

Generation of a transgenic rice seed-based edible vaccine against house dust mite allergy

Lijun Yang ^a, Hiroyuki Kajiura ^b, Kazuya Suzuki ^a, Sakiko Hirose ^a,
Kazuhito Fujiyama ^b, Fumio Takaiwa ^{a,*}

^a Transgenic Crop Research and Development Center, National Institute of Agrobiological Sciences, Kannondai 2-1-2, Tsukuba, Ibaraki 305-8602, Japan

^b The International Center for Biotechnology, Osaka University, 2-1 Yamada-oka, Suita, Osaka 565-0871, Japan

Received 9 October 2007

Available online 5 November 2007

Abstract

As an alternative approach to conventional allergen-specific immunotherapy, transgenic rice seed expressing a major house dust mite (HDM) allergen, Der p 1, was developed as an edible vaccine. The C-terminal KDEL-tagged Der p 1 allergen specifically accumulated in seed endosperm tissue under the control of the endosperm-specific *GluB1* promoter. Der p 1 reached a maximum concentration of 58 µg/grain and was deposited in the endoplasmic reticulum (ER)-derived protein body I (PB-I). Plant-derived Der p 1 was posttranslationally modified with high-mannose-type glycan structures. 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 the treatment of HDM allergy.

© 2007 Elsevier Inc. All rights reserved.

Keywords: House dust mite; Oligomannose-type glycan; Der p 1 allergen; Transgenic rice; *Oryza sativa*; Endosperm tissue; Edible vaccine

House dust mites (HDM) (*Dermatophagoides* spp.) are the most common source of indoor allergens associated with allergic diseases such as asthma, allergic rhinitis, and atopic dermatitis [1]. One of the most salient properties of allergens is their ability to induce Th2 responses, culminating in IgE antibody production. Der p 1, an allergen from the faeces 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 immuno-dominant environmental allergen involved in the expression of IgE-mediated hypersensitivity [2]. Der p 1 has cysteine protease activity that is involved in the pathogenesis of allergy through cleavage of CD23 and CD25 from the surface of immune cells, IgE independent mast cell activation, and increasing epithelial cell permeability [3,4]. Mature Der p 1 has 222 amino acid residues, with

one N-glycosylation site at Asn⁵², and Cys³⁴ plays a crucial role in its enzymatic activity [5].

Allergen-specific immunotherapy with crude extracts from HDM has been used as a highly effective treatment for patients with severe allergy [6]. The clinical effect of allergen-specific immunotherapy is the reduction of allergen-specific IgE. Conventional allergen-specific immunotherapy is primarily manifest by frequent and regular subcutaneous injections with increasing doses of natural allergen extracts for a period of 3–5 years. Conventional therapy thus has a number of drawbacks, including patient inability to sustain or complete treatment, and it carries the risk of severe side effects, including anaphylactic shock. Immunotherapy patients would therefore benefit by a safe, easy and convenient treatment, preferably by taking advantage of the mucosal immune system via oral administration as an alternative to subcutaneous injection [7].

Transgenic plants are becoming a common platform for the production of valuable pharmaceuticals, vaccines, and antibodies [8,9]. Apart from the extremely low risk of

* Corresponding author. Fax: +81 29 838 8397.

E-mail address: takaiwa@nias.affrc.go.jp (F. Takaiwa).

contamination with animal or human pathogens, as well as low production costs, transgenic plants can be used as a reliable delivery system for the oral administration of expressed proteins [10,11]. Cereal crops, especially rice seed, have already proven to be the ideal oral delivery system in animal models for clinical prophylaxis and treatment of hypertension, diabetes, and cedar pollen allergy as well as prophylaxis against infection [12–16].

Like mammalian systems, plants can posttranslationally modify newly synthesized proteins. However, proteins produced in plant systems differ in N-glycosylation from those produced in mammalian cells. The β (1,2)-xylose and α (1,3)-fucose residues characteristic to plants are probably immunogenic, and elicit glycan-specific IgE and IgG antibodies in humans as glycol-epitopes of plant allergens [17]. N-glycosylation depends on the organ and the subcellular compartment where the plant glycoprotein is deposited. Proteins in the lumen of the plant endoplasmic reticulum (ER) contain high-mannose-type N-glycans with structures common to mammals. It is thus possible to develop ‘humanized’ non-immunogenic N-glycosylated proteins in transgenic plants through the fusion of the KDEL or HDEL ER retention signal sequences to the C-terminus of a recombinant protein [18].

