Chapter 12
Plant-Based Vaccines Against Pollen Allergy

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Introduction

Plant pollens are one of the most common causes of seasonal allergic diseases including allergic rhinitis, conjunctivitis, atopic dermatitis, and asthma. These occur as a consequence of fundamental allergenic mechanisms involving the induction of pollen-specific T helper type 2 (Th2) effector cells from naïve Th0 cells. Allergic diseases are characterized by allergen-specific immunoglobulin E (IgE) production and the activation of effector cells including eosinophils, mast cells, and basophils (Bousquet et al. 1998, 2009; Frew 2010). Immunological binding of allergen-specific IgE via Fc receptors (FcεRI) leads to mast cell and circulating basophil degranulation and release of the chemical mediators of inflammation. These events are regulated by Th2 cells, which preferentially produce interleukin (IL)-4, IL-5, and IL-13. Therefore, allergic diseases have been defined as the inadequate peripheral regulation of allergy-specific T cells.

Treatment strategies for these allergic diseases generally involve pharmacotherapies including antihistamines, leukotriene receptor antagonists, and corticosteroids (Holgate and Polosa 2008). Although these approaches reduce clinical symptoms by blocking the release of the critical mediators of allergic reactions or by inhibiting allergic inflammation, they are not curative and sometimes induce impaired performance as a result of side effects. Allergen-specific immunotherapy (allergen-SIT) is the only curative and antigen-specific method to treat allergic diseases by inducing immunological tolerance to the allergens responsible for the disease through multiple cellular and molecular mechanisms (Larche et al. 2006; Till et al. 2004). Such conventional allergen-SIT has been practiced for almost a century (Akdis and Akids 2007; Noon 1911). Success can be achieved by repeated subcutaneous injections of increasing doses of native allergen extracts over a period of at least 3–5 years in order to induce desensitization to the allergen. However, this treatment is sometimes

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accompanied by severe side effects, such as anaphylaxis, which is caused by the capture of the allergen together with a specific anti-allergen IgE on the surface of mast cells and circulating basophils.

It is highly desirable to develop safer, more effective, and convenient allergen-SITs to overcome these issues. To achieve this goal, tolerogens used for desensitization to allergens must be changed from crude allergen extracts to either modified hypoallergenic tolerogens with reduced IgE binding and enzymatic activity or the T cell epitope peptides required for the recognition of specific T cells, while maintaining T cell activity or immunogenicity. Second, the route of administration should be changed from systemic to mucosal (oral administration), resulting in increased convenience and relief from the pain of an injection.

Plants offer an ideal production platform for oral mucosal vaccines in terms of low production cost, no contamination by mammalian pathogens, high stability at ambient temperatures, and easy control of the production scale (Rybicki 2010; Yosibov and Rabindran 2008; Daniell et al. 2009). Production in seeds has already been demonstrated to give rise to a high yield of recombinant proteins based on established expression systems (Stoger et al. 2005; Lau and Sun 2009; Takaiwa 2011). The antigens that accumulate in seeds can be protected from degradation by the harsh environment of the gastrointestinal tract, and thereby provides a suitable delivery vehicle to gut-associated lymphoid tissue (GALT). Oral immune tolerance to allergens is expected to be induced in a manner similar to food proteins through the administration of a continuous dose of allergens in the diet. Seed-based allergy vaccines represent an innovative oral allergen-SIT for the control of allergenic diseases. We herein describe the feasibility of seed-based allergy vaccines against two representative pollen allergies.

Key Mechanisms Behind Allergen-SIT

Successful allergen-SIT by a conventional subcutaneous injection is associated with a decrease in allergen-specific IgE antibody production in the serum concomitant with the upregulation of IgG4 and IgA, which exhibit potential blocking activity by binding to the receptors of mast cells and basophils or competing with IgE for binding to the allergen (Meiler et al. 2008; Shamji et al. 2012). This altered IgE/IgG4 antibody balance also contributes to the reduction in allergen-induced IgE-mediated histamine release by mast cells and basophils and the inhibition of IgE-facilitated allergen presentation to T cells (James and Durham 2008), resulting in significant reductions in the numbers of infiltrating T cells, eosinophils, basophils, and neutrophils (Table 12.1).The induction of peripheral T cell tolerance is achieved by altering the balance between antigen-specific Th2- and Th1-type cells and/or by the induction of regulatory T cells (Tregs), leading to a reduction in inflammatory cell recruitment and activation and the suppression of mediator secretion from mast cells and basophils. The upregulation of Tregs from both naturally occurring thymus-derived forkhead box p3 (Fox p3$^+$) CD4$^+$CD25$^+$ regulatory T cells (nReg)

Table 12.1 Mechanisms of allergen-specific immunotherapy

Mode of action

1. Reduction in specific IgE levels
2. Induction of specific IgG4 and IgA as blocking antibodies
3. Reduction in basophil and mast cell activities
4. Shift to Th1 from Th2
5. T cell anergy or deletion of Th2 effector cells
6. Induction of regulatory T cells
7. Suppression of inflammatory DCs

Substances involved in action effects

1. Suppression of Th2 cell cytokine production (IL-4, IL-5, and IL-13)
2. Upregulation of Th1 cell cytokine production (IL12 and IFN-γ)
3. Upregulation of immunosuppressive cytokine IL-10 and TGF-β production
4. Suppression of the infiltration of T cells, eosinophils, basophils, and neutrophils
5. Suppression of the chemical mediators from mast cells and basophils

Effects induced by allergen-specific immunotherapy

1. Reduction in symptom scores (sneezing number and nasal itching)
2. Reduction in medication scores
3. Improvements in the quality of life (QOL)score
4. Long-term remission of symptoms (prevention of progression)
5. Prevention of the onset of new sensitizations

and peripheral inducible Tregs (iTregs) such as type 1 Tregs (Treg 1), Th3 cells, and mucosally induced Fox p3$^+$CD4$^+$CD25$^+$ cells (Foxp3$^+$iTreg), suppresses the development of allergic diseases via several immune mechanisms including the suppression of dendritic cells (DCs), Th cells, mast cells, eosinophils, and basophils in the skin, nose, eye, and bronchial mucosa (Jutel and Akdis 2011; Ozdemir et al. 2009; Palomares et al.2010).

