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Update on the use of transgenic rice seeds in oral immunotherapy

Rice seed provides an ideal production platform for pharmaceuticals in terms of high productivity and stability, as well as the scalability, safety and economy that are expected in plant production systems. Furthermore, these therapeutic products are bioencapsulated in protein bodies, which are seed-specific storage organelles that provide protection from digestion by gastrointestinal enzymes during delivery to the gut-associated lymphoid tissue. Thus, rice seed provides an ideal delivery system for the mucosal immune system. Oral immunotherapy using unprocessed transgenic rice seed containing therapeutic products has been demonstrated to induce effective mucosal immune tolerance and immune reactions against allergies and pathogens.

KEYWORDS: allergy vaccine ■ immunotherapy ■ oral tolerance ■ protein body ■ rice seed protein

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Plants provide a promising production platform for recombinant proteins in terms of scalability, safety and cost-effectiveness, and they may be an alternative to conventional fermentation systems that use bacteria, yeast and mammalian cells [1-3]. In particular, seeds provide a stable site for the deposition of recombinant proteins that can avoid degradation, even when stored at ambient temperatures for several years [4,5]. Furthermore, the recombinant proteins deposited in specific seed organelles known as protein bodies (PBs) exhibit high resistance to the harsh conditions found in the gastrointestinal environment of the digestive tract, such as low pH and proteolytic enzyme activity, due to the double barriers of the cell wall and the characteristics of PBs in plant cells, which facilitate oral administration in the form of seeds [6-8]. Compared with conventional immunotherapy via parental administration, much higher amounts of antigen can be administered in the form of PBs in rice seed, which are then delivered to the gut-associated lymphoid tissue (GALT), where they induce effective antigen-specific immune tolerance or a protective immune response. Therefore, seed has many potential benefits as a tool for immunotherapy because seed can be used as a bioreactor and a vehicle for the oral delivery of antigens to GALT.

Production of pharmaceuticals in seed

The cereal endosperm is a highly specialized storage tissue that accumulates starch and proteins, which account for approximately 80% of

the seed dry weight [9]. The yields of recombinant proteins in transgenic plants are determined by several factors including the strength of the transcriptional activity (promoter activity), mRNA stability, post-translational modification (glycosylation), intracellular compartmentalization and the genetic background [10-12]. The expression levels of recombinant proteins are determined primarily at the transcription level. Higher levels of accumulation can be obtained by sequestering proteins into specific target tissues using tissue-specific promoters. Several strong, seed-specific promoters such as glutelin, (*GluB-1* and *GluB-4*), globulin (26-kD *Glb-1*), oleosin (18 kD) and prolamin (16 and 10 kD) promoters in rice have been exploited and used for the expression of recombinant proteins in different parts of the seed without affecting the growth of stable, transgenic rice plants [13]. A previous study showed that more than 200 µg of recombinant human IL-10 accumulated in a single rice grain (approximately 20 mg) when it was expressed under the control of the endosperm-specific glutelin *GluB-1* promoter and the expression of endogenous prolamin genes was suppressed by RNAi [14].

High levels of expression can be also achieved by modifying the 5' and 3' untranslated sequences and polyadenylation signals that are responsible for mRNA stability. Codon optimization of codon bias can also be applied to eliminate rare codons or undesirable sequences, such as mRNA destabilizing signals or polyadenylation signals, and introduce codons used by many storage protein genes. This is critical

for increasing expression via enhancing translation efficiency. The codon-optimized genes were reported to have 5–100-fold increases in yield compared with the unmodified control [15]. Furthermore, selecting a suitable intracellular compartment as a deposition site is a critical factor that determines the yield level because the subcellular localization of proteins is strongly associated with the accumulation capacity, stability and post-translational modification of expressed recombinant proteins [16,17]. Transportation to suitable intracellular compartments can be facilitated by adding a targeting signal to the recombinant protein [18]. PBs and protein storage vacuoles (PSVs) as the protein storage organelles have been used as targets for the accumulation of recombinant proteins in seeds [18]. The addition of an N-terminal signal peptide to a recombinant protein targets the recombinant protein to the endoplasmic reticulum (ER), before it is directed to the secretory pathway and sequestered in the apoplast (the exterior of the plasma membrane) according to the default pathway. The targeting of proteins to the secretory pathway is superior to accumulation in the cytosol. The ER provides good conditions that are suitable for the production of recombinant proteins due to the enrichment of protein chaperones such as BiPs and PDIs, which are involved in folding and assembly [19]. By contrast, cytosolic accumulation generally results in very low levels compared with other intracellular compartments, except in certain specific cases. It has been reported that proteins targeted to the secretory pathway generally have at least a tenfold higher yield than those targeted at the cytosol [20]. When recombinant proteins were expressed in transgenic rice seed by ligating the signal peptide and the KDEL ER retention signal at the N and C termini, they were mainly accumulated in ER-derived PBs.

The selection of organelle targets can affect the post-translational processing of recombinant proteins. Proteins targeted through the ER can be glycosylated. During the production of secretory proteins, glycan is added in the ER lumen before further modifications in the Golgi apparatus. *N*- and *O*-glycosylation occur in the secretory pathway. High mannose type *N*-glucans (5–9 Man residues) are attached to Asn-X-Ser/Thr. Complex type *N*-glucans such as β -1,2 xylose and α -1,3 fucose are different from their mammalian counterparts because they are attached in the Golgi apparatus [21]. Glycan modification should be avoided because complex types of glycosylation may have significant allergenic risks.

Intracellular localization of recombinant proteins synthesized as secretory proteins in transgenic rice

PBs are specialized storage organelles for seed proteins and the deposition site of recombinant proteins in endosperm cells. There are two functionally distinct types of PBs with different origins in the rice endosperm cell (FIGURE 1) [22,23]. The first are ER-derived type I PBs containing prolamins (10-, 13- and 16-kD prolamins), which are known as PB-Is. PB-Is are spherical, with a smooth surface structure and a diameter of 1–2 μ m. The second type are PSVs known as PB-IIIs, which contain glutelins and 26-kD globulin. PB-IIIs are irregular in shape with a high electron density and diameter of 3–4 μ m.

■ ER-derived PB-I

Prolamin is a major seed storage protein in most cereal grains, although it only comprises approximately 20% of the total seed protein in rice and oats. The rice prolamins consist of 16-, 13- and 10-kD prolamins. These prolamins are divided into cysteine (Cys)-rich (16, 13 and 10 kD) and Cys-poor (13 kD-b) types. PB-Is are known to consist of a 10-kD prolamin-rich core, 13-kD-b prolamin-inner layer, 13-kD-a and 16-kD prolamin-rich middle layer, and 13-kD-b prolamin-rich outer layers [24].

A similar PB structure has been reported in maize. Maize storage proteins are composed of α -, β -, γ - and δ -zeins. Cys-poor α -zein comprises most of the core, which is surrounded by a thin layer of β - and γ -Cys-rich zeins. The penetration of major Cys-poor α -zein into the core region during the late maturity stage leads to the enlargement of ER-derived PBs [25].

The reduction of individual storage proteins and PB formation has been investigated using knockdown transgenic rice plants [26]. The knockdown of 13-kD prolamins reduced the size of ER-derived PBs and changed them from round-shaped structures to rugged peripheral structures. By contrast, suppression of Cys-rich 10 and 16 kD prolamins resulted in a slight reduction in PB-I size.

