T-cell epitopes has been shown to be a plausible

approach for the treatment of type I allergic disease (Hoyne *et al.*, 1995; Wallner and Geffer, 1996).

Therapeutic peptides have recently been developed for immunotherapy against Japanese cedar pollinosis (Sone *et al.*, 1998; Hirahara *et al.*, 2001). Five- and seven-linked dominant human T-cell epitopes synthesized in *E. coli* were shown to be recognized by T-cell clones specific for each peptide, and to retain their potential to induce immune cell proliferation and tolerance. It has recently been demonstrated that the oral administration of one predominant epitope or a three-linked T-cell epitope peptide induces immune tolerance against individual specific T cells in a mouse model (Hirahara *et al.*, 1998; Yoshitomi *et al.*, 2002). This evidence suggests that the oral administration of seven-linked dominant human T-cell epitopes (7Crp), using rice seeds as the vehicle, has the potential to induce immune tolerance to Japanese cedar pollen allergic disease, the underlying assumption being that 7Crp levels could be increased in rice seeds to those necessary to induce tolerance.

To date, many biopharmaceutical recombinant proteins, including vaccines and antibodies, have been expressed in plants (Fischer and Emans, 2000; Daniell *et al.*, 2001; Walmsley and Arntzen, 2003). However, their expression levels are often less than 0.01% of total soluble proteins, although some very high expression levels are being achieved in nuclear transgenics, especially in seeds. Higher expression levels are required for these proteins to become of practical and commercial use.

Several approaches have been used to increase the levels of artificial 7Crp in the endosperm of rice seeds. In our study, an endosperm-specific glutelin *GluB-1* promoter, with high activity, was used to express the synthetic 7Crp gene, as it has recently been shown that the larger size (2.3 vs. 1.3 kb) of the *GluB-1* glutelin promoter (Goto *et al.*, 1999; Katsube *et al.*,

1999) leads to about a 10-fold increase in β -glucuronidase (GUS) activity in the seeds of stable transgenic rice (Qu and Takaiwa, 2004). When we tested the effectiveness of expression driven by the 1.3-kb *GluB-1* promoter, we found that it resulted in less than 16 μ g of 7Crp peptide per grain at its highest (data not shown). We found that a secretory signal peptide primarily leading to translocation of the peptide into the secretory pathway was essential for the accumulation of 7Crp. Absence of the signal peptide resulted in instability of 7Crp in the cytoplasm, irrespective of the fact that nearly the same levels of RNA were expressed as in the KDEL+ construct. Similar results have been reported for the expression of α -lactalbumin (Yang *et al.*, 2002) and Cry j 1 genes (Okada *et al.*, 2003). The addition of the ER retention signal, KDEL, to the C-terminus of 7Crp greatly increased protein levels, a finding that was consistent with reports using other genes (Wandelt *et al.*, 1992; Schouten *et al.*, 1996). Specifically, we found a greater than fourfold enhancement when KDEL was used. Synthetic 7Crp gene codons, optimized for expression in rice seeds, were used in our study, as it has been reported that such an approach results in increased protein expression (Koziel *et al.*, 1993; Hood *et al.*, 1997). Our data suggest that the combination of the use of a strong promoter, secretory and ER retention signals and codon optimization of the 7Crp gene contributed to the high level of accumulation of 7Crp in our rice seeds, even though the peptide used was artificially made. To our knowledge, the expression level observed here (about 4% of total seed protein) is the highest obtained for edible vaccines created by nuclear transformation (Fischer and Emans, 2000; Daniell *et al.*, 2001).

It has been demonstrated that 7Crp mainly accumulates in protein bodies, PBI and PBII, in the endosperm of seed, with relatively higher amounts being observed in PBI. A similar observation was reported when a recombinant single-chain antibody containing an N-terminal signal peptide derived from the murine immunoglobulin heavy-chain cDNA and an ER retention signal KDEL were expressed in transgenic rice seed endosperm (Torres *et al.*, 2001). It was suggested that the presence of the glutelin signal peptide containing the entire 5' untranslated region, the 3' untranslated region responsible for directing mRNA to cisternal ER and leading to PBII targeting (Hamada *et al.*, 2003) and the KDEL signal may be involved in the targeting to these two protein bodies, although the underlying molecular mechanism of this trafficking has not been clearly defined. Such targeting into protein bodies may also contribute to the high accumulation of 7Crp in the endosperm.

