

Long-term oral administration of transgenic rice containing cedar pollen T-cell epitopes potentially improves medication- and allergy-related quality-of-life scores

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ABSTRACT

Background: We previously developed a transgenic rice that contains seven linked human predominant T-cell epitopes (7Crp) derived from Japanese cedar (JC) pollen allergens Cry j 1 and Cry j 2. Oral administration of 80 g of transgenic rice for 20 weeks suppressed allergen-specific T-cell proliferation in participants with JC pollinosis, but their clinical symptoms did not improve.

Objective: We examined the clinical efficacy of low-dose (5 g and 20 g) intake of the transgenic rice administered for two successive seasons.

Methods: In this randomized, double-blind, placebo controlled study, transgenic rice seeds (5 g or 20 g) were orally administered to the participants for 24 weeks in each of two successive JC pollen seasons. We analyzed T-cell proliferation and cytokine expression, and monitored symptom and medication scores during the pollen season. Quality of life (QOL) was evaluated by using the Japanese Allergic Rhinitis Quality of Life Standard Questionnaire (JRQLQ).

Results: Specific T-cell proliferation after stimulation with 7Crp, Cry j 1, and Cry j 2 was significantly suppressed in the second JC pollen season. No significant differences were found among the three groups (5 g, 20 g, and placebo) with regard to clinical symptoms or medication scores in the first season. However, the medication scores and face scale for overall condition of JRQLQ improved in the 5-g transgenic rice group in the second season, although careful re-examination with a large sample size is necessary to confirm the results.

Conclusion: Low-dose oral administration of transgenic rice that contains 7Crp significantly reduced allergen-specific T-cell responses and improved medication scores during the second season of administration. Thus, oral intake of the transgenic rice has the potential to induce immune tolerance to JC pollen allergens when administered for at least two successive seasons.

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Pollinosis caused by Japanese cedar (JC) pollen is one of the most common forms of seasonal allergic rhinitis in Japan.¹ The prevalence of JC pollinosis has increased significantly over the past decade, which affects 45.6% of the population in Tokyo.² The main treatment for JC pollinosis is currently allergy medications, *e.g.*, antihistamines.³ Allergen-specific immunotherapy is the only curative treatment for allergic

diseases.⁴ Sublingual immunotherapy was recently introduced in Japan for JC pollinosis,^{5,6} but it does not avoid some adverse effects because the natural antigens cross-link with specific immunoglobulins on mast cells and basophils. To make the immunotherapy for JC pollinosis safer and more convenient, oral peptide immunotherapy may be a plausible option.

Seven major T-cell epitopes identified from major JC pollen allergens Cry j 1 and Cry j 2 were serially concatenated and designated as 7Crp.⁷ Transgenic rice that accumulates 7Crp peptide in its seeds has been developed for oral administration of 7Crp peptides. In the transgenic rice, 7Crp peptides are deposited in endoplasmic reticulum-derived protein bodies of the endosperm tissue, which are highly resistant to gastrointestinal digestion.⁸ Some of 7Crp peptides are expected to be delivered to gut-associated lymphoid tissue without degradation in the harsh environment of the gastrointestinal tract. Thus, immune tolerance to JC pollen allergens will likely be induced by oral intake of transgenic rice that contains 7Crp *via* the gut mucosal immune

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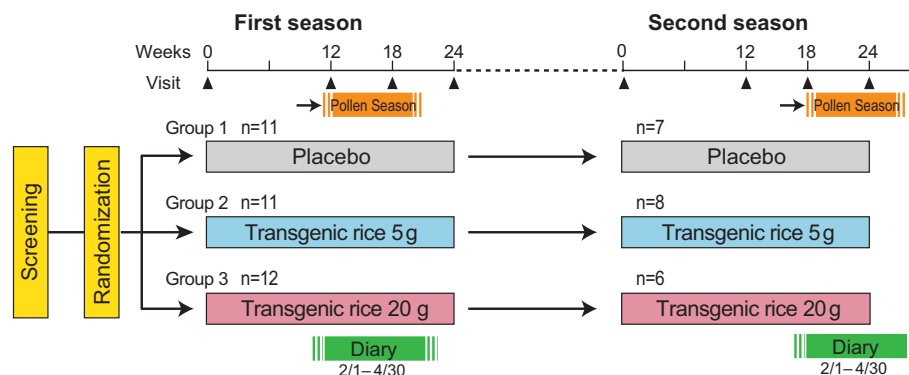