■ PSVs

Glutelin is the major seed storage protein in rice (i.e., 60–70%), while 26-kD globulin comprises approximately 10% of the total seed proteins. These glutelins (GluA, B, C and D) and globulins are mainly transported from the ER lumen to PSVs through the Golgi apparatus via dense vesicles. Some components of glutelins and globulins are trafficked directly into PSVs

via precursor accumulating vesicles measuring 0.2–0.4 μm that are derived from the ER as an alternative pathway, thereby bypassing the Golgi apparatus [27]. Precursor accumulating vesicles originate from protein aggregates in the ER. Reduction of the glutelin levels slightly decreases the PSVs, whereas the simultaneous reduction of glutelin and globulin levels affects the inner structure of PSVs, suggesting a role for globulin during PSV formation [26].

■ Generation of new PBs containing recombinant proteins

The ER is a favorable location for the storage of recombinant proteins. Recombinant proteins with a signal peptide and an ER retention signal (KDEL) generally accumulate in the ER-derived PB when they are expressed in cereal seeds under the control of endosperm-specific expression [28].

However, it is interesting to note that the intracellular deposition sites of recombinant proteins are greatly affected by endogenous seed storage proteins in seeds if recombinant proteins are produced constitutively as secretory proteins, which results in unexpected intracellular localizations that differ from the inherent destination sites observed in the vegetative tissues of the same plant. For example, *Aspergillus niger* phytase was deposited in ER-derived PBs and PSVs of rice endosperm, whereas it was secreted into the apoplasts of the rice leaf by the intrinsic secretory signal [29]. Human serum albumin containing a KDEL tag was also deposited as storage protein aggregates within the vacuoles of wheat endosperm cells, although it was targeted to the ER lumen in leaf cells [30]. Furthermore, large amounts of KDEL-tagged antibody expressed in tobacco or *Arabidopsis* seed were delivered to

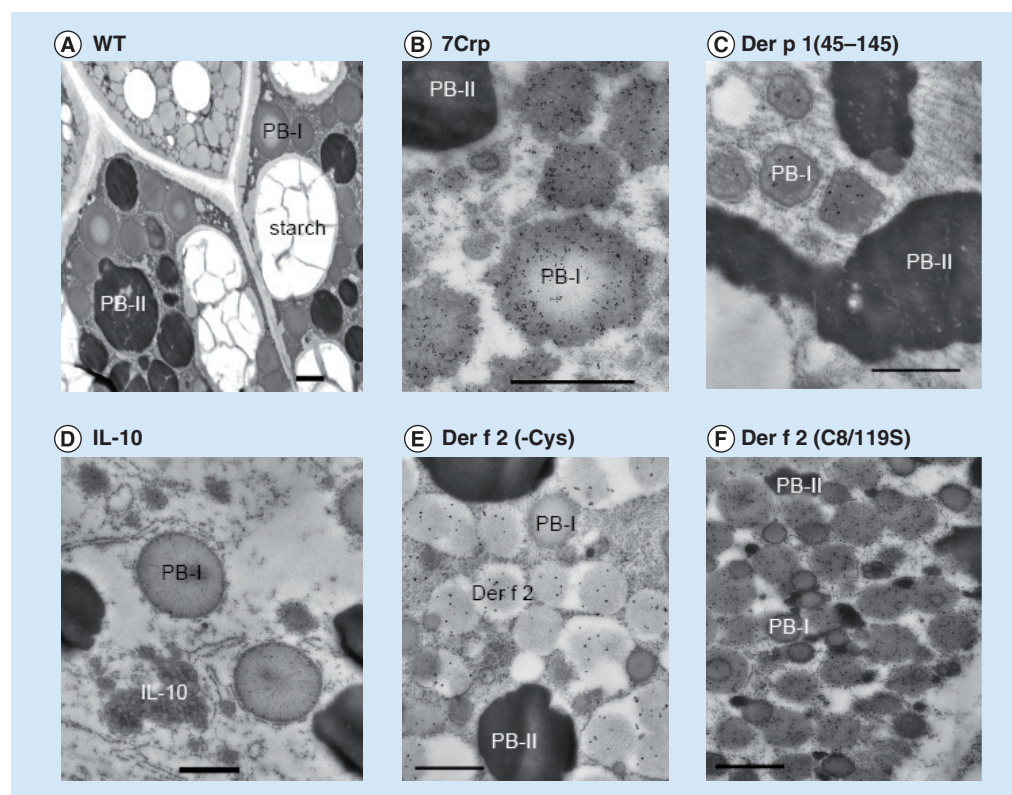


Figure 1. Differences in the structure of protein bodies containing various recombinant proteins. Recombinant proteins are deposited as unique endoplasmic reticulum-derived PBs, depending on the physical properties of the proteins accumulated in the rice endosperm cell. Intracellular localization of recombinant proteins in developing rice endosperm cells was analyzed by immunoelectron microscopy. (A) WT: nontransgenic WT rice; (B) 7Crp: seven linked T-cell epitope peptide derived from Japanese cedar pollen allergens Cry j 1 and 2; (C) Der p 1 (45–145): fragment comprising amino acid positions 45–145 of the group 1 house dust mite allergen, Der p 1; (D) IL-10: human IL-10; (E) Der f 2 (-Cys): full-length sequence of the mutagenized group 2 house dust mite allergen Der f 2, in which all six cysteine residues were changed to serine residues; (F) Der f 2 (C8/119S): full-length sequence of the mutagenized group two house mite allergen Der f 2, in which two cysteine residues involved in an intramolecular disulfide bond formation between Cys 8 and 119 were changed to serine residues. Scale bar represents 1 μm . PB: Protein body; WT: Wild-type.

the periplasmic space between the plasma membrane and the cell wall, as well as the ER lumen [31,32]. Thus, protein targeting is regulated in a tissue- or cell-specific manner. One explanation for the targeting of recombinant proteins to ER-derived PBs in transgenic rice seed is that the free cysteine residues of recombinant proteins are arrested by Cys-rich storage proteins, such as Cys-rich prolamins in the ER lumen, via intermolecular interactions and subsequently sequestered in PBs [33].

Recombinant proteins with noncovalent free cysteine residues (7Crp and Der p 1) were found to interact with various types of Cys-rich prolamins in transgenic rice seed, which facilitated the deposition of proteins into PB-Is via the formation of aggregates between the recombinant protein and Cys-rich prolamins [34–36]. These aggregates are retained in the ER, resulting in the formation of ER-derived PBs containing polymerized aggregates, which are sequestered into PB-Is (FIGURE 1). When human IL-10 with a KDEL tag was produced in rice seeds, IL-10 aggregated in the ER lumen and formed unique, small ER bodies, which then assembled with prolamins, resulting in the formation of an IL-10/prolamin assembly [14]. A similar intracellular localization to ER vesicles was also observed when GM-CSF without the KDEL tag was expressed as a secretory protein with a signal peptide in rice endosperm [37]. Modified mite allergen Der f 2 derivatives aggregated in the ER lumen where they were deposited in unique, PB-like structures, known as Der f 2 bodies [36]. The Der f 2 body differs from PB-I and -II in terms of its intracellular localization and physicochemical properties. These results suggest that the structure and formation of ER-derived PBs containing recombinant proteins are dependent on the physical properties of the protein. The digestive enzyme resistance of these ER-derived PBs is generally lower than representative PB-Is.

