# Transgenic Crop Research and Development Center

## Characterization of a new rice glutelin gene *GluD-1* expressed in the starchy endosperm

Rice endosperm has been utilized as an excellent platform for producing foreign recombinant proteins because it is a natural storage organ for starch and proteins and can offer ample deposition space for foreign recombinant products. A number of biopharmaceuticals, nutriceuticals containing health-promoting peptides, and mineral-binding proteins have been produced using rice endosperm as a bioreactor. Because seed storage proteins (SSPs) are abundant in the endosperm, promoters of native SSP genes are often employed to achieve high accumulation of those compounds in the endosperm.

Studies on the molecular mechanisms underlying endosperm-specific expression of cereal SSP genes have been carried out. It has been reported that several cis-elements, such as the prolamin box (P box: AAAG) and GCN4 (TGA(G/C)TCA), ACGT and AACA motifs are conserved in many cereal SSP genes. Transcriptional factors that recognize these cis-elements have also been isolated, such as Opaque2-like bZIP, Dof and Myb proteins. Understanding these gene regulation mechanisms in greater detail would provide useful information for fine-tuning the expression of recombinant products in transgenic crops.

We discovered a new glutelin by comparing the seed protein composition of various rice cultivars. The expression of this glutelin gene (*GluD-1*) has unique spatial and temporal features during seed development, although like the other glutelins, it is expressed in an endosperm-specific manner (Fig 1). *GluD-1* was predominantly expressed in the inner starchy endosperm from about 5 DAF and steadily increased until maturity at 30 DAF (Fig 1). Since the 0.2kb upstream region is sufficient to confer GUS reporter expression, the essential regulatory elements controlling *GluD-1* expression in the starchy endosperm are present within this region (Fig. 2). An in vitro gel-shift assay and an in vivo transient reporter assay indicated that such endosperm specificity is controlled by combinatorial interactions of a GCN4-like motif and a closely linked P box around position –200 from the ATG start site through binding by bZIP transcription factor RISBZ1 and DOF transcription factor RPBF. Its spatial specificity may be caused by deviations within the GCN4 motif of the *GluD-1* promoter.

## Development of rice-based oral vaccine against house dust mite allergy

Transgenic plants are becoming common hosts for low cost production of valuable pharmaceuticals, vaccines and antibodies. Apart from extremely low risk of contamination with animal or human pathogens, transgenic plants can be used as a reliable delivery system for the oral administration of expressed valuable proteins. Among cereal crops, rice is especially an ideal potential oral delivery system of vaccines for clinical prophylactic and treatment of allergic disease as well as protecting against infection by pathogens.

Conventional allergen-specific immunotherapy has been mainly performed by repeated subcutaneous injection of increasing concentrations of allergens throughout a period of 3-5 years with severe side effects including the risk of anaphylactic shock. A safe, easy and convenient treatment would benefit patients by taking advantage of the mucosal immune system via oral administration as an alternative to subcutaneous injection.

House dust mite (HDM) of *Dermatophagoides* species is the most common source of indoor allergens associated with various allergic diseases such as asthma, rhinitis and atopic dermatitis. One of the most important properties of allergens is their ability to induce Th2 responses culminating in IgE antibody production. Der p 1, an allergen from feces of *D. pteronyssinus*, elicits IgE antibody responses in over 80% of

patients who are sensitive to *D. pteronyssinus* and is considered to be the most immunodominant allergen involved in the expression of IgE-mediated hypersensitivity. Der p 1 has cysteine protease activity that is involved in the pathogenesis of allergies through cleavage of CD23 and CD25 from the surface of immune cells, IgE-independent mast cell activation, and increasing epithelial cell permeability. The mature Der p 1 has 222 amino acid residues possessing one N-glycosylation motif at 52nd Asn and the 34$^{th}$ Cys residue also plays a crucial role in its enzyme activity.

We developed transgenic rice seed expressing a major house dust mite allergen, Der p 1, as an oral vaccine. The C-terminal KDEL-tagged Der p 1 allergen specifically accumulated in seed endosperm tissue under the control of the endosperm-specific GluB-1 promoter. Der p 1 reached a maximum concentration of 58μg/grain and was deposited in the endoplasmic reticulum-derived protein body I (PB-I) (Fig 3). Plant-derived Der p 1 was posttranslationally modified with high-mannose-type glycan structure (Table 1). Glycosylated Der p 1 displayed reduced IgE binding capacity in comparison with its unglycosylated counterpart in vitro. Our results indicate that transgenic Der p 1 rice seeds are a safe, potential oral delivery vaccine for treatment of HDM allergy.

