

Accumulation of Japanese cedar pollen allergen, Cry j 1, in the protein body I of transgenic rice seeds using the promoter and signal sequence of glutelin GluB-1 gene

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Abstract

Recombinant Cry j 1, a Japanese cedar pollen allergen, was produced in rice seeds for potential use for oral immunotherapy. Cry j 1 cDNA was divided into two parts, an N-terminal half and a C-terminal half, and each was fused downstream to glutelin GluB-1 gene containing sequences of the promoter, 5' untranslated region and signal peptide. A gene for green fluorescent protein was also fused to the 3' end of the Cry j 1 fragment. Recombinant Cry j 1 of up to 16.6 µg per mg total protein of the seeds was expressed in transgenic rice seeds. Although the recombinant Cry j 1 was expected to be accumulated in protein body II because of the employment of glutelin signal peptide, it was demonstrated to be accumulated exclusively in protein body I. The recombinant Cry j 1 was not shown to react with IgE of allergic patients, indicating the reduction of the risk of anaphylactic reaction. These results demonstrate that the transgenic rice seeds with the recombinant Cry j 1 would be useful for the study of oral immunotherapy.

Abbreviations: 5'UTR – 5' untranslated region, GFP – green fluorescent protein, Cry j 1ΔSP – Cry j 1 without signal peptide, GluB-1 – Glutelin B-1 gene, NhCry j 1 – the N-terminal half of Cry j 1, ChCry j 1 – the C-terminal half of Cry j 1, PB-I – protein body I, PB-II – protein body II

Introduction

Transgenic plants have been used as bioreactors for the production of carbohydrates, fatty acids, industrial enzymes, biodegradable plastics, and pharmaceuticals, which has been regarded as 'molecular farming' (Goddijn and Pen 1995; Mason and Arntzen 1995). The major advantages of a plant expression system are its ability to scale up biomass with low maintenance and cost, and effective post-translational modifications. Furthermore, plants or plant products that

are edible provide an additional unique opportunity for oral delivery of the recombinant protein as pharmaceuticals. Recent examples include the production of monoclonal antibodies, vaccines and human growth hormone (Arakawa et al. 1998; Zeitlin et al. 1998; McCormick et al. 1999; Leite et al. 2000). Accumulation of provitamin A in rice seeds, which is called 'golden rice,' and improvement of the iron content of rice have also been reported (Goto et al. 1999; Ye et al. 2000). Such kinds of transgenic rice are

considered to supply essential nutrients to those who are suffering from nutritional deficiencies.

Up to 20% of the human population in developed countries is suffering from IgE-mediated atopic diseases such as allergic rhinitis, conjunctivitis and bronchial asthma. Many cDNA clones encoding pollen allergens have been isolated from trees, weeds and grasses and other plants, and recombinant proteins have been used as tools for diagnosis and for determination of epitopes and immunotherapy (Valenta and Kraft 1995). Japanese cedar pollinosis is one of the most common seasonal allergic diseases in Japan. The major allergens of Japanese cedar pollen are Cry j 1 and Cry j 2, and these cDNAs have been isolated and characterized (Komiyama et al. 1994; Sone et al. 1994). The feasibility of using Cry j 1 and Cry j 2 peptides for immunotherapy has been reported, and oral administration of Cry j 2 peptide has shown potential for peptide-based oral immunotherapy in order to treat allergic immune responses (Hirahara et al. 1998; Sone et al. 1998). Oral immunotherapy is based on the phenomenon of oral tolerance and considered to be a new approach for therapeutic administration (Weiner 1997). One of the promising sources of oral immunotherapy is the edible organs of plants in which recombinant polypeptides of pollen allergens are produced. There have been no reports of the expression of pollen allergen in edible organs of plants, although Krebitz et al. (2000) have demonstrated the expression of birch pollen allergen Bet v 1 in tobacco.

In this report, we present the expression of Cry j 1 polypeptides in rice seeds. We employed the promoter and a signal sequence of glutelin, a part of Cry j 1 and GFP. Accumulation and localization of recombinant Cry j 1 in the protein body of rice endosperm are investigated. IgE-binding activity of the recombinant Cry j 1 is also examined. The possibility of this model system for oral immunotherapy is discussed.

Material and methods

Vector constructions

Cry j 1 contains a signal peptide of 21 amino acid residues (Figure 1A). In order to remove the sequence for signal peptide and to attach the restriction enzyme sites for vector construction, Cry j 1 cDNA was PCR-amplified by using designed primers; Cry j 1A primer (TCTGGATCCATGGATAATCCCATAGACAGC, the *Bam*HI site is underlined and the inserted initia-

tion codon is shown in italics) and Cry j 1S primer (TTGAGCTCTCAACAACGTTTAGAGAGAG, *Sac*I site is underlined). The PCR fragment was cloned into a pCRII vector (Invitrogen, Tokyo, Japan). The cloned Cry j 1 cDNA fragment was confirmed by sequencing. The 1.3 kb glutelin GluB-1 promoter fragment in pUC18 (Takaiwa et al. 1996) was digested by *Hind*III and *Bam*HI and ligated into the same site of the pBI101H (Yokoi et al. 1997). A *Bam*HI – *Sac*I fragment of the Cry j 1 cDNA was inserted after the GluB-1 promoter to generate pGCryj1ΔSP (Figure 1B).