In this study, we expressed mature Der p 1 protein in transgenic rice seed under the control of a strong rice storage protein promoter. The KDEL-tagged Der p 1 protein accumulated at up to 58 μ g/grain and was mainly stored in ER-derived protein body I (PB-I). Like its natural counterpart, plant-derived Der p 1 is glycosylated. The high-mannose-type Der p 1 has less IgE binding reactivity than unglycosylated Der p 1. These results suggest that transgenic Der p 1 rice seeds have potential as a safe, edible vaccine for use against HDM allergy.

Materials and methods

Plant transformation and expression. The gene encoding mature Der p 1 was optimized for translation based on codons frequently used in rice seed storage protein genes, and synthesized by GenScript Corporation (New Jersey, USA). To eliminate cysteine protease activity, Cys³⁴ was replaced with an Ala residue. The modified gene was ligated downstream of the 2.3 kb *GluB-1* promoter [19] (Fig. 1A). A *GluB-1* signal peptide sequence and the KDEL ER retention signal were attached to the N- and C-termini of the gene, respectively, followed by a 0.65-kb fragment of the *GluB-1* terminator. The resulting construct was cloned into the HindIII and EcoRI sites of binary vector pGPTV-35S-HPT [20] (Fig. 1A).

The expression plasmid was introduced into rice (*Oryza sativa* cv. Kita-ake) by *Agrobacterium tumefaciens*-mediated transformation as described previously [20]. The resulting transgenic plants were grown in a greenhouse at 28 °C with 12 h light/dark cycles.

Expression of recombinant Der p 1 in *Escherichia coli*. The DNA sequence encoding mature Der p 1 protein was cloned into the expression plasmid pET23d (+) (Novagen) at the NcoI and SacI sites, and transformed into *E. coli* BL21 (DE3) (Novagen). The expression and purification of Der p 1 was carried out with the QIAexpressionist™ kit according to the manufacturer's instructions (Qiagen).

Antibodies. *Escherichia coli*-expressed and purified Der p 1 was used to raise rabbit anti-Der p 1 antibody (Qiagen, Tokyo). Antibodies to Glutelin A, B, and C peptides, and 10K-, 13K-, and 16K-prolamins were prepared in our laboratory previously. Anti- α -amylase inhibitors, anti- α -globulin

and anti-glyoxalase I antibodies were kindly provided by Dr. T. Matsuda (Nagoya University, Nagoya, Japan). Horseradish-conjugated peroxidase-labeled anti-human IgE(ϵ) and anti-rabbit IgG were purchased from KPL (UK) and Cell Signaling (USA), respectively.

Determination of rice seed proteins. Rice seeds were ground to a fine powder with a Multibeads shocker (Yasui Kikai, Osaka, Japan), and total proteins were extracted in Urea–SDS buffer (50 mM Tris–Cl, pH 6.8, 8 M Urea, 4% SDS, 5% 2-mercaptoethanol, 20% glycerol) [14]. After separation by 12% SDS–PAGE and transfer to PVDF membrane (Millipore, USA), Der p 1 was detected with anti-Der p 1 antibody followed by a goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase. Der p 1 accumulation levels were determined using quantitative immunodot-blots with anti-Der p 1 antibody and Der p 1 produced in *E. coli* as a standard.

N-Glycosylation analysis. The glycan structures of Der p 1 were determined using enzyme digestion, reversed-phase (RP)-HPLC, size-fractionation (SF)-HPLC and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) [21].

Total seed proteins were extracted with Urea–SDS buffer and precipitated using a chloroform/methanol method [22]. The proteins were then resuspended in reaction buffer and incubated in the presence or absence of endoglycosidase H (Endo H) at 37 °C for 2 h according to the protocol provided by the manufacturer (New England Biolabs). The treated samples were analyzed by Western blotting assays with anti-Der p 1 antibody.

