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Original Article

Efficacy of oral immunotherapy with a rice-based edible vaccine containing hypoallergenic Japanese cedar pollen allergens for treatment of established allergic conjunctivitis in mice

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ABSTRACT

Background: We have previously shown that prophylactic oral administration of transgenic rice seeds expressing hypoallergenic modified antigens suppressed the development of allergic conjunctivitis induced by Japanese cedar pollen. We have now investigated the efficacy of oral immunotherapy with such transgenic rice for established allergic conjunctivitis in mice.

Methods: BALB/c mice were sensitized with two intraperitoneal injections of Japanese cedar pollen in alum, challenged with pollen in eyedrops, and then fed for 16 days with transgenic rice seeds expressing modified Japanese cedar pollen allergens Cry j 1 and Cry j 2 or with nontransgenic rice seeds as a control. They were then challenged twice with pollen in eyedrops, with clinical signs being evaluated at 15 min after the first challenge and the eyes, blood, spleen, and lymph nodes being isolated at 24 h after the second challenge.

Results: The number of eosinophils in the conjunctiva and the clinical score for conjunctivitis were both significantly lower in mice fed the transgenic rice than in those fed nontransgenic rice. Oral vaccination with transgenic rice seeds also resulted in a significant increase in the production of IFN-γ by splenocytes, whereas it had no effect on the number of $CD4^+CD25^+Foxp3^+$ regulatory T cells in the spleen or submandibular or mesenteric lymph nodes.

Conclusions: Oral administration of transgenic rice seeds expressing hypoallergenic allergens ameliorated allergic conjunctivitis in the established setting. Such a rice-based edible vaccine is potentially both safe and effective for oral immunotherapy in individuals with allergic conjunctivitis.

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Introduction

Pollinosis including allergic conjunctivitis is one of the most common diseases worldwide. Japanese cedar (*Cryptomeria japonica*) pollinosis is a predominant allergic condition in Japan, with a prevalence of >25%.[1] Most treatments for allergic conjunctivitis are not antigen specific but rather involve the administration of antiallergy eyedrops that contain a mast cell stabilizer or antihistamine (or both agents) to relieve symptoms. Allergen-specific immunotherapy is the only available curative treatment for allergic diseases in which the clinical effects persist over the long term. Such immunotherapy was initially performed by SCIT that required frequent hospital visits for injections and was thus highly inconvenient for the patient. The subsequent introduction of SLIT, which is administered at home by patients themselves without pain, solved the inconvenience problem. However, the rate of discontinuation of SLIT is high, with <15% of patients who initiate the treatment persisting with it for >2 years.[2] Given that such early discontinuation of treatment reduces the clinical efficacy of immunotherapy, this poor compliance is an important clinical problem.

Oral administration of staple foods engineered to express allergens is one possible means of antigen delivery for immunotherapy of allergic diseases that would address both issues of

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convenience and compliance. To develop more effective, safer, and convenient immunotherapy for allergic diseases, we have prepared rice-based edible vaccines that express various antigens including those derived from Japanese cedar pollen, birch pollen, or house dust mites.[3] We recently established transgenic rice expressing the entire molecules of the major Japanese cedar pollen allergens Cry j 1 and Cry j 2 after their molecular fragmentation or shuffling in order to treat a broader range of patients with different genetic backgrounds.[4] Furthermore, we also recently showed that the development of Japanese cedar pollen–induced allergic conjunctivitis is suppressed by prophylactic feeding of such transgenic rice seeds in a mouse model.[5] In the clinical setting, a therapeutic effect of vaccination for established pollinosis is more important than a preventive effect for nonsensitized healthy individuals. We have therefore now examined whether oral immunotherapy with transgenic rice seeds expressing these hypoallergenic modified antigens suppresses established cedar pollen–induced allergic conjunctivitis in mice.

Methods

Mice

Inbred wild-type BALB/c mice were obtained from Japan SLC (Hamamatsu, Shizuoka, Japan) and were maintained under specific pathogen–free conditions at the animal facility of Kochi Medical School. Age- and sex-matched mice were subjected to experiments at 6–12 weeks of age. This study was approved by the Committee for Care and Use of Laboratory Animals at Kochi University (permit no. I-77) and was performed in strict accordance with the Statement on the Use of Animals in Ophthalmic and Vision Research of the Association for Research in Vision and Ophthalmology.