   The specific inhibition of T cell proliferation and allergenic inflammation is dependent on the suppressive cytokines IL-10 and TGF-β released by different populations of Tregs and DCs (Jutel et al. 2003). IL-10 is produced by Treg1, Fox p3$^+$ iTregs, CD8$^+$ Treg, γδ T cells, and DCs, whereas transforming growth factor-β (TGF-β) is mainly produced by Th3 cells and Fox p3$^+$ nReg. IL-10 directly acts on CD4$^+$ T cells and downregulates the production of IL-2 and IFN-γ by Th1 cells and of IL-4, IL-5, and IL-13 by Th2 cells. The production of allergen-specific IgE and expression of the IgE receptor are suppressed by IL-10, whereas it induces a class switch toward IgG4 production. Moreover, allergic inflammation in peripheral tissue is also suppressed by decreasing proinflammatory cytokine release from mast cells and depressing eosinophil activities (Ozdemir et al. 2009; Palomares et al. 2010). On the other hand, TGF-β is involved in the conversion of naïve T cells in Tregs, inhibition of the proliferation of T cells and B cells, downregulation of effector cytokine production, and suppression of macrophages, DCs, and natural killer cells (Yoshimura et al. 2010). Moreover, it has also been implicated in the induction of class switching to IgA.

   Therefore, the basic principle of allergen-SIT is to induce immune tolerance to the allergens causing the disease through multiple cellular and molecular mechanisms, leading to a reduction in inflammatory cell recruitment and activation and also mediator secretion from mast cells and basophils. The induction of a tolerant state in peripheral T cells represents an essential step in allergen-SIT.

Advantage of Seed-Based Allergy Vaccines as a Delivery System to GALT

When crude antigen extracts used in subcutaneous injections are orally administrated, they are generally subjected to proteolysis in the gastrointestinal tract before they arrive at immune cells in GALT, resulting in little significant efficacy. Thus, to achieve the same level of efficacy as that of a subcutaneous injection, oral administration requires doses that are hundreds of times higher. This limitation makes it impossible to establish allergen-SIT for allergic diseases via oral administration (Polovic and Velickovic 2008).

   However, irrespective of the markedly lower concentrations than those required from naked allergen proteins, plant-based antigens are expected to be effectively delivered to mucosal immune cells in GALT through protection from harsh conditions in the gastrointestinal tract, and immune tolerance can be easily induced when vaccines are produced in plant cells and directly administered through the oral route (Streatfield 2006; Pelosi et al. 2012; Paul and Ma 2010). This may be explained by the bioencapsulation of antigens with double barriers, consisted of the cell wall and intracellular compartments such as the protein body (PB). Furthermore, because cells containing antigens are filled with starch or lipid, the antigen may be gradually released to the gastrointestinal tract. Thus, when antigen presentation to antigen-presenting cells (APC) is prolonged under these conditions, the efficiency of immune responses (tolerance) is improved.

   When digestibility and immune tolerance-inducing capacity were compared between endoplasmic reticulum (ER)-derived PB (PB-I)-containing and protein-storage vacuole (PSV; PB-II)-containing antigens, the resistance of the ER-derived PB in cereal grain to gastrointestinal digestion enzymes was previously shown to be stronger than that of the PSV (Takagi et al. 2010). This physical difference may be related to the polymerized or aggregated formulation of the antigen observed in ER-derived PBs, which are formed by disulfide bonds through cys-rich prolamins. Interestingly, several prolamins with different physical properties in cereal grains have been shown to be tightly packaged in a specific arrangement in ER-derived PBs during seed maturation (Lending and Larkins 1989; Saito et al. 2012; Takaiwa 2013a). Taken together, the resistance of antigens to proteolysis against digestive enzymes is associated with the efficacy of edible vaccines. Physical bioencapsulation within plant cells provides an effective delivery system for an oral vaccine.

Induction Mechanisms of Oral Immune Tolerance

Immune tolerance levels through the oral route have been reported to vary depending on the dose, frequency, or formulation of the administrated antigen (Mayer and Shao 2004; Burks et al. 2008; Weiner et al. 2011). A soluble antigen is known to be more tolerogenic than a particulate one, although the latter is more resistant to the harsh conditions in the gastrointestinal tract than the former. However, protection from proteolysis by digestive enzymes has a crucial effect on the efficacy of antigens. Furthermore, oral administration can induce both mucosal and systemic immunity, whereas parenteral delivery cannot. Thus, the selection of an oral route is rational as an administration method because immune tolerance is more prone to be induced than by immune stimulation.

The high-dose administration of an antigen (100–500 mg) was shown to result in the deletion or anergy of lymphocytes (T cells) (Mayer and Shao 2004). Fas (CD95)-dependent apoptosis was responsible for the deletion of effector T cells (Marth et al. 1998). Allergen-specific T cell anergy occurs when incomplete activation signals are sent through T cell receptor (TCR) interactions between factors such as B7-1 (CD80) or B7-2 (CD86) (B7 family) on APC with CD28 (B28 family) on T cells or when a lack of costimulatory molecules occurs during this activity (Appeman and Boussiotis 2003). In addition to the original B7/CD28 family members, several B7 family members such as inducible costimulator ligand (ICOSL (B7-H2)), PDL-1 (B7-H1), PDL-2 (B7-DC), and B7-H3 have been identified to date. Several CD28 families including B7-1 (CD80), PD-1, cytotoxic T lymphocyte antigen-4, and inducible costimulator (ICOS) have also been identified (Greenwald et al. 2005; Keir et al. 2008). Downregulation of the costimulation molecule CD80/CD86 on APC was observed with an increase in programmed death ligand (PDL-1) as an inhibitory costimulator through the induction of anergy in the process of oral immune tolerance (Piconi et al. 2010). High PDL-1 and PDL-2 expression levels in DCs in the gut have been implicated in oral tolerance through the induction of antigen-specific Tregs. The ICOSL was also shown to be involved in the induction of Tregs. Thus, interactions involving costimulatory molecules play a key role in the regulation of T cell activation and tolerance.

In contrast, the repeated administration of a low-dose antigen (1–5 mg) was shown to be mediated by active immune suppression through the induction of Tregs, such as $CD4^+$ iTregs, Fox $p3^+$ iTregs, Th3 cells, Tr1 cells, Fox $p3^+$ $CD4^+$ $CD25^+$ nTregs, $CD8^+$ T cells, and $\gamma\delta$ T cells (Weiner et al. 2011). These Tregs express anti-inflammatory IL-10 or TGF-β cytokines as well as CTRA-4 as the costimulation factor. The CTLA-4 released from Fox $p3^+$ $CD4^+$ $CD25^+$ is known to bind to CD80 and CD86 on APCs with a higher affinity than CD28 and to counteract the activation delivered by the TCR. Foxp3$^+$ $CD4^+$ $CD25^+$ nTregs in particular involve the expression of cell-surface-bound TGF-β, which can act as a cognate suppressive factor. IL-10-producing immature DCs ($CD11b^+$) and $CD103^+$ DC in the gut have been shown to contribute to immune tolerance through the induction of Tr1 and Foxp3$^+$ iTregs, respectively (Lafaille and Lafaille 2009; Belkaid and Oldenhave

2008). Lamina propria (LP) macrophages can efficiently induce Foxp3^{+} T cells in the presence of TGF-β. The tolerogenic capacity of DCs depends on the maturation stage. Immature or partially mature DCs have the ability to induce peripheral tolerance through the generation of Tregs, whereas fully mature DCs prime naïve T cells to different effector Th cells. It is generally accepted that myeloid DC and plasmacytoid DC (pDC) are different functional subsets. The pDCs play an essential role in the prevention of allergy sensitization by inducing IL-10-producing Tregs.