For preparation of glycans, the bands corresponding to 27 kDa glycosylated Der p 1 were excised from SDS–PAGE gels. The procedures for releasing N-glycans by hydrazinolysis, pyridylamination (PA) of oligosaccharides and purification/fractionation of PA-sugar chains on RP-HPLC or SF-HPLC were carried out as previously described [21].

The molecular masses of PA-sugar chains were determined by MALDI-TOF-MS. Elution positions of the sugar chain in each fraction of the RP-HPLC profile were compared with the elution positions of authentic PA-sugar chains of M9, M8A, M8B, M8C, M7A, and M5 (Takara Bio., Shiga, Japan). An authentic PA-sugar chain G1M9 was purchased from Masuda Chemical Industries (Kagawa, Japan). The sugar chain of G1M8 was prepared from G1M9 by digestion with recombinant α -1,2-mannosidase from *Aspergillus phoenicis* (Seikagaku Co., Tokyo, Japan). The sugar chain of M3X was prepared from *Arabidopsis thaliana* Col-0.

In vitro IgE binding capacity. Urea–SDS buffer-extracted seed proteins were separated on 12% SDS–PAGE, and bands corresponding to Der p 1 glycoprotein were excised and extracted with Urea–SDS buffer at 60 °C for 10 min. Identical amounts (0.5 μ g/lane) of the resulting protein and unglycosylated Der p 1 isolated directly from *E. coli* culture were loaded onto 12% SDS–PAGE gels under reducing conditions, and electroblotted onto a membrane as previously described [23]. The blot was probed with sera of seven mite-sensitive patients (1:1000 dilution) and further detected with horseradish peroxidase-conjugated anti-human IgE antibody (1:5000 dilution). Band density was quantified with NIH image software (U.S. National Institutes of Health).

Immunocytochemical electron microscopy. Eighteen to 20-day-old immature transgenic rice seeds were collected and used for immunocytochemical electron microscopic analysis. The procedure for preparing samples was exactly as described previously [11], except that the sections were reacted with primary anti-Der p 1 antibody (1:100 dilution) followed by secondary 20 nm gold-labeled goat anti-rabbit IgG Fc SP (EY Laboratories Inc., San Mateo, CA, USA) at a 1:200 dilution.

Results

Der p 1 is specifically accumulated in transgenic rice seed and is located in the ER-derived PB-I of endosperm cells

The KDEL ER retention signal attached to the C-terminus of Der p 1 stabilizes the protein and confines it within the ER, thus avoiding posttranslational modification with glycans by passage through the Golgi apparatus