Immunodominant epitopes of Japanese cedar pollen allergens Cry j 1 and Cry j 2 have been identified, and the

feasibility of using these epitopes for peptide-based immunotherapy in humans has been proposed (Hirahara *et al.*, 2001). The artificial polypeptides designated 7Crp, containing seven major human T-cell epitopes derived from Cry j 1 and Cry j 2, showed higher induction of T-cell proliferation than a mixture of the individual epitopes, suggesting that they had a higher potential efficacy for regulating cedar pollen allergy. It is generally accepted that the ability of an epitope peptide to induce the proliferation of T cells correlates with its ability to generate immune tolerance.

A mouse model designed to evaluate the effectiveness of 7Crp as a peptide vaccine against Japanese cedar pollen allergy has not yet been developed, because, although the seven dominant T-cell epitopes that it contains are known to be recognized by human specific T cells, they are not recognized by BALB/c mice T cells, for which Cry j 1 P1-277-290 and Cry j 2 P2-246-259 are the dominant T-cell epitopes (Yoshitomi *et al.*, 2002). Therefore, a human feeding trial will be required in order to evaluate whether rice seed containing human 7Crp can be effective as a tolerogen. However, it has recently been reported that a dominant T-cell epitope of Cry j 1 P1-211-225 in B10.S mice is identical to a major epitope in 7Crp (Ohno *et al.*, 2000). Thus, we tentatively preclinically evaluated the tolerogenic potential of rice that accumulated 7Crp in B10.S mice.

When 7Crp synthesized in rice seeds was subjected to a T-cell proliferation assay, it induced a T-cell proliferative response to Cry j 1 P1-211-225 that was similar to that seen with whole Cry j 1. This result suggested that 7Crp produced in rice seeds functioned in the same manner as the synthesized peptide and that the individual T-cell epitopes in 7Crp had the potential to activate specific T cells *in vitro*, even if the epitope was linked as a peptide.

Oral administration of rice seeds containing 7Crp to B10.S mice down-regulated not only the T-cell proliferative response to Cry j 1, but also serum IgE levels in mice sensitized with Cry j 1. It is notable that in addition to T-cell effects, our immunotherapeutic regimen resulted in a reduction in IgE production. These results showed that immune tolerance to the Cry j 1 allergen was clearly induced by feeding mice transgenic rice seeds containing 7Crp. Furthermore, as each T-cell epitope in 7Crp has the potential to induce specific T-cell tolerance in humans, it is likely that immune tolerance against the Cry j 1 and Cry j 2 allergens would occur by oral feeding of the above transgenic rice seeds containing 7Crp and, as such, this approach could be used to treat Japanese cedar pollen allergic disease.

It has been reported that feeding mice 40–200 μ g of an epitope peptide four times during a period of 2 weeks (total

amount, 160–800 µg) induces immune tolerance (Hirahara *et al.*, 1998). Tolerogenicity was shown to be more effective when linked epitope peptides were used rather than one epitope (Yoshitomi *et al.*, 2002). It can be calculated that the effective dose of 7Crp required for the induction of oral immune tolerance to Japanese cedar pollen allergy in humans will be 80–400 mg (total amount, 320–1600 mg), based on the ratio of the body weight of the mouse (30 g) to that of a human (60 kg). This translates to an amount per day that would require the consumption of about 30–160 g of high-expressing rice seed (about 50 µg per 20 mg of grain). Most Japanese citizens eat about 100–150 g of rice as a staple food every day. Considering that the linked epitope peptide is more effective than a single epitope peptide, it is possible that even smaller amounts of rice would be required. In addition, long-term treatment with small amounts of transgenic rice containing 7Crp may also be effective. It was important to examine whether the 7Crp in rice seed was stable after boiling, as rice seeds are usually eaten in the form of steamed rice. When rice seeds containing 7Crp were boiled in water at 100 °C for 20 min and then assayed by Western blotting analysis, it was observed that 7Crp was resistant to heating and present as an intact form after boiling (data not shown). Furthermore, the T-cell proliferative response was little affected by boiling (data not shown). These results indicate that oral administration of 7Crp is possible in the form of steamed rice. Taken together, it can be concluded that steamed rice containing epitope peptide can be utilized as an oral delivery system.