The increased levels of IL-10 and TGF-β potently suppress allergen-specific IgE production from B cells by inducing a class switch toward the non-inflammatory isotype IgG4 and mucosal IgA production, respectively. Tregs are able to form aggregates around DCs, which inhibit their maturation and also downregulate their costimulatory molecules. In addition, Tregs directly or indirectly suppress the effector cells of allergic inflammation such as mast cells, basophils, and eosinophils as well as Th2, by producing the regulatory cytokines TGF-β and IL-10 (Ozdemir et al. 2009; Palomares et al. 2010; Jutel et al. 2003). The suppression of mast cells (FcεRI-dependent mast cell degranulation) by Tregs was previously shown to be mediated through cell–cell direct contact involving OX40–OX40 ligand interactions (Gri et al. 2008). In conclusion, peripheral tolerance to allergens is controlled by multiple active suppression mechanisms. (Table 12.2)

The differentiation of IL-10-producing Treg 1 is induced by IL-6, IL-27, and TGF-β, whereas IL-6, IL-23, and TGF-β are responsible for the full differentiation of Th17 implicated in autoimmunity and pathogen attacks (Bettelli et al. 2006; Mucida et al. 2007; Awasthi et al. 2007; McGeachy et al. 2007). Both IL-6 and IL-27 are produced by DCs and macrophages. The conversion of Fosp3^{+} iTregs from naïve CD4^{+} T cells is also induced by TGF-β and retinoic acid (RA), which are produced in DCs (CD103^{+} DC) with retinal dehydrogenase activity in the small intestine and mesenteric lymphoid nodes (MLN) of the gut (Mucida et al. 2007). Therefore, DCs from the small intestine and MLN are efficient in the peripheral conversion of Tregs. RA suppresses differentiation from naïve T cells to Th17, while IL-6 inhibits TGF-β-induced Foxp3^{+} iTreg induction, resulting in the differentiation of Th17 (Mucida et al. 2007; McGeachy et al. 2007). Th3 cells contribute to the induction of Fox p3[+] iTregs through the production of TGF-β. TGF-β also induces the Runt-related transcription factors RUNX1 and RUNX3 that bind to the *FOXP3* promoter, which participates in the development and function of Tregs. Inactivation of the RUNX cofactors leads to a decreased number of iTregs.

Uptake of Antigens and Immune Reactions in GALT

When antigens capsulated in the aggregate formulation such as PBs were orally administered, they were demonstrated to cross the intestinal epithelial cell barrier in several ways (Mayer and Shao 2004; Burks et al. 2008). Particulates of <10 μm such as PBs (usually 1–2 μm) are principally taken up by M cells in the follicle-associated epithelium (FAE) covering Peyer's patches (PPs) as part of

Table 12.2 Regulatory T cells and dendritic cells involved in immune tolerance and their effects

Regulatory T cells	Secreted cytokines
Thymus-derived natural Fox p3^{+} T cell (nFox p3)	TGF-β
Inducible Foxp3^{+} T cell (iFox p3)	IL-10, TGF-β
Treg1(Tr1)	IL-10
Th3	TGF-β
CD8^{+} Treg	IL-10

Effect of regulatory T cells

1. Suppression of effector Th2 cells
2. Suppression of effector Th1 cells
3. Suppression of effector Th17 cells
4. Anergy of T cells
5. Suppression of T cell migration to tissues
6. Direct and indirect suppressive effects on mast cells, basophils, and eosinophils
7. Suppression of inflammatory DC and the induction of IL-10-producing DCs
8. Induction of IgG4 by B cell class switch (IL-10)
9. Induction of IgA by B cell class switch (TGF-β)

Dendritic cells (DCs)	Induced effector T cells
CD103^{+} DC (retinoic acid (RA)+TGF-β)	iFoxp3^{+}Treg.
DC (IL-10+RA)	Tr1
DC (TGF-β+IL-6+IL-27)	Tr1
DC (TGF-β+IL-6)	Th17
CD11c^{+} DC (TGF-β)	Th3
CD11b^{+} DC (IL-10, IL-27)	Tr1
Plasmacytoid DC	Tr1
CD11b[+](TGF-b^{+}IL-6^{+}IL10)	Th2
IDO^{+}CD11c^{+}	iFoxp3^{+}Treg

GALT in the small intestine and are then presented to APCs such as DCs in adjacent mucosal T cell areas. M cells act as a portal for the uptake of antigens from the intestinal lumen that are transferred into DCs in the PPs, which are composed of 5–10 % of cells in FAE. As examples of other routes, antigens up to the size of a few micrometers or nanometers are processed and presented by intestinal epithelial cells. CD103^{+} DCs themselves are capable of extending their dendrites through the epithelium (tight junctions) in the LP into the gut lumen to catch antigens without disrupting the tight junctions and function as a conduit for the delivery of antigens. Subsequently, antigens sampled by CD103^{+} DCs are carried to the local MLN and are then presented to specific T cells, which indicates that MLN plays a central role in the acquisition of oral tolerance (Weiner 2011; Tsuji and Kosaka 2008). Moreover, liver pDCs can also contribute to the acquisition of tolerance against oral antigens because the liver stores up to 80 % of total body retinol.