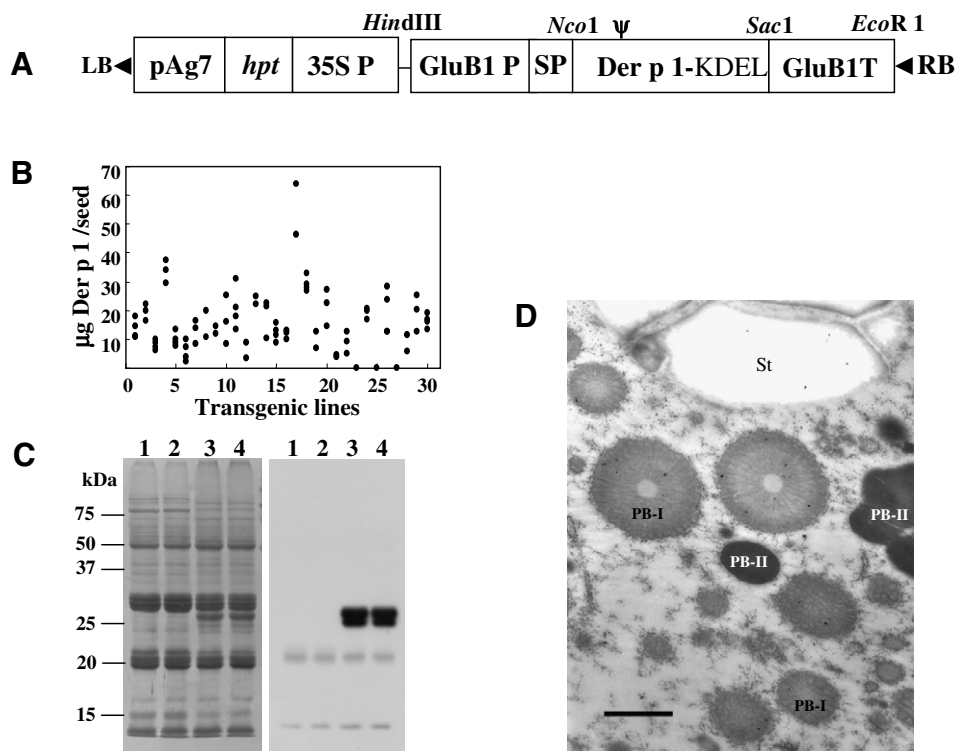


Fig. 1. Characterization of transgenic rice seed. (A) Diagram of the binary vector used for transformation. *hpt*, hygromycin phosphotransferase gene; pAg7, 3' terminal region of the agropine systrase gene; 35S P, CaMV 35S promoter; GluB1 P, SP, and GluB1 T represent the 2.3 kb glutelin B1 promoter, its signal peptide sequence, and 3' UTR, respectively. Der p 1-KDEL, Der p 1 containing a KDEL ER retention signal at its C-terminus; RB and LB are the right and left borders; Ψ , glycosylation site in Der p 1. (B) Accumulation levels. Four seeds from each independent line were used for the assay. (C) Western blotting analysis. Total proteins extracted from two independent non-transgenic cv. Kita-ake (lanes 1 and 2) and homozygous seeds of transgenic line 17 (lanes 3 and 4) were separated by 12% SDS-PAGE and immuno-detected with anti-Der p 1 antibody. Molecular size markers are indicated on the left. (D) Immuno-electron microscopy. Subcellular localization of Der p 1 in developing transgenic rice endosperm cells. 20 nm gold-labeled particles are specifically present inside ER-derived protein body I. PB-I, protein body I; PB-II, protein body II; St, starch granules; Bar = 1 μm .

(Fig. 1A) [18]. Thirty independent transgenic plants were regenerated, and four seeds from each line were used to determine accumulation levels by quantitative immunodot blot analysis. The accumulation levels varied among the transgenic lines examined, with the highest expression at 58 $\mu\text{g Der p 1/grain}$, or about 4% of total seed protein in line 17 (Fig. 1B). This line was self-pollinated through three generations and the resulting homozygous seeds were further analyzed by 12% SDS-PAGE. There were two additional CBB-visible bands with molecular masses of ca. 25–27 kDa that specifically immuno-reacted to anti-Der p 1 antibody in Western blots (Fig. 1C, lanes 3 and 4). These results suggest that the upper band was glycosylated, since there is a conserved N-linked glycosylation site in Der p 1 [5], whereas the 25 kDa lower band is the unglycosylated form of Der p 1. Immunocytochemical electron microscopy revealed that Der p 1 was specifically localized in ER-derived PB-I (Fig. 1D).

Der p 1 is posttranslationally modified with high-mannose-type glycans

To determine whether the upper band observed on SDS-PAGE gels was glycosylated Der p 1, total seed pro-

tein was treated with Endo H, which releases all mannose-type glycans. The upper, 27-kDa band shifted to the 25-kDa region, at about the same position as the lower band, indicating that the 27 kDa Der p 1 was N-glycosylated (Fig. 2).

To investigate the glycans linked to Der p 1 in more detail, PA-sugar chains were fractionated and characterized by both RP- and SF-HPLC, and a total of 16 fractions were obtained (data not shown). The molecular masses of compounds in the fractions were analyzed by MALDI-TOF-MS. Elution positions of each glycan in the RP-HPLC profile were compared with authentic PA-glycans (Table 1). The most abundant glycan structures released from Der p 1 were $\text{Man}_8\text{GlcNAc}_2$ [fraction 1 (m/z) 1821.56, ratio 26.3%], $\text{Man}_9\text{GlcNAc}_2$ [fraction 2 (m/z) 1983.39, ratio 12.9%], $\text{Glu}_1\text{Man}_8\text{GlcNAc}_2$ [Fraction 4-c (m/z) 1983.03, ratio 26.8%] and $\text{Glu}_1\text{Man}_9\text{GlcNAc}_2$ [fraction 5-b (m/z) 2145.26, ratio 25.4%]. Altogether they comprised 91.4% of the total glycans released from Der p 1. Some minor glycans, including $\text{Man}_7\text{GlcNAc}_2$ (1.6%), $\text{Man}_5\text{GlcNAc}_2$ (2.1%), and $\text{Man}_3\text{XylGlcNAc}_2$ (4.9%) were also found (Table 1). Modification with high-mannose-type oligosaccharides (mostly Man 8 and 9) clearly demonstrated that most of the Der p 1 protein was retained in