We also employed half of Cry j 1 in order to disrupt the native conformational structure of Cry j 1 still maintaining some T-cell epitopes. The N-terminal half of Cry j 1 contains four of five typical T-cell epitopes and the C-terminal half of Cry j 1 contains another T-cell epitope (Figure 1A). The N-terminal half of Cry j 1 (NhCry j 1) was PCR-amplified by using a forward primer (CTTCCATGGCTGATAATCCATAGACAGC, *Nco*I site is underlined) and a reverse primer (GCACCATGGGTCCACAGTTAGGTCCAAATTG, *Nco*I site is underlined). The C-terminal half of Cry j 1 (ChCry j 1) was also amplified by using a forward primer (CTACCATGGCAATTGGTGGGAGTTCAAATCC, *Nco*I site is underlined) and reverse primer (GCGCCATGGAACGTTTAGAGAGAGAGC, *Nco*I site is underlined). All the PCR fragments were cloned into the pCRII vector and confirmed by sequencing. Each cDNA was fused to a part of GluB-1 and GFP. The 0.6 kb fragment of GluB-1 gene containing sequences of promoter, 5' non-coding region (5'UTR) and signal peptide was PCR-amplified from rice (*Oryza sativa* L. cv. Sasani-shiki) genome as a template by using a forward primer (ATCTCTAGACAAGTTATATAGTCAATAAAG; *Xba*I site is underlined) and a reverse primer (GGATCTAGAACCATGGCCATAGAACCGTGGCATAATAG AAGAAC; the *Nco*I site is underlined). The GluB-1 promoter with a 5'UTR and signal peptide sequence was inserted into the *Xba*I and *Nco*I site upstream of GFP cDNA in pBluescript (Chiu et al. 1996). Then, NhCry j 1 and ChCry j 1 were ligated between the GluB-1 promoter and GFP at the *Nco*I site. The chimeric genes consisting of the GluB-1 promoter, NhCry j 1 or ChCry j 1 cDNA and GFP were inserted into pBI101H to generate pGNh-GFP and pGChGFP (Figure 1B).

A

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MDSPCLVALLVFESEVIGSCFSDNPIDSCWRGDSNWAQNRMKLADCAVGFG 50
      SP                                     1
SSTMGKGGLDYTVTNSDDDPVNPAPGTLRYGATDRPLWIIFSGNMNIK 100
LKMPMYIAGYKTFDGRGAQVYIGNGGPCVFIKRVSNTIHLGLYLYGCSTS 150
      2                                     3
VLGNVLINESFGVEPVHPQDGDALTLRTATNIWIDHNSFSNSSDGLVDVT 200
LTSTGVTISNNLFFNHKKVMLLGHDDAYSDDKSMKVTVAFNQEGPNCQQR 250
      4
MPRARYGLVHVANNNDPWTIYIAIGGSSNPTILSEGSFTAPNESYKKQV 300
TIRIGCKTSSSCSNWVWQSTQDVFYNGAYFVSSGKYEGGNIYTKKEAFNV 350
      5
ENGNATPQLTKNAGVLTCSLSKRC 375

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B

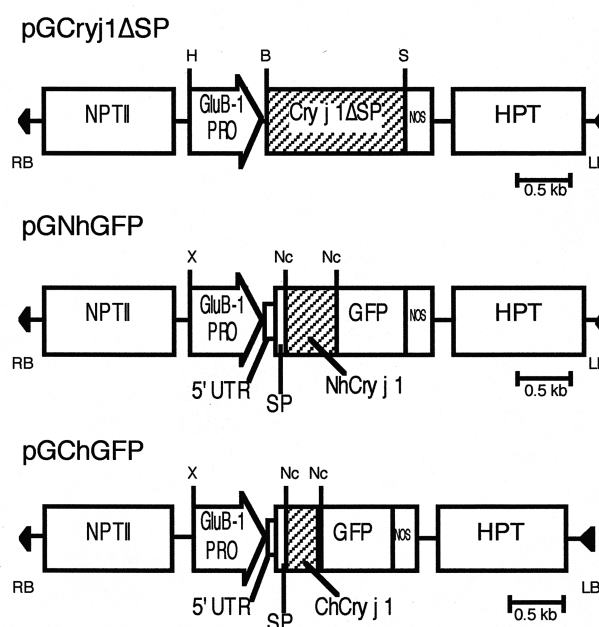


Figure 1. (A) Amino acid sequence of Cry j 1 and five typical T-cell epitopes of Cry j 1. T-cell epitopes were double-underlined and numbered under the sequence according to Sone et al. (1998). Shaded box and open box indicate the region used for the construction of GFP fusion, NhCry j 1 and ChCry j 1, respectively. Amino acids labeled by SP indicate signal sequence of Cry j 1. (B) A schematic representation of the T-DNA region of transformation vector pGCryj1ΔSP, pGNhGFP and pGChGFP. NPTII, gene for neomycin phosphotransferase II; HPT, gene for hygromycin resistance; GluB-1 PRO, rice glutelin GluB-1 promoter; 5'UTR, 5' non-coding region of rice GluB-1 gene; SP, sequence for signal peptide of rice GluB-1 gene; Cry j 1ΔSP, Cry j 1 without signal peptide; NhCry j 1, the N-terminal half of Cry j 1; ChCry j 1, the C-terminal half of Cry j 1; GFP, gene for green fluorescent protein; NOS, nopaline synthase gene terminator; RB and LB, right and left borders of T-DNA; B, *Bam*HI; H, *Hind*III; Nc, *Nco*I; S, *Sac*I; X, *Xba*I.

Agrobacterium-mediated rice transformation

pGCry j 1ΔSP, pGNhGFP and pGChGFP were transferred to *Agrobacterium tumefaciens* strain EHA101

(Hood et al. 1986). Each T-DNA was introduced into rice plants (*Oryza sativa* L. cv. Yamahoshi and cv. Sasanishiki) by *Agrobacterium*-mediated gene transfer (Hiei et al. 1994; Yokoi et al. 1997). Since hygro-

mycin resistance was found to be most effective for obtaining transgenic rice, we used hygromycin for the selection of transformants.

