

Seed-based oral vaccines as allergen-specific immunotherapies

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Plant-based vaccines have advantages over conventional vaccines in terms of scalability, lack of requirement for cold chain logistics, stability, safety, cost-effectiveness and needle-free administration. In particular, when antigen is expressed in seeds, high production is possible and immunogenicity is not lost even if stocked at ambient temperature for several years. Induction of immune tolerance (desensitization) to allergen is a principle strategy for controlling allergic diseases, and is generally carried out by subcutaneous injection. Seed-based oral administration offers a straightforward and inexpensive alternative approach to deliver vaccines effectively to the GALT without loss of activity. Consumption of transgenic seeds containing modified hypo-allergenic tolerogen or T-cell epitope peptides derived from allergens has no or very few severe side effects and can induce immune tolerance leading to reduction of allergen-specific IgE production, T-cell proliferation and release of histamine. Suppression of allergen-specific clinical symptoms results. Thus, seed-based allergy vaccines offer an innovative and convenient allergen-specific immunotherapeutic approach as an alternative to conventional allergen-specific immunotherapy.

Introduction

Type I immunoglobulin (Ig)E-mediated allergy, a state of hypersensitivity to allergens, is becoming an important global health problem. In industrialized countries, more than 25% of the population suffers from some kind of allergic disorder such as asthma, allergic rhinitis, conjunctivitis, atopic dermatitis, etc., the prevalence of which is steadily increasing.^{1,2} Treatment strategies for these allergic diseases generally involve pharmacotherapy using different drugs such as systemic antihistamines and intranasal corticosteroids. However, although these approaches reduce the clinical symptoms, they are not curative. T-cells are key regulators of the pathogenesis of allergic disease³ and it is well-established that allergen-specific immunotherapy (SIT) is the only way to modulate these immune reactions. When successful, therapeutic effects persist for many years without further treatment.^{4,5} Conventional SIT has been practiced by systemic immunization using crude allergen extracts for almost a century.⁶

Success can be achieved by repeated subcutaneous injections of increasing doses of native allergen extracts over a period of at least 2 to 3 years. This treatment is sometimes accompanied by severe side-effects, such as anaphylaxis, which is caused by capture of allergen together with specific anti-allergen IgE on the surface of mast cells and basophils via Fc receptors (FcεRI) for IgE. The development of safer, more effective and convenient SIT has therefore long been desired. Safer and noninvasive sublingual, intranasal and oral immunotherapy is considered an alternative to subcutaneous application. To address these requirements, seed-based edible allergy vaccines have been developed recently, containing modified recombinant allergens with reduced or no allergenicity, or allergen-derived T-cell epitope peptides. These oral vaccines offer simple, safe and cost-effective immunotherapy.

Allergic Disease and Allergen-specific Immunotherapy

Allergic inflammation is caused by T helper 2 (Th2) cytokines (e.g., IL-4, IL-5, IL-13) secreted from CD4⁺ Th2 cells in response to recognition of inhaled allergens, and is characterized by allergen-specific enhanced IgE production by B-cells, degradation of mast cells and eosinophil-mediated inflammation.⁷ CD4⁺ T cell activation requires the interaction of the antigen-specific T-cell receptor with peptide-MHC (major histocompatibility complex) class II complexes on antigen-presenting cells (APCs) and the ligation of costimulatory receptors by ligands present on APCs. Initial exposure of naïve T cells to allergens by APCs mainly leads to the activation of allergen-specific Th2 cells and to IgE synthesis, is known as allergic sensitization. Subsequent exposures to the same allergen cause inflammatory cell activation and mediator release, responsible for early and late allergic responses.

The mechanisms of SIT are complex and may differ depending on the particular allergens and route of immunization. Immune tolerance is believed to be a consequence of either immune deviation of T cells to an anergic state or the induction of suppressive mechanisms mediated by regulatory T cells. Inflammatory cell recruitment and activation and mediator secretion, are reduced by immunotherapy.

An imbalance between Th2 and Th1 cells responses is involved in allergy. Regulatory T cells (Tregs) such as Tr1, Th3 and CD25⁺ cell subsets of CD4⁺ T cells are implicated in suppression of allergic responses. They may control Th2 cell responses either through production of the inhibitory cytokines IL-10 and

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TGF β , or by direct cell-cell contact through membrane-bound TGF β or cytotoxic T-lymphocyte antigen-4 (CTLA-4).⁸ In addition, Tregs potently suppress IgE production and directly or indirectly suppress the activity of effector cells of allergic inflammation, such as eosinophils, basophils and mast cells. Another newly identified type of CD4⁺ T cell, the Th17 cell, associated with neutrophilic inflammation, may also be involved.

Mucosal Vaccines

Mucosal immunization is advantageous as a vaccination strategy because of its simplicity and low expense. The administration route using mucosal application (oral, sublingual, nasal delivery) is preferable to subcutaneous injection, and is convenient and safe for treatment of allergic patients. The necessity to visit the clinic frequently for injections is obviated and there is no pain or side-effects of injecting. This makes the immunization practice more acceptable to many patients.⁹

It has been estimated that the surface area of human mucosae is up to 400 m² and that the mucosal-associated lymphoid tissue represents about 50% of the total immune system, producing 70% of the body's immunoglobulin in the form of sIgA. The mucosal immune system not only provides a front line of defense against invading pathogens by its induction of positive immune responses but must also maintain immunologic unresponsiveness (immune tolerance) to most dietary proteins and commensal bacteria.¹⁰⁻¹² Mucosal immunization has the potential advantage of stimulating the induction of both mucosal and systemic antigen-specific immune responses or to result in the generation of a state of systemic unresponsiveness termed oral tolerance.