GALT contains an organized macro-architecture of B and T lymphocyte zones that respond to the antigens presented by DCs, which can induce memory B and T cells (Tsuji and Kosaka 2008; Neutra and Kozlowski 2006). B cells are a major

component of PP cells, comprising more than 70%, and are preferentially located in the follicle region. Formation of the germinal center occurs in the PPs, in which class switching of B cells from IgM to IgA is achieved. T cells account for approximately 20% of PP cells and are mainly located in the FAE region that mainly contains naïve T cells (Kunisawa et al. 2012). Differentiation into IFN-γ-producing Th1, IL-4-producing Th2, or IL-10-producing Fox p3$^+$ Tregs is induced depending on the presentation of various types of DCs. There are at least three subsets of DCs with distinct tissue distribution in PPs (Milling et al. 2010). CD11b$^+$ (CD11b$^+$CD8α$^-$) myeloid DCs are present in subepithelial domain (SED) regions, CD8$^+$ (CD11b$^-$CD8α$^+$) lymphoid DCs in the T cell-rich interfollicular region (IFR), and double-negative CD4$^-$CD8α$^-$DCs in both SED and IFRs (Neutra and Kozlowski 2006). DCs in the latter two regions produce IL-12 and induce IFN-γ-producing Th1 cells, which are responsible for pathogen clearance. The production of IL-10 and IL-27 by CD11b$^+$ cells (myeloid DCs) plays a critical role in oral tolerance by inhibiting the differentiation of naïve T cells to Th 17 cells as well as enhancing IL-10 production by Tregs, since CD11b$^+$ cell-deficient animals have a defect in oral tolerance. In contrast, CD11c$^+$ mucosal DCs preferentially produce anti-inflammatory cytokines (Fig. 12.1).

Another important component of GALT, which serves to regulate intestinal homeostasis, is intraepithelial lymphocytes (IELs), which constitute 10–20% of epithelial cells. Approximately 30–40% of all peripheral T cells are present in IELs. The lack of IELs results in the loss of oral immune tolerance, which indicates their significant participation in gut oral immune tolerance. The majority of IELs contain CD8$^+$ T cells with regulatory activity, which express αβ or γδ TCRs Kunisawa et al. 2007). CD8$^+$ Tregs are involved in oral tolerance by suppressing Th1 and Th17 responses. γδ T cells are important mediators of mucosal tolerance.

Benefits of Hypoallergenic Tolerogens

Many advantages are associated with replacing the natural allergen extracts used to induce immune tolerance (desensitization) with recombinant proteins having reduced allergenicity (low IgE binding activity), because the former can bind to specific IgE on mast cells and basophils, leading to anaphylactic side effects (Valenta et al. 2010). Therefore, new approaches to SIT using modified hypoallergenic antigen derivatives (so-called hypoallergens) are required in order to increase the safety of immunotherapy by reducing the risks of anaphylactic reactions relative to the corresponding natural allergen (Focke et al. 2010). Binding of an allergen to the specific IgE was previously shown to be determined by a continuous stretch of amino acids (B cell epitope) or conformational structures. Thus, in order to develop such ideally hypoallergenic tolerogens as immune modulators, deletion, site-directed mutagenesis, fragmentation, oligomeric formation, or molecular shuffling have all been tested (Valenta et al. 2011; Linhart and Valenta 2005; Cromwell et al. 2011). Recombinant hypoallergenic allergen derivatives exhibit reduced IgE
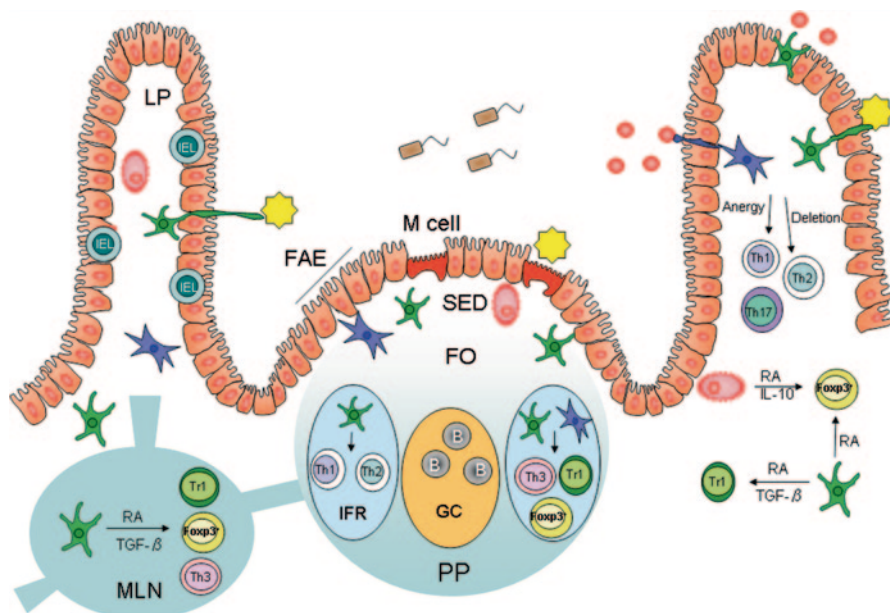
Fig. 12.1 Mechanisms of oral tolerance induction. Orally delivered antigens can be captured by antigen presentation cells (*APCs*) such as DCs and macrophages in the GALT. DCs and macrophages in the subepithelial dome of peyer's patches (*PPs*) and in the lamina propria (*LP*) of the intestine take up fed antigens via M cell overlying PPs. LP DCs can directly sample luminal antigens by extending their dendrites between IECs. DCs can present the antigens to T cells or after migration to the mesenteric lymph node (*MLN*), an attending lymph node in the intestinal mucosa. A number of different types of Tregs (Foxp3$^+$iTregs, nTregs, Tr1 cells, Th3 cells, CD8$^+$ Tregs, and γδT cells) are induced or expanded in the gut and are involved in oral tolerance. *FO* follicle, *FAE* follicle-associated epithelium, *GALT* gut-associated lymphoid tissue, *GC* germinal center, *IEL* intraepithelial lymphocytes, *IFR* intrafollicular region, *LP* lamina propria, *MLN* mesenteric lymph node, *PP* peyer's patch, *SED* subepithelial domain

reactivity; hence, they do not induce IgE-mediated side effects when administered to allergic patients.

Vaccinations with these genetically modified recombinant allergen derivatives induced the production of protective IgG4 antibodies against specific IgE, which was accompanied by improvements in clinical symptoms and skin sensitivity. Furthermore, hybrid molecules consisting of multiple allergens have been developed for several important allergen sources such as birch pollen allergens and timothy glass pollen allergens. Vaccinations with hybrid molecules composed of five timothy grass pollen allergens significantly improved grass pollen-induced immediate-type allergic symptoms and resulted in the reduced consumption of medication (Linhart et al. 2002).

On the other hand, peptide immunotherapy using dominant T cell epitopes derived from allergens offers a safe and ideal treatment for controlling allergic diseases because regions involved in allergenicity can be completely excised while

ensuring the retention of immunogenicity (Larché 2007; Moldaver and Larche´ 2011). It should be noted that designing effective peptide vaccines for human immunization is complicated by the polymorphism of MHC and antigenic complexity of allergens. Hybrid peptides composed of multiple major T cell epitopes from bee venom (Api ml) and cat allergens (Fel d 1) have been created to compensate for genetic viability. Hybrid peptides can also be engineered to preserve most allergen-specific T cell epitopes derived from a few molecules by linking several T cell epitopes.