Protein extraction and immunoblot analysis

To extract the proteins, one grain of rice seed was homogenized and extracted either in 300 μ l of total protein extraction buffer (125 mM Tris-HCl pH 6.8, 140 mM SDS, 8 M urea, 200 mM glycerol, 4.5% 2-mercaptoethanol) or soluble protein extraction buffer (50 mM Tris-HCl pH 7.5, 2 μ g ml⁻¹ aprotinin). After centrifugation (12,000 $\times$ g, 15 min, 4 °C), the supernatants were used for immunoblot analysis. The amount of protein was estimated by the Bradford protein assay (BIO-RAD, CA, USA.). Sixty μ g of protein extract was loaded onto 12% SDS-polyacrylamide gels and the proteins were electroblotted onto PVDF membrane. Immunoblot analysis was performed as previously described in Okada et al. (1998). For the primary antibody, anti-Cry j 1 polyclonal antibody (Hayashibara Biochem Lab Inc, Okayama, Japan), the anti-GFP peptide antibody (CLONTECH, CA, USA.) or serum of a patient allergic to Japanese cedar pollen were used at a 1:1300, a 1:200 or 1:5 dilution, respectively. The blots were incubated with alkaline phosphatase-conjugated secondary antibody and visualized by incubation with 5-bromo-4-chloro-3-indolyl-phosphate p-toluidine salt and nitroblue tetrazolium chloride solution.

Fluorescence microscopy

Rice seeds, 4 weeks after flowering, were embedded in 5% agar and sliced into 100- μ m thick cross sections by a microslicer. Slides were mounted with Perma Fluor Aqueous Mountant (Shandon, PA, USA). Sections were viewed by fluorescent microscopy using a blue filter. Observations and photography were conducted on a Zeiss Axioskop.

Immunogold transmission electron microscopy

Rice seeds with NhCry j 1-GFP at three weeks after flowering were dehusked and dissected into three pieces transversely by a razor blade. The seed samples were fixed with 0.5% glutaraldehyde (GA) in 0.05 M sodium phosphate buffer (pH 7.4) at 4 °C overnight in a refrigerator. The specimens were dehydrated in a graded ethanol series (15 min each in 10, 30, 50, 70, 90, 95% aqueous ethanol, and in 100%

ethanol). The dehydrated samples were then treated with 50% LR White resin in ethanol for overnight. The samples were finally embedded in LR White resin at 60 °C for two days to harden. The specimens were sectioned with a diamond knife on a Lize ultramicrotome. Nickel grids coated with formvar membrane and carbon were used. Sections from LR White resin embedded samples were blocked with 1% bovine serum albumin (BSA) in PBS (pH 7.4) and incubated with mouse anti-GFP antibody and rabbit anti-prolamin polyclonal antibody overnight at 4 °C, followed by 5-nm of colloidal gold anti-mouse IgG (EY Labs, Inc., San Mateo, CA) and 10-nm of colloidal gold anti-rabbit IgG (Amersham Biosciences Corp, Piscataway, NJ) for 1 h.

Results

Construction of vector plasmids and transformation of rice plants

To produce recombinant Cry j 1 in the rice endosperm, we used the promoter of rice glutelin GluB-1 gene (Takaiwa et al. 1996). The Cry j 1 cDNA without signal peptide (Cry j 1 Δ SP) was expressed under the control of GluB-1 promoter, in which the accumulation of Cry j 1 protein in the cytoplasm of rice endosperm was expected (pGCryj1 Δ SP in Figure 1). For targeting Cry j 1 to the protein storage particles, the protein bodies, we used the GluB-1 promoter, sequences for 5'UTR and signal peptide of GluB-1 gene. Cry j 1 cDNA was divided into two parts, *NhCry j 1* and *ChCry j 1* (Figure 1), expecting that the splitting of the Cry j 1 molecule would reduce reactivity to IgE of pollen allergic patients because of the loss of the native structure of Cry j 1. To assist the stability of Cry j 1 peptide and to visualize it in rice endosperm, GFP was fused to the 3' end of NhCry j 1 or ChCry j 1, generating pGNhGFP and pGChGFP (Figure 1B).

Twenty-two transgenic plants with pGCryj1 Δ SP, 23 plants with pGNhGFP and 6 plants with pGChGFP were obtained. The insertion of the T-DNAs was confirmed by Southern blot analysis. The copy number of the T-DNA was estimated to be one to five (data not shown).

Expression of Cry j 1 polypeptide in rice seeds

Expression of Cry j 1 was investigated by immunoblot analysis of protein extracts from transgenic rice seeds. Since Cry j 1 was hardly detected in the total protein extracts of GCryj1ΔSP seeds, we used soluble protein extracts for the analysis of GCryj1ΔSP plants. A 30 kD band was detected by anti-Cry j 1 polyclonal antibody in all 5 transgenic plants tested, though the predicted molecular size of Cry j 1ΔSP was 38.5 kD (Figure 2A). The bands of native Cry j 1 from Japanese cedar pollen were observed at 43 kD and 46 kD as expected. Any modification of exogenous Cry j 1 in rice endosperm might have occurred in GCry j 1ΔSP.