Taken together, our data suggest that oral administration of the transgenic rice seeds described in this paper may be an effective allergen-specific immunotherapeutic approach for the prevention of human cedar pollen allergy. Furthermore, our work supports the generalized use of rice seeds as a vehicle for the production and delivery of peptide vaccines, which would open up new markets for agrochemical and pharmaceutical industries.

Experimental procedures

Plasmid construction and generation of transgenic rice plants

A 2.3-kb promoter sequence of rice glutelin *GluB-1* was amplified by PCR from a rice (*Oryza sativa* L. cv. Mangetsumochi) genomic λ9 clone (Takaiwa *et al.*, 1991) with the primers ATTCTAGACAGATTCTTGCTACCAAC (2.3K *GluB-1* XbaF) and AACCATGGCTATTTGTACTTGCTTATGGAA (*GluB-1* NcoR). Another primer set containing the forward primer,

2.3K *GluB-1* XbaF, and the reverse primer, AACCATGGGCTGGCCATAGAACCGTGGCATAATA (*GluB-1* SigR), was used to amplify the 2.3-kb promoter and a coding sequence for the signal peptide of *GluB-1*. These PCR products were subcloned at an EcoRV T-cloning site of pT7blue (Novagen) to form pGluB and pGluBsig, respectively.

To generate a DNA fragment encoding the 7Crp peptide, three pairs of cDNAs (F1-up/F1-low, F2-up/F2-low, F3-up/F3-low) coding for parts of 7Crp were synthesized according to codon usage preferred by rice storage protein genes. For the linkage of these DNAs, a subcloning vector was generated by inserting a synthetic linker, Bcl-up/Bcl-low, at the *XbaI/HindIII* sites of pUC18, forming m-pUC18. The F1 DNA fragment was then cloned into m-pUC18 at the *EcoRI/XbaI* sites, the F2 fragment was inserted at the *NheI/BclI* sites and the F3 fragment was cloned at the *BclI/HindIII* sites to produce pUCF1-3. Finally, the complete DNA sequence for the 7Crp peptide was amplified by PCR using pUCF1-3 as a template with the primer set, 7Crp-F and 7CrpSacR. Another primer set, 7Crp-F and 7Crp-2R, was used to add a sequence code for KDEL amino acid residues at the 3' end of the 7Crp gene.

To fuse the 2.3-kb *GluB-1* promoter sequence and the 7Crp gene, the PCR products coding for 7Crp ± KDEL were inserted into pGluB and pGluBsig using the *NcoI/SacI* sites to generate three plasmids, pGluBpro/7CrpKDEL, pGluBpro sig/7Crp and pGluBpro sig/7CrpKDEL. These plasmids were digested with *HindIII* and *SacI*, after which the *HindIII/SacI* fragments were inserted into the pGPTV-HPT-*GluB* 3' binary vector, containing a 0.6-kb 3' non-coding region of the *GluB-1* gene, to produce pGPTV-HPT *GluBpro*/7CrpKDEL, pGPTV-HPT *GluBpro* sig/7Crp and pGPTV-HPT *GluBpro* sig/7CrpKDEL. These transformation plasmids were transferred into the rice genome (*Oryza sativa* cv. Kita-ake) by *Agrobacterium tumefaciens*-mediated transformation (Goto *et al.*, 1999) and transgenic rice plants were generated by hygromycin selection.

Northern and Southern blot analyses

Total RNA was extracted as described previously (Takaiwa *et al.*, 1987) from frozen rice seeds, leaves or stems, after which it was precipitated with 2 M LiCl. Ten micrograms of RNA were subjected to electrophoresis on 1.2% (w/v) formaldehyde-agarose gels. Rice genomic DNA was prepared from leaves using the cetyltrimethylammonium bromide (CTAB) extraction method (Murray and Thompson, 1980). Ten micrograms of DNA were digested with *HindIII* or *SacI*, and then subjected to electrophoresis on 0.8% (w/v) agarose

gels. The gels were blotted onto Hybond N+ membranes (Amersham), and a double-stranded DNA for the full length of the 7Crp coding region, labelled with [α - ^{32} P]dCTP using the Megaprime DNA labelling system (Amersham Biosciences), was used as the probe.

