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REVIEW

Transgenic plants for allergen-specific immunotherapy

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Abstract

Allergen-specific immunotherapy (IT) is an effective treatment for allergic diseases. Although subcutaneous and sublingual ITs are currently used, safer, easier, and more effective IT is under development. Induction of immune tolerance by oral administration of allergen has been proven, though oral IT has not been applied clinically. It is mainly because a large amount of purified allergen is required to induce oral tolerance. To overcome this problem, plants, peculiarly rice, have been investigated as allergen vehicles for oral IT. Rice can store a considerable amount of expressed allergen in its seeds and the accumulated allergen is stable and resistant to gastrointestinal digestion. Therefore, we have developed transgenic rice seeds (Tg rice) in which major epitopes of cedar pollen or house dust mites are expressed. We are establishing Tg rice with demonstrated efficacy in murine models of allergic rhinitis and bronchial asthma by oral administration at practical doses. In addition, the amount, distribution, and allergenicity of the expressed allergen have been improved in our Tg rice. Rice-based oral IT is a promising new concept in IT for the treatment of allergic diseases.

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Key words: Allergic disease; Asthma; Oral immunotherapy; Rhinitis; Transgenic rice

Core tip: We aim to establish clinically applicable oral immunotherapy by employing transgenic rice seeds (Tg rice) in which allergen epitopes are expressed. We have identified a suitable allergen packaging system, modified allergens to reduce their allergenicity, selected high allergen-producing lines, and evaluated their efficacy in allergic disease models. We are thus nearly ready to start clinical trials of our Tg rice for the treatment of Japanese cedar pollinosis.

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INTRODUCTION

The prevalence of allergic diseases such as allergic rhinitis and bronchial asthma has markedly increased in industrialized countries, and it is a major health concern[1-3]. Allergen-specific immunotherapy (IT) leads to immune tolerance, a state of immune unresponsiveness, by repeated allergen administration, and has been recognized as an effective therapeutic method[4,5]. Oral IT, which is based on oral allergen administration, is a relatively conventional method to induce allergen-specific immune tolerance. However, this IT has yet to be applied clinically because of several problems[6-8], particularly the lack of an efficient oral allergen delivery system. We recently

showed that rice seeds can be used as a carrier for oral IT. The factors favoring their use are ease of consumption for individuals of all ages, the ability to store a considerable amount of allergen, stability at room temperature for 2-3 years, resistance to digestive enzymes and low pH[9-12]. Therefore, to establish clinically applicable oral IT, we have been developing transgenic rice seeds (Tg rice) expressing full-length allergen or epitopes of Japanese cedar pollen or house dust mites. Oral IT with Tg rice is set to become one of the most useful tools to treat allergic diseases. This review summarizes the progression and prospects of allergen-specific ITs for allergic diseases, with a focus on Tg rice-based oral IT.

SUBCUTANEOUS AND SUBLINGUAL IMMUNOTHERAPY

Currently, subcutaneous IT (SCIT) and sublingual IT (SLIT) are two major IT modes used for clinical treatment of pollen- or house dust-mediated allergic diseases[13-16]. Since SCIT was initially described by Noon in 1911[17], it has been the most popular mode of IT for allergic diseases. Although the immunological mechanisms by which SCIT induces immune tolerance are not completely understood, researchers have demonstrated the production of IgE-blocking antibody, alteration of the Th1/Th2 balance in favor of Th1 responses, and the induction of regulatory T cells by this therapy[18-22]. Nevertheless, repeated systemic injection of allergens by SCIT is inconvenient and heightens the risk of severe side effects, including anaphylactic shock[23,24]. Therefore, SLIT has been developed as an alternative, noninvasive IT[25,26]. Since it was initially described by Scadding *et al*[27] in 1986, its clinical market share has become approximately 45% of the total allergen-specific IT treatments performed in Europe[28]. Although the incidence of anaphylaxis is reduced in SLIT, several side effects associated with allergenicity have been reported[23,29]. In addition, SLIT requires the patient to retain the allergen solution or tablet under the tongue, something that is difficult for small children. Therefore, several trials are in progress to develop safer, easier, and more efficient IT by modifying allergens, adjuvants, and routes of administration[30-34]. For example, to avoid IgE-dependent side effects, peptide IT using dominant T-cell epitopes has been proposed in both SCIT and SLIT[35,36], and various alternative routes of allergen administration such as oral[37-39], epicutaneous[40], and intralymphatic[41,42] have been investigated.