As shown in Figure 2B, NhCry j 1-GFP was detected at 49 kD in the total protein extracts of GNhGFP seeds. The size of the band was almost consistent with the calculated molecular size (52.2 kD) from the DNA sequence. It was observed that transgenic lines with high copy numbers of the transgene tended to show higher accumulation of NhCry j 1-GFP (cf. 4 copies in line 12, 3 copies in line 15, and one copy in the other lines). ChCry j 1-GFP protein was detected at 42 kD as expected in the GChGFP seeds (Figure 2C). NhCry j 1-GFP and ChCry j 1-GFP proteins were also detected by anti-GFP antibody and found to be the same size of the bands detected by anti-Cry j 1 antibody, indicating the successful expression of the fusion protein of Cry j 1 polypeptide and GFP (Figure 2D).

A transgenic line showing the highest amount of the recombinant protein by immunoblot analysis was chosen, and the amount of Cry j 1 protein accumulated in seeds was calculated. Different amounts of purified native Cry j 1 were loaded along with the extracts of transgenic seeds, and the intensities of the bands detected by anti-Cry j 1 antibody were compared (data not shown). The calculated amounts of Cry j 1 proteins in one grain of transgenic seed on average were 0.15 μg/mg protein for the GCryj1ΔSP line, 16.6 μg/mg protein for the GNhGFP line and 7.6 μg/mg protein for the GChGFP line. Although NhCry j 1-GFP and ChCry j 1-GFP protein contain GFP polypeptide within themselves, accumulation of Cry j 1 polypeptide in both transgenic seeds is still 15- to 50-fold higher than that in GCryj1ΔSP seeds.

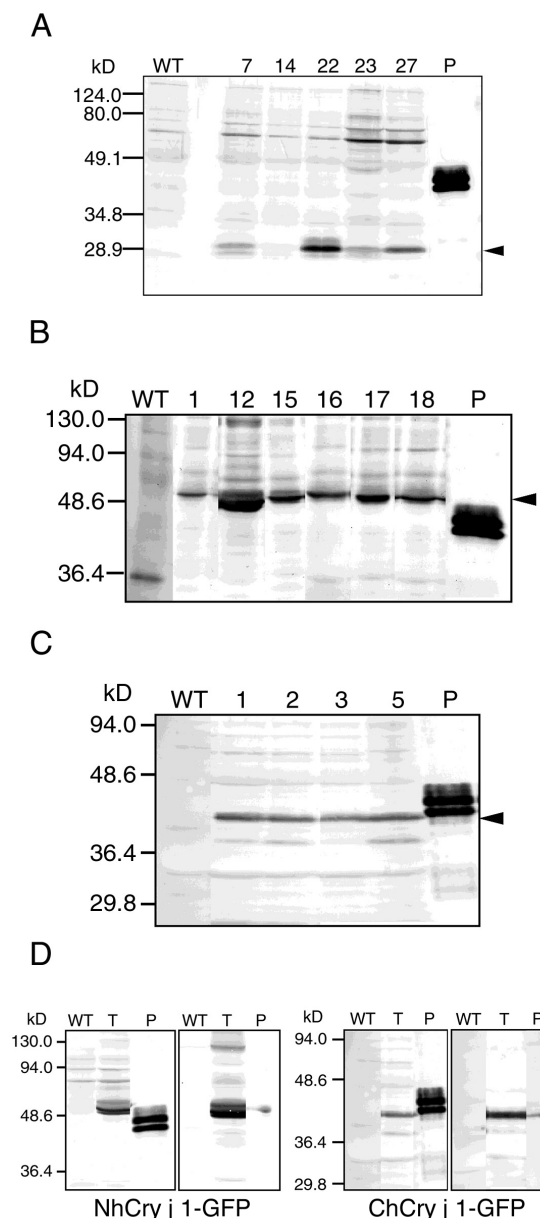


Figure 2. Expression of Cry j 1 in transgenic rice seeds detected by anti-Cry j 1 polyclonal antibody. (A) Soluble protein extracts of GCryj1ΔSP seeds were used for immunoblot analysis. Total proteins were extracted from GNhGFP (B) and GChGFP (C) and reacted with anti-Cry j 1 antibody. Arrowheads indicate the transgene products of Cry j 1. (D) Detection of NhCry j 1-GFP and ChCry j 1-GFP protein by anti-GFP antibody. Total protein of GNhGFP and GChGFP extracts was reacted with either anti-Cry j 1 antibody (left) or anti-GFP antibody (right). Numbers on top of each figure indicate the independent lines of transgenic plants. WT, wild type plant; P, cedar pollen; T, transgenic plant. Molecular markers are indicated on the left.