## Enhanced oral tolerogenicity of T cell epitopes coupled with cholera toxin B subunit

Oral peptide immunotherapy using dominant T-cell epitopes provides a safe, easy and effective strategy against allergic and autoimmune diseases. We have recently reported that feeding mice with transgenic rice seed containing T-cell epitopes derived from major Japanese cedar pollen allergens Cry j 1 and Cry j 2 suppressed pollen-induced allergic responses and clinical symptoms. These results demonstrated the clinical potential of T-cell epitopes accumulated in rice seed for immunotherapy of allergic disorders.

Cholera toxin B (CTB), a GM1 ganglioside-binding subunit of cholera toxin, has been shown to function as an effective carrier for mucosal antigens, as well as a non-toxic oral cholera vaccine antigen. Here, we applied this CTB molecule for the induction of oral tolerance against Japanese cedar pollen allergens. Allergen-derived tolerogenic T cell epitopes (3Crp) were expressed in transgenic rice seed as a fusion protein with either CTB or rice glutelin as a control (Fig 4). In an experimental mouse model of pollen allergy, a relatively high dose of CTB-free T cell epitopes (equivalent to 15μg 3Crp) was required for the suppression of allergen-specific IgE antibody responses (Fig. 4). In contrast, a significantly lower dose of the CTB-coupled T cell epitopes (equivalent to 0.3μg 3Crp) was sufficient for IgE suppression (Fig 4). Furthermore, the development of clinical symptoms such as pollen allergen-induced sneezing was suppressed by oral feeding of rice seed that accumulate T cell epitopes fused with CTB. Thus, our findings demonstrate that CTB enhances oral tolerogenicity of T cell epitope 3Crp peptide, and support a promising potential role for CTB in peptide-based immunotherapy of Japanese cedar pollen allergy.

## Improvement of CoQ10-enriched rice

We previously produced CoQ10-enriched transgenic rice plants by introducing the expression vector construct of S14:ddsA, in which the gene encoding DdsA, an enzyme involved in the CoQ10 biosynthesis pathway, was driven by the CaMV35S promoter. Here, in order to obtain a transgenic line accumulating higher concentration of CoQ10, we first expressed the S14:ddsA construct using the rice ubiquitin promoter as a much stronger promoter. In this approach, the highest expression line resulted in 1.3-fold enhancement of CoQ10 in mature seed than the previous construct. We next produced another type of CoQ10-enriched rice plant by introducing two constructs simultaneously; one is *DdsA* driven by the rice ubiquitin promoter and the other is *COQ2*, the enzyme involved in CoQ biosynthesis pathway, driven by the CaMV 35S promoter. Although the COQ2 gene was successfully expressed in the newly generated transgenic plants, CoQ10 level was very similar to that of Ubi:S14:ddsA plants. This result indicates that co-expression of DdsA and CoQ2 does not result in an additive effect and that another approach is required to

