

NOTE

Transgenic rice accumulating modified cedar pollen allergen Cry j 2 derivatives

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In order to create a safe tolerogenic antigen with reduced IgE reactivity, we developed transgenic rice that accumulates in the seed endosperm a sufficient amount of Cry j 2, the cedar pollen allergen, in a restructured form of tail-to-head, providing a feasible mucosal allergy vaccine against cedar pollinosis.

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[**Key words:** Cedar pollen allergen; Cry j 2; Pollinosis; Transgenic rice; Tolerogen]

Japanese cedar pollinosis is an important public health problem in Japan. About 27% of Japanese people are afflicted with this pollinosis from February to April each year, with the number of patients steadily increasing (1). The major allergens of Japanese cedar pollinosis have been characterized to be Cry j 1 and Cry j 2 (2,3). Cry j 1 is a basic glycoprotein with a molecular mass of about 41 to 45 kDa that is located in pollen cell walls and has pectate lyase activity (2). Mature Cry j 2 protein has a molecular mass of 45 kDa, is located in pollen starch granules and has polymethylgalacturonase activity (3). More than 90% of cedar pollen allergy patients have IgE specific to these allergens.

Allergen-specific immunotherapy is the only curative treatment that interferes with the basic pathological mechanisms of allergic diseases (4,5). Desensitization treatment by subcutaneous injection using a crude pollen extract is conventionally performed as allergen-specific immunotherapy. However, the repeated injection of allergen over three to five years is laborious and inconvenient, thus establishment of a convenient, effective protocol for the prevention and treatment of Japanese cedar pollinosis is highly desired.

In order to control this pollinosis, we have generated transgenic rice that accumulates cedar pollen allergens in the edible part (endosperm) of the seeds, providing a new immunotherapy using a rice-based allergy vaccine (6). As a model experiment, mouse major T-cell epitopes from Japanese cedar pollen allergens, Cry j 1 and Cry j 2, were expressed as fusion proteins with soybean seed protein glycinin in transgenic rice seed to remove the B-cell epitopes responsible for specific IgE binding. The feasibility of immunotherapy using the rice seed-based peptide vaccine has been demonstrated by feeding BALB/c mice transgenic rice containing mouse T-cell epitopes from Japanese cedar pollen allergens (7). Oral administration of transgenic rice containing the major T-cell epitopes inhibited the allergy-associated Th2 reaction including allergen-specific IgE and CD4⁺T cell proliferative responses, Th2 cytokine (IL4, IL5 and IL13) production and histamine release, and

also alleviated clinical symptoms such as sneezing. As the next step toward the development of specific immunotherapy for humans, transgenic rice containing a construct composed of the seven major T-cell epitopes from Cry j 1 and Cry j 2 (7Crp) was exploited for the control of pollen allergy in humans (8). The 7Crp protein accumulates to high levels of 50 to 60 µg/grain deposited predominantly in endoplasmic reticulum (ER)-derived prolamin protein bodies (PBs) in transgenic rice seed (9).

Although peptide immunotherapy using T-cell epitopes derived from allergens can provide very safe treatment without side effects due to the lack of B-cell epitopes (10), the efficacy of this treatment is limited to some extent, because several subdominant T-cell epitopes are not completely included due to genetic diversity in humans (11). Thus, recombinant modified antigen derivatives with reduced or no IgE binding (allergenicity) formed by deconstructing the conformational structure of the target antigen involved for IgE recognition, while maintaining immunogenicity, are ideal tolerogens to induce immune tolerance against pollen allergies that will avoid side effects such as anaphylactic shock (12,13). Here, we aimed to express the entire modified Cry j 2 sequence in transgenic rice seed to create a rice-based allergy vaccine.

When wild type Cry j 2 cDNA was expressed under the control of the endosperm-specific glutelin 2.3 kb *GluB-1* promoter containing its signal peptide, no proteins were detected by immuno-blot analysis after SDS-PAGE using the Cry j 2 antibody ((Figs. 1, lane 1 in Fig. 2). lane 1). Little or no accumulation of the wild type Cry j 2 antigen in rice seed may have resulted from either the presence of rare codons in its coding sequence or from the enzymatic activity (polymethylgalacturonase) responsible for degradation of cell walls. To test the hypothesis, codon optimization was performed by replacing rare codons observed in the native *Cry j 1* sequence with codons frequently used in many rice seed storage protein genes (14), and aspartic acid (D) at position 183 corresponding to the enzymatic active site was mutagenized to alanine (A). The complete codon-optimized Cry j 2 sequence was synthesized and the mutagenized gene was produced from the synthetic gene. As shown in Fig. 2B (lane 2), full length Cry j 2