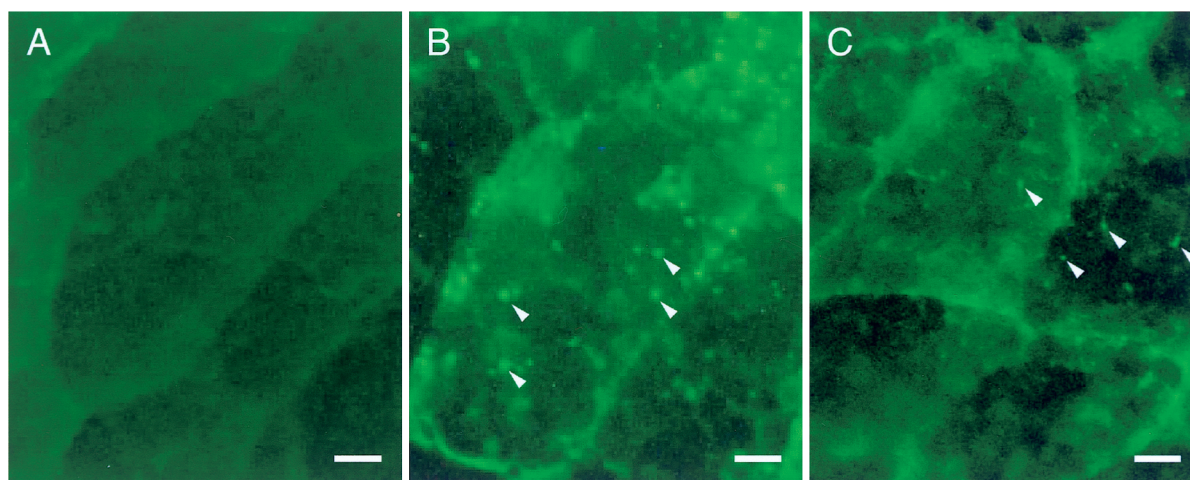


Figure 3. Subcellular localization of Cry j 1-GFP fusion protein in transgenic rice seeds. Rice seeds were sliced and observed under fluorescent microscope with blue light excitation. (A) Wild type seed. (B) GNhGFP seed. (C) GChGFP seed. Arrowheads indicate the green fluorescence in endosperm cell, representing the localization of Cry j 1-GFP fusion protein. Bars in all panels = 10 μ m.

Subcellular localization of GFP fluorescence in rice endosperm

We investigated the subcellular distribution of GFP fluorescence in cells of the transgenic rice seeds using fluorescent microscopy. In the cross sections of rice seeds, spots of green fluorescence were observed in the transgenic rice endosperm, whereas no fluorescence was detected in wild type plants (Figure 3). These green fluorescent spots were distributed throughout the endosperm cytoplasm and formed a number of spherical granules which were 1–3 μ m in diameter in GNhGFP and GChGFP seeds (Figures 3B and 3C). It has been reported that there are two types of protein bodies (PB-I and PB-II) which are 1–3 μ m in diameter in rice endosperm, and rice glutelin is accumulated in PB-II (Yamagata and Tanaka 1986; Ogawa et al. 1987). According to the size of the green spots, NhCry j 1-GFP and ChCry j 1-GFP are considered to be localized in the protein body of rice endosperm.

Immunogold electron microscopic localization of NhCry j 1-GFP in rice endosperm

Since the localization of GFP was not sufficiently clear to determine whether it was in PB-I or PB-II, we performed immunogold labeling of thin sections of rice seeds to elucidate the further localization of NhCry j 1-GFP. Anti-GFP antibody was used for immunodetection because Cry j 1 antibody resulted in a

high background. Anti-prolamin antibody was also used at the same time to identify PB-I, since the main protein component of PB-I is prolamin (Ogawa et al. 1987). As shown in Figure 4A, PB-I was observed as lightly stained protein bodies, whereas PB-II showed dark stained structures, both of which had a diameter of 1–3 μ m. The 10-nm gold particles that bound to anti-prolamin antibody were found only in lightly stained protein bodies confirming that these lightly stained ones are PB-Is (Figures 4A and 4B). The 5-nm gold particles indicating the localization of NhCry j 1-GFP were observed only at PB-I (Figures 4A and 4C), whereas none of the 5-nm particles were found in PB-II of transgenic seeds and PB-I of wild type (Figures 4A and 4B). It is noteworthy that a higher number of NhCry j 1-GFP proteins was found at the edge of PB-I than in the center, although prolamin was uniformly distributed throughout PB-I (Figure 4C).

IgE reactivity to NhCry j 1-GFP

For use with allergen-specific immunotherapy, low IgE-binding activity to Cry j 1 polypeptide is essential. NhCry j 1 has 195 amino acids and contains three T-cell epitopes that are necessary for recognition by T cells, whereas ChCry j 1 has 102 amino acids and only one T-cell epitope. Sone et al. (1998) demonstrated that T-cell epitopes no. 3 and 4 revealed an immunodominant determinant among five major T-cell epitopes of Cry j 1. In addition, GNhGFP plants