Transgenic rice

The major Japanese cedar pollen allergens Cry j 1 and Cry j 2 were deconstructed by fragmentation and molecular shuffling, respectively. Transgenic rice that accumulates such modified forms of Cry j 1 and Cry j 2 in the edible portion (endosperm) of the seed was generated by transformation as described previously.[4] Cry j 1 was thus divided into three overlapping fragments that were expressed as fusion proteins with rice seed storage glutelins (GluA2, GluB1, and GluC), whereas Cry j 2 deconstructed by shuffling was expressed as a secretory protein by attachment of an NH_2-terminal signal peptide and COOH-terminal endoplasmic reticulum retention signal (Lys-Asp-Glu-Leu). The modified antigens are deposited in endoplasmic reticulum–derived protein bodies and are thereby rendered resistant both to hydrolysis by intestinal enzymes and to harsh environments. The transgenic rice is thus suitable for oral delivery of the antigens to the mucosal immune system in gut-associated lymphoid tissue.[6]

Feeding and sensitization of mice

Mice had access to a powder diet and drinking water ad libitum. Experimental allergic conjunctivitis was induced by a modified version of a previously described protocol (Fig. 1A).[5,7] In brief, mice were injected intraperitoneally twice with 0.2 mg of Japanese cedar pollen (Hayashibara, Okayama, Japan) mixed with alum (2.5 mg), with an interval of 7 days between injections. Six days after the second sensitization, both eyes of each mouse were challenged with Japanese cedar pollen in PBS (1.2 mg per 2 μl per eye) and clinical signs such as lid swelling, tear production or discharge, chemosis, and redness were evaluated 15 min later in a double-blind manner on the basis of previously described criteria.[8] The