Production of Tolerogens in Plants

To produce the allergy vaccines (tolerogens) required to induce immune tolerance by oral administration in plants, it is important to increase their production levels as much as possible. Enhancing strategies for vaccine levels include strong tissue-specific promoters, codon optimization, translation fusion, targeting expression to specific tissues or subcellular localization, and introgression of transgenes into the germplasm more suitable for high-level expression (Streatfield 2007; Sharma and Sharma 2009; Kawakatsu and Takaiwa 2010).

Several parameters that determine transcription, translation, and posttranslational modifications have to be optimized to boost accumulation levels. Expression levels are primarily determined by transcription levels; therefore, it is critical to employ strong tissue-specific promoters to enable higher levels of gene expression in targeted deposition tissue. Seed-specific promoters are desirable for the high-level accumulation of recombinant proteins because the constitutive expression of recombinant proteins by CaMV 35S, ubiquitin, or actin promoters has sometimes detrimental or vital effects on vegetative growth (Lau and Sun 2009; Sharma and Sharma 2009).

The endosperm of cereal grain accounts for more than 80 % of total seed weight and is a specialized storage organ for starch and proteins, whereas the embryo or cotyledon is a storage organ of dicot seeds. Rice major seed storage protein (SSP) glutelin (*GluB-1, GluB-2, GluA-2*), 26-kDa globulin, or 10-kDa and 16-kDa prolamin promoters have been used as strong endosperm-specific promoters for the expression of recombinant proteins in transgenic rice seed (Qu and Takaiwa 2004). Foreign products highly accumulated as detectable proteins in the CBB-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels of total SSPs, accounting for up to 12 % of total seed proteins (10 mg/g dry seeds). In maize and barley seeds, γ-zein, globulin, and hordein promoters have been used for these transgenic plants. When expressed in dicot seeds, soybean glycinin and β-conglycinin, common bean β-phaseolin and arcelin-5 promoters, pea legumin (legA), and broad bean unknown seed protein (USP) promoters have been employed and resulted in high yields of target recombinant proteins, which reached a level 36.5 % of total seed proteins at the maximum yield (Lau and Sun 2009; Sharma and Sharma 2009).

   To improve accumulation levels, it is critically important to optimize codons in the coding sequence by exchanging rare codons with those frequently used in highly expressed plant genes taking the difference in codon bias into consideration, and further accumulation can be achieved by eliminating the A/U-rich mRNA-destabilizing motif, AUUUA motif, and the polyadenylation signal, AAUAAA motif. Codon optimization resulted in a 5–100-fold enrichment of the accumulation yield over that of the original one (Gustafsson et al. 2004).

   Furthermore, the 5′ and 3′ untranslated regions (UTRs), which are involved in translation efficiency or mRNA stability, must also be considered. The complete 5′ and 3′ UTRs derived from SSPs are usually attached to the coding sequence of target recombinant proteins (Sharma and Sharma 2009). Use of the 3′ UTR derived from the SSP gene terminated more specifically at a few sites than the noparine synthase (Nos) terminator, leading to improvements in accumulation level by stabilizing transcripts.

Targeting to Intracellular Compartments

The trafficking process and intracellular localization site of expressed recombinant proteins have a crucial influence on folding, assembly, and posttranslational modifications such as glycosylation (Benchabane et al. 2008). Therefore, it is important to deposit recombinant proteins into the suitable intracellular site, which allows stable and ample storage. The accumulation capacity may be affected by the individual intracellular compartment determining the deposition space (Khan et al. 2012). For example, the endosperm cells of cereal seeds are dominated by starch granules and PBs, whereas dicotyledonous legumes or rape seed cells are filled with protein and oil bodies.

   The ER is an entry point for the secretory pathway and secretory proteins are correctly folded in the ER lumen before sorting. Attachment of the signal peptide to the N-terminus of the recombinant protein is essential for the expression of recombinant proteins as secretory proteins. When pharmaceutical proteins such as vaccines and antibodies are produced in plants, targeting to the secretory pathway generally improves accumulation levels as a result of the higher folding ability of ER-resident chaperons, such as binding proteins (BiP) and protein disulfide isomerase (PDI), or the low proteolytic activity in the ER lumen. Secretory proteins are subject to assessment by protein quality control in the ER lumen prior to trafficking to the destination site via the endomembrane system (Vitale and Boston 2008). If they cannot be properly folded or assembled, unfolded or misfolded proteins are finally degraded by the ER-associated degradation (ERAD) system. In the case of plant-based allergy vaccines, high amounts of destructed hypoallergenic allergens have to be produced for efficacy; therefore, there is a high possibility that ER stress or the unfolded protein response may be highly induced by the accumulation of unfolded or misfolded proteins. At least two signaling pathways have been implicated in the ER stress response in plants, the orthologs of IRE1/XBP1 and ATF6, whereas

the plant counterpart of PERK has not been identified (Howell 2013). Many genes coding for chaperons and folding enzymes are upregulated through these ER stress signaling pathways as a result of the unfolded protein response. Therefore, enriching the ER quality control capacity (protein folding capacity) is necessary to control the ER stress signaling pathway determining chaperon levels to enhance the accumulation levels of foreign proteins.

As a unique strategy, higher levels of recombinant proteins could be obtained by their expression in specific tissues and targeting to intracellular compartments such as PB or PSV through the simultaneous suppression of endogenous seed proteins by an RNAi-based approach because the deposition space is enlarged by the vacancy of endogenous seed proteins and compensatory mechanisms work to maintain total nitrogen and sulfur levels in seed proteins (Takaiwa 2013b; Wu et al. 2012). This improvement in the recombinant protein yield was also observed with the expression of recombinant proteins in a host (germplasm) deficient or suppressed in some seed proteins. This has been attributed to the rebalancing or compensatory effect of proteins to maintain the same nitrogen levels in seeds and to lessen competition for deposition in a limited space between endogenous and foreign proteins.

When proteins are transported as secretory proteins into the ER, high mannose type glycans are added to accessible asparagines in the amino acid sequence context Asn-Xaa-Ser/Thr and more complex glycans are built on the mannose framework in the Golgi apparatus, including the addition of β-1,2-xylose and α-1,3-fucose at particular sites in the glycan (Gomrd et al.2010). Plants can be genetically modified to produce glycans that are more similar to those found in animals by deleting or suppressing plant-specific glycosylating enzymes genes such as α-1,3 fucosylransferase and β-1,2 xyltransferase through mutation or homologous recombination (gene knockout), suppressing their expression by RNAi (knock down), or by predominantly targeting the ER lumen through retrieval from the Golgi apparatus by ligating the C-terminal KDEL ER retention signal.