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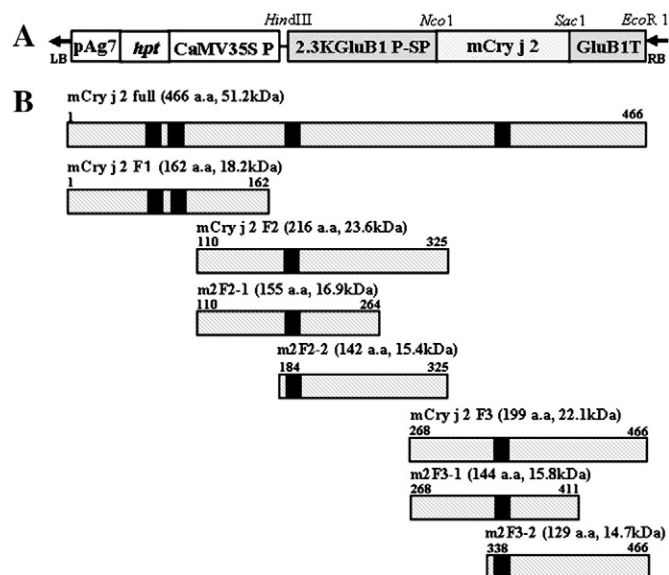


FIG. 1. Construction of expression vectors for rice transformation. Wild type and codon-optimized entire Cry j 2 coding region without a signal peptide derived from the full-length cDNA were linked to the 2.3 kb glutelin *GluB-1* promoter containing its signal peptide sequence and then were inserted into pGTV35S HPT binary vector. Overlapped three fragments (F1, F2 and F3) covering the full length of the Cry j 2 coding region and F2 (F2-1, F2-2) and F3 (F3-1, F3-2) derivatives, whose sequences were derived from the codon-optimized full-length Cry j 2, were attached to the KDEL ER retention signal at the C-termini of all constructs to enhance accumulation levels. These fragments were ligated to the glutelin 2.3 kb *GluB-1* promoter containing its signal peptide. The binary vector harboring the expression cassette of Cry j 2 was introduced into the rice genome (*Oryza sativa* L. cv. Kitaake) by *Agrobacterium*-mediated transformation and transgenic plants were selected by resistance to hygromycin. Major human T-cell epitopes of Cry j 2 are indicated by black solid boxes.

was detected as a band with a molecular mass of about 50 kDa by immuno-blot analysis, indicating that the accumulation level was enhanced by codon optimization. In contrast, mutagenesis of the enzymatic active site using the codon-optimized sequence had little effect on accumulation level (Fig. 2B, lane 3).

This enhancing effect on Cry j 2 accumulation by codon optimization was observed, even when the Cry j 2 antigen was fragmented so as to destruct the conformational structure of Cry j 2 antigen required for specific IgE antibody binding. The Cry j 2 antigen was divided into three overlapping fragments (F1: amino acid position (p)1–162; F2: p110–325; F3: p268–466) as shown in Fig. 1B. The glutelin signal peptide and KDEL ER retention signal followed by a stop codon, which are

required by targeting to the endoplasmic reticulum (ER) for high level accumulation (8,15), were artificially attached at the N- and C-termini of the individual fragment that was then specifically expressed in endosperm tissue under the control of the 2.3 kb glutelin *GluB-1* promoter. In the transgenic lines with highest expression of these individual constructs, these fragments accumulated at levels detected as faint bands in CBB-stained SDS-PAGE gels after electrophoresis of total seed proteins extracted from mature transgenic rice seed (Fig. 2A). Therefore, in order to create transgenic rice accumulating the entire Cry j 2 sequence with a deconstructed structure by fragmentation, conventional crossing of high expression lines accumulating individual Cry j 2 fragments will permit the stacking of the three overlapped fragments covering the entire mature Cry j 2 sequence. Alternatively, introduction of a binary vector harboring the expression cassettes capable of simultaneous expression of a few fragments spanning the entire Cry j 2 in endosperm tissue.