Rice seed PBs are an effective system for the delivery of vaccines to GALT

The oral administration of pharmaceuticals has several advantages over traditional parenteral (systemic) routes because of its convenience, the absence of a requirement for administration by medical personnel and the lack of pain caused by injections. Furthermore, parenteral and mucosal immune reactions can be induced if antigens are administered via the oral route, resulting in prophylactic and therapeutic effects. Therefore, the administration of vaccines to mucosal surfaces is expected to be more effective than

their parenteral administration. Most pathogens invade the host via the mucosal route, so mucosal immune responses are considered to be the most effective means of preventing infectious diseases. With oral vaccines, however, the naked antigens are degraded by digestive enzymes in the GI tract before they arrive at the GALT. Oral products require a 100- to 1000-fold higher dose to produce an injectable dose equivalent when using a crude allergen extract or purified allergen. Resistance to digestive enzymes in the stomach and small intestine, and from other harsh conditions is effectively promoted by bioencapsulation using the double barriers provided by the plant-specific cell wall and the seed-specific protein body [8]. The efficacy of this route appears to be similar to the use of encapsulating agents such as liposomes. In addition to the two barriers of the cell wall and PBs, cereal grains also contain carbohydrates that confer protein stability, which may account for the slow release of proteins and their ability to survive into the gut.

The stability was compared between the two different types of PBs (i.e., ER-PB [PB-I] and PSV [PB-II]) found in rice endosperm, and the former showed higher resistance to digestive enzymes than the latter in simulated gastric fluid [38]. The dose of PB-I localized antigen required for suppression of allergen-specific IgE was significantly lower ($\sim 1/3$) when fed in a PB-II localized form. This is because PB-Is are tightly packaged by several types of prolamins with different physical properties that are spatially and systematically arranged during seed maturation. These findings indicate the advantages of bioencapsulation in PB-Is for achieving efficient mucosal delivery of oral protein therapeutics.

Immune response in the gut

Oral immunization stimulates the induction of mucosal and systemic antigen-specific immune responses, which result in the generation of a state of immune tolerance. Thus, the mucosal immune system provides a front line of defense against invading pathogens via its induction of positive immune responses, as well as inducing immunological unresponsiveness [39]. The gut is an entry site for numerous antigens, where tolerance is required for a healthy immune response. Vaccines should be evaluated carefully on a case-by-case basis to balance oral tolerance with the immune response (FIGURE 2).

Immune tolerance is caused by anergy or the deletion of specific T cells and the induction of Tregs, such as thymus-derived natural Foxp3⁺ CD4⁺CD25⁺, and adaptive or inducible

Tregs (iTregs) related to peripherally generated Foxp3⁺ inducible T cells and Foxp3⁺ iTregs, such as Tr1 and Th3 cells, depending on the antigen concentration administered and the duration and frequency of treatment [40–42]. PBs containing antigens with a diameter less than 10 μ m are taken up by M cells in Peyer's patches (PPs) and presented to dendritic cells (DCs), taken up directly by epithelial cells, or sampled by the extending dendrites of DCs in the intestinal lamina propria (LP) via the epithelium [43]. There are at least three distinct subsets of DCs in PP:

- CD11c⁺ DCs in the subepithelial domain region;
- CD8⁺ DCs in the T-cell-rich interfollicular regions;
- Double-negative DCs in both the subepithelial domain and interfollicular regions [44,45].

Furthermore, DCs from the LP play a substantial role in the induction of antigen-specific responses. CD103⁺ DCs in the LP can induce conversion of Foxp3⁺ iTregs via their capacity for releasing TGF- β and retinoic acid [46]. IL-10-producing immature DCs contribute to immunological tolerance by inducing Tr1 regulatory cells.

High-dose tolerance is mediated by anergy or deletion [47]. Fas (CD95)-dependent apoptosis is responsible for the deletion of effector

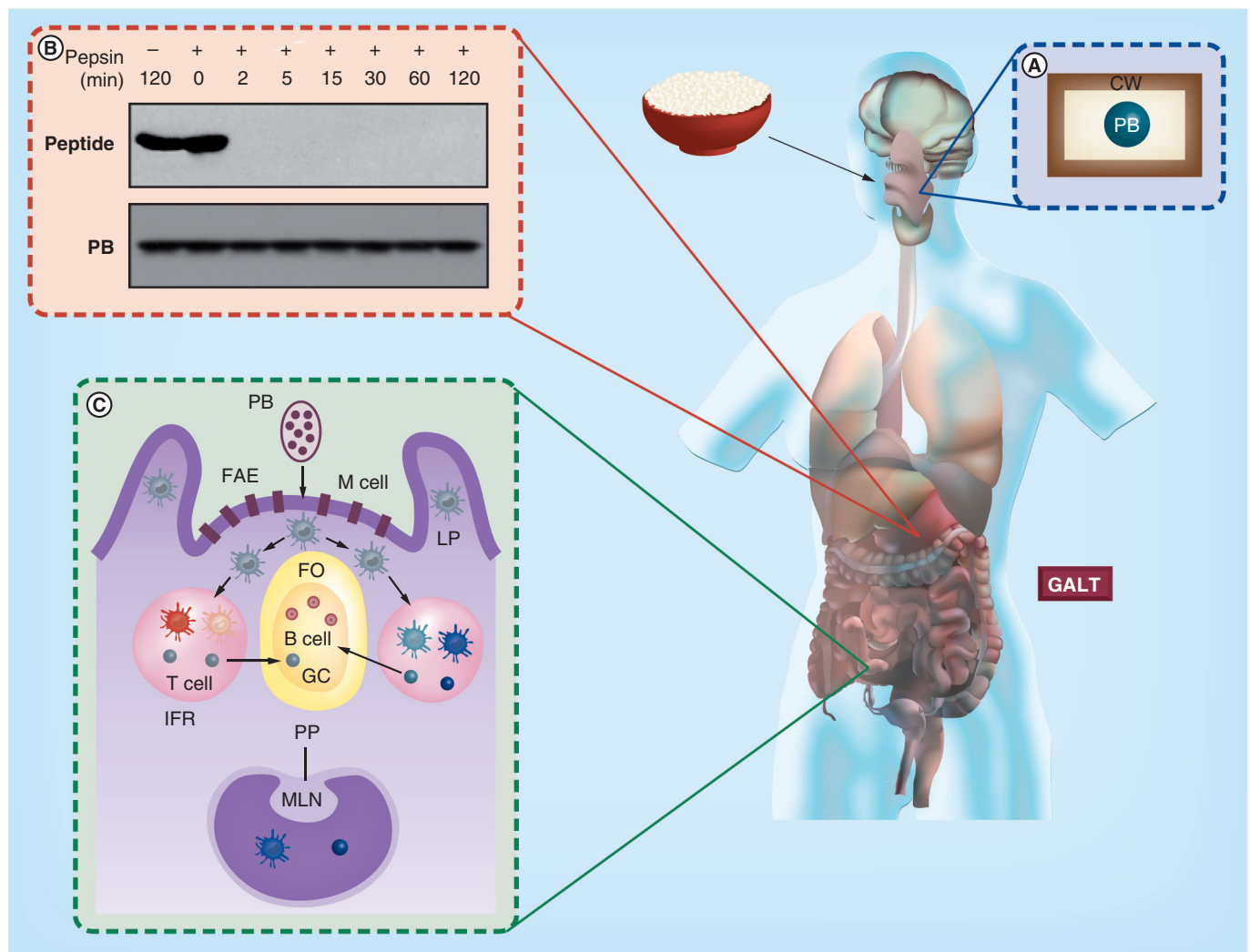


Figure 2. Advantage of seed-based vaccine via protein body as a delivery vehicle to gut-associated lymphoid tissue.