Antibody preparation

The coding region of 7Crp was amplified by PCR with the primer set, 7Crp-FCATGCCATGGGCATCATCGCAGCTTAC-CAAAATCCAGC and 7Crp-R-His CCGCAAGCTTCAACTCGTC-CTTGCGTCCCATGAGAGTGAAGC, which contains restriction sites *Nco*I and *Hind*III, respectively. The PCR product was inserted into the pET23d expression vector (Novagen). The manufacturer's protocol was followed to express the 7Crp peptide tagged with 6 $\times$ His residues in *E. coli* strain BL21(DE3) (Novagen), which was purified by affinity chromatography using an Ni^{2+} -nitrilotriacetate (Ni-NTA) agarose column (Qiagen). The purified protein was used to raise antibodies in a rabbit.

SDS-PAGE and immunoblotting assay

Rice seeds and leaves were ground to fine powder using a Multi-beads Shocker (Yasui Kikai, Osaka, Japan), and proteins from each tissue were extracted in buffer containing 4% (w/v) SDS, 8 M urea, 5% (v/v) β -mercaptoethanol, 50 mM Tris-HCl (pH 6.8) and 20% (w/v) glycerol, with vigorous shaking at room temperature for 15 min, as described previously (Tada *et al.*, 2003). After centrifugation at 5000 *g* for 2 min at 25 °C, the samples recovered in the supernatant fraction were separated by SDS-PAGE and electrophoretically transferred to poly(vinylidene difluoride) (PVDF) (Millipore) membranes for Western blot analysis. The membranes were probed with anti-7Crp antibody (1 : 2000 dilution), after which they were incubated with anti-rabbit IgG antibody labelled with alkaline phosphatase (1 : 3000) or horseradish peroxidase (1 : 3000) (Promega) in order to visualize the signals.

The amount of 7Crp peptide in protein extracts from a seed of an independent transgenic plant was estimated on the basis of the intensity of signals detected by Western blot analysis, using purified 7Crp-6 $\times$ His fusion protein as the calibration control.

Tissue-specific expression of 7Crp was examined using the *in situ* hybridization method (Qu *et al.*, 2003). Developing rice seeds that were cut in half vertically using a razor blade were probed with anti-7Crp (1 : 2000 dilution) antibody, followed by anti-rabbit IgG antibody labelled with alkaline phosphatase (1 : 3000 dilution).

Immunogold electron microscopic observation

The endosperm, dissected from developing immature seeds of transgenic rice plants, was sandwiched between carriers, set into a holder and then frozen with a high-pressure freezing machine (HPM010S, BAL-TEC, Balzers, FL, USA). For freeze substitution, samples were kept for 2 days at -80 °C in acetone, after which their temperature was gradually increased to -20 °C. The samples were placed in dimethylformamide at -20 °C and embedded in LR-White resin (London Resin, Berkshire, UK) that was polymerized in an ultraviolet polymerizer (TUV-200; Dosaka EM, Kyoto, Japan) at -20 °C for 24 h. Ultrathin sections were prepared using an ultramicrotome and mounted on nickel grids. The sections were treated with blocking solution, i.e. phosphate-buffered saline (PBS) containing 1% (w/v) bovine serum albumin and 0.1% (w/v) sodium azide, for 30 min at room temperature. They were then incubated with primary antibodies in blocking solution at 4 °C overnight. Sections were washed and then incubated with secondary antibodies (goat anti-rabbit IgG 15 nm gold conjugate, Biocell, Cardiff, UK) in blocking solution at room temperature for 30 min. The sections were then washed again and were stained with 2% (w/v) uranyl acetate and lead citrate.

Mice

Male B10.S mice were purchased from Sankyo Laboratories (Tokyo, Japan) and were housed in our facilities under conventional conditions. They were used for experiments at the age of 6–10 weeks. The care and handling of the mice followed the Animal Experimentation Guidelines of Jikei University School of Medicine.