Figure 1. Study design. Thirty-four enrolled participants were randomly divided into three groups (placebo, 5 g, or 20 g transgenic rice) in a 1:1:1 ratio. The participants were asked to consume one prepacked serving of cooked rice per day for 24 weeks, overlapping with the Japanese cedar (JC) pollen season (February to April) for two seasons. The participants were asked to keep an allergy diary during each pollen season from February 1 to April 30 (green bars). The number of participants in the trials is denoted as *n*.

system, similar to food and intestinal microbes.⁸ The transgenic rice seed offers an ideal production platform for the 7Crp peptide in terms of robust production levels, ample deposition space, and high stability at an ambient temperature for several years, which leads to low cost of production.⁹ Recently, transgenic plant systems were used successfully to express recombinant proteins, such as human serum albumin produced in rice seeds¹⁰ and hemagglutinin-based influenza virus-like vaccine particles produced in tobacco leaves.¹¹

We previously observed no adverse events with the oral administration of transgenic rice that contained 7Crp.¹² Furthermore, oral intake of 80 g of transgenic rice for 20 weeks in the JC pollen season resulted in significant suppression of allergen-specific T-cell proliferation in a randomized, single-blind, placebo controlled study. The suppression of specific T-cell proliferative activities against Cry j 1, Cry j 2, and 7Crp was also observed with the intake of 5 g and 20 g of transgenic rice for 8 weeks during the off-season. The clinical symptom scores against allergic rhinitis exhibited a trend of improvement but not significant results.¹² One plausible reason for this uncertainty was that the clinical trial was carried out for only one season, which contradicted the clinical efficacy of allergen-specific immunotherapy evaluated in multiyear interventions.^{5,6} Therefore, low doses (5 g and 20 g) of transgenic rice were orally administered in two successive JC pollen seasons for 24 weeks each in a randomized, double-blind, placebo controlled study.

METHODS

Participants

The participants were patients with JC pollinosis for at least 2 years during JC pollen season and who underwent medical examination. Patients who had a JC pollens-IgE score (CAP system fluorescein-enzyme

immunoassay) >0.70 IU/ml and a positive response to JC pollens in the provocation test and intradermal reaction were enrolled in the study. Exclusion criteria are described in the Supplemental data.

Study Design

The study was conducted at Jikei University Hospital as a double-blind, placebo controlled trial from August 2016 to April 2018. The protocols were approved by the ethics committee of Jikei University School of Medicine (registered clinical trial 28-043 [8286]) and carried out in accordance with the Declaration of Helsinki and the ethics guidance with regard to clinical studies from the Ministry of Health, Labor and Welfare. Written informed consent was obtained from each patient before participation in the study. This work was supported by JSPS KAKENHI (grant 16K20282) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by the “Research for Agri-Health Translational Research Project” from the Ministry of Agriculture Forestry and Fisheries of Japan.

Thirty-four enrolled patients were randomly divided 1:1:1 into three groups: placebo, 5-g transgenic rice, or 20-g transgenic rice. The participants were asked to consume one prepacked serving of cooked rice per day for 24 weeks, which overlapped with the JC pollen season (February to April) for two seasons (from 2016 to 2018). The participants did not consume the prepacked cooked rice between the two experimental periods (Fig. 1). The participants were asked to keep an allergy diary during each pollen season, from February 1 to April 30.

Lymphocyte Proliferation Assays and Analysis of Cytokines and Antigen-Specific Serum Antibodies

Blood was collected from the participants at each follow-up visit and proliferation in peripheral blood mononuclear cells (PBMCs) were obtained as des-

cribed previously.¹³ Allergen-specific lymphocyte proliferative responses to 7Crp, Cry j 1, and Cry j 2 were measured by using an *in vitro* radioactively labeled thymidine incorporation assay.¹³ The concentrations of IL-4, IL-5, IL-10, IL-13, and IFN- γ secreted by PMBCs after stimulation with 7Crp, Cry j 1, and Cry j 2 were measured by using standard sandwich ELISA protocols. Antigen-specific immunoglobulin E (IgE) antibody titers were measured by IgE capture ELISA as described previously.¹⁴ The antigen-specific IgG4 antibody titers were measured by using N-Assay LA IgG4 Nittobo (Nittobo Medical Co., Ltd., Fukushima, Japan).