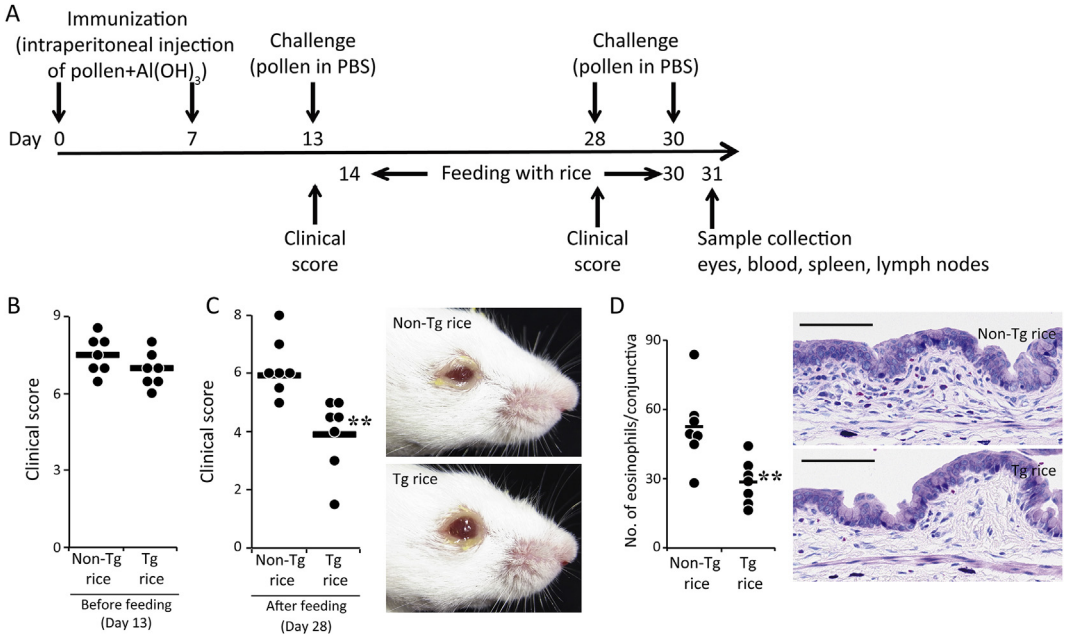

Fig. 1. Therapeutic feeding with transgenic rice suppresses both early-phase signs of and late-phase inflammation in experimental allergic conjunctivitis. As shown in **(A)**, mice were injected intraperitoneally twice (day 0 and 7) with Japanese cedar pollen grains mixed with alum. On day 13, both eyes of each mouse were challenged with Japanese cedar pollen grains suspended in PBS and clinical scores were evaluated 15 min later **(B)**, with circles representing the mean values for the two eyes of each mouse and the horizontal lines indicating overall median values. The mice were then randomly divided into two groups that were provided each day for 16 days with nontransgenic rice seeds or transgenic rice seeds. All mice were challenged twice with pollen in eyedrops on days 28 and 30. The clinical score was evaluated 15 min after allergen challenge on day 28, with representative photographs also being shown **(C)**. The eyes, blood, spleen, and lymph nodes of the mice were isolated on day 31. Eyes were subjected to histological analysis with Giemsa staining to determine the number of eosinophils in the conjunctiva. Representative photographs for mice fed nontransgenic or transgenic rice are shown in **(D)**; scale bars, 200 μm. Individual circles and bars in **(D)** represent mean values for the number of eosinophils in conjunctival sections from the two eyes of each mouse and the overall median values, respectively. Data are representative of two independent experiments with similar results. $^{**}P < 0.01$ (Student's *t* test) versus the value for nontransgenic rice.

mice were then randomly divided into two groups, one of which was provided each day for 16 days with a fine powder prepared from a mixture of 2.78 g of nontransgenic rice seeds and 0.22 g of transgenic rice seeds; the daily amount of recombinant proteins consumed by each mouse was estimated to be 0.11–0.29 mg. The second group of mice was provided with a fine powder prepared from 3 g of nontransgenic rice seeds as a control. All mice were challenged twice with pollen in eyedrops on days 28 and 30. At 15 min after the first challenge, clinical signs were again evaluated in a double-blind manner. Twenty-four hours after the second challenge, the eyes, blood, and spleen of the mice were isolated for histological analysis, measurement of serum Ig levels, and cytokine production assays, respectively. The spleen as well as submandibular and mesenteric lymph nodes were also analyzed for the number of T_{reg} cells.

Histological analysis

Each eye, including the conjunctiva, was fixed in 10% buffered formalin. Vertical sections with a thickness of 2 μm were cut and stained with Giemsa solution. Infiltrating eosinophils in the lamina propria mucosa of the tarsal and bulbar conjunctiva in sections of the central portion of the eye, including the pupil and optic nerve head, were counted by two observers in a blinded manner.[9]

Measurement of serum IgE levels

Serum isolated from blood samples was assayed for both total IgE and Cry j–specific IgE and IgG2a with the use of ELISAs, essentially as described previously.[5] In brief, 96-well EIA plates (Corning-Costar, Corning, NY, USA) were coated overnight at 4 °C with either affinity-purified rat antibodies to mouse IgE (2 μg/ml; Southern Biotech, Birmingham, AL, USA) or Japanese cedar pollen extract (5 μg/ml; Cosmo Bio, Tokyo, Japan). The plates were then washed, incubated with blocking buffer (1% bovine serum albumin in PBS) for 3 h at room temperature, and washed again before the addition of samples or IgE standard (TNP-KLH–specific IgE; BD Biosciences, San Jose, CA, USA). After incubation for 2 h at room temperature, the plates were washed and biotin-conjugated rat antibodies to mouse IgE (BD Biosciences) or alkaline phosphatase–conjugated rabbit antibodies to mouse IgG2a (Thermo Fisher Scientific, Waltham, MA, USA) were added to each well. The plates were incubated for 1 h at room temperature, washed, and, for measurement of IgE, incubated for an additional 1 h with avidin-conjugated alkaline phosphatase (Sigma–Aldrich, St. Louis, MO, USA) and then washed again. *p*-Nitrophenyl phosphate substrate (Sigma–Aldrich) was added to each well, the plates were incubated for 15 min, and absorbance was measured at 405 nm. The concentration of total IgE was determined with reference to the known concentrations of the IgE standard. For Cry j–specific IgE, the results were expressed as absorbance units because a Cry j–specific IgE standard is not available.

Flow cytometric analysis

Cells isolated from the spleen as well as from submandibular and mesenteric lymph nodes were washed with flow cytometry staining buffer (PBS containing 1% fetal bovine serum and 0.1% sodium azide), incubated for 30 min on ice with LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit reagent (Thermo Fisher Scientific), washed again with flow cytometry staining buffer, and fixed for 25 min on ice with Foxp3 Staining Buffer (Thermo Fisher Scientific). After washing with Permeabilization Buffer (Thermo Fisher Scientific), the cells were stained for 25 min on ice with fluorescein isothiocyanate–labeled antibodies to CD4, phycoerythrin-labeled antibodies to CD25, and phycoerythrin-Cy5–labeled antibodies to Foxp3 (Thermo Fisher Scientific). The cells were then washed with flow cytometry staining buffer before determination of the percentage of $CD25^+Foxp3^+$ cells among gated $CD4^+$ T cells with the use of an Attune flow cytometer (Thermo Fisher Scientific).