Oral tolerance is clearly the more common immunological response than the generation of an antigen-specific active immunity, because it is the default response to food antigens and commensal bacteria.¹³ Oral administration of large or continuous doses of antigens has been shown to be inclined to induce immune tolerance rather than sensitization. Taken together, administration of plant (seed)-based oral vaccine containing allergen would be likely to represent a potentially powerful strategy for the control of allergic disease caused by aeroallergens such as pollens.¹⁴

Mucosal Immune System

At least three unique subsets of dendritic cells (DCs) with a distinct tissue distribution in PPs (CD11⁺ DC in the subepithelial domain region (SED), CD8a⁺ DC in the T cell-rich interfollicular regions (IFRs) and double-negative CD4⁺CD8a⁻ DC in SED and IFRs) are implicated in immune tolerance as well as in the induction of immune responses against pathogens.^{15,16} IL-10-producing immature DCs contribute to immunologic tolerance through inducing Tr1 regulatory cells, whereas fully mature DCs stimulate regulatory CD4⁺CD25⁺ T cells. It has recently been demonstrated that retinoic acid (RA) produced by intestinal DCs is involved in the induction of Foxp3⁺ Tregs in cooperation with TGF β .¹⁷ Especially DCs from the lamina propria promote high level conversion of this Treg cell relative to lymphoid organ-derived DCs. In addition, either IL-23 or IL-27 plus IL-6 and

TGF β is implicated in determining differentiation into effector Th17 or IL-10-producing type 1 regulatory cells (Tr1) from naïve T cells, respectively.^{18,19} Activation of Th17 cells leads to inflammation, whereas induction of Tr1 results in immune suppression. Foxp3⁺ Tregs and Tr1 participate in immune tolerance and therefore induction of these regulatory T cells is very important for controlling allergy through mucosal routes.²⁰

Antigens administered via the oral route are taken up preferentially by M cells in the follicle-associated epithelium (FAE) covering the Peyer's patches as part of the gut-associated lymphoid tissue (GALT) of the small intestine, and are then presented (delivered) to subepithelial DCs in adjacent mucosal T cell areas. As examples of other routes, mucosal DCs may directly sample antigen by extending processes through the epithelium (tight junctions) into the lumen or epithelial cells may act as non-professional APC.^{21,22}

Mechanisms Involved in the Induction of Oral Tolerance

Several immunological mechanisms have been implicated in the induction of oral tolerance, depending on the frequency, duration and dose of antigen administered.^{23,24} Repeated low dose administration of antigen favors tolerance driven by Treg cells, whereas single high doses favor anergy²⁵ or cell deletion-driven tolerance.²⁶ T lymphocyte anergy occurs through T cell antigen receptor ligation in the absence of co-stimulatory signals, and is prevented by T cell receptor ligation together with cognate interactions between CD80 and/or CD86 (B7 family) on the APC with CD28 or CTLA-4 (CD28 family) on the T cells.^{27,28} Interactions involving co-stimulatory molecules of the B7/CD28 families play a key role in the regulation of T cell activation and tolerance. Participation of additional, new B7 and CD28 family members as co-stimulation factors has been discovered recently, and their involvement in immune regulation has been reported. Cell deletion by apoptosis is caused by CD95 (FAS)-dependent caspase activation.²⁹ In contrast, repeated administration of low doses of allergens results in mucosal immune tolerance termed active suppression. This is mediated by induction of regulatory T cells such as Tr1, Th3, Foxp3⁺CD4⁺CD25⁺ cells and CD8⁺ regulatory T cells.^{30,31} IL-10-producing immature DCs contribute to this form of immunologic tolerance through the induction of Tr1 regulatory cells, whereas fully mature DCs stimulate regulatory CD4⁺CD25⁺ T cells.

It has been shown that peripheral conversion of CD4⁺ T cells to Tregs occurs primarily in the GALT. DCs purified from the lamina propria promote a high level of Treg cell conversion relative to lymphoid organ-derived DCs. This enhanced conversion is dependent on TGF β and retinoic acid.

Mechanisms Underlying Allergen-Specific Immunotherapy

SIT has been conventionally performed by subcutaneous injection with increasing dose of allergen extract as the only curative treatment for type I allergic disease. Clinical effects obtained by SIT are (1) decrease in allergen-specific IgE, (2) increase in the

level of allergen-specific IgA and IgG4 isotypes which block the binding of the allergen-specific IgE to mast cells and basophils and (3) reduction of specific T-cell proliferation.³²⁻³⁵ These lines of evidence regarding impact of treatment on immune reactions are explained as follows: in response to parenteral (systemic) administration of allergen, different populations of regulatory T cells (CD4⁺CD25⁺, Tr1 and Th3) producing the suppressive cytokines IL-10 and TGFβ are induced. This is linked to the downregulation of allergen-specific T cell proliferation and decreased secretion of Th1-type (IFNγ, IL-2) and Th2-type (IL-4, IL-5, IL-13) cytokines, resulting in reduction of allergen-specific IgE and upregulation of IgG4 and IgA. This leads to alleviation of clinical symptoms as the hallmark of conventional SIT. It is known that IL-10 promotes immunoglobulin isotype switching to IgG4, and that TGFβ mediates switching to IgA. IL-10 also suppresses the activity of a number of effector cells including mast cells, eosinophils and basophils, resulting in decreased amounts of mediator release and depressed T-cell responses. Furthermore, immune deviation in favor of allergen-driven Th1 responses has also been observed. Recently, a new subset of Th cells, designated Th17 cells, which has major functions in tissue inflammation and host protection against extracellular pathogens, has been reported to be involved in allergic inflammation. The differentiation of precursors into Th17 or Treg cells follows a reciprocal pathway, with either IL-27 or IL-23 in addition to IL-6 and TGFβ giving rise to Tregs suppressing allergy or Th17 cells initiating tissue inflammation, respectively.

Advantages of Plant-Based Oral Vaccines

Plant-based vaccines can be generated by three production strategies (1) stable transgenic plants (2) chloroplast expression (3) transient expression using RNA virus vectors or Agrobacterium-mediated transient expression by Agro-infection.³⁶⁻³⁸ Depending on which expression systems or host plants are used, expression levels can be adjusted as required. When the latter two strategies are used for expression, higher yields can generally be obtained. Furthermore, an advantage of using virus vectors is rapidity. Transgene products can be obtained by transient expression within one week of inoculation. However, host plants suitable for use with these RNA virus vector systems or Agrobacterium-mediated transient expression systems are restricted to a few crops, because introduction of the gene is performed by vacuum infiltration or syringe. Furthermore, antigen products have to be extracted and purified. Downstream processing of recombinant products is very expensive, amounting to more than 80% of the total expense.