Clinical Symptoms

Clinical symptoms were evaluated by symptom scores and medication scores self recorded by the participants in their daily allergy diary. When daily life was severely affected by JC pollinosis, 20 mg of epinastine hydrochloride was used as the first-choice treatment. If this treatment was insufficient, then cromoglycate nasal spray or cromoglycate eye drops were added to alleviate nasal or eye symptoms, respectively. Use of one of the drugs described above was defined as a medication score of 1, and the use of all three drugs was defined as a medication score of 3. We used the Japanese Allergic Rhinitis Quality of Life Standard Questionnaire (JRQLQ)^{15,16} to assess the quality of life (QOL) of patients with allergic rhinitis according to the Practical Guideline for the Management of Allergic Rhinitis.¹⁶ Nasal and ocular symptom scores, QOL-related questionnaire scores for pollinosis, and the face scale score for overall condition were calculated and analyzed statistically.

Statistical Analysis

Statistical analyses were carried out by using GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA) and IBM SPSS version 23 (IBM Corp., Armonk, NY). Symptom scores were compared among the three groups by using one-way ANOVA or the Kruskal–Wallis test with the *post hoc* test. T-cell proliferation and cytokine production were analyzed by repeated measures ANOVA and the Friedman test. Differences in categorical variables were evaluated by using the χ^2 test. Significance was defined as $p < 0.05$.

RESULTS

Participant Enrollment and Baseline Characteristics

Thirty-four participants with JC pollinosis were randomly allotted to the placebo group ($n = 11$), 5-g transgenic rice group ($n = 11$), or 20-g transgenic rice group ($n = 12$) (Fig. 1). One patient withdrew from participating halfway through the first pollen season due to relocation. In the first pollen season, 33 participants completed the 24-week rice intake. Eleven participants

(placebo group, $n = 4$; 5-g group, $n = 3$; and 20-g group, $n = 4$) could not participate in the second pollen season, one participant from the 20-g group withdrew because she wanted to get pregnant during the off-season, and one participant in the 20-g group dropped out during the second pollen season due to asthma. Therefore, 20 participants (placebo group, $n = 7$; 5-g group, $n = 8$; and 20-g group, $n = 5$) completed the two pollen seasons (Fig. 1). The demographic (gender, age, and disease duration) and baseline characteristics (total IgE, peripheral eosinophil count, specific IgE levels for JC pollen in serum, baseline score for nasal and ocular symptoms, QOL, and face scale) of the participants who completed each of the seasons are shown in Supplemental Tables 1 and 2. The demographic and average clinical characteristics were similar among the three groups.

T-Cell Proliferation Responses Induced by Oral Intake of Different Doses of Transgenic Rice during the Pollen Season

The effects of transgenic rice intake were initially examined based on the T-cell proliferation responses of the participants with JC pollinosis during the pollen season. Notably, the pollen seasons started at different times between the first and second experimental periods: from the 12th week in the first season and from the 18th week in the second season. In addition, the cedar pollen count in the second season was approximately twice the count as in the first season (Supplemental Fig. 1). In the first season, T-cell proliferation in response to 7Crp was significantly suppressed 18 weeks after intake of 5 g and 12, 18, and 24 weeks after intake of 20 g of transgenic rice compared with placebo (Fig. 2A). No significant differences in T-cell proliferation were observed among the three groups after stimulation with Cry j 1 and Cry j 2, with the exception of 18 weeks after stimulation with Cry j 2 in the 20-g group.

In the second pollen season (Fig. 2B), the T-cell proliferation in response to 7Crp was significantly suppressed at 12 and 18 weeks in both transgenic rice groups compared with placebo. After stimulation with Cry j 1, T-cell proliferation was suppressed only at 18 weeks in the 5-g group, whereas Cry j 2-induced stimulation levels were diminished at 12 and 18 weeks in the 5-g group and at 18 weeks in the 20-g group compared with placebo. Analysis of these results suggested that 5 g of transgenic rice was sufficient to suppress 7Crp-specific T-cell proliferation, but its effects on Cry j 1- and Cry j 2-specific T-cell proliferation are limited.