Furthermore, a reassembled Cry j 2 that was divided into the three fragments and then rearranged in the order of C-terminus to N-terminus was expressed as a single tail-to-head recombination molecule (P3–P2–P1) under the control of the endosperm specific glutelin *GluB-1* promoter (Fig. 3B). Disruption of conformational structure required for recognition of the specific IgE was designed to retain the major T cell epitope sequences (p77–89, p96–107, p192–204 and p357–367) (16). This restructured Cry j 2 molecule contains the mature sequence of the Cry j 2 molecule (1 to 388), indicating that all T-cell epitopes (immunogenicity) are preserved (16,17). As shown in Fig. 3C, the restructured Cry j 2 accumulated in the majority of the 48 independent transgenic lines. About 10 to 13 µg of reassembled Cry j 2 accumulated in one mature grain in the highest expression line. The modified protein was clearly detected as an extra band on the CBB-stained gel and was confirmed by immuno-blot analysis (Fig. 3D). When the copy number of the restructured Cry j 2 gene in the highest expression line (no. 39) was examined by Southern blot analysis using the *hpt* coding region as a probe, two copies were found to be integrated in the genome of the transgenic line (Fig. S1). When this artificially shuffled Cry j 2 was expressed as a secretory protein in rice endosperm tissue, accumulation levels of chaperone proteins such as binding proteins (BiPs) were shown to be significantly enhanced as ER stress response in mature seed (Fig. S2).

We demonstrated here that the second major Japanese cedar pollen allergen, Cry j 2, accumulated in transgenic rice seed by expressing its codon-optimized sequence under the control of a glutelin promoter. In addition, in order to develop transgenic rice accumulating sufficient quantities of the modified Cry j 2 antigen, Cry j 2 should be divided into several overlapping fragments or be reassembled as a mosaic molecule.

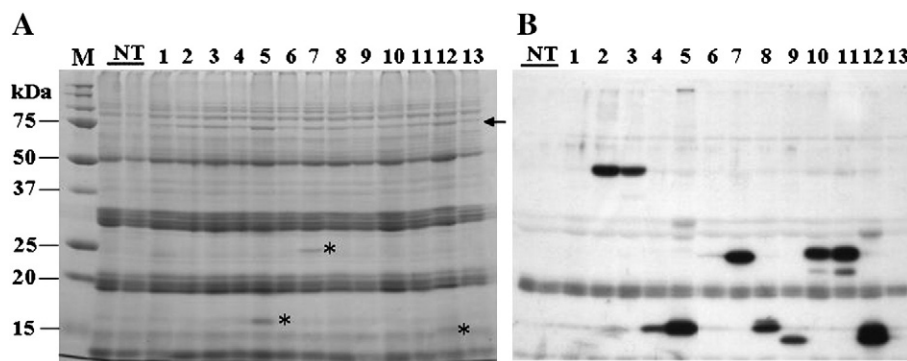


FIG. 2. Expression of Cry j 2 full and fragmented Cry j 2 derivatives in transgenic rice seeds. (A) SDS-PAGE analysis of total proteins extracted from non-transgenic 'Kitaake' control (NT) and transgenic rice seeds of the original full (lane 1), codon-optimized modified full (m full) (lane 2), mutagenized modified full (D183A m full) (lane 3), original F1 (lane 4), mF1 (lane 5), mF2 (lane 6), mF2-1 (lane 7), F2-2 (lane 8), mF2-2 (lane 9), original F3 (lane 10), mF3 (lane 11), mF3-1 (lane 12) and mF3-2 (lane 13). Seeds from highest expressing transgenic lines for each construct were analyzed by SDS-PAGE. (B) Immuno-detection of Cry j 2 with anti Cry j 2 antibody. The samples are the same as panel A. Asterisks and arrow represent the faint bands of Cry j 2 fragment derivatives and BiP, respectively, that are detected by CBB staining of seed proteins. M, molecular markers.