(A) Schematic presentation of rice endosperm cell. **(B)** Comparison of digestibility between native peptide and bioencapsulated peptide in PBs by *in vitro* pepsin digestion. **(C)** Sites of antigen uptake and immune reaction in the gut. Particle antigens, such as PBs, are taken up by M cells overlying PPs and then delivered to intestine APCs, such as dendritic cells, macrophages and B cells, to present antigen to T cells in the GALT (PP and LP) or draining lymph nodes such as the MLN.

CW: Cell wall; FAE: Follicle-associated epithelium; FO: Follicle; GALT: Gut-associated lymphoid tissue; GC: Germinal center; IFR: Intrafollicular region; LP: Lamina propria; M cell: Microfold cell; MLN: Mesenteric lymph node; PB: Protein body; PP: Peyer's patch.

T cells. Anergy is associated with the expression of costimulation factors (interactions between costimulatory receptors on T cells (CD28/CTRA-4) and counter-receptors on APCs [CD80/86]). CTRA-4 is released by CD4⁺CD25⁺ and is involved in the suppression of APCs by competing with CD28 for binding to CD80/86 on the APC. The ICOS–ICOS-L pathway or the PD-1–PD-L costimulatory pathways are also implicated in oral tolerance. PD-L1 (B7-H1) and PD-L2 (B7-DC) expressed on activated DCs play a substantial role in tolerance induction [48,49].

Peripheral conversion of CD4⁺ T cells to Tregs occurs primarily in GALT. DCs from LP promotes a high level of Treg conversion relative to the lymphoid organ-derived DC. CD103⁺ DCs isolated from the small intestine and the mesenteric lymph nodes of the gut produce both TGF- β and retinoic acid, which efficiently mediate the differentiation of naive T cells into Foxp3⁺ Tregs. Oral tolerance is established via the ability to enhance IL-10 production from PP-CD11c⁺ cells and the generation of Ag-specific suppressive T cells. IL-10-producing T cells are highly abundant in the intestine and frequently observed in the PP and intraepithelial lymphocyte in the small and large intestines. After peripheral T-cell tolerance is triggered, allergen-specific Tregs produce elevated levels of IL-10 and TGF- β . IL-10-producing T cells can be induced from Foxp3⁺ or Treg 1 after induction by TGF- β and IL-27 [50]. The iTregs found in the peripheral tissue are essential for mucosal immune tolerance and the control of severe chronic allergic inflammation.

NK regulatory cells have the capacity to suppress antigen-specific T-cell responses. $\gamma\delta$ T cells are important mediators of mucosal tolerance that regulate autoimmunity. CD8⁺ Tregs are also involved in oral tolerance. Double-negative T cells promote tolerance induction and regulate autoimmunity [51].

Immune regulation via the oral administration of rice seed-based pharmaceuticals

■ Allergy vaccines

Allergen-specific immunotherapy is the only known curative treatment for allergic diseases [52,53]. Conventional immunotherapy using subcutaneous injection of increasing doses of crude allergens has been used for the last 3–5 years, resulting in a reduction of clinical symptoms and the prevention of disease progression.

Japanese cedar pollen allergy

Peptide immunotherapy is an ideal allergen-specific immunotherapy because of its high safety, so high-dose administration is possible [54,55]. The oral administration of T-cell epitopes from Japanese cedar pollen allergen is reported to specifically induce immune tolerance against the pollen allergen [56]. Rice seed can express and accumulate remarkably high levels of T-cell epitope peptides compared with the doses used in conventional immunotherapy via the oral route. To evaluate the feasibility of seed-based peptide immunotherapy, two major T-cell epitopes derived from the Japanese cedar major pollen allergens, Cry j 1 and Cry j 2, were expressed as a translational fusion protein with the soybean seed storage protein glycinin in the endosperm of transgenic rice seeds (TABLE 1) [57]. Transgenic rice seeds containing the major T-cell epitopes were administered orally and followed by challenge with cedar pollen allergens, which led to a specific T-cell proliferation response that was significantly reduced compared with mice fed with nontransgenic rice seeds. The clinical symptoms (i.e., the specific IgE level and frequency of sneezing) were also reduced. The Th-2 type cytokines IL-4, -5 and -13 were also suppressed compared with the wild-type seed. These results suggest that allergen-specific immune tolerance can be induced by oral administration of transgenic rice seed containing major T-cell epitopes.

Based on positive results using a model mouse, human versions of a rice-based peptide vaccine against cedar pollen allergy have been generated. However, the T-cell epitopes derived from cedar pollen allergens are different in humans due to the genetic divergence of MHC. To compensate for this genetic diversity, seven major T-cell epitopes were linked as a hybrid peptide. The seven linked T-cell epitopes are known as 7Crp, which is composed of three and four major T-cell epitopes derived from Cry j 1 and 2, respectively. This hybrid T-cell peptide produced a similar level of immunogenicity to the native allergens but no allergenicity (IgE-binding activity), therefore, providing an ideal peptide vaccine for Japanese cedar pollen allergy [58].

The 7Crp peptide was highly accumulated in transgenic rice seed (approximately 60 μ g/grain) and was deposited in ER-derived PBs, which are suitable for delivery to GALT [59]. To test its safety, the chronic and reproductive toxicity were evaluated by feeding transgenic rice seeds to monkeys and rats, with no adverse effects detected [60].

Table 1. Examples of seed-based antigens and cytokines.

Recombinant protein	Promoter	Expression level/yield, intracellular localization	Immunogenic reaction	Ref.
T-cell epitope peptide from Cry j 1 and 2, inserted into soybean glycinin (A1aB1b)	Rice glutelin, <i>GluB-1</i>	7 µg per grain (0.5% TSP), PB-II	Suppression of allergen-specific T-cell proliferation, IgG and IgE in mice following oral administration; reduction of histamine levels and sneezing frequency as a result of decrease in Th2 cytokines	[57]
Artificial peptide (7Crp): 7 linked major T-cell epitope peptides from Cry j 1 and 2	Rice glutelin, <i>GluB-1</i>	60 µg per grain, PB-I	Suppression of allergen-specific T-cell proliferative response and serum IgE in mice following oral administration	[59]
Destructured Cry j 1 and 2	Rice glutelin, <i>GluB-1</i> , <i>GluB-4</i> , prolamin: 10 and 16 kD	5–20 µg per grain, PB-I	Suppression of allergen-specific T-cell proliferation, IgG and IgE in mice following oral administration; reduction of sneezing frequency and infiltration of inflammatory cells as a result of decrease in Th2-type cytokines	[61]
Dust mite allergen Der p 1(45–145)	Rice glutelin, <i>GluB-1</i>	90 µg per grain (7.5% of TSP)	Inhibition of Der f 1-specific IgE and IgG, T-cell response and Th2-type cytokines; inhibition of allergen-induced inflammatory cell infiltration in mice following oral administration	[35]
Dust mite allergen hypoallergenic Der f 2	Rice glutelin, <i>GluB-1</i>	15–30 µg per grain; Der f 2 body	Suppression of Der f 2-specific IgG and IgE production in mice following oral administration	[36]
CTB subunit	Rice glutelin, <i>GluB-1</i>	30 µg per grain, PB-I and PB-II	Induction of CTB-specific serum IgG and mucosal IgA with neutralizing activity in mice and macaques following oral administration; CTB-specific IgA-mediated protection against CT or LT-induced diarrhea	[6]
Nontoxic dmCT	Rice glutelin, <i>GluB-1</i> , <i>GluB4</i>	5 µg per grain	Induction of CTB-, but not CTA-specific serum IgG and mucosal IgA antibodies in mice following oral administration; diarrhea was protected by oral administration	[70]
Truncated HBVs fused with hepatocyte receptor binding presurface 1 region	Rice glutelin, <i>GluB-4</i>	31.5 ng/g dry weight grain	Specific antibody responses were elicited in mice after intraperitoneal injection	[64]
Surface glycoprotein F from Newcastle disease virus	Maize ubiquitin and rice glutelin, <i>Gt1</i>	2.5–5.5 µg/g TSP from seed or leaf	Induces antigen-specific antibodies in mice following oral administration; specific serum antibody responses were elicited	[65]
16-kD antigen of the roundworm (<i>Ascaris suum</i>) fused to CTB	Rice glutelin, <i>GluB-1</i>	1.5 µg per grain	Induction of At16-specific serum IgG in mice by oral administration in combination with CT; reduction of number of larvae	[67]
IBDV host-protective VP2	Rice glutelin, <i>Gt1</i>	4.5% of total soluble protein	Induction of serum antibody responses against IBDV in chickens vaccinated with transgenic rice seeds	[66]
45 kDa C-terminal half of the heavy chain of Botulinum type A neurotoxin	Rice prolamin: 13 kD, simultaneous suppression of glutelin A and 13-kD prolamins by RNAi	100 µg per grain, cytoplasm	Induction of protective immunity against a challenge with <i>botulinum</i> type A neurotoxin in mice following oral administration	[71]
hIL-10	Rice glutelin, <i>GluB-1</i> , simultaneous suppression of 10-, 16- and 13-kD prolamins by RNAi	200 µg per grain, IL-10 body	Suppression of cedar pollen allergen-specific IgG and IgG in mice following oral administration	[11]
hGM-CSF	Rice glutelin, <i>Gt1</i>	14 µg per grain, ER-derived unique body	Mice fed transgenic seeds showed a 27% increase in leukocytes	[37]