When vaccine antigens are expressed in stable transgenic plants by nuclear transformation, product yields are usually <1% of total soluble proteins.³⁹ Enhancement of accumulation levels is therefore crucial for practical use (commercialization). Nonetheless, seed-based production offers an ideal production platform as a bioreactor for recombinant proteins,^{40,41} because the seed is inherently specialized for storage of proteins, carbohydrates and oils, which are used as nutrients for the germinating seedling. The most important point is that seed containing antigen can be directly used as a mucosal oral vaccine without

purification. Edible transgenic plant tissue used as food or feed supplement offers the possibility of allowing direct oral delivery of plant-based vaccines, eliminating the need for expensive downstream protein purification and processing.

Seed storage proteins (SSP) are specifically and predominantly synthesized at markedly higher levels during seed maturation.⁴² That is, SSP genes are some of the most highly expressed genes in the plant genome. Furthermore, SSPs are stably deposited in protein bodies, which protect the contents from proteolysis by proteases, thus ensuring that PBs provide ample space for storage of recombinant product. Desiccation of seeds observed in the dormancy stage is also closely related to long-term room temperature stability of the recombinant product expressed in seed.

Endosperm in cereal monocot crop seed is a highly specialized dedicated tissue, and accounts for 80 to 90% of grain weight. It is important to note that the specific expression of transgene product in this tissue has no potentially detrimental effects on development of the host plant due to lack of expression in vegetative tissues. This is in contrast to recombinant products driven by dicot SSP gene promoters which are expressed in the embryo and cotyledon of seeds and sometimes have inhibitory effects on embryo growth. Thus, such properties of endosperm as a deposition site afford a good production platform for high level accumulation of recombinant proteins.⁴³ Screening of strong endosperm-specific promoters is critical to enable higher levels of accumulation of recombinant proteins in seed to be achieved.

Molecular Approaches to Enhance Accumulation Levels

The yield of recombinant products in transgenic plants has been increased by optimizing parameters that determine transcription, translation and post-translational modification.^{44,45} Enhancing strategies include strong seed-specific promoters, codon optimization, translation fusion with storage proteins, targeting expression to specific cell types or sub-cellular localization, introgression of transgenes into germplasm more suitable for high-level expression.

Accumulation levels are primarily determined at the transcription level, which can be enhanced by employing tissue-specific promoters to enable expression of genes in targeted deposition tissues of the plants. Selection of a strong tissue-specific promoter capable of expression in the desired tissue (endosperm) is preferable for high level expression of antigens. For expression of antigens in transgenic rice seed, strong endosperm-specific promoters such as those for the major storage protein glutelin (GluB-1, GluB-4), the 26 kDa globulin or 10 kDa and 16 kDa prolamin have been utilized⁴⁶ (Fig. 1).

In order to improve the rate and fidelity of translation, it is very important to adapt the coding sequence of the target gene to the codon bias used in the highly expressed genes in the host plant seed. It must be noted that codon bias varies even among different tissues of the same host species. Several rare codons should be avoided in codon optimization. A 5–100-fold increase in protein expression has been observed after codon optimization. Furthermore, the 5' and 3' untranslated regions which are involved in translation efficiency or mRNA stability must also be

