

A Rice-Based Edible Vaccine Expressing Multiple T-Cell Epitopes to Induce Oral Tolerance and Inhibit Allergy

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Japanese cedar (*Cryptomeria japonica*) pollinosis is the predominant seasonal allergic disease in Japan, with rhinitis and conjunctivitis as clinical symptoms from February to April each year. Epidemiologic studies indicate that approximately 20% of the Japanese population is afflicted with this allergic disease [1]. Furthermore, more than half of the general population has specific circulating IgE for cedar pollen allergens. The number of patients who have *Cryptomeria* pollinosis and the economic costs associated with this disease are expected to increase steadily, resulting in a strong social demand for the development of a reliable and effective way to control it.

In general, allergen avoidance, pharmacotherapy, and immunotherapy are the three principle treatments for allergic diseases [2]. The main strategy for treating allergic diseases is based on pharmacologic therapy. Many therapeutic medicines against allergic diseases have been developed that block the release of chemical mediators of the inflammatory response, such as histamine, leukotrienes, and prostaglandins. Intranasal corticosteroids and antihistamines have been used to ameliorate allergy symptoms in the mucous membranes, where the inflammatory response is propagated.

However, treatment using these pharmacologic therapies reduces clinical symptoms but does not provide a cure for allergic diseases, and several contraindications and side effects are associated with these drugs. Therefore, a treatment that addresses the immunologic causes of allergic disease would be a significant boon to society. Allergen-specific immunotherapy is a cornerstone in the management of allergic diseases and is the only treatment that

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can interfere with the basic pathophysiologic mechanisms of allergic disease [3].

Systemic immunization with protein extracts from several pollen sources has been shown to be an effective therapeutic treatment [4]. The clinical effects obtained through allergen-specific immunotherapy are the result of a decrease in allergen-specific IgE, an increase in blocking IgG, an increase in type 1 T-helper (Th1) cytokines, and a reduction in allergen-specific T-cell responsiveness. However, in conventional allergen-specific immunotherapy, repeated hypodermic administration with increasing doses of natural allergen extracts over the course of up to 3 years is required for effective and long-term results. Missing or delaying a treatment requires a regression in dose, thus extending the required treatment period. Because this regimen can be difficult to maintain, many patients drop out of treatment. This regimen also has a risk for severe adverse events, such as anaphylactic shock and pain from inflammation at the injection site, because intact allergens are subcutaneously administered. Because of the risks involved, systemic administration of allergens must be performed in a clinical setting. Therefore, development of a safe, effective, and convenient allergen-specific immunotherapy is needed. An edible vaccine (oral treatment) could be a desirable alternative to current treatments.

To avoid IgE-mediated complications and decrease the length of treatment, oral peptide immunotherapy using T-cell epitopes derived from allergens has been applied in mice as an animal model. This alternative therapy is possible because the specific T-cell activity responsible for the antigen-specific immune reaction can be modulated through not only subcutaneous and intradermal injection but also oral administration of specific T-cell epitopes [5,6].

This article reviews progress in the area of oral administration of rice plants containing T-cell epitopes derived from Japanese cedar pollen allergens. In an important series of proof-of-concept experiments, transgenic rice seeds were fed to mice to induce oral immune tolerance before challenging with pollen allergens. This study showed a significant reduction of allergy symptoms in the test mice and a decrease in allergen-specific IgE and histamine levels from suppression of the specific T-cell reaction. Transgenic rice seed that accumulates allergen-specific T-cell epitopes can thus be expected to act as a new type of tolerogen for suppressing allergen-specific CD4⁺ T-cell reactivity and to provide a new approach for the immunotherapy of allergic diseases. Furthermore, advantages of rice seed-based peptide vaccines include low cost of production, simple administration, negligible risk for contamination with human or animal pathogens, and low risk for negative immune reactions.