Assay of cytokine production by splenocytes

Splenocyte preparations depleted of red blood cells were incubated at 37 °C for 48 h at a density of 1×10^7 cells/ml with Japanese cedar pollen extract (Cosmo Bio) at a concentration of 20 μg/ml in 96-well flat-bottom plates containing 0.2 ml per well of RPMI 1640 medium supplemented with 20% fetal bovine serum and 0.1 mM 2-mercaptoethanol. The levels of IL-2, IL-4, IL-5, IL-10, IL-12p70, and IFN-γ in the culture supernatants were then measured with the use of the Bioplex system (Bio-Rad, Hercules, CA, USA).[10]

Statistical analysis

Quantitative data are presented as means ± SEM, unless indicated otherwise, and were compared between groups with the use of the unpaired Student's *t* test. A *P* value of <0.05 was considered statistically significant.

Results

Therapeutic feeding with transgenic rice suppresses established allergic conjunctivitis

Mice were sensitized twice (days 0 and 7) and then challenged with pollen in eyedrops on day 13 for assessment of early-phase clinical signs (Fig. 1A). The mice were then divided randomly into two groups and fed daily for 16 days with 0.22 g of transgenic rice seeds expressing modified Japanese cedar pollen allergens Cry j 1 and Cry j 2 or with nontransgenic rice seeds as a control. They were again challenged on days 28 and 30 and were assessed for early-phase signs after the first of these challenges. Whereas the clinical score was similar for both groups of mice on day 13, it was significantly lower on day 28 for mice fed the transgenic rice seeds than for those fed nontransgenic rice (Fig. 1B, C). Histological assessment of late-phase inflammation revealed that the extent of eosinophil infiltration into the conjunctiva on day 31 was significantly reduced in mice fed the transgenic rice compared with those fed nontransgenic rice (Fig. 1D).

Therapeutic feeding with transgenic rice alters systemic immune responses

The serum concentrations of both total and allergen-specific IgE (Fig. 2A, B) as well as that of allergen-specific IgG2a (data not shown) did not differ significantly between the two groups on day 31. We next determined the number of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in the spleen as well as in both submandibular and mesenteric lymph nodes by flow cytometry. The number of T_{reg} cells at each of these sites did not differ significantly between mice fed with transgenic or nontransgenic rice seeds (Table 1). Finally, to examine the effects of oral vaccination on allergen-specific T cell responses, we isolated splenocytes from mice fed the transgenic or nontransgenic rice seeds and stimulated them with cedar pollen extract. The concentrations of cytokines in culture supernatants were then measured with a multiplex bead array (Fig. 2C). Oral vaccination with transgenic rice resulted in significant up-regulation of the production of IFN-γ by splenocytes. Whereas the production of other Th1 cytokines (IL-2 and IL-12p70) also tended to be increased for mice fed the transgenic rice, these effects