CT: Cholera toxin; CTA: Cholera toxin A; CTB: Cholera toxin B; dm: Double mutant; ER: Endoplasmic reticulum; h: Human; IBDV: Infectious bursal disease virus; LT: Escherichia coli heat-labile enterotoxin; PB: Protein body; TSP: Total seed protein.

However, despite its low allergenicity, peptide immunotherapy using T-cell epitopes may not be applicable to all Japanese cedar pollinosis patients. Thus, to treat a broader range of allergy patients, the Cry j 1 and 2 entire molecules were destroyed by a molecular shuffling and fragmentation process that perturbed the tertiary structure required for recognition of an allergen-specific IgE.

Transgenic rice seeds containing accumulations of these destructed whole Cry j 1 and 2 molecules were fed to mice daily, which were then challenged using crude cedar pollen allergen [61]. It was found that the allergen-specific CD4⁺ T-cell proliferation and IgE and IgG levels were markedly suppressed compared with mice fed nontransgenic rice seeds. The sneezing frequency, which is a clinical symptom of pollinosis, and the infiltration of inflammatory cells in the nasal tissue, such as eosinophils and neutrophils, were also significantly reduced. These results suggest that oral administration of transgenic rice seeds containing structurally disrupted Cry j 1 and 2 antigens provides a promising therapeutic allergy vaccine, which could be applicable to all patients who suffer with Japanese cedar pollinosis.

House dust mite allergy

House dust mites (HDMs) are the most common source of indoor allergens and are associated with allergic diseases worldwide. To benefit allergic patients, safer and noninvasive mucosal routes of oral administration are considered to be the best alternative to conventional allergen-specific immunotherapy. A strategy using rice-based allergy vaccines was examined to test whether it could be applicable to other allergies. HDM allergy is the main cause of bronchial asthma. The main allergens responsible for HDM allergy are classified into group 1 and 2, which contain cysteine protease and similarity to a family of epididymal proteins, respectively. A damaged Der p 1 fragment (p45–145) that included most human and mice major T-cell epitopes and Der f 2 derivatives, which had reduced IgE reactivity due to disrupted intramolecular disulfide bonds, were produced in transgenic rice seeds and used as tolerogens to induce oral immune tolerance to mite allergens [35]. Mite allergen transgenic rice seeds containing the Der p 1 fragment (p45–145) were fed to mice that were subsequently immunized using a recombinant Der p 1, and the allergen-specific CD4⁺ T-cell proliferation and production of allergen-specific IgE and IgG were suppressed compared with mice fed nontransgenic rice seeds. Measureable

clinical symptoms such as the development of allergic airway inflammation due to infiltration by eosinophils and lymphocytes, and bronchial hyper-responsiveness were inhibited by prophylactic vaccination with transgenic rice seeds [35].

Transgenic rice seeds containing Der f 2 derivatives were fed to mice and immune tolerance to the Der f 2 antigen was induced in a similar way to other allergens. Interestingly, the inhibition of Der f 2-specific IgE and IgG production indicated that the efficacy of these derivatives was related to the resistance of Der f 2-containing PBs to digestive enzymes in the GI tract [36].

■ Vaccines against pathogens

Cholera toxin B

Cholera is an acutely dehydrating, watery diarrheal disease caused by *Vibrio cholerae*. Cholera affects the small intestine through its secreted exotoxin, which is composed of five receptor-binding B subunits and a single catalytic A subunit. Transgenic rice expressing cholera toxin (CT)B in rice grains was developed as a rice-based oral vaccine. When MucoRice-expressed CTB was orally administered to mice, specific immune responses and neutralizing activity were detected in the systemic and mucosal compartments (induction of secretory IgA and serum IgG responses) [6]. Interestingly, animals immunized with MucoRice-CTB exhibited better protection from an oral challenge with CT compared with control animals. In a similar study conducted using a nonhuman primate model, cynomolgus macaques were orally administered MucoRice-CTB [62]. The animals were found to have CT-specific, neutralizing antibodies and high levels of systemic IgG and intestinal IgA antibodies [6,62].

MucoRice CTB is taken up by the microfold cells and induces a CTB-specific serum IgG and intestinal IgA response to confer protection against oral challenges with CT, thereby providing two layers of defense against mucosal pathogens. No serum IgE antibodies against rice storage proteins were induced, even after multiple immunizations. In addition, rice that was preserved at room temperature for 3 years had an equivalent CTB-specific mucosal immunogenicity to freshly harvested rice [63]. This demonstrated the stability of rice seed-based vaccines at room temperature, which can avoid difficult cold-chain requirements.

HBV

The modified HBV surface antigen gene SS1 is a fusion protein that contains amino acids 21–47

from the hepatocyte receptor-binding presurface 1 region fused to the truncated C-terminus of the major HBV surface protein and it has been specifically expressed in rice endosperm under the control of the glutelin *GluB-4* promoter. The recombinant SS1 protein was observed to form virus-like particles in the cytoplasm of transgenic seeds, although its accumulation level was very low (approximately 31.5 ng/g dry weight grain). The production of antibodies against the pre-surface 1 region protected humans from HBV infection by preventing HBV from binding to hepatocytes, when BALB/c mice were immunized with the recombinant SS1 protein [64]. This is a promising candidate as an alternative HBV vaccine for preventing hepatitis B.

Newcastle disease virus

The Newcastle disease virus F protein was expressed and accumulated in the leaf and seed tissues of transgenic rice plants. The immunogenicity of the expressed proteins was tested using a mouse model and specific antibodies were shown to be elicited in mice that had been immunized intraperitoneally using crude protein extracts from transgenic rice plants [65].