Effects of Transgenic Rice Intake on Allergen-Specific IgE and Cytokine Secretion by PMBCs

As shown in Fig. 3, when 20 g of transgenic rice was administered, JC pollen-specific IgE was not increased during the first pollen season (from 12 to 20 weeks)

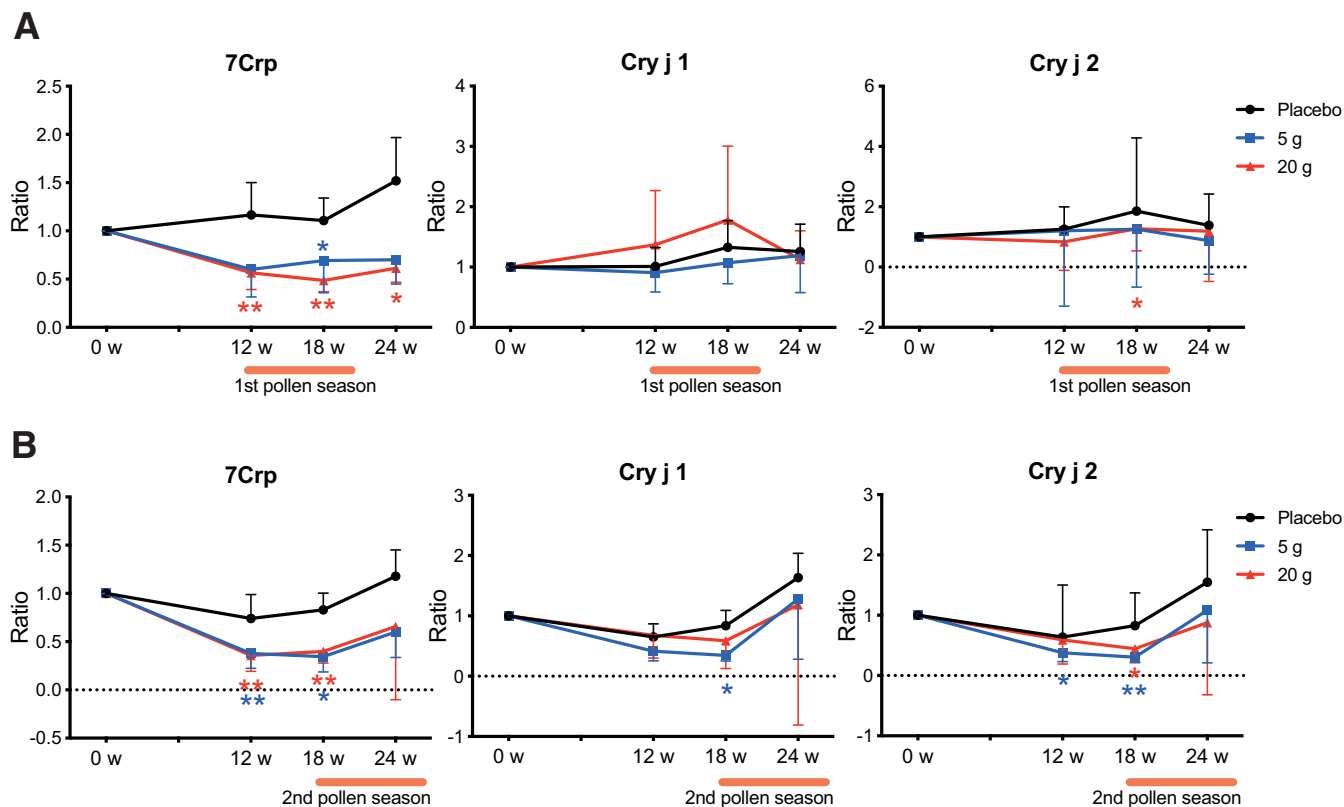


Figure 2. Changes in T-cell proliferation responses to seven linked human predominant T-cell epitopes (7Crp), Cry j 1, and Cry j 2 after oral administration of 5 g and 20 g of transgenic or nontransgenic rice for 24 weeks. Results were expressed as the Stimulation index (SI), which was calculated as the mean counts/minute in the presence of the antigen divided by the mean counts/minute in the absence of the antigen. Intergroup comparisons were based on the mean proportion of antigen-specific responses in the group at the indicated times during treatment, which was calculated as the SI at the indicated date divided by the SI on day 0. (A) First pollen season. (B) Second pollen season. Data are presented as mean $\pm$ 95% confidence interval. * $p < 0.05$, ** $p < 0.01$.