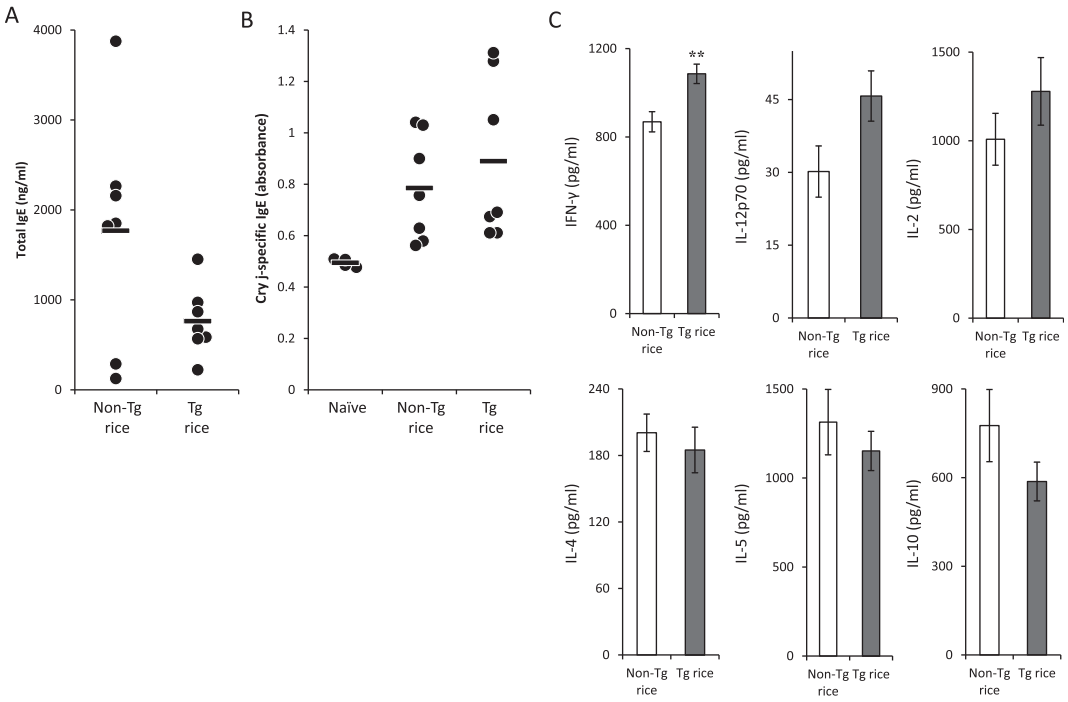

Fig. 2. Effects of therapeutic feeding with transgenic rice on systemic immune responses. The total IgE **(A)** and antigen-specific IgE **(B)** levels in serum were determined 24 h after allergen challenge on day 30 for mice treated as in Fig. 1. Circles and bars represent values from individual mice (seven mice fed transgenic or nontransgenic rice as well as four naïve control mice) and overall median values, respectively. **(C)** The spleen was isolated 24 h after allergen challenge on day 30 for mice treated as in Fig. 1, and the antigen-specific production of cytokines by splenocytes was measured. Data are means ± SEM for three mice per group and are representative of two independent experiments with similar results. $^{**}P < 0.01$ (Student's *t* test) versus the corresponding value for nontransgenic rice.

Table 1
Number of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in mice fed transgenic rice or nontransgenic rice.

	Spleen	Submandibular lymph nodes	Mesenteric lymph nodes
Nontransgenic rice	1.22 ± 0.05	2.42 ± 0.09	1.98 ± 0.15
Transgenic rice	1.21 ± 0.06	2.38 ± 0.09	1.53 ± 0.15

Data are means ± SEM (three mice per group) for tissue isolated 24 h after allergen challenge on day 30 for mice treated as in Fig. 1 and they represent the percentage of $CD4^+CD25^+Foxp3^+$ cells among all $CD4^+$ cells.

were not statistically significant. The production of the Th2 cytokines IL-4, IL-5, and IL-10 by splenocytes also did not differ significantly between the two groups.

Discussion

We have shown here that oral immunotherapy with transgenic rice seeds expressing hypoallergenic forms of the major Japanese cedar pollen allergens suppressed established pollen-induced allergic conjunctivitis in mice. We previously showed that prophylactic feeding of this transgenic rice had a protective effect against the development of allergic conjunctivitis in mice.[5] Poor patient adherence to treatment protocols is a key problem for both SCIT and SLIT in the clinical setting.[11] Rice is a staple food for Asian populations and is therefore a potential route for antigen delivery that could improve adherence to oral immunotherapy in this region of the world.

Oral tolerance suppresses immune responses by various mechanisms including the induction of T cell deletion or anergy and the activation of T_{reg} cells.[12] The mechanisms of immune suppression by immunotherapy likely depend on various factors such as the duration, frequency, dose, and specificity of antigen administration. We have previously shown that high-dose oral antigen administration suppressed antigen-induced cytokine production by splenocytes as well as serum Ig levels through the induction of T cell anergy or deletion, whereas low-dose antigen treatment induced T_{reg} cell–mediated oral tolerance, in a mouse model of ovalbumin-induced allergic conjunctivitis.[13] We have also shown that prophylactic feeding with transgenic rice resulted in the uniform suppression of antigen-induced cytokine (both Th1 and Th2 cytokines including IL-10) production by splenocytes as well as in inhibition of antigen-specific IgE production in a mouse model of cedar pollen–induced conjunctivitis.[5] In the present study, systemic immune responses appeared to differ from those in mice subjected to prophylactic feeding. We detected a significant increase in antigen-stimulated IFN-γ production by splenocytes from mice fed transgenic rice seeds. The production of other Th1 cytokines by splenocytes from mice fed the transgenic rice also tended to be increased, whereas that of Th2 cytokines tended to be decreased. We previously showed that IFN-γ suppressed the infiltration of eosinophils into the conjunctiva induced by IL-4,[14] suggesting that increased IFN-γ production might contribute to suppression of conjunctival eosinophil infiltration in mice fed the transgenic rice seeds in the present study. A shift from a Th2 to Th1 cytokine milieu accompanied by clinical improvement in symptoms has also been demonstrated after SCIT or SLIT.[15–18] SLIT was also found to induce an early increase in IFN-γ production related to clinical improvement,[19] similar to our present results. Although the number of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in the spleen or lymph nodes did not differ significantly between mice fed transgenic or nontransgenic rice seeds, our data do not rule out a possible role for allergen-specific $IL\text{-}10^+Foxp3^-$ adaptive T_{reg} cells in the immunosuppressive effect of the transgenic rice. Further study is warranted to establish a clear immunologic or other effect as a marker of clinical efficacy for oral immunization with transgenic rice.