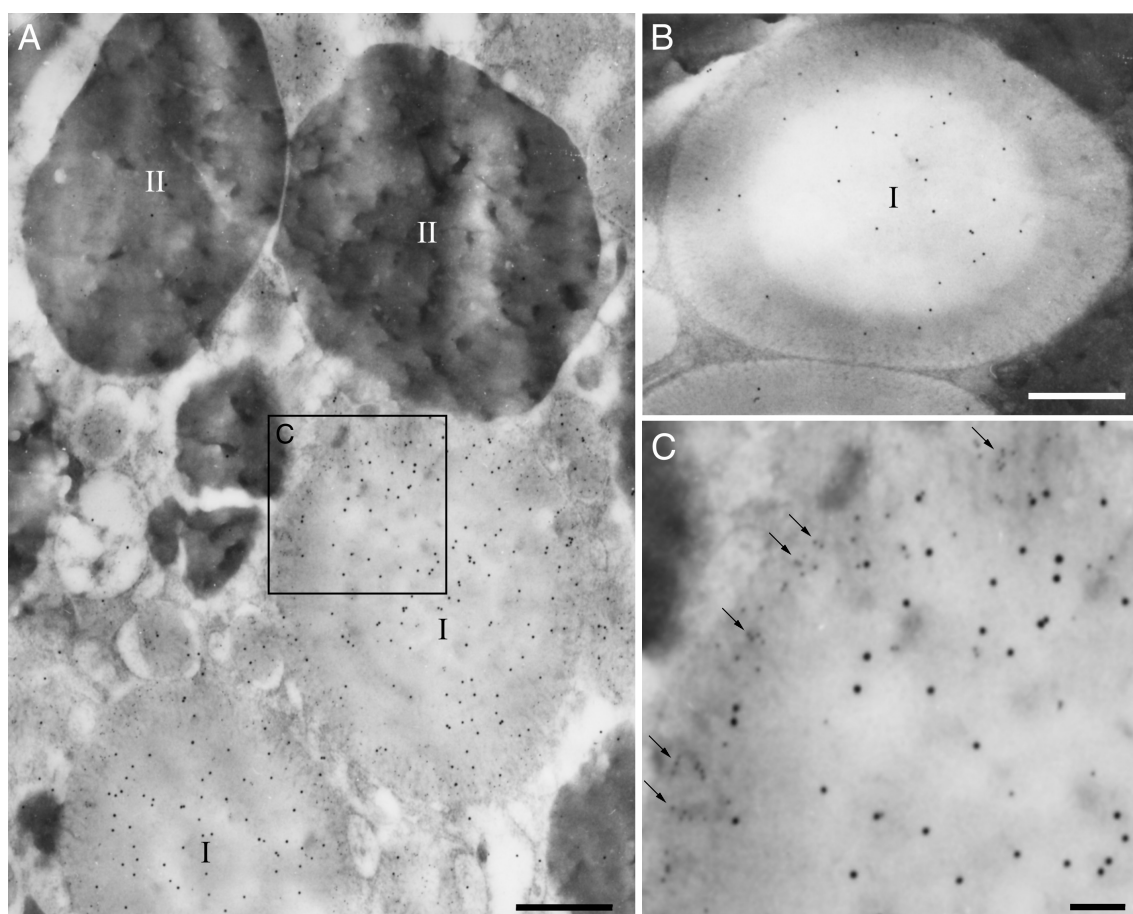


Figure 4. Immunogold electron microscope localization of NhCry j 1-GFP in rice endosperm. Rice seeds were reacted with anti-GFP antibody (5 nm-particles) and anti-prolamin antibody (10 nm-particles). (A) GNhGFP transgenic rice. (B) Wild type rice. (C) The box region in (A) is shown at higher magnification. Arrows indicate 5 nm-gold particles, representing the localization of NhCry j 1-GFP fusion protein. I, protein body I; II, protein body II. Bars in A and B = 500 nm and bar in C = 100 nm.

accumulated more Cry j 1 polypeptides in their seeds than GChGFP plants as mentioned above. Therefore, we considered that the transgenic seeds with NhCry j 1 are more useful than those of ChCry j 1 for immunotherapy purpose, and we therefore investigated IgE reactivity to NhCry j 1 by immunoblot analysis using the serum of a patient allergic to cedar pollen. The patient serum IgE did not react with NhCry j 1-GFP, but in contrast, it reacted to native Cry j 1 of cedar pollen (Figure 5 right panel). The blotting of NhCry j 1-GFP was confirmed by detection using anti-Cry j 1 polyclonal antibody (Figure 5 left panel). This result indicates that NhCry j 1 has less IgE-binding activity than native Cry j 1.

Discussion

Transgenic plants have become attractive systems for production of human therapeutic proteins because of the reduced risk of mammalian viral contaminants, the ability to produce them at a low cost, and their low maintenance requirements. (Arakawa et al. 1998; Kapusta et al. 1999; Staub et al. 2000). Recombinant birch pollen allergen Bet v 1 has been produced in tobacco leaves, and a broad utility of a transgenic system for the development of new and low-cost vaccination strategies against allergy has been suggested (Krebitz et al. 2000). However, there have been no reports of the expression of pollen allergens in edible organs of plants. Cry j 1 is the major pollen allergen of Japanese cedars, and more than 20% of the population is suffering from the pollinosis caused by ex-

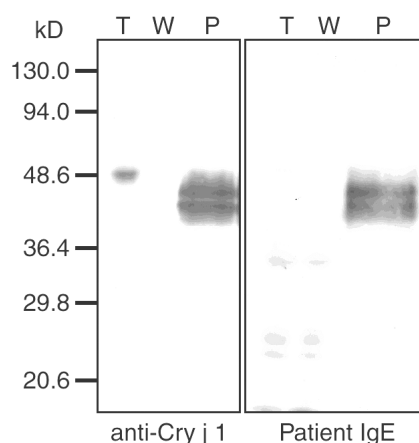


Figure 5. IgE-reactivity to NhCry j 1-GFP fusion protein of transgenic seeds. SDS-gel blots of total proteins of seeds were reacted with anti-Cry j 1 antibody (left) and patient serum allergic to cedar pollen (right). T, transgenic plant; W, wild type plant; P, cedar pollen. Molecular markers are indicated on the left.

posure to the cedar pollen. In the present study, we reported for the first time that Cry j 1 polypeptide was expressed in rice seeds and efficiently accumulated in the protein body, PB-I. Rice is one of the most popular cereals in the world, and transgenic rice is considered to be a suitable food to supply nutrients, pharmaceuticals and vaccines to those who are suffering from pollen allergies.