and gradually decreased until just before the second pollen season started, whereas the placebo control group presented a typical induction pattern of specific IgE due to pollen dispersal. With the oral intake of 5 g of transgenic rice, the specific IgE level was suppressed at the beginning of the first pollen season (at 12 weeks). Although the specific IgE levels then increased in a similar pattern as the placebo group, the levels were lower than in the placebo group and finally decreased to a similar level as the 20-g group at the beginning of the second pollen season (18 weeks). In both transgenic rice groups, specific IgE levels were increased at 24 weeks of the second pollen season. We examined the production of allergy-related cytokines by PBMCs in response to 7Crp, Cry j 1, or Cry j 2. We found no significant difference in the secreted levels of IL-4, IL-5, IL-10, and IFN- γ compared with placebo, which indicated that low doses of transgenic rice had little effect on the production of these cytokines, even over two seasons (data not shown).

Effect of Transgenic Rice Intake on Symptoms Related to JC Pollinosis

To evaluate the clinical effects of oral administration of transgenic rice, we compared clinical symptoms, medication scores, and the combined symptom-medication score (total nasal symptom and medication score [TNSMS]) during two successive pollen seasons between the intake and placebo groups. We observed no significant difference in clinical symptoms between the transgenic rice intake and placebo groups during the first pollen season (Supplemental Fig. 2A). In the second season, we observed no significant differences in the clinical symptom scores (Fig. 4A) or the TNSMS (Fig. 4B) among the three groups. However, medication scores were clearly improved in the transgenic rice groups; the average medication score in the 5-g transgenic rice group significantly decreased, to approximately one-third the score of the placebo group. The average medication score for the 20-g intake group was also

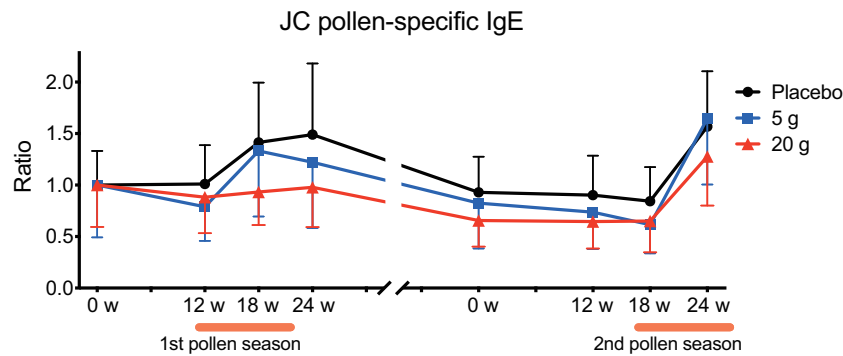


Figure 3. Changes in Japanese cedar (JC) pollen-specific immunoglobulin E (IgE) levels in the participants orally administered 5 g or 20 g of transgenic rice or nontransgenic rice for two seasons (24 weeks each). Blood was collected from the participants at each follow-up visit, and JC pollen-specific IgE was measured. The results are expressed as the ratio of the values at each visit to those on day 0 of the first season. Intergroup comparisons were based on the mean proportion of antigen-specific responses in the group at the indicated times during treatment. Data are presented as mean $\pm$ standard error of the mean (SEM).