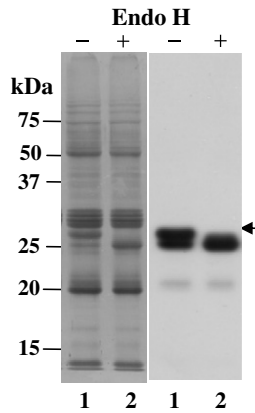


Fig. 2. Analysis of Der p 1 glycoprotein derived from rice seed. SDS-PAGE (left) and Western blot analysis (right) showed increased electrophoretic mobility after deglycosylation with Endo H (lane 2) in comparison with lane 1 (without Endo H digestion). The arrow indicates the glycosylated Der p 1 band.

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 ₈ GlcNAc ₂	26.3
2	1983.39	1983.78	Man ₉ GlcNAc ₂	12.9
4	a 1659.36	1659.50	Man ₇ GlcNAc ₂	1.6
	c 1983.03	1983.78	Glu ₁ Man ₈ GlcNAc ₂	26.8
5	b 2145.26	2145.92	Glu ₁ Man ₉ GlcNAc ₂	25.4
6	b 1143.90	1143.05	Man ₃ XylGlcNAc ₂	4.9
	e 1335.40	1335.22	Man ₅ GlcNAc ₂	2.1

ER and further deposited in ER-derived PB-I, or retrieved from the *cis*-Golgi by retrieval machinery. Furthermore, these MS data confirm the results of Endo H digestion.

Glycosylated Der p 1 had less *in vitro* IgE binding capacity than unglycosylated Der p 1

The binding reactivity of Der p 1-specific IgE in patient sera to glycosylated Der p 1 from transgenic rice seed and unglycosylated Der p 1 from *E. coli* culture was examined by immunoblotting [23]. Glycosylated Der p 1 showed significantly reduced IgE binding capacity in comparison with unglycosylated Der p 1 in the sera of all seven HDM patients (Fig. 3). The reduction in IgE binding due to glycosylation was highly variable among sera (Fig. 3B). Subjects P5 and P6, and to a lesser extent P7, had IgE binding activity to glycosylated Der p 1 only equal to about 30% that of unglycosylated protein.

Evaluation of inherited storage and allergic proteins of transgenic rice seed

To determine if there were any changes in internal seed proteins after the introduction of foreign recombinant Der p 1, Western blots were used to examine the expression levels of the major glutelin and prolamin storage proteins, and the α -amylase inhibitors, glyoxalase I and α -globulin, which have been identified as major rice seed allergens [24–26] (Fig. 4). There were no obvious changes among the mature acidic subunits of GluA, GluB or GluC, except for a slight increase of the GluC precursor in transgenic seed (Fig. 4A, lanes 3 and 4). The levels of 10K-, 13K-, and 16K-prolamins remained similar to those of non-transgenic seed (Fig. 4C). Among the three allergens, α -amylase inhibitor expression levels were reduced by about two-thirds in comparison with the non-transgenic control. Glyoxalase I and α -globulin accumulation was comparable with the non-transgenic control (Fig. 4B).