Antigens

Cry j 1, a major allergen of Japanese cedar pollen, was purchased from Hayashibara Biochemical Laboratories (Okayama, Japan). Synthesized peptide (p211–225) of Cry j 1 was a kind gift from Sankyo Co., Ltd. Fine powder of transgenic or non-transgenic rice seeds was made with a mixer (Millser IFM-700G, Iwatani Co., Ltd.).

Mixed feeds

Mixed feeds were hardened by adding water to a cylinder of 1.2 cm in diameter and 3.0 cm in length containing a mixed powder of pellet foods and rice seeds at a rate of 19 : 1, followed by baking in a microwave oven.

T-cell proliferation assay

Seed protein was extracted from fine powder of transgenic or non-transgenic rice seeds. Each powder was suspended to 10% (w/v) in 0.125 M NaHCO₃. The mixtures were incubated at room temperature for 2 h, centrifuged at 20 000 *g* for 10 min and the supernatants were evaluated for the induction of T-cell activation using Cry j 1 p211–230-specific T-cell line (SCR1) established from the lymph node cell (LNC) of B10.S mice (Ohno *et al.*, 2000). SCR1 cells (10⁴/well) were cultured in 96-well flat-bottomed plates in the presence of 6 × 10⁵ APCs without antigen or with 5 µL of seed protein extracts, Cry j 1 epitope peptide p211–225 (1.0 µg/mL) or Cry j 1 (2.5 µg/mL) for 48 h at 37 °C, and were pulsed for the last 16 h with 0.5 µCi of [³H]thymidine. The cells were harvested with a Labo harvester. The incorporated radioactivity was measured using a liquid scintillation counter. The results are shown as the mean counts per minute (c.p.m.) with standard deviation. To examine the tolerogenic function of transgenic rice seeds containing 7Crp after oral feeding, spleen cells were isolated from mice 7 days after the last immunization. A T-cell proliferation assay was carried out in 96-well flat-bottomed plates at a concentration of 8 × 10⁵ cells per well in a similar manner to that described previously (Ohno *et al.*, 2000).

Induction of tolerance

In a protective approach, four mice in the tolerance group and four mice in the control group were fed freely on mixed feeds containing transgenic (from line 10) or non-transgenic (*Oryza sativa* L. cv. Kita-ake) rice seeds, respectively, for 32 days. The amount of 7Crp peptide consumed by the mice was calculated at the end of the feeding period from the amount of mixed feed eaten by the mice. In the tolerance group, the mice consumed 560 µg of 7Crp peptide per day for this period. Mice were then immunized intranasally with 1 µg of Cry j 1 adsorbed onto 10 µg of aluminium hydroxide (alum), a regimen which stimulates the T-helper-2 (Th2) response, in 10 µL of PBS every other day for a total of nine immunizations. Seven days after the last immunization, the mice were sacrificed and their immune responsiveness to these allergens was examined.

In a therapeutic approach, each mouse was first immunized intranasally with 1 µg of Cry j 1 adsorbed onto 10 µg of alum in 10 µL of PBS every other day for a total of nine immunizations, after which they were fed mixed feeds for 31 days. After their last feeding, mice were re-immunized intranasally with 1 µg of Cry j 1 adsorbed onto 10 µg of alum in 10 µL of PBS every other day for a total of three immunizations. Seven

days after their final immunization, the mice were sacrificed and their immune responsiveness to these allergens was examined. The tolerance group in the therapeutic approach consumed 516 µg of 7Crp peptide per day for this period.

Serum IgE levels

Seven days after the last immunization, mice were sacrificed and their serum IgE levels were analysed using enzyme-linked immunosorbent assay (ELISA; Shibayagi Inc., Gunma, Japan). Briefly, an ELISA plate coated with anti-mouse IgE-captured monoclonal antibody (mAb) was blocked with bovine serum albumin (BSA), and diluted standards and samples were applied for 2 h. After washing, biotinylated anti-mouse IgE was added to each well with further incubation for 1 h at room temperature. The plate was washed and streptavidin–horseradish peroxidase was added for 1 h. After washing the plate, MTB (3,5',5,5'-tetramethylbenzidine) substrate buffer was added and incubated for 20 min. The optical density for each well was measured with a microplate reader set to 450 nm.

Statistics

Statistical significance was determined by Student's *t*-test and analysis of variance.

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