ORAL IMMUNOTHERAPY FOR ALLERGIC DISEASES

Induction of immune tolerance by oral administration of specific allergen has been recognized for a century[43-45]. Even though the mechanisms of oral tolerance have not been fully defined, many studies have suggested that low-dose allergens induce cellular activation, which was adoptively transferable *in vivo*, and that high-dose allergens induce nontransferable clonal anergy and/or deletion[46,50]. The characteristics of the immune system in the intestinal mucosa suggest some patients unresponsive to SCIT or SLIT are curable by oral IT[51,52]. Thus, several attempts have been made to use oral IT to treat allergic diseases since the 1920s[53]. Recently, clinical trials of oral IT for patients with autoimmune diseases have also been performed[37-39], although this therapy is yet to be used for human diseases. The greatest obstacle to its clinical application is the amount of allergen required. Oral IT requires about 200 times larger quantities of allergens to induce immune tolerance in comparison to SCIT[54]. Therefore, researchers hope to develop systems for effective allergen delivery to the intestinal mucosa to achieve clinical applications of oral IT. In this regard, there is concern that oral administration of a large amount of allergen may lead adverse effects such as impaired clearance of secondary bacterial infections due to excess suppression of immune function. However, almost all of adverse effects reported in clinical studies of oral IT were relatively mild, and no systemic immunological suppression have been observed[43,44,54]. Therefore, such risks in oral IT seem to be negligible.

PLANT-BASED VACCINES

In order to meet the requirement of large amounts of allergen in oral IT, several allergen-expressing plants have been developed. During early studies in the 1990s, tobacco was used as a model transgenic plant for expressing allergen[55]. Since then, the feasibility of plants such as potato, banana, tomato, and rice has been explored by introducing heat-labile enterotoxin, hepatitis B antigen, respiratory syncytial virus antigen, and cholera toxin[9,56-59]. The use of plants as allergen vehicles offers several advantages over genetically engineered and/or purified allergens. The cost of production is lower, no refrigeration is required for storage, and contamination with mammalian pathogens hardly occurs in transgenic plants[60]. In addition, the walls of plant cells in which the expressed allergen accumulate are resistant to the acidic environment of the stomach[60]. The edible parts of plants such as fruits and crop seeds are easy to administer to people of all ages.

RICE SEED AS AN ALLERGEN CARRIER

Rice is the major food staple commonly eaten daily throughout Asia. Allergens expressed in rice seeds are stable at room temperature for 2-3 years[9,10]. Rice seeds mainly express three endogenous proteins: alcohol-soluble prolamin, acid- and alkaline-soluble glutelin, and saline-soluble globulin. Two characteristic organs, protein body (PB)- I and PB-Ⅱ, mediate storage of these proteins in rice seeds[61-63]. Prolamin is composed of three isoforms (10, 13, and 16-kDa) synthesized in the endo-

plasmic reticulum (ER) and retained in the ER lumen; then, it forms the smooth and spherical protein body PB-I[61,64]. Four glutelin isoforms (GluA, GluB, GluC, and GluD) are initially synthesized in the ER, and then ~~they are~~ transported to the protein storage vacuole, PB-Ⅱ[65,66]. The α-globulin is also deposited in PB-Ⅱ. It has been reported that exogenous proteins can be made to accumulate in PB-Ⅰ and/or PB-Ⅱ of rice seeds by expressing them with the promoters of glutelin (GluB-1, GluB-4), 26-kDa globulin, or 10-kDa and 16-kDa prolamin[67]. Allergen accumulated in PBs is protected from the gastrointestinal digestive enzymes and low pH environment[10,12,13]. In particular, PB-Ⅰ is characterized by higher digestion resistance and it seems to be an ideal capsule for efficient allergen delivery to the intestinal immune system[68]. For these reasons, rice seeds have been recognized as one of the most feasible allergen carriers for oral IT.