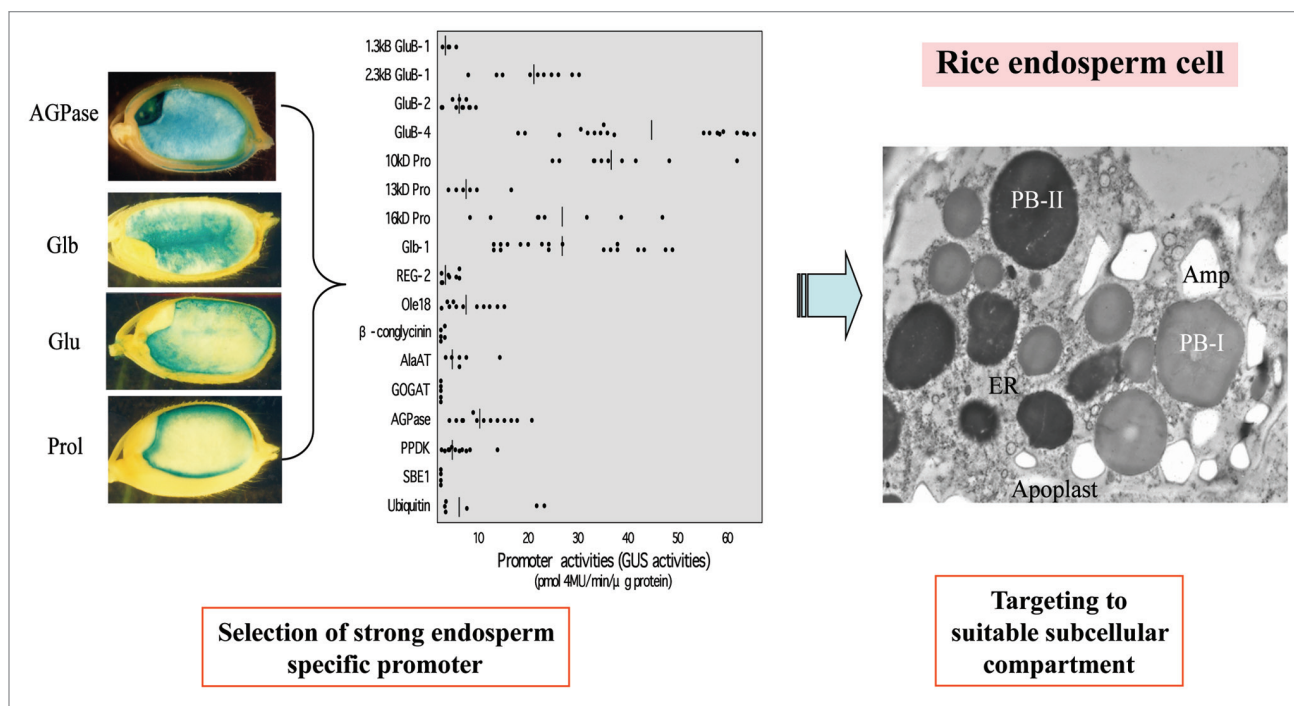


Figure 1. Tools for high expression of antigen in rice endosperm. (A) Histochemical analysis of GUS expression directed by rice ADP-glucose pyrophosphorylase (AGPase), 26 kD globulin (Glb), 2.3 kb glutelin GluB-1 (Glu), 10 kD prolamin (Pro) promoters. (B) GUS activities in maturing seeds of transgenic rice 17 days after flowering, driven by promoters of seed protein genes. (C) Intracellular structure of rice maturing endosperm 17 days after flowering.

considered. The complete 5' and 3' untranslated regions derived from SSPs are usually attached to the coding sequence of recombinant proteins (allergens).

Post-translational glycosylation may be affected by subcellular localization. N-glycans of recombinant products localized in the ER-derived PB are high mannose type, different from those (complex type N-glycans) deposited in PSV trafficked through Golgi-dependent transport pathways. Differences in N-glycan structure can influence function (safety, efficacy) of the recombinant product. Removal of the plant-specific α 1,3-linked fucose or β 1,4-linked xylose from complex N-glycans of the high mannose glycoproteins in plant-made vaccines can be carried out by suppression of the genes encoding these specific enzymes or by targeting to the ER by addition of KDEL. This is possible because complex N-glycans from plants still elicit glycan-specific IgE and IgG responses in humans.⁴⁷

Targeting to specific intracellular compartments has been recognized as a key factor determining the overall stability and yield of recombinant proteins in plants, because subcellular destination strongly influences the process of folding, assembly and post-translational modification. Targeting of recombinant proteins to the desired intracellular compartments can be controlled by attaching the targeting signal sequences at the N- or C-terminal end of the target protein.^{48,49} In general, when expressed as secretory proteins by virtue of the N-terminal ER signal peptide, recombinant proteins are relatively highly accumulated. An advantage of production as secretory proteins may be due to the presence of high amounts of chaperones and folding enzymes required for folding and assembly in the ER lumen. In contrast, cytosolic accumulation

generally results in very low levels compared to other intracellular compartments, except in certain specific cases. In the case of expression in seed, targeting to PBs is suitable for high and stable accumulation. In cereal seed there are two types of PBs, protein storage vacuoles (PSV) and the ER-derived protein body (ER-PB). Recombinant products deposited in the PSV are transported by Golgi- or by precursor accumulating (PAC) vesicle mediated trafficking. These are the largest subcellular compartments in the cell, thus providing ample deposition space for transgenic products. Extracellular (intercellular) spaces, termed apoplasts, which are surrounded by the cell wall, have a limited capacity in seed, but can be targeted by bulk flow of secretory protein using chitinase or α -amylase signal peptide. Retention at the ER caused by adding an ER retention signal (KDEL or HDEL) at the C-terminus of the recombinant protein has been reported to increase the accumulation of recombinant product by retrieving proteins from cis-Golgi to the ER. When targeted to the ER by the ER retention signal, products are accumulated in ER-derived PB. Where there is no retention signal, recombinant protein follows the default pathway and may undergo substantial degradation. The endosperm cell contains large amounts of starch in amyloplasts, which may also be candidate deposition compartments for foreign products. It was reported that *E. coli* LTB were unexpectedly targeted to amyloplasts as starch granules of maize endosperm by using a common signal peptide sequence as the targeting signal.⁵⁰ However, accumulation levels were extremely low, although targeting recombinant proteins to starch granules could still potentially be very useful for the oral or mucosal delivery of vaccines. Similar alterations of intracellular localization have often been observed

irrespective of the presence of target signal, when recombinant proteins are expressed in seeds.⁵¹ This is attributed to the fact that aggregation properties of storage proteins can influence traffic in the endomembrane system through intermolecular interactions via disulfide bonds.⁵²

Tissues suitable for stable deposition of recombinant products are known to be different between rice and maize.⁴³ Recombinant products expressed under the control of the constitutive promoter are highly and stably accumulated in endosperm tissue comprising about 90% of the total rice seed weight. In contrast, the embryo, representing only 20% of the total maize kernel weight, rather than the endosperm, provides the main deposition site of recombinant products (80–90% of total product) in this species. Stability of expressed recombinant proteins in the tissue may also differ on a case-by-case basis.

Recombinant Products with Reduced Allergenicity

There would be a great advantage to replacing natural allergen extracts used for induction of immune tolerance (desensitization) by recombinant proteins with reduced allergenicity, because the former can bind to specific IgE on mast cells and basophils, leading to anaphylactic side-effects. Therefore, new approaches to SIT using modified hypoallergenic antigens are required in order to increase the safety of immunotherapy by reducing the risks of anaphylactic reactions.^{53–56} It has been reported that binding of allergen to the specific IgE is determined by a continuous stretch of amino acids or by conformational structures. Thus, in order to develop such ideally hypoallergenic tolerogens with low IgE binding activity to use as immune modulators, deletion, site-directed mutagenesis, fragmentation, oligomeric formation or molecular shuffling have all been tested. Recombinant hypoallergenic allergen derivatives exhibit reduced IgE reactivity and hence do not induce IgE-mediated side effects when administered to allergic patients. For example, mutagenesis of the major birch pollen allergen, Bet v 1,⁵⁷ and group 5 pollen allergen of timothy grass, Phl p 5b,⁵⁸ and ryegrass, Lol p 5,⁵⁹ resulted in significant reduction of IgE binding activity. Disruption of conformational epitopes (tertiary structure required for specific IgE binding) can be achieved either by expressing allergen molecules as fragments, reconstruction (reassembling) by molecular shuffling or by mutagenesis of conserved cysteine residues involved in disulphide bonds. Thus, deletion of disulphide bonds required for tertiary structure formation from grass pollen allergen Phl p 7,⁶⁰ house dust mite allergen Der f 2,⁶¹ and rubber latex allergen Hev b 6.01,⁶² resulted in a reduction of between 40% to 100% of IgE reactivity. Reconstruction of timothy grass pollen allergens, Phl p 1,⁶³ and Phl p 2,⁶⁴ reduced their IgE reactivity to more than 85%. Fragmentation or formation of oligomers composed of multiple copies of the same allergen also led to marked reduction of allergic properties, as tested in the Bet v 1 allergen (Bet v