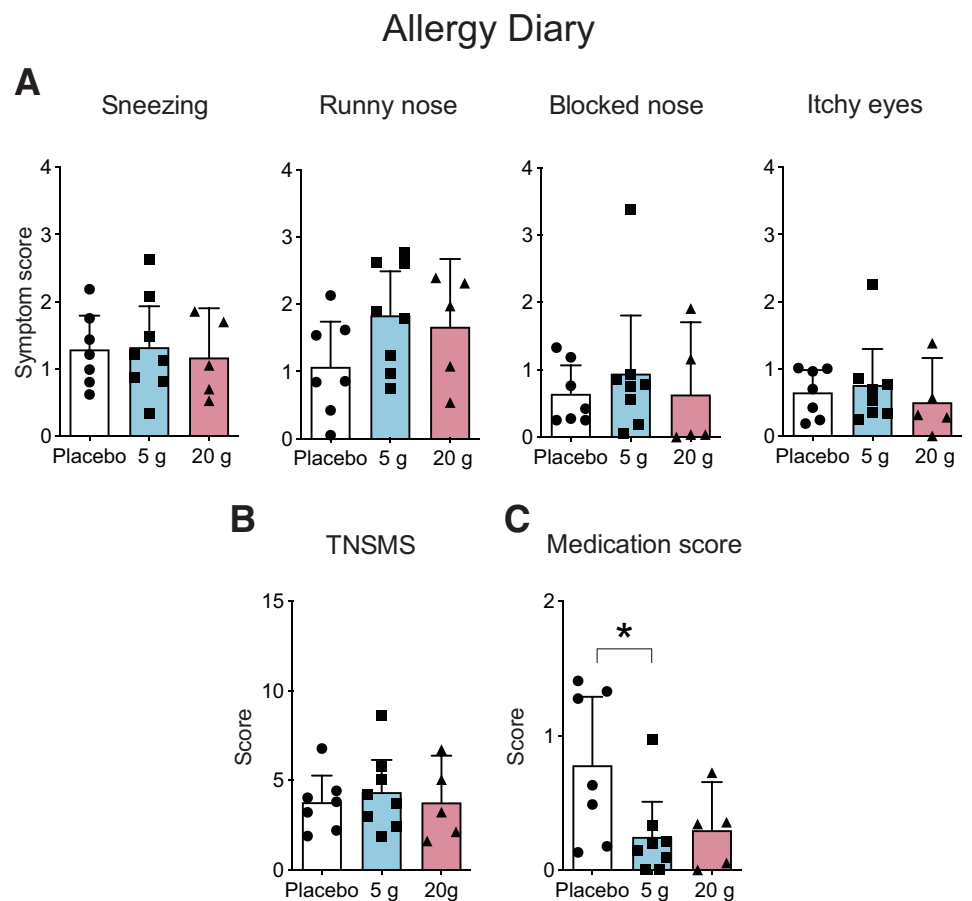


Figure 4. Average scores for clinical symptoms, total nasal symptom and medication score (TNSMS), and medication scores during the oral administration of 5 and 20 g transgenic or nontransgenic rice for 24 weeks in the second pollen season. (A) Symptom scores, (B) TNSMS, and (C) medication scores based on the allergy diary. Data are presented as mean $\pm$ 95% confidence interval. * $p < 0.05$.

reduced to less than half the score of the placebo group, although the difference was not significant (Fig. 4C).

QOL symptom scores were also evaluated by using the JRQLQ. Although no significant difference was

observed in the first season (Supplemental Fig. 2B), a significant alleviation of sneezing was observed in the second season in the 5-g transgenic rice group compared with the placebo group (Fig. 5A). In addition, some other QOL symptom scores, such as runny nose