RICE SEED-BASED ORAL IMMUNOTHERAPY FOR ALLERGIC RHINITIS

Seasonal allergic rhinitis caused by Japanese cedar pollen is a serious health concern in Japan. Over 26% of the Japanese population has this disease[69-71]. Two major allergens, *Cryptomeria japonica* (Cry j) 1 and Cry j 2, have been identified in the pollen[72,73]. To avoid the IgE-mediated side effects seen with SCIT and SLIT, we have developed several types of Tg rice that accumulate modified fusion proteins of Cry j 1 and Cry j 2 epitope peptides (Table 1)

Cry j Tg rice

First, we established Tg rice expressing a fusion protein of mouse T-cell epitope peptides in Cry j 1 and Cry j 2, and a soybean storage protein, glycinin A1aB1b (Cry j Tg rice)[74]. A1aB1b is located in PB-Ⅱ when expressed in Tg rice seeds with the GluB-1 promoter[75]; the fusion protein was expected to be accumulated in PB-Ⅱ[74]. Oral administration of Cry j Tg rice to cedar pollen-immunized mice inhibited allergen-induced IgE and IgG responses, $CD4^+$ T-cell proliferation, and T helper 2 (Th2) cytokine synthesis (IL-4, IL-5, and IL-13). In addition, allergen-induced serum histamine elevation and sneezing response were suppressed[74].

7Crp Tg rice

Since Cry j Tg rice was effective in the mouse model of allergic rhinitis, we developed Tg rice for application in the human body. Seven dominant human T-cell epitopes were identified in Cry j 1 and Cry j 2[76-78]. We have demonstrated that 92% of 48 patients with Japanese cedar pollinosis showed positive T-cell responses to the fusion protein in which the seven epitopes were linked (7Crp). This 7Crp was not reactive to cedar pollen-specific IgE in the patients' sera[79]. These findings suggest 7Crp has the potential to modulate cedar pollen-mediated T-cell responses without inducing IgE-dependent side effects. We developed three lines of Tg rice accumulating 7Crp[79]. The first line expressed 7Crp under GluB-1 promoter regulation without its signal peptide and failed to express the allergen protein, although its transcript was detectable. We concluded that absence of the signal peptide caused 7Crp instability. By adding GluB-1 signal peptide, 7Crp was accumulated in the Tg rice. In addition, high 7Crp expression in the Tg rice was achieved by adding a C-terminal ER retention signal (KDEL sequence). Finally, we established Tg rice expressing 7Crp with GluB-1 signal peptide as well as the KDEL sequence (7Crp Tg rice). After verifying accumulation of 7Crp in PB-Ⅰ and PB-Ⅱ, the effect of 7Crp Tg rice on allergic responses was examined in the mouse model[79]. Oral administration of 7Crp Tg rice to B10.S mice that recognize one of the seven epitopes suppressed allergen-induced IgE and T-cell responses[79].