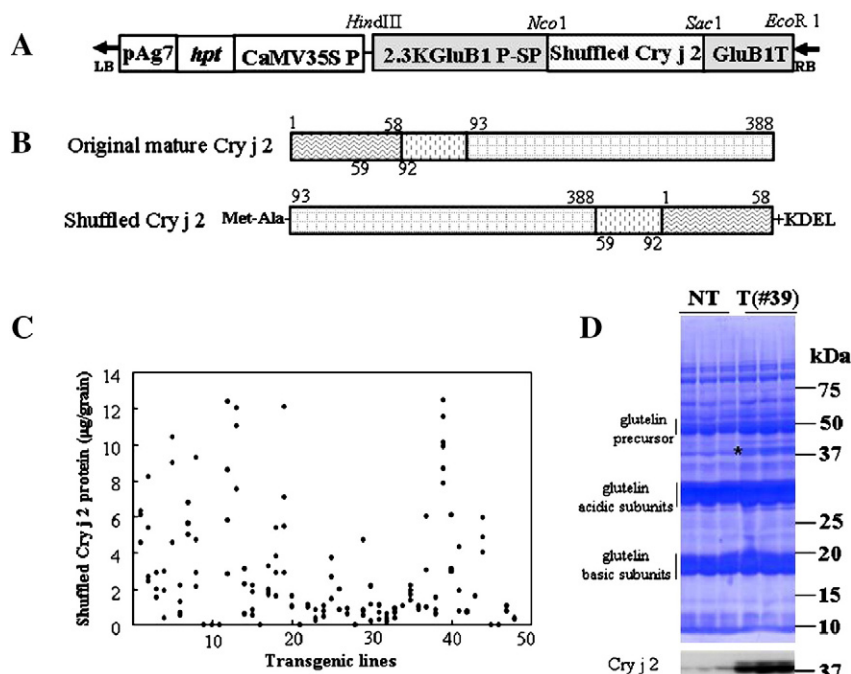


FIG. 3. Expression of shuffled Cry j 2 in transgenic rice seeds. (A) Schematic representation of the construct used for the expression of shuffled Cry j 2. (B) Comparison of structure between the original and shuffled Cry j 2 antigen. (C) Accumulation levels of shuffled Cry j 2 in transgenic rice seed. Amounts of shuffled Cry j 2 in mature seeds were examined by immuno-blot analysis for individual transgenic rice line. Each dot represents the concentration of shuffled Cry j 2 in a seed (μg/seed) for an individual transgenic line. (D) SDS-PAGE and immuno-blot analysis showing the accumulation level of shuffle Cry j 2 in mature seed of transgenic line no. 39. Seed proteins in wild type (NT) and transgenic rice grains. SDS-PAGE (upper panel) and immuno-blot using the Cry j 2 antibody (lower panel). The asterisk represents the band of shuffled Cry j 2 visible by CBB staining of transgenic seed proteins.

Since these treatments disrupt the conformational structure required for specific IgE binding, while retaining T-cell epitopes responsible for immunogenicity, transgenic rice accumulating significant amounts of destructured whole Cry j 2 sequence in seed may provide a safe and effective tolerogenic antigen for inducing mucosal immune tolerance as an allergen-specific immunotherapy against cedar pollen allergy. However, further work will be required to examine using sera from Japanese cedar pollinosis patients for practical use whether specific IgE binding ability of the destructured Cry j 2 is lower than the wild type.

Supplementary materials related to this article can be found online at doi:10.1016/j.jbiosc.2011.10.005.

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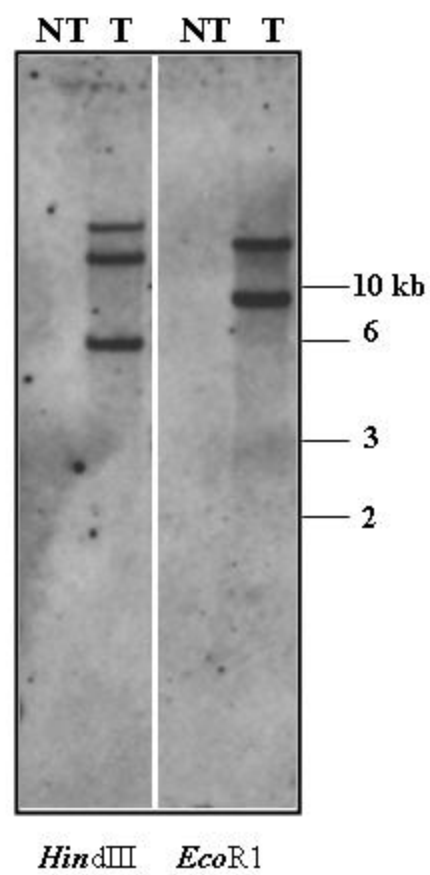


Figure S1

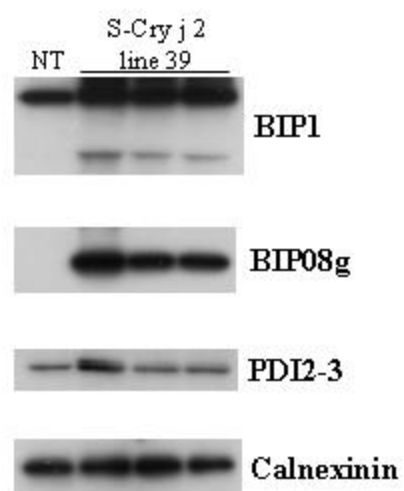


Figure S2