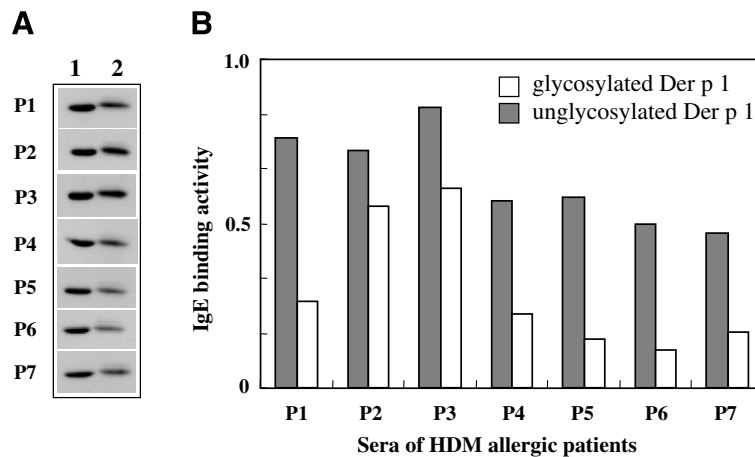


Fig. 3. Comparison of IgE-binding capacities between glycosylated and unglycosylated Der p 1 proteins. (A) Immunoblot assay of IgE binding reactivity. 0.5 μ g of rice seed-derived glycosylated Der p 1 and unglycosylated Der p 1 isolated from *E. coli* culture were analyzed using sera of seven HDM allergic patients. Lane 1 is unglycosylated and lane 2 is glycosylated Der p 1 protein. (B) Histogram of the IgE binding activity. Quantification of binding assays showing the variation in patient serum response to glycosylation of Der p 1.

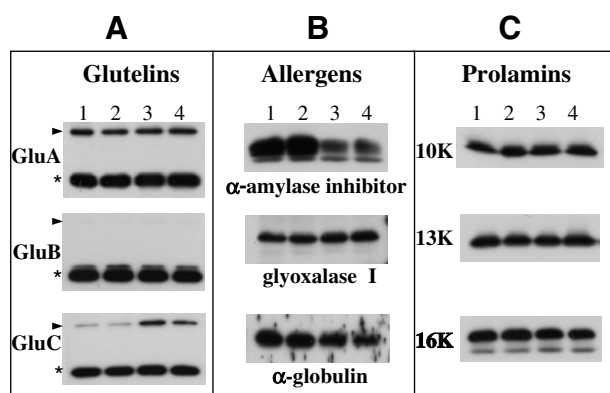


Fig. 4. Western blot analysis of native host seed proteins. 5 μ g of Urea–SDS buffer-extracted total seed protein was applied to each lane and membranes were immuno-probed with corresponding antibodies. Lanes 1 and 2 represent non-transgenic controls, and lanes 3 and 4 represent transgenic rice seed. Arrow head and asterisk represent glutelin precursor and acidic subunit, respectively.

Discussion

Allergen-specific immunotherapy is broadly recognized as the only curative treatment for the worldwide problem of allergic diseases caused by inhalant allergens from HDM. To benefit allergic patients, safer, and noninvasive mucosal routes of oral administration are considered to be the best alternative to conventional allergen-specific immunotherapy. In the present study, we developed transgenic rice plants that specifically express modified mature Der p 1 in seeds, thus providing a viable option for direct oral delivery for treatment of HDM allergy.

The clinical use of a rice seed-based edible vaccine containing HDM allergen Der p 1 will depend on the accumulation of the allergen at pharmacologically appropriate levels. Der p 1 protein accumulation in transgenic rice seed under the control of the strong endosperm specific *GluB-1* promoter [19] accounted for about 4% of total seed protein (Fig. 1B). To prevent the protein from being transported out of the ER lumen into protein storage vacuoles (PSV), known as PB-II, by a Golgi-dependent pathway where it would be further posttranslationally modified with complex-type N-glycans containing plant-specific β (1,2)-xylose and core α (1,3)-fucose residues that might be immunogenic in humans [17], an ER retention sequence (KDEL) was attached to the C-terminus of Der p 1. As expected, the Der p 1 proteins were efficiently restricted within ER-derived PB-I and carried high mannose glycans with structures common to mammalian cells (Figs. 1D and 2, Table 1). Subcellular targeting of Der p 1 protein could thus be achieved by the combination of a specific signal sequence and the KDEL retention sequence.

It should be noted that Der p 1 modified with high-mannose glycans showed significantly reduced IgE binding activity in comparison with its unglycosylated counterpart *in vitro* (Fig. 3). One possible explanation for this result is that the consensus motif for N-glycosylation may reside just inside the IgE epitope region, thus glycosylation with

high-mannose glycan chains would certainly alter IgE binding capacity. Alternatively, the attachment of glycan to Der p 1 may cause a conformational change, resulting in reduced IgE binding activity.