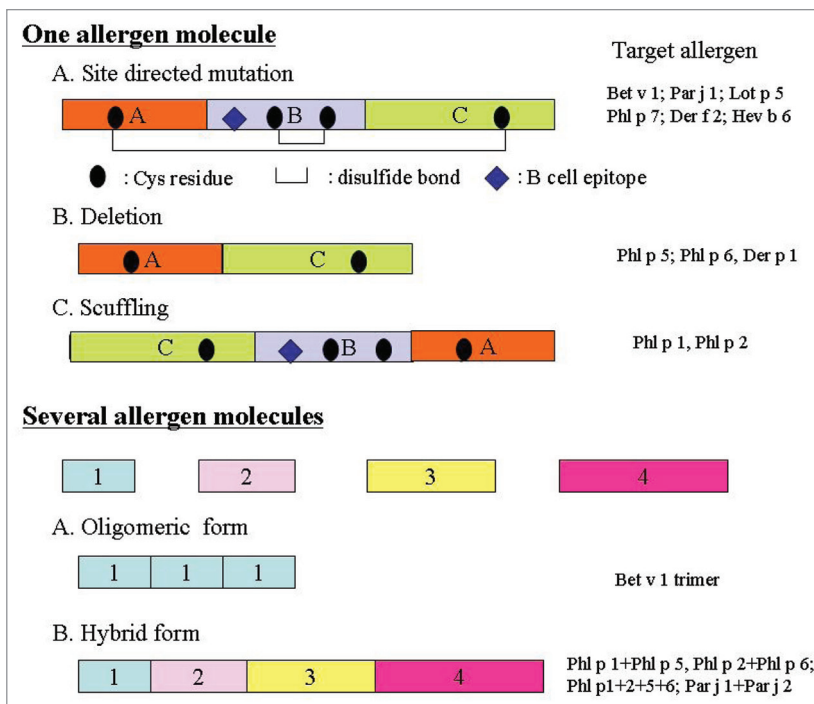


Figure 2. Design of hypoallergy vaccines.

1 trimer) system.⁶⁵ Vaccination with these genetically-modified recombinant birch pollen allergen derivatives induced production of protective IgG4 antibodies against specific IgE, which was accompanied by improvement of clinical symptoms and skin sensitivity. Furthermore, hybrid molecules consisting of multiple allergens have been developed for several important allergen sources.⁶⁶ Vaccination with hybrid molecules composed of five timothy grass pollen allergens significantly improved grass pollen-induced immediate-type allergic symptoms and resulted in reduced consumption of medication⁶⁷ (Fig. 2).

On the other hand, peptide immunotherapy using dominant T-cell epitopes derived from allergens offers a safe treatment for controlling allergic disease because regions involved in allergenicity (i.e., responsible for reactivity with IgE) can be completely excised while ensuring retention of immunogenicity.^{68,69} It should be noted that designing effective peptide vaccines for human immunization is complicated by the polymorphism of the MHC and the antigenic complexity of allergens. To overcome this problem, hybrid peptides comprising several major T-cell epitopes suitable for different genetic backgrounds have been generated. Peptide immunotherapy has been performed by systemic injection or intra-nasal application to control several allergic diseases. Efficacy by oral administration was first demonstrated in a mouse model with a T cell epitope peptide derived from cedar pollen Cry j 2.⁷⁰ Clinical trials have been performed with peptides from the cat major allergen (Fel d 1) and bee venom PLA (Api ml) allergens. When evaluated as vaccines for subcutaneous immunotherapy of allergic patients, Tr1-type allergen-specific immune responses were induced, together with decreased production of the Th2-type cytokines IL-4, IL-5 and IL-13.