Allergenomic analysis showed that >100 spots reacted with IgE in the plasma of patients with allergies to Japanese cedar pollen and eight allergens have been isolated and characterized. IgE reactive allergens differ among individuals with pollinosis, with 45% of patients manifesting IgE-binding reactivity with many allergens other than these two major allergens Cry j 1 and Cry j 2.[20,21] Whereas transgenic rice accumulates modified forms of Cry j 1 and Cry j 2, we used Japanese cedar pollen extract that include not only these two major allergens but also other antigens to measure antigen-specific IgE and to stimulate splenocytes. It is thus possible that the amounts of Cry j 1– or Cry j 2–specific IgE or the Th2 cytokine response of spleen cells stimulated with Cry j 1 and Cry j 2 (rather than with pollen extract) might have been significantly decreased in mice fed with transgenic rice.

The transgenic rice used in this study contains molecularly modified forms of Japanese cedar pollen antigens—Cry j 1 having been fragmented and Cry j 2 having been deconstructed by molecular shuffling—that have altered conformational structures but which retain all T cell, but not B cell, epitopes.[4] The modified antigens in the transgenic rice are thus thought not to be recognized by pollen antigen–specific IgE, and indeed they were found not to bind to Cry j 1– or Cry j 2–specific IgE.[4] IgE-mediated side effects such as anaphylaxis or asthma are thus minimized whereas the T cell–dependent immunogenicity of the allergens is preserved, a highly advantageous situation in the clinical setting of immunotherapy. The reduced risk of anaphylaxis may allow high-dose administration of the modified antigens and therefore a shortening of the duration of immunotherapy.

One of the disadvantages of oral therapy is the degradation of antigen by enzymes in the gastrointestinal tract. However, the modified antigens in the transgenic rice seeds are highly resistant to enzymatic degradation as a result of their deposition in protein bodies. The antigens in rice seed are therefore delivered effectively to the gut. Other practical advantages of transgenic rice–based immunotherapy are that large amounts of antigen accumulate in the endoplasmic reticulum–derived protein bodies in rice grains (~10–25 μg of antigens per grain) and that the antigens are stable at room temperature for several years, have no toxicity, and are edible. Given that rice is a staple food of Asian populations including Japanese, oral immunotherapy with transgenic rice is convenient and may improve treatment adherence in this region of the world.

Conjunctival antigen challenge tests have recently been adopted to evaluate antiallergy eyedrops in clinical trials. The conjunctival antigen challenge test is an allergen provocation test for the conjunctiva that is simple and safe to perform and which can provide valuable clinical information. Optimization of the dose and duration of antigen administration during immunotherapy is key to improvement of clinical efficacy in vaccine development. Further studies to determine the optimal dose and duration of transgenic rice administration for achievement of maximal efficacy are warranted before clinical application. The clinical efficacy of immunotherapy can be readily and repeatedly evaluated with the conjunctival antigen challenge test, and such testing may therefore contribute to determination of the appropriate dose and duration of antigen administration in immunotherapy for pollinosis.

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Conflict of interest

KF has received funding from the charitable Trust Fund for Ophthalmic Research in Commemoration of Santen Pharmaceutical's Founder. The rest of the authors have no conflict of interest.

Authors' contributions

KF and AF designed research; KF, WI, and YH performed experiments; YW, HT, and FT produced transgenic rice; and KF, FT, and AF wrote the paper. All authors read and approved the final manuscript.

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