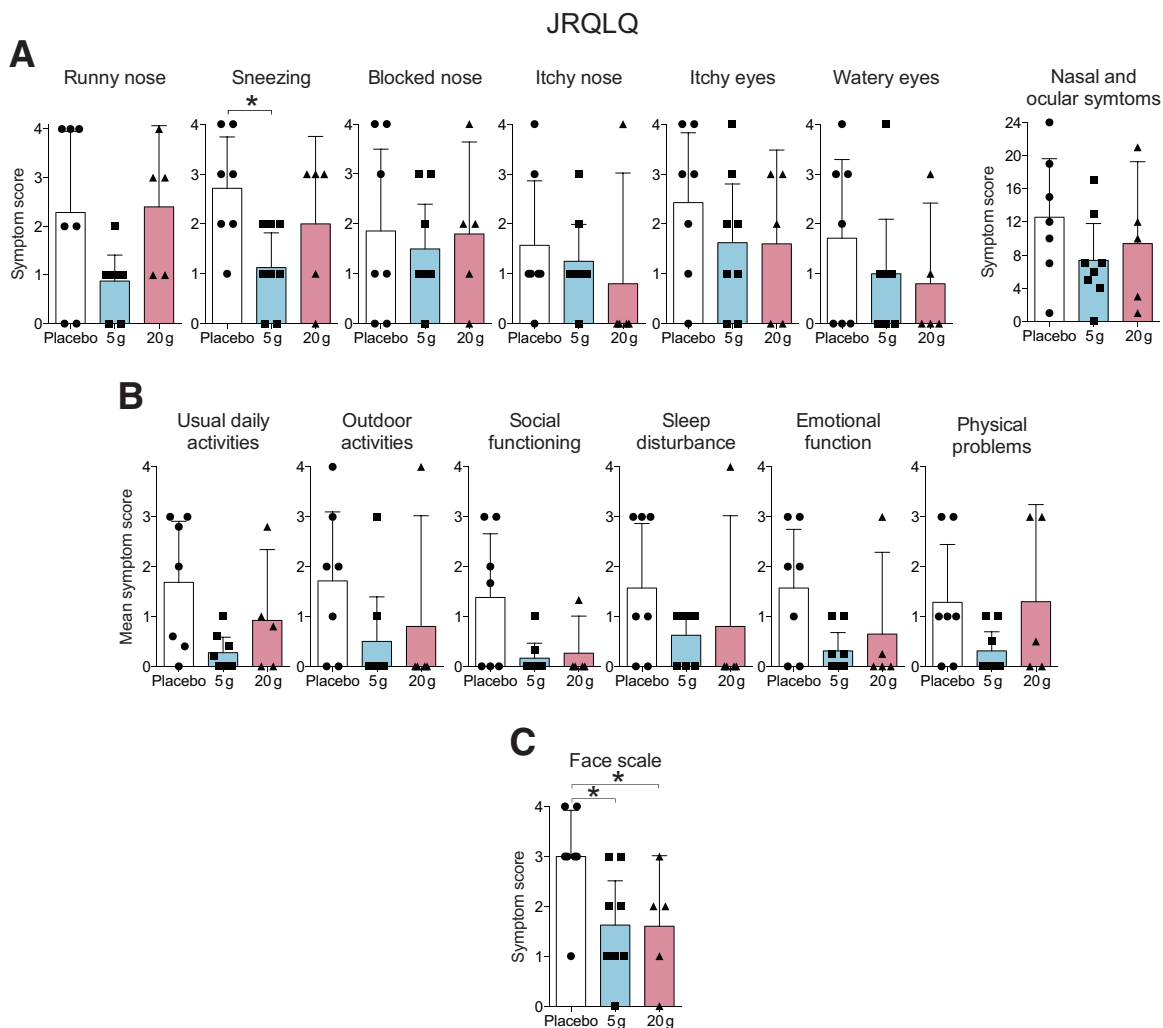


Figure 5. Average quality of life (QOL) scores and the face scale during the oral administration of 5 and 20 g of transgenic or nontransgenic rice for 24 weeks in the second pollen season. (A) Nasal and ocular symptom scores, and (B) QOL scores, and (C) face scale score by using the Japanese Rhinoconjunctivitis Quality of Life Questionnaire (JRQLQ). Data are presented as mean $\pm$ 95% confidence interval. * $p < 0.05$.

and itchy and watery eyes, presented some signs of improvement in the transgenic rice groups, and the majority of QOL-related questionnaire scores showed some signs of improvement in the 5-g transgenic rice group, although the differences were not significant (Fig. 5B). Furthermore, the overall face scale was significantly improved in the 5-g and 20-g transgenic rice groups compared with the placebo group (Fig. 5C). Thus, analysis of the results indicates that some clinical symptoms may be improved by the intake of 5 g of transgenic rice.

DISCUSSION

The oral route is safe and convenient, and routinely used for immunotherapy against food allergies, with positive results.¹⁷ However, oral immune tolerance has been fundamentally abandoned for allergic rhinitis¹⁸

because satisfactory clinical efficacy could not be obtained for several allergens (e.g., birch pollen allergen¹⁹ and timothy pollen allergen²⁰) in the 1980s. However, these negative results may depend on inadequate formulation of the administered antigens. This problem could be overcome by producing antigens in rice seeds. The present study showed that 7Crip-specific T-cell proliferation was significantly decreased by the oral administration of transgenic rice from the first season and that Cry j 1- and Cry j 2-specific T-cell proliferation was suppressed in the second season (Fig. 2). Cedar pollen-specific IgE also tended to decrease after intake of the transgenic rice compared with the placebo (Fig. 3), although the differences were not significant in either intake group. The specific IgE levels were increased in the second pollen season (24 weeks in Fig. 3), even in the 20-g transgenic rice group, which may be due to a large quantity of pollen dispersal in

the second season (Supplemental Fig. 1). In our previous study,²¹ we found that cedar pollen-specific IgE levels increased during the pollen season, even in the participants whose symptoms had improved after immunotherapy, possibly due to the heavy exposure to cedar pollen.