Infectious bursal disease virus

Infectious bursal disease virus (IBDV) causes severe immunosuppression in young chickens by destroying the immature B lymphocytes in the bursa of Fabricius. The IBDV host-protective immunogen VP2 protein was expressed in rice seeds. Chickens were orally vaccinated with transgenic rice seeds and challenged with a highly virulent IBDV strain, and they produced neutralizing antibodies against IBDV that conferred protection [66]. These results demonstrated that transgenic rice seeds expressing IBDV VP2 conferred protection against infectious agents in an animal model.

Protection against the roundworm

As16 is characterized as an antigen that confers protection against the swine roundworm *Ascaris suum* and was produced in transgenic rice seed as a chimeric protein fused with the mucosal carrier molecule CTB. When transgenic rice seeds were orally fed to mice with or without CT, the specific serum antibody (IgG) against As16 was only elicited by combination with CT as the mucosal adjuvant. When the immunized mice were subsequently infected with *A. suum* embryonated eggs by oral inoculation, lung worm burden (the number of larvae in the lungs of mice) was significantly reduced compared

with mice fed with the Tg rice without CT or regular food [67].

■ Production of cytokines in rice seed IL-10

Plants can correctly process human IL-10 as biologically active dimers and they provide a suitable platform for the production of cytokines. The accumulation levels are sufficiently high to allow the delivery of an immunologically relevant dose of IL-10.

The functional efficacy of the IL-10 produced as a stable dimer in rice seeds was evaluated by oral administration in pollen- and food-allergy mouse models [11]. Allergen-specific IgE and T-cell proliferation were significantly suppressed by consumption of the transgenic rice seeds, which showed that the IL-10 functional dimer retained its biological activity in rice seed.

GM-CSF

GM-CSF is used to treat the leukopenia caused by cancer chemotherapy or radiotherapy. GM-CSF was expressed under the control of an endosperm-specific glutelin promoter and was localized in PBs and apoplasts [37]. It stimulated the growth of TF-1 cells *in vitro*. Rice seed-based GM-CSF generates its biological activity in a dose-dependent manner, which is identical to that of GM-CSF by injection. Immunohistochemistry demonstrated that after oral consumption in a seed-based form, GM-CSF can pass through the small intestine mucosal wall into the bloodstream of mice, which suggests that GM-CSF can be absorbed as a functional molecule.

Conclusion & future perspective

Rice seed-based oral vaccines offer significant advantages over needle-based vaccines. The expression of vaccine antigens in transgenic plants, such as rice, has the potential to provide a convenient, safe approach for oral vaccination and to provide a feasible alternative to traditional parenteral vaccines. The absence of a need for processing and purification makes the process very cost-effective. In particular, seed-based vaccines circumvent the limitations of other oral vaccines because they are stable for several years at room temperature. It is important to note that not only mucosal, but also systemic immune reactions, are induced by oral administration. Furthermore, recombinant proteins deposited in PBs in rice seed (bioencapsulated vaccines) can be effectively delivered to GALT because they are protected from digestive enzymes in

the GI tract. Improved antigen expression has been established in transgenic rice seed, which serves as a factory for vaccine production. Rice seed-based pharmaceuticals can be cultivated in the field or closed compartments. The plant production system has the advantage of scalability, which permits the large-scale and inexpensive production of vaccines dependent on requirement of many patients. During open-field cultivation, however, there are major concerns regarding out-crossing with wild-type rice. It is also possible that the contamination with nontransgenic rice grains could occur by direct mixing during the harvest and distribution. Zero tolerance of food chain contamination is strictly demanded by consumers and environmental organizations. Thus, contamination via gene transfer via pollen must be avoided using physical or biological methods [68]. Physical containment can be achieved by geographical isolation and different plantings, although rice is a self-pollinating plant and its pollen viability and out-crossing rates are very low. More severe physical segregation can be achieved by cultivation in greenhouses or closed molecular farming facilities (closed glasshouses). Biological methods include the use of sterile male plants or cleistogamy (self-fertilization without flower opening). It would also be helpful to use discriminative markers such as color-coded rice to ensure traceability. Furthermore, it may also be important to take measures to prevent the agent entering the food chain by using specific facilities for processing the seed and establishing a standard operation protocol for the management of transgenic rice.

It has been proposed that pharmaceutical crops should be produced under the control of specific regulations and guidelines. Rice seed-based mucosal vaccines are a new form of medical drug that has to be manufactured according to good manufacturing practice standards, including extensive quality assurance and

control procedures before the start of clinical trials (Phase I) [69]. However, standardization of the dosage (antigen) is a major problem in transgenic rice seeds. The expression levels may vary among plants (and seeds) and generations, depending on physiological conditions. It is necessary to establish a production system where the content and quality of the vaccine antigen expressed by the plants are affected as little as possible, even when cultivated in natural environment conditions, such as in Japan, good manufacturing practice validation of all processes from cultivation to processing of the packaged rice as a final product is required from the Pharmaceuticals and Medical Devices Agency. Allergen-specific immunotherapy is the only curative treatment for allergies. It is based on mechanisms that induce immune tolerance against allergens that induce IgE-mediated type I allergy. The consumption of a rice-based allergy vaccine containing a modified hypoallergenic tolerogen induces very few severe side effects. This allows the administration of high doses of the vaccine from the start of treatment, resulting in a reduction in the length of treatment. At present, preclinical experiments are underway to test rice-based allergy vaccines against cedar pollen allergy in Japan. If seed-based allergy vaccines can be accepted as seed-based mucosal pharmaceuticals, these vaccines will provide an innovative immunotherapeutic approach.

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Executive summary

- Pharmaceuticals (antigens and cytokines) can be accumulated at high levels in seed protein bodies (PBs), particularly when they are expressed in rice seeds under the control of a strong endosperm-specific promoter to be produced as a secretory protein.
- The bioencapsulated antigens in PBs are resistant to proteolysis in the GI tract and delivered much more effectively to the gut-associated lymphoid tissue than those accumulated in other tissues.
- Mucosal and systemic antigen-specific immune responses have been shown to be induced effectively when antigens deposited in rice seed PBs were orally administered. The immune activation or tolerance may be determined by the biological properties of antigens.
- The oral administration of modified allergens with reduced IgE-binding activities in the PBs of rice seeds led to allergen-specific immune suppression, which indicated the efficacy of rice seed-based allergy vaccines as oral tolerogens against aeroallergenic disease.
- Various vaccine antigens against pathogens were produced using transgenic rice seeds. The administration of the rice seed-based vaccines via oral consumption elicited neutralizing serum antibody and protective immunity.
- Cytokines produced in rice seeds retained their biological functions after oral administration.