The use of a transgenic plant as a platform for the production of pharmacologically active products is limited by the ability of the plant not only to produce the product, but also to maintain its own viability, reproductive capacity, and other agronomic characteristics. To determine whether native seed proteins would be affected by the expression and accumulation of heterologous Der p 1, transgenic and non-transgenic rice seed proteins were compared. The expression of two major storage proteins, glutelins and prolamins, did not differ significantly between transgenic and non-transgenic rice seeds. However, the allergenic protein α -amylase inhibitor was significantly reduced, a finding that will need to be further explored (Fig. 4). Plant-derived Der p 1 has the desirable properties of high accumulation in rice seed, modification with non-immunogenic high-mannose glycans, and markedly reduced IgE binding activity, making rice oral delivery a much safer approach than conventional immunotherapy. Animal model studies are the next stage of clinical evaluation of the immunogenicity and possible allergenicity of transgenic rice containing Der p 1.

Acknowledgments

This work was supported by the research grant, “Functional analysis of genes relevant to agriculturally important traits in the rice genome IP2001” from the Ministry of Agriculture, Forestry and Fisheries of Japan to F. Takaiwa.

References

- [1] W.R. Thomas, W. Smith, House-dust-mite allergens, *Allergy* 53 (1998) 821–832.
- [2] E.R. Tovey, M.D. Chapman, T.A. Platts-Mills, Mite faeces are a major source of house dust allergens, *Nature* 289 (1981) 592–593.
- [3] L. Gough, E. Campbell, D. Bayley, G. Van Heeke, F. Shakib, Proteolytic activity of the house dust mite allergen Der p 1 enhances allergenicity in a mouse inhalation model, *Clin. Exp. Allergy* 33 (2003) 1159–1163.
- [4] T. Takai, T. Kato, M. Ota, H. Yasueda, T. Kuhara, K. Okumura, H. Ogawa, Recombinant Der p 1 and Der f 1 with *in vitro* enzymatic activity to cleave human CD23, CD25 and α_1 -antitrypsin, and *in vivo* IgE eliciting activity in mice, *Int. Arch. Allergy Immunol.* 137 (2005) 194–200.
- [5] K.Y. Chua, G.A. Stewart, W.R. Thomas, R.J. Simpson, R.J. Dilworth, T.M. Plozza, K.J. Turner, Sequence analysis of cDNA coding for a major house dust mite allergen, Der p 1. Homology with cysteine proteases, *J. Exp. Med.* 167 (1988) 175–182.
- [6] G. Hoyne, T. Bourne, N. Kristensen, C. Hetzel, J. Lamb, From epitopes to peptides to immunotherapy, *Clin. Immunol. Immunopathol.* 80 (1996) S23–S30.
- [7] T. Hiroi, F. Takaiwa, Peptide immunotherapy for allergic diseases using a rice-based edible vaccine, *Curr. Opin. Allergy Clin. Immunol.* 6 (2006) 455–460.
- [8] G. Giddings, G. Allison, D. Brooks, A. Carter, Transgenic plants as factories for biopharmaceuticals, *Nat. Biotechnol.* 18 (2000) 1151–1155.