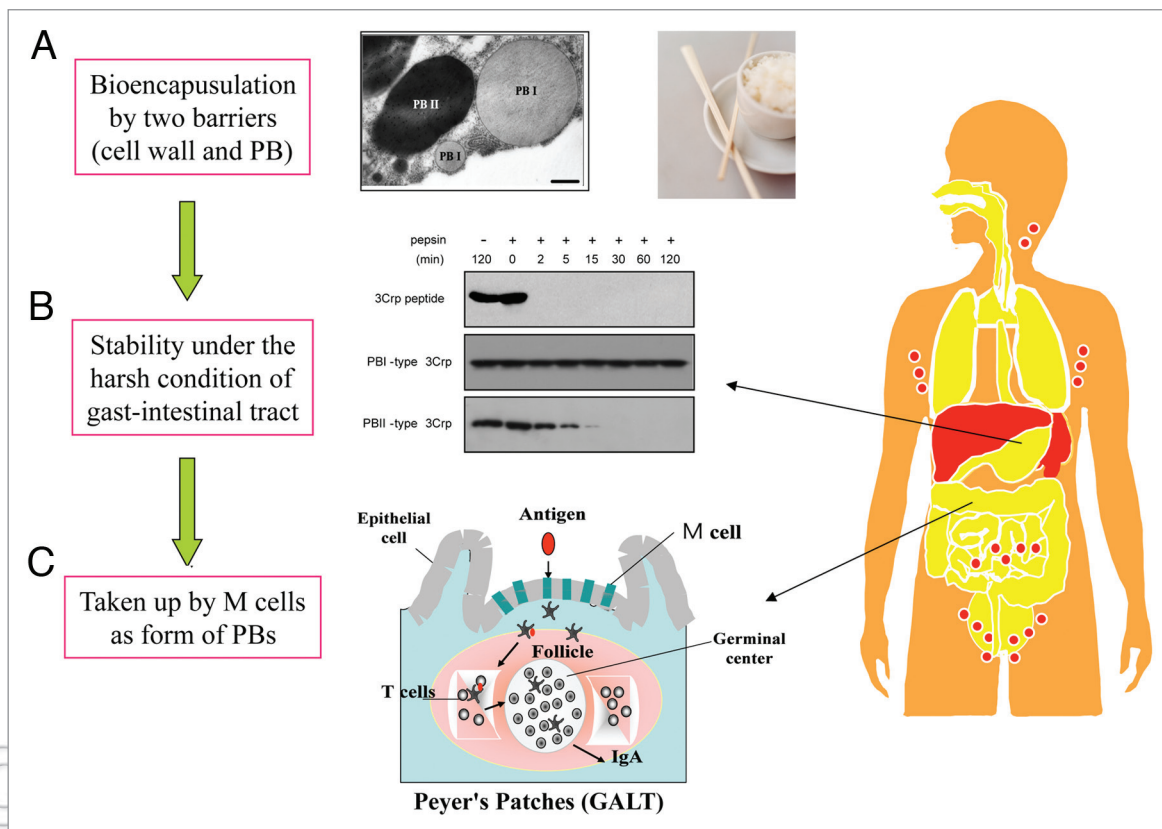


Figure 3. Advantages of seed-based allergy vaccines via PB as a delivery vehicle to GALT. (A) PB structure in rice endosperm cell. (B) In vitro digestion of the three forms of 3Crp with pepsin. (C) Morphological structure of Peyer's patches.

PB Provides an Excellent Delivery System to GALT

Oral delivery of plant-based antigens induces not only mucosal but also systemic immune responses. Repeated oral administration is inclined to induce immune tolerance to the fed antigen, especially when the antigen is deposited in PBs as the storage organelle in seed. PBs <10 nm are principally taken up by M cells covering the Peyer's patches and presented to APC such as DCs. The GALT contains an organized macroarchitecture of zones of B and T lymphocytes that respond to antigens presented by the DC which can induce memory B and T cells (Fig. 3).

It has been demonstrated that oral delivery requires >100-fold the amount antigen to achieve the same level of efficacy via oral mucosal immunotherapy as subcutaneous injection. This is because the antigen is degraded in the gastrointestinal tract before arrival at the mucosal immune cells in the GALT, due to exposure to the harsh environment in the stomach (low pH, pepsin). When orally delivered via cereal seeds such as rice grains, bio-encapsulation of antigen by the double barriers of PBs and the cell walls characteristic of plant cells has the advantage of protecting the antigen from proteolysis. This is not the case when antigen is delivered by other plant vegetative cells. It should be noted that resistance of ER-derived PB to gastrointestinal enzyme digestion is stronger than PSV, as shown by comparing digestibility and immune tolerance-inducing capacity between ER-derived PB (PB-I) and PSV (PB-II) localized

antigens. This physical difference may be related to the polymerized or aggregated form of antigen observed in ER-derived PBs, formed by disulphide bonds through Cys-rich prolamines. Capture of ER-derived PB by M-cells in the GALT is observed on feeding transgenic rice containing cholera toxin B subunit (CTB) to mice.⁷¹ In fact, when transgenic rice seeds containing T cell epitopes of pollen allergens in ER-derived PB or PSV are fed to mice, PB-I-localized epitopes manifested greater resistance to enzymatic digestion than PB-II-localized or synthesized naked forms. The dose of PB-I-localized T-cell epitope required for suppression of allergen-specific IgE in mice was about 20- and 3-fold lower than the amount of synthetic peptide or PB-II-localized form, respectively (Fig. 4).⁷²

There may be a threshold level for each single dose of effective antigen (minimum amount) required for induction of immune tolerance, because repeated administration of a lesser amount was unable to induce tolerance even though the total amount of antigen administered exceeded that required for induction of oral tolerance.

Rice Seed-Based Japanese Cedar Pollen Allergy Vaccine

Japanese cedar pollen allergy is an important public health problem in Japan. At present, about 25% of the population is afflicted with this pollinosis from February to April each year. People with

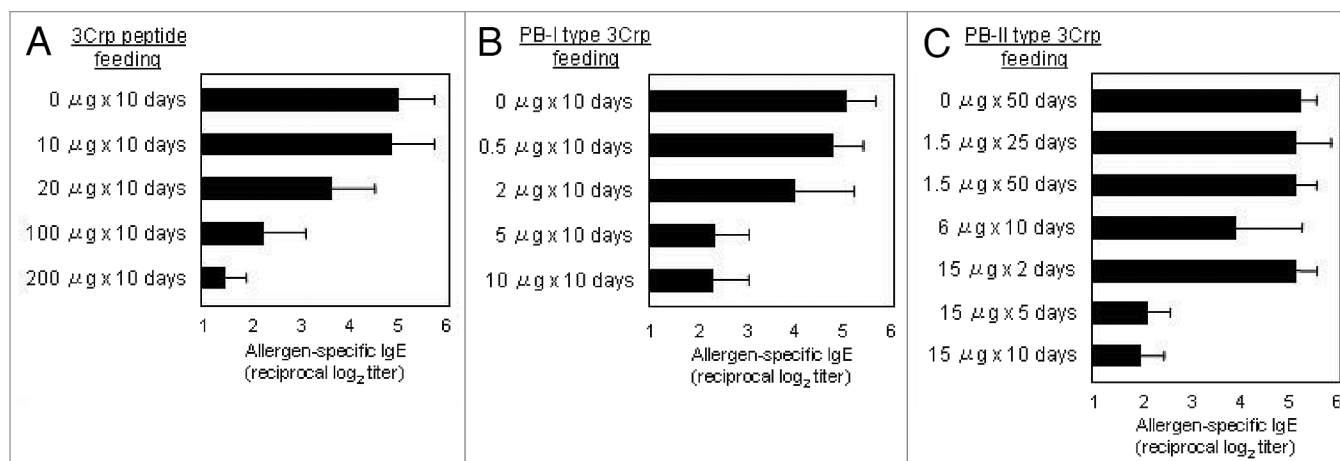


Figure 4. Suppression of allergen-specific IgE responses after oral administration of the three forms of 3Crp. Mice were orally given either chemically synthesized 3Crp peptide (A) or seeds containing PB-I type 3Crp or PB-II type 3Crp (C). Mice were then intraperitoneally challenged with total protein extract of Japanese cedar pollen, and allergen-specific serum IgE titers were determined by ELISA.