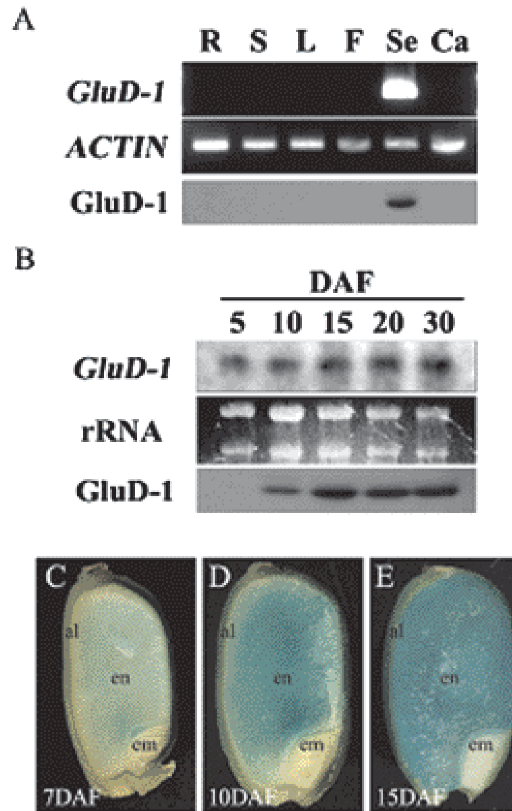


Fig 1. Temporal and spatial expression patterns of *GluD-1*.
(A) Organ-specific expression pattern of *GluD-1*. R: root, S: shoot apex, L: leaf blade, F: flower, Se: seed at 15 days after flowering (DAF), and Ca: callus. (B) Temporal expression pattern during seed maturation of *GluD-1*. C–E) GUS expression driven by the 1.6kb *GluD-1* promoter during seed maturation, at 7, 10, 15 DAF, respectively. al: aleurone and subaleurone layers, en: starchy endosperm, and em: embryo.

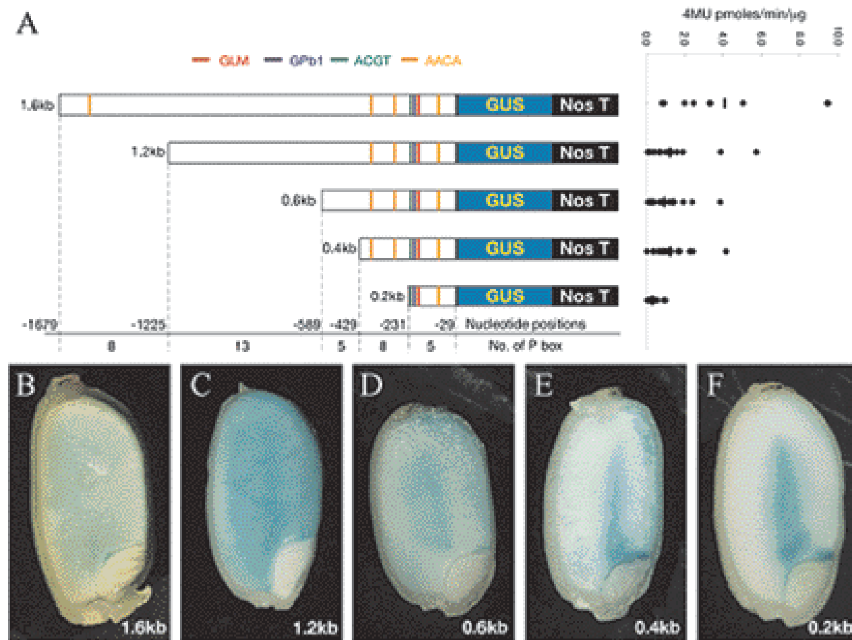


Fig 2. Truncation analysis of the *GluD-1* promoter.
(A) Schematic representation of truncated promoter:GUS constructs for generating transgenic rice (left), and expression strength (right). (B)-(F) GUS expression at 7 DAF, driven by 1.6kb, 1.2kb, 0.6kb, 0.4kb, and 0.2kb *GluD-1* promoters, respectively.