Glutelin is a seed storage protein whose gene and promoter have been isolated and well characterized (Takaiwa et al. 1996; Wu et al. 1998). In the current study, GluB-1 promoter was used for the expression of Cry j 1 polypeptide in rice endosperm (Figures 2 and 3). Targeting of Cry j 1 to the protein body of endosperm increased the accumulation in rice seed (16.6 µg/mg protein for GNhGFP and 7.6 µg/mg protein for GChGFP), which is 15- to 50-fold higher than the accumulation of GCry j 1ΔSP in the cytoplasm (0.15 µg/mg protein for GCryj1ΔSP). The higher accumulation of Cry j 1 polypeptide might have been due to the targeting of the recombinant protein into the protein body by addition of 5'UTR and signal peptide of GluB-1. Glutelin is normally accumulated in PB-II which is thought to originate from vacuoles (Yamagata and Tanaka 1986); however, NhCry j 1-GFP was shown to be localized exclusively in PB-I (Figure 4). The signal peptide of GluB-1 we used contained the 1st to 24th amino acids of GluB-1, and it lacked the following sequences similar to the vesicular transport signal (Matsuoka and Nakamura 1991; Nakamura and Matsuoka 1993). This might be

the reason for the ectopic localization of NhCry j 1 in PB-I. Localization of NhCry j 1 confined to the edge of PB-I would also be explained by the ectopic localization (Figure 4). This is the first report to demonstrate the targeting of a protein to PB-I by using the signal peptide of the GluB-1 gene.

Treatment of allergic diseases is performed as allergen-specific immunotherapy, in which gradually increased doses of allergens are injected successively. However, the risk of anaphylactic reaction increases as well. To solve this problem, it is desirable that recombinant proteins have low IgE reactivity and still retain T-cell epitopes (Takai et al. 1997; Ferreira et al. 1998). The conformation of allergen is more important for the recognition by IgE than the sequences of continuous peptides. The splitting of Cry j 1 protein into two domains, NhCry j 1 and ChCry j 1, was expected to result in the loss of the native structure of Cry j 1. Figure 5 clearly shows that IgE-binding activity of NhCry j 1 was significantly reduced in contrast to intense IgE reactivity to native Cry j 1. Sone et al. (1998) reported that Cry j 1 has five typical T-cell epitopes (Figure 1A) and epitopes no. 3 and 4 were immunodominant epitopes, which demonstrated the feasibility of peptide-based immunotherapy for Cry j 1. Falk et al. (2000) demonstrated that a polypeptide comprising 16 copies of a T-cell epitope induced T-cell proliferation much more efficiently than single T-cell epitope. Furthermore, an artificial polypeptide containing 7 T-cell epitopes of Cry j 1 and Cry j 2 revealed higher induction of T-cell proliferation than individual peptides containing a single epitope (Hirahara et al. 2001). Therefore, NhCry j 1 which contains three T-cell epitopes would work efficiently for the induction of T-cell proliferation but scarcely bind to patient IgE, demonstrating its low risk for immunotherapy.

The clinical efficacy of oral administration of mite extract and Cry j 1 peptides to allergic patients has also been reported and is expected to be a safer and more practical method for immunotherapy than the standard subcutaneous administration (Suko et al. 1995; Hirahara et al. 1998). For oral immunotherapy, an efficient dose of allergen has not been elucidated yet. The transgenic rice expressing Cry j 1 polypeptide in this study would supply several mg of peptide per bowl of rice for each meal which would be sufficient for oral administration. Hirahara et al. (1998) demonstrated that oral administration four times of 200 µg of Cry j 2 polypeptide containing dominant T-cell determinants to mice before or after the sensi-

tization induced immunologic tolerance in both T_{H1} and T_{H2} cell response against the whole protein allergen. Thus, transgenic rice seeds expressing NhCry j 1 could possibly be used for oral immunotherapy of pollinosis caused by cedar pollen.

In conclusion, we have produced Cry j 1 polypeptide in rice seeds regulated by the GluB-1 promoter. Targeting of the polypeptide to PB-I of endosperm improved the accumulation in rice seeds. No IgE-binding activity to NhCry j 1 was observed by immunoblot analysis. These results suggest the feasibility of oral immunotherapy in this model system. The T-cell response against NhCry j 1, and the effect of oral administration of transgenic rice seeds on immune response should be investigated in future studies.