Shuffled Cry j Tg rice

There are inter-individual differences in the sequence recognition of T-cell epitopes because of a variety of MHC class Ⅱ haplotypes[80-82]. Therefore, peptide IT with only major T-cell epitopes does not seem to be effective for all patients. The molecular shuffling method has been used to preserve immunogenicity/tolerogenicity (T-cell reactivity) and reduce allergenicity (IgE reactivity)[32,83]. Based on this strategy, we improved our Tg rice to induce immune tolerance in a more efficient manner. Cry j 1 was divided into three overlapped fragments to disrupt its tertiary structure, and these fragments were shuffled and inserted into the middle of glutelins. The tertiary structure of Cry j 2 was also destroyed following its reconstruction to be a mosaic molecule by insertion of the KDEL sequence. After the abrogation of allergenicity in these fragments was verified[84], Tg rice accumulating three Cry j 1/glutelin fusions and one restructured Cry j 2 was established (shuffled Cry j Tg rice)[84]. The expressed allergens were successfully localized in PB-I. Oral administration of shuffled Cry j Tg rice to cedar pollen-immunized mice inhibited allergen-induced IgE and IgG responses, $CD4^+$ T-cell proliferation, and Th2 cytokine synthesis. Consistent with this finding, allergen-induced sneezing response, serum histamine elevation, and infiltration of eosinophils in the nose were attenuated[84].

RICE SEED-BASED ORAL IMMUNOTHERAPY FOR BRONCHIAL ASTHMA

House dust mites (HDM) are strongly associated with the development of allergic diseases, such as bronchial asthma, allergic rhinitis and atopic dermatitis[85,86]. Specifically, *Dermatophagoides pteronyssinus* (Der p)- and *Dermatophagoides farinae* (Der f)-derived allergens are important components of indoor allergens associated with bronchial asthma[87,88]. The major HDM allergens are classified into two groups: group 1 (Der p 1 and Der f 1), mainly derived

Table 1 Characteristics of transgenic rices for oral immunotherapy against allergic rhinitis and bronchial asthma

Tg rice name	Target disease	Target allergen	Expression plasmid construction			Allergen accumulation		Pharmacological effect		Ref.
			Promoter	Coding protein	KDEL sequence	Localization	Yield			
Cry j	Allergic rhinitis	*Cryptomeria Japonica* pollen (Japanese cedar pollen)	GluB-1	Fusion protein of mouse T-cel epitopes in Cry j 1 and Cry j 2, and glycinin A1aB1b	absence	presumaply PB-II	7 μg/ grain	IgE and IgG CD4+ T-cell proliferation Th2 cytokine Histamine Sneezing	↓ ↓ ↓ ↓ ↓	[74]
7Crp	Allergic rhinitis	*Cryptomeria Japonica* pollen (Japanese cedar pollen)	GluB-1	Fusion protein of seven human T-cell epitopes in Cry j 1 and Cry j 2	presence	PB-I and PB-II	62 μg/ grain	CD4+ T-cell proliferation IgE	↓ ↓	[79]
Shuffled Cry j	Allergic rhinitis	*Cryptomeria Japonica* pollen (Japanese cedar pollen)	Prolamin (16-kDa) GluB Prolamin (10-kDa) GluB-1	Fusion protein of deconstructed full-length Cry j 1, Cry j 2, and glutelins	presence	PB-I	10-25 μg/grain	IgE and IgG CD4+ T-cell proliferation Th2 cytokine Histamine Sneezing Eosinophilia	↓ ↓ ↓ ↓ ↓ ↓	[84]
Der p 1	Bronchial asthma	*Dermatophagoides Pteronyssinus* (House dust mite)	GluB-1	Human and mouse T-cell epitopes in Der p 1	absence	PB-I	90 μg/ grain	IgE and IgG CD4+ T-cell proliferation Th2 cytokine Eosinophilia Bronchial hyperreactivity	↓ ↓ ↓ ↓ ↓	[89-91]
Der f 2	Bronchial asthma	*Dermatophagoides Farinae* (House dust mite)	GluB-1	Cysteine residue-mutated full-length Der f 2	presence	Der f 2 body	15-30 μg/grain	IgE and IgG	↓	[99]

from feces, and group 2 (Der p 2 and Der f 2) derived from the bodies. Therefore, for application to treatment for bronchial asthma, two types of Tg rice accumulating modified Der p 1 and Der f 2 peptides have been established (Table 1).