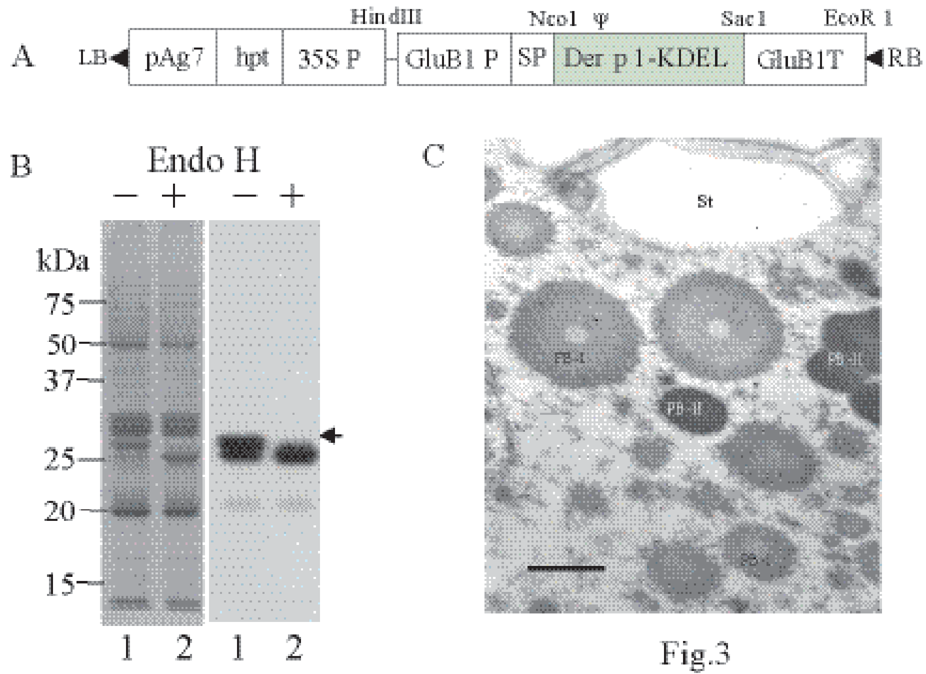


Fig 3. Characterization of transgenic rice expressing Der p 1.
(A) Construct of binary vector used for expression of mite allergen Der p 1. (B) Analysis of Der p 1 glycoprotein derived from rice seed after treatment with Endo H. (C) Analysis of intra-cellular localization of Der p 1 by immuno-electron microscopy.

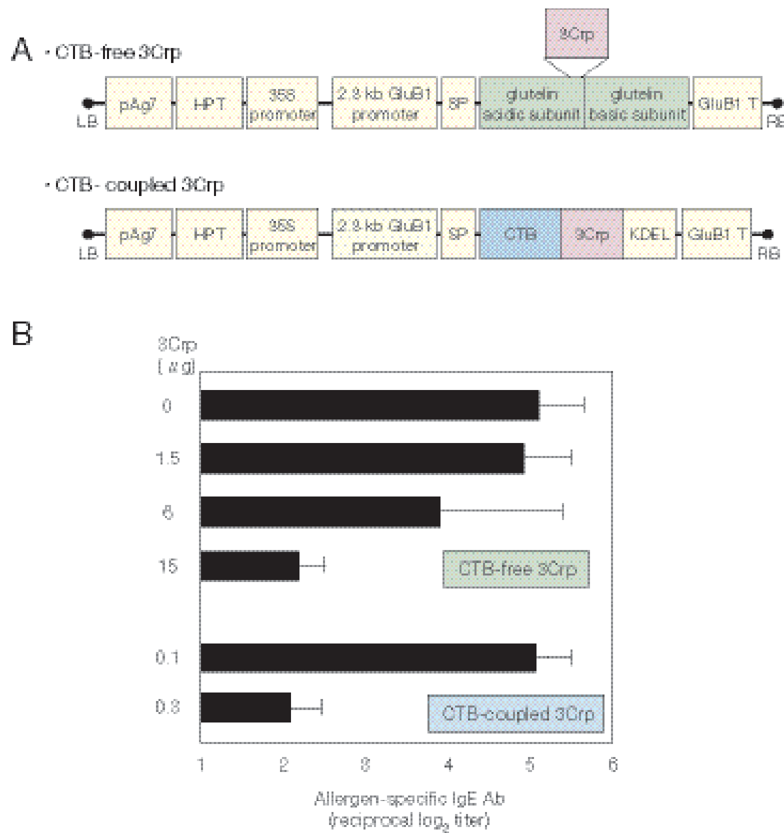


Fig 4. Efficacy of CTB-T cell epitope fusion protein expressed in rice seed.
(A). Constructs for expression of CTB-free and CTB-coupled T cell epitope 3Crp peptide. (B) Suppression of allergen-specific IgE antibody responses by oral feeding of transgenic rice seed.

Table 1. Relative abundances of N-glycans detected in Der p 1

| Fraction | | m/z (M + Na)+ | | Glycoform | Ratio (%) |
|---|---|---|---|---|---|
| | | MALDI -TOF -MS | Calculated | | |
| 1 | | 1821.56 | 1821.64 | $Man_8GlcNAc_2$ | 26.3 |
| 2 | | 1983.39 | 1983.78 | $Man_9GlcNAc_2$ | 12.9 |
| 4 | a | 1659.36 | 1659.50 | $Man_7GlcNAc_2$ | 1.6 |
| | c | 1983.03 | 1983.78 | $Glu_1Man_8GlcNAc_2$ | 26.8 |
| 5 | b | 2145.26 | 2145.92 | $Glu_1Man_9GlcNAc_2$ | 25.4 |
| 6 | b | 1143.90 | 1143.05 | $Man_3XylGlcNAc_2$ | 4.9 |
| | e | 1335.40 | 1335.22 | $Man_5GlcNAc_2$ | 2.1 |