circulating specific IgE against cedar pollen allergens, who could be viewed as potential patients, make up as high a proportion as 60% of the general population.⁷³ The main allergens causing this pollinosis are Cry j 1 and Cry j 2, the major T cell epitopes of which have been well-characterized. To confirm the efficacy of rice-based oral vaccines for inducing immune tolerance against cedar pollen allergens by allergen-specific immunotherapy, major mouse T cell epitopes derived from Cry j 1 (p 277–290) and Cry j 2 (p245–259) were inserted into the C-terminal highly variable regions of soybean storage protein glycinin A1bB1b acidic and basic subunits. They were then expressed as fusion proteins in the endosperm of transgenic rice seed under the control of the endosperm-specific glutelin *GluB-1* promoter.⁷⁴ Modified glycinin A1aB1b containing T cell epitopes specifically accumulated in mature dry seed at the level of 7 μ g/grain. In a preclinical model, daily oral administration of transgenic rice seeds (200 mg) to mice for 4 weeks prior to systemic challenge with crude pollen allergens inhibited proliferative responses of allergen-specific CD4⁺ T-cells, synthesis of specific IgE and production of histamine compared to mice fed non-transgenic control rice seeds (Fig. 5). The production of allergy-associated Th2-type cytokines such as IL-4, IL-5 and IL-13 was also inhibited by rice-based T-cell epitope peptide feeding. Furthermore, allergy symptoms such as sneezing were alleviated after exposure to cedar allergens. These results indicate that mucosal immunization with rice seeds containing T-cell epitopes efficiently induced immune tolerance (Fig. 5).

Based on this proof of the feasibility of an oral peptide immunotherapy program in this animal model, a human version of a rice-based peptide vaccine against cedar pollen allergy was developed.⁷⁵ An artificial hybrid peptide 7Crp gene composed of seven linked dominant human T-cell epitopes (96 amino acids) derived from the Cry j 1 and Cry j 2 allergens was synthesized using seed-optimized codons for each amino acid. The hybrid 7Crp peptide elicited a positive response in 92% of 48 volunteers with pollinosis without binding to specific IgE,

indicating that it could be used as a safe and effective tolerogen.⁷⁶ Notably, the 7Crp peptide had a greater effect on T cell proliferation than a mixture of the seven individual T cell epitope peptides.

This 7Crp peptide was specifically expressed in transgenic rice seed as a secretory protein under the control of several strong endosperm-specific promoters. To increase accumulation of the 7Crp peptide, the GluB-1 signal peptide and the KDEL ER retention signal were included.⁷⁵ The 7Crp peptide was remarkably highly accumulated at about 60 mg/grain as determined in CBB-stained SDS PAGE gels, accounting for 5% to 6% of total seed protein. It was mainly deposited in ER-derived PB-I in the endosperm.

Transgenic rice seeds containing 7Crp were orally administered to Cry j 1-sensitized B10.S mice, which recognize only one epitope derived from Cry j 1 as a major epitope. The mice were then nasally challenged with intact Cry j 1 allergen. Both the T cell proliferative responses against Cry j 1 and specific serum IgE levels were depressed compared with control mice fed with non-transgenic rice seeds. T cell proliferative activity is retained even after boiling 7Crp transgenic rice seeds for 20 min at 100°C or autoclaving them for 20 min, indicating that oral immune tolerance would still be effective when used with steamed or boiled rice. Safety evaluation of 7Crp transgenic rice seed showed that amino acid, lipid, carbohydrate, protein, fatty acid, mineral and vitamin composition of the transgenic seeds was essentially identical to the non-transgenic control counterpart.⁷⁷ An oral safety study was performed by administering high and low doses of steamed rice to cynomolgus macaques for 26 weeks.⁷⁸ No adverse effects were observed.

Rice Seed-Based House Dust Mite Allergy Vaccine

House dust mites are the most common sources of indoor allergens associated with diseases such as asthma, allergic rhinitis and atopic dermatitis. Group I and II allergens are the major factors