Recombinant proteins can currently be artificially targeted to various subcellular compartments such as ER lumen, Golgi complex, PB, PSV, or apoplast as well as chloroplast and mitochondria by fusing to the targeting signal peptide such as the transit peptide, vacuolar sorting signals (VSS), or ER retention signal (Benchabane et al. 2008; Vitale and Hinz 2005). Proteins with no N-terminal signal peptide result in targeting to the cytoplasm. Ligation of the signal peptide and the KDEL/HDEL ER retention signal at the N- and C-termini of recombinant proteins generally enables accumulation in the ER lumen by retrieval from the Golgi apparatus, resulting in more than a 10-fold enhancement in yield over that without the ER retention signal.

A fusion strategy further ensures transportation to the expected intracellular site as well as the enrichment and stability of products. Hybrid proteins with seed proteins, zera (proline rich N-terminal domain of the maize γ-zein), elastin, ubiquitin, and immunoglobulins (HIV-1 p24-immunoglobulin fusion) have been reported to be useful in enhancing stability and folding (Conley et al. 2011; Torrent et al. 2009; Floss et al. 2009; Obregon et al. 2006; Hondred et al. 1999). Fusion with the N-terminal portion of γ-zein or elastin-like polypeptide, the multiple repeats of the

elastin motif (VPGXG), resulted in PB formation even in vegetative tissue and enhanced production.

As an alternative, fusion with native SSP, in which foreign proteins are inserted in a highly variable region such as the C-terminal region of the rice glutelin acidic subunit and 26-kD globulin, stably accumulated as part of the seed protein and resulted in targeting to inherent PSVs, allowing enrichment of the recombinant protein in the PB (Wakasa et al. 2006). On the other hand, when recombinant protein was fused to the C-terminus of various prolamins, they were also stably deposited to ER-derived PBs. Therefore, recombinant proteins can be targeted to the desired compartment according to the properties of the fusion partner.

Rice Seed-Based Birch Pollen Allergen Against Birch Pollen Allergy

Birch pollen is widely distributed in Europe, North America, Russia, and northern Japan. The major pollen allergens of Fagales trees belonging to the same order as birch are known as the Bet v 1 family because of the strong IgE cross-reactivity between Bet v 1, a major birch pollen allergen, and the homologous allergens observed in alder (Aln g 1), hazelnut (Cor a 1), hornbeam (Car b 1), and oak (Que a 1) pollen (Weber 2006). Bet v 1 is a glycoprotein, with a molecular weight of approximately 20 kDa that belongs to the PR10 protein group. More than 90 % of birch pollen allergy patients are sensitized to Bet v 1-specific IgE. The Bet v 1 allergy is also known to cause oral allergy syndrome, as the following food allergens are cross-reactive with Bet v 1-specific IgE: apple Mal d 1, soybean Gly m 4, carrot Dau c 1, and peanut Aha h 8 (Vieths et al. 2002).

The creation of versatile recombinant hypoallergenic allergens retaining all T cell epitopes and immunogenicity should provide an ideal tolerogen for allergen-SIT. Hypoallergenic Bet v 1 derivatives against multiple Fagales pollen allergens were previously generated through in vitro random recombination by means of DNA shuffling (Wallner et al. 2007). Importantly, TPC7 and TPC9 not only exhibited lower allergenicity than native Bet v 1, as determined by IgE reactivity and basophil activation assays, but TPC7 also showed a 20-fold reduction in IgE binding capacity and an 8- to 10-fold reduction in basophil degradation activity relative to wild-type Bet v 1. Therefore, this TPC7/TPC9 chimera should be a suitable tolerogen for SIT not only against birch pollen allergy but also against allergies caused by other cross-reactive tree pollen.

A codon-optimized TPC7 synthetic gene, in which the GluB-1 signal peptide and the KDEL ER retention signal were fused to the N- and C-termini, respectively, was then expressed in stable transgenic rice seeds under the control of the endosperm-specific 2.3-kb glutelin *GluB-1* promoter (Wang et al. 2013). Recombinant TPC7 was produced as a glycoprotein with high mannose-type N-glycan, but without β-1,2-xylose or α-1,3-fucose, which suggests that TPC7 is retained in the ER. TPC7 strongly accumulated in the endosperm tissue to approximately 200 µg/grain, lead-
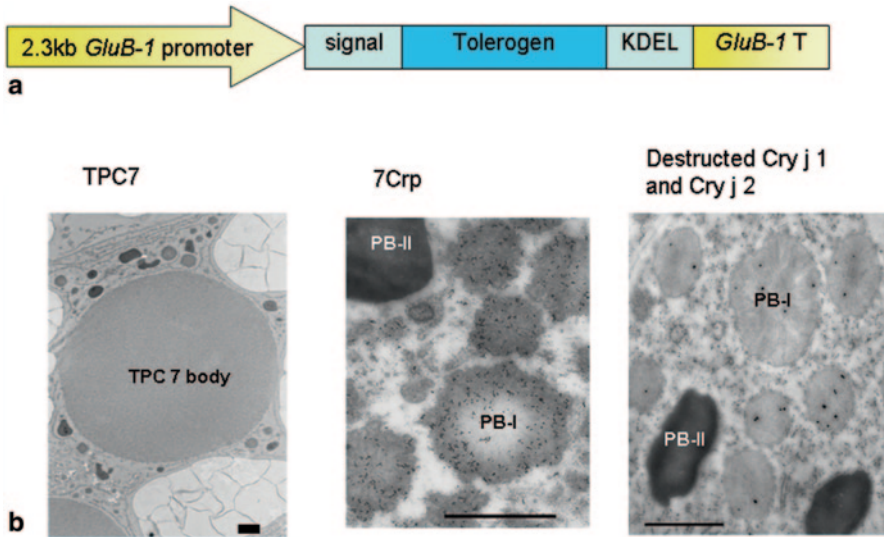
Fig. 12.2 Expression system of various tolerogens in transgenic rice seeds and the intracellular structure of endosperm cells containing PBs. **a** General expression system used for the production of tolerogens (pollen allergen derivatives) in transgenic rice seeds. **b** Intracellular structure of developing endosperm cells containing PBs depositing tolerogens in transgenic seeds. The intracellular localization of recombinant proteins was analyzed using immunoelectron microscopy. *PB-I* protein body I, *PB-II* protein body II, Bar 1 μm

ing to the suppression of cysteine-poor 13-kDa prolamin and 26-kDa globulin production. Interestingly, when the intracellular location of TPC7 was examined, it was found to be deposited into a very large organelle, more than 20 μm in diameter and is referred to as the TPC7 body, and was distinct from PB-I and PB-II (Fig. 12.2). The TPC7 body is predominantly filled with TPC7 proteins and interactions rarely occur with endogenous SSPs. Transgenic rice seed with high amounts of TPC7 may be a potential candidate as an oral tolerogen against birch pollen allergy.