- [9] E. Stoger, J.K. Ma, R. Fischer, P. Christou, Sowing the seeds of success: pharmaceutical proteins from plants, *Curr. Opin. Biotechnol.* 16 (2005) 167–173.
- [10] A.M. Walmsley, C.J. Arntzen, Plants for delivery of edible vaccines, *Curr. Opin. Biotechnol.* 11 (2000) 126–129.
- [11] F. Takaiwa, H. Takagi, S. Hirose, Y. Wakasa, Endosperm tissue is good production platform for artificial recombinant proteins in transgenic rice, *Plant Biotechnol. J.* 5 (2007) 84–92.
- [12] H. Takagi, T. Hiroi, L. Yang, Y. Tada, Y. Yuki, K. Takamura, R. Ishimitsu, H. Kawauchi, H. Kiyono, F. Takaiwa, A rice-based edible vaccine expressing multiple T cell epitopes induces oral tolerance for inhibition of Th2-mediated IgE responses, *Proc. Natl. Acad. Sci. USA* 102 (2005) 17525–17530.
- [13] K. Sugita, S. Endo-Kasahara, Y. Tada, L. Yang, H. Yasuda, Y. Hayashi, T. Jomori, H. Ebinuma, F. Takaiwa, Genetically modified rice seeds accumulating GLP-1 analogue stimulate insulin secretion from a mouse pancreatic β -cell line, *FEBS Lett.* 579 (2005) 1085–1088.
- [14] L. Yang, Y. Tada, M.P. Yamamoto, H. Zhao, M. Yoshikawa, F. Takaiwa, A transgenic rice seed accumulating an anti-hypertensive peptide reduces the blood pressure of spontaneously hypertensive rats, *FEBS Lett.* 580 (2006) 3315–3320.
- [15] T. Nochi, H. Takagi, Y. Yuki, L. Yang, T. Masumura, M. Mejuma, U. Nakanishi, A. Matsumura, A. Uozumi, T. Hiroi, S. Morita, K. Tanaka, F. Takaiwa, H. Kiyono, Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination, *Proc. Natl. Acad. Sci. USA* 104 (2007) 10986–10991.
- [16] F. Takaiwa, Transgenic rice seed as a nutraceutical delivery system, *CAB reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources* 2 (2007) 041.
- [17] V. Gomord, P. Chamberlain, R. Jefferis, L. Faye, Biopharmaceutical production in plants: problems, solutions and opportunities, *Trends Biotechnol.* 23 (2005) 559–565.
- [18] V. Gomord, C. Sourrouille, A.C. Fitchette, M. Bardor, S. Pagny, P. Lerouge, L. Faye, Production and glycosylation of plant-made pharmaceuticals: the antibodies as a challenge, *Plant Biotechnol. J.* 2 (2004) 83–100.
- [19] L.Q. Qu, F. Takaiwa, Evaluation of tissue specificity and expression strength of rice seed component gene promoters in transgenic rice, *Plant Biotechnol. J.* 2 (2004) 113–125.
- [20] F. Goto, T. Yoshihara, N. Sugimoto, S. Toki, F. Takaiwa, Iron fortification of rice seed by the soybean ferritin gene, *Nat. Biotechnol.* 17 (1999) 282–286.
- [21] K. Fujiyama, A. Furukawa, A. Katsura, R. Misaki, T. Omasa, T. Seki, Production of mouse monoclonal antibody with galactose-extended sugar chain by suspension cultured tobacco BY2 cells expressing human β (1,4)-galactosyltransferase, *Biochem. Biophys. Res. Commun.* 358 (2007) 85–91.
- [22] D. Seigneurin-Berny, N. Rolland, J. Garin, J. Joyard, Differential extraction of hydrophobic proteins from chloroplast envelope membranes: a subcellular-specific proteomic approach to identify rare intrinsic membrane proteins, *Plant J.* 19 (1999) 217–228.
- [23] T. Takai, T. Yokota, M. Yasue, C. Nishiyama, T. Yuuki, A. Mori, H. Okudaira, Y. Okumura, Engineering of the major house dust mite allergen Der f 2 for allergen-specific immunotherapy, *Nat. Biotechnol.* 15 (1997) 754–758.
- [24] M. Nakase, T. Adachi, A. Urisu, T. Miyashita, A.M. Alvarez, S. Nagasaka, N. Aoki, T. Matsuda, Rice (*Oryza sativa* L.) α -amylase inhibitors of 14–16 kDa are potential allergens and products of a multigene family, *J. Agric. Food Chem.* 44 (1996) 2624–2628.
- [25] G.G. Limas, M. Salinas, I. Moneo, S. Fischer, B. Wittmann-Liebold, E. Mendez, Purification and characterization of ten new rice NaCl-soluble proteins: identification of four protein-synthesis inhibitors and two immunoglobulin-binding proteins, *Planta* 181 (1990) 1–9.
- [26] Y. Usui, M. Nakase, H. Hotta, A. Urisu, N. Aoki, K. Kitajima, T. Matsuda, A 33-kDa allergen from rice (*Oryza sativa* L. *Japonica*). cDNA cloning, expression, and identification as a novel glyoxalase I, *J. Biol. Chem.* 276 (2001) 11376–11381.