References

Papers of special note have been highlighted as:

■ of interest

■ of considerable interest

- 1 Twyman RM, Stoger E, Schillberg S *et al.* Molecular farming in plants: host systems and expression technology. *Trends Biotech.* 21, 570–578 (2003).
- 2 Daniell H, Singh ND *et al.* Plant-made vaccine antigens and biopharmaceuticals. *Trends Plant Sci.* 14, 669–679 (2009).
- 3 Sharma AK, Sharma MK. Plants as bioreactors: recent developments and emerging opportunities. *Biotech. Adv.* 27, 811–832 (2009).
- 4 Stoger E, Ma JK, Fischer R *et al.* Sowing the seeds of success: pharmaceutical proteins from plants. *Curr. Opin. Biotechnol.* 16, 167–173 (2005).
- 5 Lau OS, Sun SS. Plant seeds as bioreactors for recombinant protein production. *Biotech. Adv.* 29, 1015–1022 (2009).
- 6 Nochi T, Takagi H, Yuki Y *et al.* Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination. *Proc. Natl Acad. Sci. USA* 104, 10986–10991 (2007).
- Describes novel strategies of foreign antigen expression in rice seeds for the development of needle-free and cold chain-free mucosal vaccines.
- 7 Streatfield SJ. Mucosal immunization using recombinant plan-based oral vaccines. *Methods* 38, 150–157 (2006).
- 8 Takaiwa F. Seed-based oral vaccines as allergen-specific immunotherapy. *Hum. Vaccine* 7, 357–366 (2011).
- 9 Shewry RP, Halford NG. Cereal seed storage proteins: structures, properties and role in grain utilization. *J. Exp. Bot.* 53, 947–958 (2002).
- 10 Streatfield SJ. Approaches to achieve high-level heterologous protein production in plants. *Plant Biotechnol. J.* 5, 2–15 (2007).
- 11 Kawakatsu T, Takaiwa F. Cereal seed storage protein synthesis: fundamental processes for recombinant protein production in cereal grains. *Plant Biotechnol. J.* 8, 939–953 (2010).
- 12 Desai PN, Srivastava N, Padh H. Production of heterologous proteins in plants: strategies for optimal expression. *Biotech. Adv.* 28, 427–435 (2010).
- 13 Qu LQ, Takaiwa F. Tissue specific expression and quantitative potential evaluate of seed storage component gene promoters in transgenic rice. *Plant Biotechnol. J.* 2, 113–125 (2004).
- 14 Yang L, Hirose S, Takahashi H, Kawakatsu T, Takaiwa F. Recombinant protein yield in rice seed is enhanced by specific suppression of endogenous seed proteins at the same deposit site. *Plant Biotechnol. J.* 10, 1035–1045 (2012).
- 15 Gustafsson C, Govindarajan S, Minshull J. Codon bias and heterologous protein expression. *Trends Biotechnol.* 22, 346–353 (2004).
- 16 Benchabane M, Goulet C, Rivard D *et al.* Preventing unintended proteolysis in plant protein biofactories. *Plant Biotechnol. J.* 6, 633–648 (2008).
- 17 Doran, PM. Foreign protein degradation and instability in plants and plant tissue cultures. *Trends Biotechnol.* 24, 426–432 (2006).
- 18 Vitale A, Hinz G. Sorting of proteins to storage vacuoles: how many mechanisms? *Trends Plant Sci.* 10, 315–323 (2005).
- 19 Schroder M. Engineering eukaryotic protein factories. *Biotechnol. Lett.* 30, 187–196 (2008).
- 20 Conrad U, Fiedler U. Compartment-specific accumulation of recombinant immunoglobulins in plant cells: an essential tool for antibody production and immunomodulation of physiological function and pathogen activity. *Plant Mol. Biol.* 18, 101–109 (1998).
- 21 Gomrd V, Fitchette A-C, Menu-Bouaouiche L *et al.* Plant-specific glycosylation patterns in the context of therapeutic protein production. *Plant Biotechnol. J.* 8, 564–587 (2010).
- 22 Tanaka K, Sugimoto T, Ogawa M *et al.* Isolation and characterization of two types of protein bodies in rice endosperm. *Agric. Biol. Chem.* 44, 1633–1639 (1980).
- 23 Krishnan HB, Franceschi VR, Okita TW. Immunochemical studies on the role of the Golgi complex in protein body formation in rice seeds. *Planta* 169, 471–480 (1986).
- 24 Saito Y, Shigemitsu T, Yamazaki *et al.* Formation mechanism of the internal structure of type I protein bodies in rice endosperm: relationship between the localization of prolamin species and the expression of individual genes. *Plant J.* 70, 1043–1055 (2012).
- 25 Lending CR, Larkins BA. Changes in the zein composition of protein bodies during maize endosperm development. *Plant Cell* 1, 1011–1023 (1989).
- 26 Kawakatsu T, Hirose S, Yasuda H, Takaiwa F. Reducing rice seed storage protein accumulation leads to changes in nutrient quality and storage organelle formation. *Plant Physiol.* 154, 1842–1854 (2010).
- 27 Takahashi H, Saito Y, Kitagawa T *et al.* A novel vesicle derived directly from endoplasmic reticulum is involved in the transport of vacuolar storage proteins in rice endosperm. *Plant Cell Physiol.* 46, 245–249 (2005).
- 28 Takaiwa F, Takagi H, Hirose S, Wakasa Y. Endosperm tissue is good production platform for artificial recombinant proteins in transgenic rice. *Plant Biotechnol. J.* 5, 84–92 (2007).
- 29 Drakakaki G, Marcel S, Arcalis E *et al.* The intracellular fate of a recombinant protein is tissue dependent. *Plant Physiol.* 141, 578–586 (2006).
- 30 Arcalis E, Marcel S, Altmann F *et al.* Unexpected deposition patterns of recombinant proteins in post-endospermic reticulum compartments of wheat endosperm. *Plant Physiol.* 136, 3457–3466 (2004).
- 31 Van Droogenbroeck B, Cao J, Stadlmann J *et al.* Aberrant localization and underglycosylation of highly accumulating single-chain Fv–Fc antibodies in transgenic *Arabidopsis* seeds. *Proc. Natl Acad. Sci. USA* 104, 1430–1435 (2007).
- 32 Loos A, Van Droogenbroeck B, Hillmer S *et al.* Expression of antibody fragments with a controlled N-glycosylation pattern and induction of endoplasmic reticulum-derived vesicles in seeds of *Arabidopsis*. *Plant Physiol.* 155, 2036–2048 (2011).
- 33 Kawagoe Y, Suzuki K, Tasaki M *et al.* The critical role of disulfide bond formation in protein sorting in the endosperm of rice. *Plant Cell* 17, 1141–1153 (2005).
- 34 Takaiwa F, Hirose S, Takagi H, Yang L, Wakasa Y. Deposition of a recombinant peptide in ER-derived protein bodies by retention with cysteine-rich prolamins in transgenic rice seed. *Planta* 229, 1147–1158 (2009).
- 35 Suzuki K, Kaminuma O, Yang L *et al.* Prevention of allergic asthma by vaccination with transgenic rice seed expressing mite allergen: induction of allergen-specific oral tolerance without bystander suppression. *Plant Biotechnol. J.* 9, 982–990 (2011).
- Demonstrates that oral administration of transgenic rice containing Der p 1 fragments induced allergen-specific immune suppression without associated bystander suppression. Airway inflammation was inhibited by prophylactic oral vaccination of transgenic rice seeds.
- 36 Yang L, Hirose S, Suzuki K, Hiroi T, Takaiwa F. Expression of hypoallergenic Der f 2 derivatives with altered intramolecular disulphide bonds induces the formation of novel ER-derived protein bodies in transgenic rice seeds. *J. Exp. Bot.* 63, 2947–2959 (2012).
- 37 Ning T, Xie T, Qiu Q *et al.* Oral administration of recombinant human granulocyte macrophage colony stimulating factor expressed in rice endosperm can increase leukocytes in mice. *Biotechnol. Lett.* 30, 1679–1686 (2008).