Medication scores and some clinical symptoms were improved in the second season. Notably, the 5-g transgenic rice group had a significantly lower medication score in the second pollen season compared with the placebo group. The 20-g transgenic rice group also showed an improvement in medication scores, although there was no significant difference due to a rather large standard error ($p=0.061$). The large variation may be attributed, at least in part, to the properties of 7Crp. Because 7Crp contains only seven major T-cell epitopes,⁷ the participants who were sensitized by other epitope types could not acquire immune tolerance against JC pollen allergens from the transgenic rice. The cedar pollen counts in the second season were approximately twice the counts in the first season (Supplemental Fig. 1). Despite the high cedar pollen counts, a significant reduction in the medication score was found in the transgenic rice groups during the second pollen season, which suggested the efficacy of the transgenic rice, even with the small number of cases in our analysis.

Various clinical symptoms and the TNSMS were not improved by oral intake of 5 or 20 g of transgenic rice throughout the two JC pollen seasons. Such little difference in clinical symptoms between the transgenic rice and placebo groups may be attributed to the participants in the placebo group receiving many concomitant drugs to alleviate their symptoms, as is evident from the medication score. However, when using the JRQLQ to evaluate clinical symptoms and QOL scores during the pollen season,¹⁵ we found that sneezing was significantly reduced in the 5-g transgenic rice group, and the face scale was significantly improved in both transgenic rice groups during the second pollen season compared with the placebo group. These observations indicated that oral administration of transgenic rice that contained 7Crp for at least two seasons reduced medication usage and improved QOL in some participants during the JC pollen season.

The transgenic rice was given only seasonally because this study was designed as a follow-up study of our previous trial¹² to evaluate the efficacy of lower doses with long-term administration. Other reasons are to relieve the burden on patients and to promote compliance by limiting the duration of the intake. In our previous trial, it seemed that the pollen season may provide patients with pollinosis with a good incentive to take the transgenic rice, although it did not work in this study.

We presented the possibility that oral administration of transgenic rice that contained 7Crp induced immune tolerance against JC pollinosis when administered for at least two successive seasons. Importantly, medical scores and allergen-specific T-cell proliferation were abrogated, even by oral intake of a low dose of transgenic rice (*i.e.*, 5 g) in the second season, when the cedar pollen counts were approximately twice the count in the first season. In addition, the face scale score, which indicates the general state of the participants, improved in the second season. Thus, oral intake of transgenic rice has potential clinical benefits.

Unfortunately, limitations in the number of enrolled participants made it difficult to obtain reliable results (especially in the 20-g group during the second season). The high dropout rate and small sample size may have introduced bias, which should be taken into consideration when interpreting the findings of this study. To confirm the clinical efficiency of rice-based oral immunotherapy against JC pollinosis, oral administration of the transgenic rice may have to be studied for several years in a large sample. Furthermore, the efficacy of transgenic rice will have to be investigated under constant conditions by using an environmental exposure unit (chamber) because symptoms change, depending on the pollen count exposure and the scattered JC pollen count changes each year.

CONCLUSION

Low-dose oral administration of transgenic rice that contains 7Crp significantly reduced allergen-specific T-cell responses and improved medication scores in the second season of administration. Thus, oral intake of the transgenic rice has the potential to induce immune tolerance to JC pollen allergens when administered for at least two successive seasons. However, larger-scale studies are needed to confirm these results.

Author Contributions:

T. Endo, D. Asaka, and Saburo Saito were involved in the conception and design of the study; H. Takagi, Y. Wakasa, K. Ozawa, M. Takano, and F. Takaiwa provided the transgenic rice and technical support; T. Endo, D. Asaka, H. Kodama, R. Mitsuyoshi, N. Sugimoto, S. Omae, H. Kojima, and Saburo Saito were involved in the acquisition of data; T. Endo, F. Takaiwa, M. Takano, D. Asaka, T. Nakayama, Shota Saito, and Saburo Saito analyzed and interpreted the data; T. Endo, M. Takano, F. Takaiwa, T. Nakayama, and Saburo Saito drafted the article, and all the other authors critically revised the article for important intellectual content. All the authors read and approved the final manuscript.

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