Der p 1 Tg rice

First, we created Tg rice expressing both human and mouse T-cell epitopes of Der p 1 (Der p 1 Tg rice)[89,90]. By using the technique established to develop Cry j-related Tg rice, the expressed allergen was efficiently deposited in PB-I[90]. Oral administration of Der p 1 Tg rice to Der p 1-immunized mice suppressed allergen-induced IgE and IgG responses, $CD4^+$ T-cell proliferation, and Th2 cytokine synthesis. In addition, allergen-induced eosinophil infiltration into the lungs and bronchial hyperreactivity were diminished[90], suggesting that rice seed-based oral IT is useful for the treatment of allergic rhinitis and bronchial asthma.

Administration of lower doses (approximately 5 g/kg per day) also suppressed allergen-induced lung eosinophilia[91]. Interestingly, the production of allergen-specific IgE was not affected unlike in the high-dose experiment (approximately 50 g/kg per day)[91]. Although we cannot explain why the low-dose Der p 1 Tg rice was not effective for IgE production, it was suggested that the efficacy of oral IT is not mainly caused by suppression of IgE responses. Most importantly, effective attenuation of allergic inflammation was accomplished at a dose of Tg rice that is achievable through daily consumption.

Der f 2 Tg rice

Der f 2 contains three disulfide bonds (Cys8-Cys119, Cys21-Cys27, and Cys73-Cys78), two of which (Cys8-Cys119 and Cys73-Cys78) are critical for IgE-binding[92-96]. In contrast, T-cell epitopes of the group 2 allergens are distributed over the entire protein[94,97]. We constructed three Der f 2 derivatives in which cysteine residues were mutated: ΔC lacked all three disulfide bonds, C8/119S lacked the Cys8-Cys119 bond, and 8-119C lacked the Cys21-Cys27 and Cys73-Cys78 bonds[98]. Binding activity with HDM-specific IgE was markedly decreased in ΔC, followed by C8/119S and 8-119C. Then, three lines of Tg rice expressing these Der f 2 derivatives were established (Der f 2 Tg rice). The localization of the expressed allergen in the Tg rice was unique. Thus, Der f 2 derivatives aggregated and formed a PB-like structure, named the Der f 2 body, distinguishable from PB- I and PB- Ⅱ by its electron density. Oral administration of Der f 2 Tg rice containing C8/119S, 8-119C, or both to Der f 2-immunized mice inhibited allergen-induced IgE and IgG responses[98], suggesting the potential of this Tg rice

to treat HDM-mediated allergic responses. Interestingly, IgE and IgG responses were not affected by Der f 2 Tg rice containing ΔC. ΔC was water-soluble and rapidly degraded by digestive enzymes in comparison to other Der f 2 derivatives[98]. These data strongly suggest allergen digestibility is critical for the efficacy of oral IT using Tg rice. Further studies are needed to investigate the effectiveness of Der f 2 Tg rice in treating asthma symptoms.

SAFETY OF RICE SEED-BASED ORAL IMMUNOTHERAPY

So far a series of oral IT trials have raised several safety concerns including gastrointestinal symptoms[43,44,54]. However, such adverse effects were not observed in cynomolgus macaques at least by daily oral administration of high-dose 7Crp Tg rice for 26 wk[99]. Although further investigation into its safety is required, Tg rice-based oral IT may be safer than other modes of IT.

CONCLUSION

We have demonstrated the potential of Tg rice-based oral IT for treating allergic diseases such as allergic rhinitis and bronchial asthma in preclinical animal studies. Because rice-based oral IT is highly effective an does not produce side effects, this mode of treatment promises to become a new approach to oral IT that will improve the quality of treatment for allergic diseases, replacing established drug therapies and other types of IT. A clinical trial of Tg rice is under development to evaluate the efficacy and safety of Tg rice-based oral IT in human subjects.

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