Rice Seed-Based Allergy Vaccine Against Japanese Cedar Pollen Allergy

Japanese cedar pollen allergy is an important public health problem in Japan. Approximately 30 % of the population is currently afflicted with this pollinosis between February and April each year (Okamoto et al. 2009). People with circulating specific IgE against cedar pollen allergens (potential patients) account for up to 60 % of the general population. 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. Cry j 1 has pectate lyase activity and is specifically localized in the pollen cell wall. Cry

j 2 has polygalacturonase activity and is localized in the amyloplast of pollen. Oral administration of the dominant T cell epitope of Cry j 2 inhibited specific T cell responses in Cry j 2-sensitized mice in a mouse model of Japanese cedar pollinosis (Hirahara et al. 1998). Sneezing frequency as a measurable clinical symptom was decreased not only by systemic injections but also by oral administration of the dominant T cell epitope of Cry j 2. To confirm the efficacy of rice-based oral vaccines for inducing immune tolerance against cedar pollen allergens by allergen-SIT, 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 the 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 (Takaiwa 2007). Modified glycinin A1aB1b containing T cell epitopes specifically accumulated to 7 μg/grain in mature dry seeds. In a preclinical model, the proliferative responses of allergen-specific CD4$^+$ T cells, synthesis of specific IgE, and production of histamine were less in mice orally administered with transgenic rice seeds (200 mg) daily for 4 weeks prior to a systemic challenge with crude pollen allergens than in mice fed non-transgenic control rice seeds (Takaiwa 2007). 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. Histamine release from mast cells was also suppressed. Furthermore, allergy symptoms such as sneezing were alleviated after exposure to cedar allergens. These results indicated that mucosal immunization with rice seeds containing T cell epitopes efficiently induced immune tolerance.

Based on this confirmation of the feasibility of an oral peptide immunotherapy program in this animal model, a human version of a rice-based peptide vaccine was developed against cedar pollen allergy. 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 (Takagi et al. 2005a). The hybrid 7Crp peptide elicited a positive response in 92 % of 48 volunteers with pollinosis without binding to specific IgE, which indicated that it could be used as a safe and effective tolerogen (Hirahara et al. 2001). Notably, the 7Crp peptide had a greater effect on T cell proliferation than that of a mixture of the seven individual T cell epitope peptides.

This 7Crp peptide was specifically expressed in transgenic rice seeds as a secretory protein under the control of several strong endosperm-specific promoters. The GluB-1 signal peptide and KDEL ER retention signal were included to increase the accumulation of the 7Crp peptide. Accumulation of the 7Crp peptide was markedly higher at approximately 60 μg/grain, as determined in CBB-stained SDS-PAGE gels, and accounted for 5–6 % of total seed protein. It was mainly deposited in ER-derived PB-I in the endosperm (Takagi et al. 2005b).

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 the major epitope. Mice were then nasally challenged with intact Cry j 1 allergen. Both the T cell proliferative response against Cry j 1 and specific serum IgE levels were lower than those in control mice fed non-transgenic rice seeds (Takagi et al. 2005b).

T cell proliferative activity was retained even after boiling 7Crp transgenic rice seeds for 20 min at 100 °C or autoclaving for 20 min, which indicates that oral immune tolerance may still be effective when used with steamed or cooked rice. A safety evaluation of 7Crp transgenic rice seeds showed that the amino acid, lipid, carbohydrate, protein, fatty acid, mineral, and vitamin composition of the transgenic seeds was essentially identical to the non-transgenic control counterpart (Takagi et al. 2006). An oral safety study was performed by administering high and low doses of steamed rice to cynomolgus macaques for 26 weeks (Domon et al. 2009). No adverse effects were observed.

However, since humans with different genetic backgrounds respond differently to various T cell epitopes, peptide immunotherapy using T cell epitopes may not be applicable to all Japanese cedar pollinosis patients in spite of its proven safety. Thus, to treat a broader range of allergy patients, the entire Cry j 1 and Cry j 2 molecules were destroyed by a molecular shuffling and fragmentation process that perturbed the tertiary structure to identify an allergen-specific IgE. The full length of mature Cry j 1 (1–353 aa) was divided into three overlapped fragments. These three fragments with lengths of 131–144 amino acids were inserted into highly variable regions of the acidic subunits of GluA-2, GluB-1, and GluC and were then expressed as fusion proteins with these glutelins under the control of the rice endosperm-specific glutelin *GluB-4* and 16-kDa and 10-kDa prolamin promoters (Wakasa et al. 2013). On the other hand, the coding sequence of Cry j 2 was restructured in the form of a tail to top inverse orientation to disrupt the tertiary structure. This shuffled Cry j 2 was attached to the KDEL ER retention signal at the C-terminus and expressed under the control of the *GluB-1* promoter with its signal peptide sequence (Takagi et al. 2006). A binary vector harboring four expression cassettes (three Cry j 1/glutelin fusions and one shuffled Cry j 2) was introduced into the good-taste rice variety genome lacking three glutelin genes by *Agrobacterium*-mediated transformation (Fig. 12.3). Three chimeric glutelin-Cry j 1 fragments were detected as glutelin precursors with molecular masses from 56 to 60 kDa, but were not processed into mature acidic glutelins containing Cry j 1 subunits. The shuffled Cry j 2 accumulated as a visible CBB-stained band with a molecular mass of 38 kDa. Four individual antigens of 10–25 μg accumulated in one dry grain (approximately 20 mg) and were deposited into ER-derived PB-I. Little or no allergenicity by fragmentation and shuffling was confirmed by binding capacity to specific IgE or the basophil degradation assay (Wakasa et al. 2013).