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References

- Arakawa T., Chong D. and Langridge W. 1998. Efficacy of a food plant-based oral cholera toxin B subunit vaccine. *Nat. Biotech.* 16: 292–297.
- Chiu W., Niwa Y., Zeng W., Hirano T., Kobayashi H. and Sheen J. 1996. Engineered GFP as a vital reporter in plants. *Curr. Biol.* 6: 325–330.
- Falk K., Rotzschke O., Santambrogio L., Dorf M.E., Brosnan C. and Strominger J.L. 2000. Induction and suppression of an autoimmune disease by oligomerized T cell epitopes: enhanced in vivo potency of encephalitogenic peptides. *J. Exp. Med.* 191: 717–730.
- Ferreira F., Ebner C., Kramer B., Casari G., Briza P., Kungl A.J. et al. 1998. Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. *FASEB J.* 12: 231–242.
- Goddijn O.J.M. and Pen J. 1995. Plants as bioreactors. *Trends in Biotechnology* 13: 379–387.
- Goto F., Yoshihara T., Shigemoto N., Toki S. and Takaiwa F. 1999. Iron fortification of rice seed by the soybean ferritin gene. *Nat. Biotech.* 17: 282–286.
- Hiei Y., Ohta S., Komari T. and Kumashiro T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J.* 6: 271–282.
- Hirahara K., Saito S., Serizawa N., Sasaki R., Sakaguchi M., Inoue S. et al. 1998. Oral administration of a dominant T-cell determinant peptide inhibits allergen-specific TH1 and TH2 cell responses in Cry j 2-primed mice. *J. Allergy Clin. Immunol.* 102: 961–967.
- Hirahara K., Tatsuta T., Takatori T., Ohtsuki M., Kirinaka H., Kawaguchi J. et al. 2001. Preclinical evaluation of an immunotherapeutic peptide comprising 7 T-cell determinants of Cry j 1 and Cry j 2, the major Japanese cedar pollen allergens. *J. Allergy Clin. Immunol.* 108: 94–100.
- Hood E., Helmer G., Fraley R. and Chilton M. 1986. The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. *J. Bacteriol.* 168: 1291–1301.
- Kapusta J., Modelska A., Figlerowicz M., Pniewski T., Letellier M., Lisowa O. et al. 1999. A plant-derived edible vaccine against hepatitis B virus. *FASEB J.* 13: 1796–1799.
- Komiyama N., Sone T., Shimizu K., Morikubo K. and Kino K. 1994. cDNA cloning and expression of Cry j II the second major allergen of Japanese cedar pollen. *Biochem. Biophys. Res. Commun.* 201: 1021–1028.
- Krebitz M., Wiedermann U., Essl D., Steinkellner H., Wagner B., Turpen T. et al. 2000. Rapid production of the major birch pollen allergen Bet v 1 in *Nicotiana benthamiana* plants and its immunological *in vitro* and *in vivo* characterization. *FASEB J.* 14: 1279–1288.
- Leite A., Kemper E., Silva M., Luchessi A., Siloto R., Bonaccorsi E. et al. 2000. Expression of correctly processed human growth hormone in seeds of transgenic tobacco plants. *Mol. Breed.* 6: 47–53.
- Mason H.S. and Arntzen C.J. 1995. Transgenic plants as vaccine production systems. *Trend. Biotech.* 13: 388–392.
- Matsuoka K. and Nakamura K. 1991. Propeptide of a precursor to a plant vacuolar protein required for vacuolar targeting. *Proc. Natl. Acad. Sci. USA* 88: 834–838.
- McCormick A., Kumagai M., Hanley K., Turpen T., Hakim I., Grill L. et al. 1999. Rapid production of specific vaccines for lymphoma by expression of the tumor-derived single-chain Fv epitopes in tobacco plants. *Proc. Natl. Acad. Sci. USA* 96: 703–708.
- Nakamura K. and Matsuoka K. 1993. Protein targeting to the vacuole in plant cells. *Plant Physiol.* 101: 1–5.
- Ogawa M., Kumamaru T., Satoh N., Iwata N., Omura T., Kasai Z. et al. 1987. Purification of protein body-I of rice seed and its polypeptide composition. *Plant Cell Physiol.* 28: 1517–1527.
- Okada T., Swoboda I., Toriyama K., Bhara P.L. and Singh M.B. 1998. Engineering of hypoallergenic mutant of Ca²⁺-binding pollen allergen, Bra r 1, for immunotherapy. *FEBS Lett.* 434: 255–260.
- Sone T., Komiyama N., Shimizu K., Kusakabe T., Morikubo K. and Kino K. 1994. Cloning and sequencing of cDNA coding for Cry j I, a major allergen of Japanese cedar pollen. *Biochem. Biophys. Res. Commun.* 199: 619–625.
- Sone T., Morikubo K., Miyahara M., Komiyama N., Dhimizu K., Tsunoo H. et al. 1998. T cell epitopes in Japanese cedar pollen allergens: Choice of major T cell epitopes in Cry j 1 and Cry j 2 toward design of the peptide-based immunotherapeutics for the management of Japanese cedar pollinosis. *J. Immunol.* 161: 448–457.
- Staub J., Garcia B., Graves J., Hajdukiewicz P., Hunter P., Nehra N. et al. 2000. High-yield production of a human therapeutic protein in tobacco chloroplasts. *Nat. Biotech.* 18: 333–338.

- Suko M., Mori A., Ito K. and Okudaira H. 1995. Oral immunotherapy may induce T cell anergy. *Int. Arch. Allergy Immunol.* 107: 278–281.
- Takai T., Yokota T., Yasue M., Nishiyama C., Yuuki T., Mori A. et al. 1997. Engineering of the major house dust mite allergen Der f 2 for allergen-specific immunotherapy. *Nat. Biotech.* 15: 754–758.
- Takaiwa F., Yamanouchi U., Yoshihara T., Washida H., Tanabe F., Kato A. et al. 1996. Characterization of common cis-regulatory elements responsible for the endosperm-specific expression of members of the rice glutelin multigene family. *Plant Mol. Biol.* 30: 1207–1221.
- Valenta R. and Kraft D. 1995. Recombinant allergens for diagnosis and therapy of allergic diseases. *Curr. Opin. Immunol.* 7: 751–756.
- Weiner H.L. 1997. Oral tolerance: immune mechanisms and treatment of autoimmune diseases. *Immunol. Today* 18: 335–343.
- Wu C., Suzuki A., Washida H. and Takaiwa F. 1998. The GCN4 motif in a rice glutelin gene is essential for endosperm-specific gene expression and is activated by Opaque-2 in transgenic rice plants. *Plant J.* 14: 673–683.
- Yamagata H. and Tanaka K. 1986. The site of synthesis and accumulation of rice storage proteins. *Plant Cell Physiol.* 27: 135–146.
- Ye X., Al-Babili S., Kloti A., Zhang J., Lucca P., Beyer P. et al. 2000. Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* 287: 303–305.
- Yokoi S., Tsuchiya T., Toriyama K. and Hinata K. 1997. Tapetum-specific expression of the Osg6B promoter-β-glucuronidase gene in transgenic rice. *Plant Cell Rep.* 16: 363–367.
- Zeitlin L., Olmsted S., Moench T., Co M., Martinell B., Paradkar V. et al. 1998. A humanized monoclonal antibody produced in transgenic plants for immunoprotection of the vagina against genital herpes. *Nat. Biotech.* 16: 1361–1364.