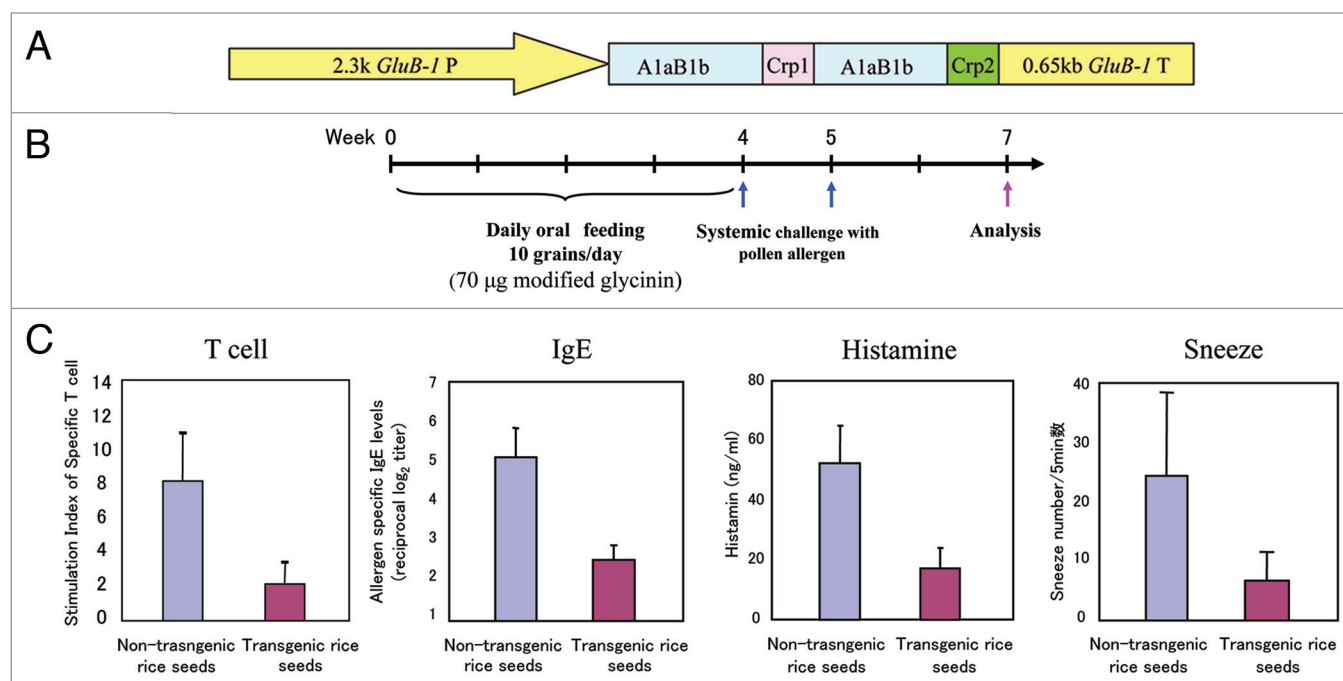


Figure 5. Oral immune suppression induced by feeding transgenic rice seeds accumulating A1aB1b soybean glycinin storage protein containing major T cell epitopes derived from Cry j 1 (Crp1) and Cry j 2 (Crp2) allergens. A. Schematic representation of the expression construct. B. Feeding schedule and challenge with cedar pollen allergens. C. Inhibition of allergen-specific T cell proliferation, allergen-specific serum IgE, serum histamine and the number of sneezes by oral administration of transgenic rice seeds compared with non-transgenic control seeds.

eliciting IgE antibody response in over 80% of patients afflicted with mite allergy. The Group I allergen Der p 1 has cysteine protease activity involved in the pathogenesis of allergy through cleavage of CD23 and CD25 from the surface of immune cells.

The entire molecule (p1-222) as well as a fragment (p45-145) of mite allergen Der p 1 containing most mouse (BALB/c) and human major T-cell epitopes were expressed specifically in the endosperm tissue of transgenic rice seed under the control of the glutelin *GluB-1* promoter.⁷⁹ In addition, modified mite allergen Der f 2, in which all the Cys residues are mutated to Ser was expressed in the same manner. Genes were synthesized using optimized codons, and as secretory proteins, by ligating the glutelin signal peptide and ER retention signal at the N- and C-termini to enhance accumulation. The products were highly accumulated in the endosperm, deposited in ER-derived protein bodies or in specific compartments. These genes were consistently inherited over several subsequent generations without silencing. Furthermore, these antigens remain stably present without loss of immunogenicity even when the seed is stocked at ambient temperature for several years. It is interesting to note that plant-derived Der p 1 was post-translationally modified with a high-mannose type glycan structure. Glycosylated Der p 1 displayed reduced IgE binding capacity in comparison with its unglycosylated counterpart.⁷⁹

When mite allergen transgenic rice seeds were fed to mice, prevention of asthma caused by mite allergen exposure was achieved.^{80, 81} Oral administration of this transgenic rice clearly reduced the levels of antigen-specific serum IgE and IgG through suppression of IL-4 and IL-13 production. Furthermore, infiltration of eosinophils into the airways and

development of bronchial hyperresponsiveness was also inhibited through suppression of IL-5.

Future Perspectives

Seed-based allergy vaccines must be produced in conformity with specific guidelines and regulations for pharmaceutical products, under conditions of good manufacturing practice (GMP). It may be necessary to develop processing steps (purification) for each product, because only purified antigens are presently approved by the WHO. The downstream processing stage of production requiring many steps of extraction and purification is generally expensive, representing up to 80% of overall production costs. Special facilities and equipment are also required for processing antigens in this way. It is important to note that direct oral vaccination using products of transgenic crops without any further processing is highly advantageous in terms of efficient delivery to the GALT and their stability. Crop seeds containing antigen act as vehicles for mucosal delivery (natural coated oral formulations). Validation to be observed in plant production systems is one other obstacle to commercialization as a pharmaceutical product. There are usually variations in accumulation of product due to physiological differences in biological systems.

Furthermore, there are concerns about out-crossing with wild-type crops in the case of open field cultivation. Pharmaceuticals should be produced under the control of specific regulations and guidelines. Closed farming facilities (closed glasshouses) with strictly-controlled environmental conditions would be ideal and are recommended in order to reduce variation of product

concentration in targeted tissue as well as to prevent out-crossing. However, in the case of allergy vaccines, very large quantities of seeds will be required in practice. For example, daily oral consumption of cooked transgenic rice (about 50 to 100 g) for a minimum of three months and continuing for up to a few years may well be required for desensitization against cedar pollen allergens—and such transgenic rice will have to be produced for about 20 million patients afflicted with Japanese cedar pollinosis. It would be quite impossible to cultivate the required amount of transgenic rice in closed glasshouses like most plant-made pharmaceuticals. Allergen-specific immunotherapy based on mechanisms inducing immune tolerance against allergens for IgE-mediated type I allergy can be controlled more easily than that using immune induction for vaccines against infectious diseases. In the case of oral vaccines against pathogens, it would be very difficult to control the administration regimen, because of the danger of inducing immune tolerance of pathogens. Different production systems such as cultivation in physically isolated fields

(areas) and biological containment such as closed flowers will have to be considered and specific guidelines and regulations will have to be developed for this purpose. Additionally, entry into the food chain during transportation and handling will need to be strictly avoided. The cultivation of crop plants for the production of biopharmaceuticals such as vaccines and medical products should be strictly separated from non-transgenic crop plants. There would need to be zero-tolerance for cross-contamination of plants used for food and medicine in the all processes including transportation from production to processed products. In order to guarantee traceability, inclusion of discriminative markers, such as color coding, may also be required.

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