Transgenic rice seeds (0.6 g) containing accumulations of these destructed whole Cry j 1 and 2 molecules were fed daily to mice for 21 days, which were then challenged twice using a crude cedar pollen allergen. Allergen-specific CD4$^+$ T cell proliferation and IgE and IgG levels were markedly lower than those in mice fed non-transgenic rice seeds (Fig. 12.3). The production of Th2-type cytokines such as IL-4, IL-5, and IL-13 was decreased by the oral administration of transgenic rice grains. 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 the oral administration of transgenic rice seeds containing structurally disrupted Cry j 1 and Cry j 2 antigens
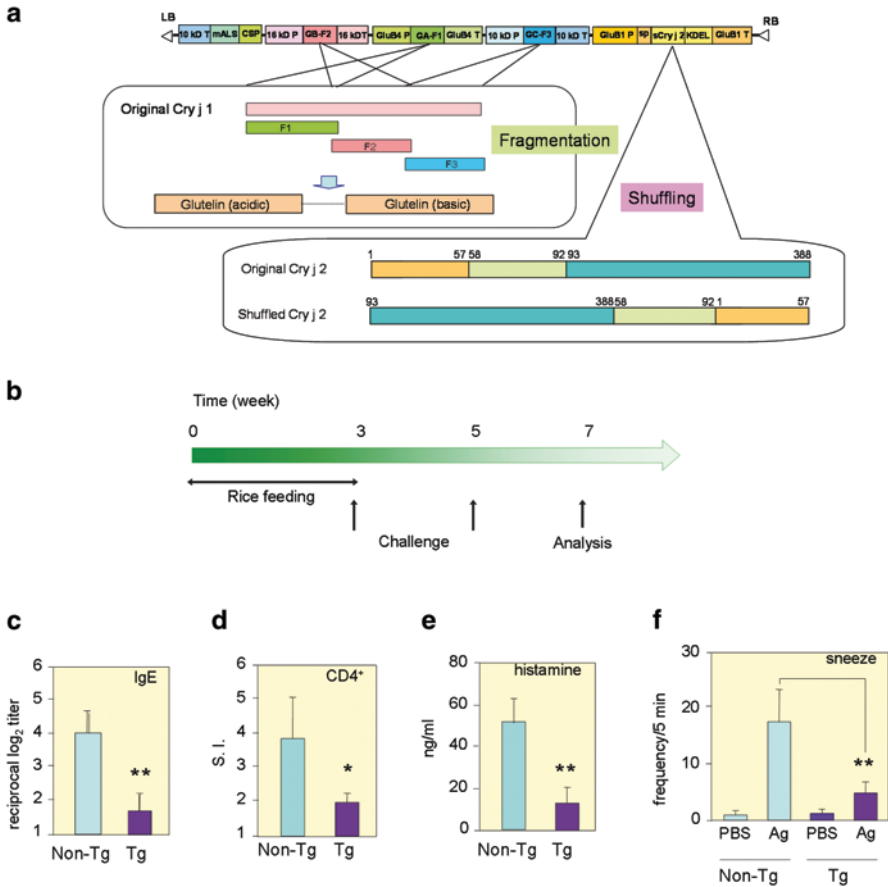
Fig. 12.3 Vector construct used for the expression of hypoallergenic Cry j 1 and Cry j 2 in transgenic rice seeds and the induction of oral immune tolerance by the oral administration of transgenic rice seeds. **a** The Cry j 1 allergen was divided into three overlapped fragments, each of which was inserted into the C-terminal highly variable region of the acidic subunit of three different glutelins. The Cry j 2 allergen was shuffled in the form of a tail to top orientation. The three glutelin/Cry j 1 fragment fusions and one shuffle Cry j 2 were linked to endosperm promoters and were then introduced into the binary vector. Four expression constructs were introduced into the rice genome via *Agrobacterium*-mediated transformation. **b** Experimental time line used for the induction of oral immune tolerance in mice administrated with transgenic rice seeds. **c** Allergen-specific IgE levels. **d** Allergen-specific splenic CD4$^+$ T cell proliferative responses. **e** Serum histamine levels. **f** Frequency of sneezes (sneeze number/5 min after exposure to cedar pollen) sky blue column: mice fed with normal non-transgenic rice seeds, magenta column: mice fed with transgenic seeds

is a more promising approach than with those containing the major T cell epitopes (7Crp) for the induction of immune tolerance against Japanese cedar pollinosis due to its applicability to a broader range of patients.

Conclusions

Plant seeds are a good production platform for tolerogens used in allergen-SIT. Even though destructed antigens or T cell epitopes were produced in seeds, they accumulated securely and at large quantities without degradation. Furthermore, the seed containing the tolerogen acts as an edible vaccine that induces immune tolerance. Tolerogens that accumulate in seeds can be protected from degradation by digestive enzymes in the gastrointestinal tract, resulting in more effective delivery to immune cells in GALT. This is mainly attributed to the bioencapsulation of tolerogens by two barriers, the cell wall and PB, as natural formulation characteristics of the plant cell. Therefore, the oral administration of seeds containing tolerogens is expected to be a promising approach to induce immune tolerance. It is important to note that the immunogenicity of tolerogens stocked in seeds is not lost even by cocking, which is in marked contrast to vaccines against infectious diseases. This is attributed to immunogenicity being fundamentally determined by T cell epitopes between 8 and 15 amino acids in length. Oral administration is simpler and more convenient and comfortable than a conventional subcutaneous injection of the crude extract. Moreover, seed-based edible vaccines are very cost-effective because there is no requirement of downstream processing such as isolation and purification, which represents up to 80 % of the overall production cost. The continuous oral administration of tolerogens is more likely to induce immune tolerance than parenteral administration. Furthermore, not only mucosal but also systemic immune reactions are induced by oral administration. Taken together, seed-based allergy edible vaccines represent an innovative allergen-SIT as an alternative to conventional subcutaneous injections.

The efficacy of seed-based allergy vaccines has been demonstrated by their oral administration to model mice. The production of allergen-specific IgE and IgG and T cell proliferation was significantly lower in these mice than in control mice fed non-transgenic seeds. Furthermore, clinical symptoms such as sneezing frequency were downregulated as a result of a decrease in histamine levels. These results indicate that the oral administration of seed-based allergy vaccines can induce mucosal immune tolerance.

However, there are hurdles to overcome prior to the commercialization of seed-based edible allergy vaccines. One concern is entry into the food chain during processing and handling through the process from production to consumption or by gene flow into non-transgenic plants though outcrossing of pollens. Contamination by gene transfer via pollen can be avoided using physical or biological methods. Another concern is the many practical processes regarding the clinical development of pharmaceuticals according to the existing regulations under Good Manufacturing Practice (GMP) regulations (Fischer et al. 2012). Significant changes through discussions with the Japanese Pharmaceuticals and Medical Devise Agency (PMDA) will be required for the commercialization of seed-based edible vaccines.

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