- 38 Takagi H, Hiroi T, Hirose S, Yang L, Takaiwa F. Rice seed ER-derived protein body as an efficient delivery vehicle for oral tolerogenic peptides. *Peptides* 31, 1421–1425 (2010).
- Reports that bioencapsulation of antigens in endoplasmic reticulum-derived protein bodies has advantages in digestibility and immunological efficacy as mucosal delivery vacuoles or chemically synthesized peptides.
- 39 McGhee JR, Michalek SM, Kiyono H *et al.* Mucosal immunoregulation: environmental lipopolysaccharide and GALT T lymphocytes regulate the IgA response. *Microbiol. Immunol.* 28, 261–280 (1984).
- 40 Faria AM, Weinrer HL. Oral tolerance: therapeutic implications for autoimmune diseases. *Clin. Dev. Immunol.* 13, 143–157 (2006).
- 41 Dubois B, Goubier A, Joubert G, Kaiserlian D. Oral tolerance and regulation of mucosal immunity. *Cell. Mol. Life Sci.* 62, 1322–1332.
- 42 Tsuji NM, Kosaka A. Oral tolerance: intestinal homeostasis and antigen-specific regulatory T cells. *Trends Immunol.* 29, 532–540 (2008).
- 43 Kunisawa J, Kurashima Y, Kiyono H. Gut-associated lymphoid tissues for the development of oral vaccines. *Adv. Drug Delivery Rev.* 64, 523–530 (2012).
- 44 Iwasaki A, Kelsall BL. Unique functions of CD11b⁺, CD8a⁺, and double-negative Peyer's patch dendritic cells. *J. Immunol.* 166, 4884–4890 (2001).
- 45 Kelsall BL, Leon F. Involvement of intestinal dendritic cells in oral tolerance, immunity to pathogens, and inflammatory bowel disease. *Immunol. Rev.* 206, 132–148 (2005).
- 46 Mucida D, Park Y, Cheroutre H. From the diet to the nucleus: vitamin A and TGF- β join efforts at the mucosal interface of the intestine. *Semin. Immunol.* 21, 14–21 (2009).
- 47 Mayer L, Shao L. Therapeutic potential of oral tolerance. *Nature Rev. Immunol.* 4, 407–419 (2004).
- 48 Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. *Ann. Rev. Immunol.* 23, 515–548 (2005).
- 49 Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-I and its ligands in tolerance and immunity. *Ann. Rev. Immunol.* 26, 677–704 (2008).
- 50 Awasthi A, Carrier Y, Peron JP *et al.* A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. *Nature Immunol.* 12, 1380–1390 (2007).
- 51 Ozdemir C, Akids M, Akids CA. T regulatory cells and their counterparts: masters of immune regulation. *Clin. Exper. Allergy* 39, 626–639 (2009).
- 52 Frew AJ. Immunotherapy of allergic diseases. *J. Allergy Clin. Immunol.* 111, S712–S719 (2003).
- 53 Larche M, Akdis CA, Valenta R. Immunological mechanisms of allergen-specific immunotherapy. *Nature Rev. Immunol.* 6, 761–766 (2006).
- 54 Ali FR, Larche M. Peptide-based immunotherapy: a novel strategy for allergic disease. *Expert Rev. Vaccines* 4, 881–889 (2005).
- 55 Larche M. Peptide immunotherapy for allergic diseases. *Allergy* 62, 325–331 (2007).
- 56 Hirahara K, Saito S, Serizawa N *et al.* Oral administration of a dominant T-cell determinant peptide inhibits allergen-specific Th1 and Th2 cell response in Cry j 2-primed mice. *J. Allergy Clin. Immunol.* 102, 961–967 (1998).
- 57 Takagi, H, Hiroi T, Yang L *et al.* A rice-based edible vaccine expressing multiple epitopes induces oral tolerance for inhibition of Th2-mediated IgE response. *Proc Natl Acad. Sci. USA* 102, 17525–17530 (2005).
- Reports, for the first time, that feeding of transgenic rice seeds containing T-cell epitopes could prevent the immune response that triggers allergies, providing the potential for a rice-based allergy vaccine.
- 58 Hirahara K, Tastuta T, Takatori T *et al.* Preclinical evaluation of an immunotherapeutic peptide comprising 7 T-cell determinants of Cry j 1 and Cry j 2, the major Japanese cedar pollen allergens. *J. Allergy Clin. Immunol.* 108, 94–100 (2001).
- 59 Takagi H, Saito S, Yang L *et al.* Oral immunotherapy against a pollen allergy using a seed-based peptide vaccine. *Plant Biotechnol. J.* 3, 521–533 (2005).
- 60 Domon E, Takagi H, Hirose S *et al.* 26-week oral safety study in macaques for transgenic rice containing major human T-cell epitope peptides from Japanese cedar pollen allergens. *J. Agric. Food Chem.* 57, 5633–5638 (2009).
- 61 Wakasa Y, Takagi H, Hirose S *et al.* Oral immunotherapy with transgenic rice seed containing structurally disrupted Japanese cedar pollen allergens, Cry j 1 and Cry j 2, against Japanese cedar pollinosis. *Plant Biotechnol. J.* 11(1), 66–76 (2013).
- 62 Nochi T, Yuki Y, Katakai Y *et al.* A rice-based oral cholera vaccine induces macaque-specific systemic neutralizing antibodies but does not influence pre-existing intestinal immunity. *J. Immunol.* 6538–6544 (2009).
- 63 Tokuhara D, Yuki Y, Nochi T *et al.* Secretory IgA-mediated protection against *V. cholerae* and heat-labile enterotoxin-producing enterotoxigenic *Escherichia coli* by rice-based vaccine. *Proc. Natl Acad. Sci. USA* 107, 8794–8799 (2010).
- 64 Qian B, Shen H, Liang W *et al.* Immunogenicity of recombinant hepatitis B virus surface antigen fused with preS1 epitopes expressed in rice seeds. *Transgenic Res.* 17, 621–631 (2008).
- 65 Yang Z, Liu Q, Pan Z, Yu HX, Jiao XA. Expression of the fusion glycoprotein of Newcastle disease virus in transgenic rice and its immunogenicity in mice. *Vaccine* 25, 591–598 (2007).
- 66 Wu J, Yu L, Li L *et al.* Oral immunization with transgenic rice seeds expressing VP2 protein of infectious bursal disease virus induces protective immune responses in chickens. *Plant Biotechnol. J.* 5, 570–578 (2007).
- 67 Matsumoto Y, Suzuki S, Nozoye T *et al.* Oral immunogenicity and protective efficacy in mice of transgenic rice plants producing a vaccine candidate antigen (As16) of *Ascaris suum* fused with cholera toxin B subunit. *Transgenic Res.* 18, 185–192 (2009).
- 68 Murphy DJ. Improving containment strategies in biopharming. *Plant Biotechnol. J.* 5, 555–569 (2007).
- 69 Fischer R, Schillberg S, Hellwig S, Twyman RM, Drossard J. GMP issues for recombinant plant-derived pharmaceutical proteins. *Biotech. Adv.* 30, 434–439 (2012).
- 70 Yuki Y, Tokuhara D, Noichi T *et al.* Oral MucoRice expressing double-mutant cholera toxin A and B subunits induces toxin-specific neutralizing immunity. *Vaccine* 27, 5982–5988 (2009).
- 71 Yuki Y, Mejima M, Hiroiwa T *et al.* RNAi suppression of rice endogenous storage proteins enhances the production of rice-based Botulinum neurotoxin type A vaccine. *Vaccine* 30, 4160–4166 (2012).