Characterization of Japanese cedar pollen allergen proteins and their T-cell epitope peptides

Two major allergens of Japanese cedar pollen, Cry j 1 and Cry j 2, have been isolated and characterized. More than 90% of patients who have

Japanese cedar pollinosis had IgE specific to both allergens [7]. Cry j 1 is a basic glycoprotein pectate lyase with an apparent molecular weight of 41 to 45 kd and isoelectric point (pI) of 8.9 to 9.2 [8]. Cry j 2 is also a basic protein with polygalacturonate activity and a molecular weight of 37 kd and pI of 8.6 to 8.8 [9]. Both proteins are specifically localized in the cell walls of papilla and amyloplasts in cedar pollen. The nucleotide sequences of the Cry j 1 [8,10] and Cry j 2 cDNAs [9,11] have been determined and their amino acid sequences have been deduced from their nucleotide sequences. T-cell determinants in Cry j 1 and Cry j 2 have been characterized by measuring the proliferative responses of peripheral blood mononuclear cells (PMBC) from patients who have Japanese cedar pollinosis to overlapping peptide fragments covering the entire length of the allergen peptides. Optimized T-cell epitopes without IgE binding were determined by two Japanese research groups [12,13]. Three amino acid sequences from Cry j1 (p212–224, p235–247, and p312–330) and four amino acid sequences from Cry j 2 (p77–89, p96–107, p192–204, and p356–367) were identified as being of a suitable size for major human T-cell epitopes using a primary proliferative response assay of freshly isolated PBMC [12]. Two amino acid sequences from Cry j 1 (p108–120, p211–225) and three amino acid sequences from Cry j 2 (p66–80, p182–200, p344–355) were determined by other groups [13]. Both groups identified four of the five T-cell epitopes as potential tolerogens.

T-cell epitopes derived from allergens, which are recognized by specific T cells, vary because they have different haplotypes of the human leukocyte antigen (HLA) II class molecules. This variation is a serious problem for the development of peptide immunotherapy that takes advantage of T-cell epitope peptides. To overcome this problem, artificial hybrid epitope peptides composed of several T-cell epitopes derived from the same allergen or a few allergens were created. Several major T-cell epitopes were chosen to be included in the design of an artificial hybrid polypeptide in which these five or seven candidate T-cell epitopes, derived from different allergen molecules, were linked together [13,14]. Hybrid T-cell epitope peptides have several benefits when used as immunotherapeutic tolerogens [14]. These peptides exhibit little IgE binding activity because B-cell epitopes are eliminated, and the novel combinations result in conformational changes. For example, the hybrid seven-linked epitope (7Crp epitope) showed no binding to specific IgE in the serum of 48 patients who had Japanese cedar pollinosis symptoms [14]. The 7Crp peptide induced T-cell proliferation with an average concentration 100-fold lower than a mixture of the T-cell epitopes. Immunogenicity comparable to the response to native Cry j 1 and Cry j 2 allergens is retained even by the form of the hybrid peptide composed of several T-cell epitopes. The major T-cell epitopes from Cry j 1 and Cry j 2 were also determined using T cells from mice immunized with these antigens. The T cells of B10.S mice immunized with Cry j 1 were reported to recognize three amino sequences, p111–130, p211–230, and p310–330 [15], whereas the two amino acid sequences (Cry j 1 p277–290 and Cry j 2 p246–258)

were identified as major T-cell epitopes in BALB/c mice using overlapping peptides [16]. Cry j 2 p70–83 was identified as a minor T-cell epitope [16].

Oral administration of the immunodominant Cry j 2 p246–258 T-cell epitope peptide in BALB/c mice four times at intervals of 3 or 4 days for 14 days before challenging with Cry j 2 allergen results in suppression of T-cell proliferation, specific IgE, and histamine release by mast cells, indicating that oral feeding of T-cell epitopes can result in immune tolerance [17]. The potential for peptide-based oral immunotherapy was first shown by this experiment. The immunogenicity and tolerogenicity of each T-cell epitope and in the linked epitope peptide were apparently also retained, because oral administration of three linked BALB/c mouse T-cell epitopes inhibited T-cell proliferation against the three T-cell epitopes [16].

Mucosal immune tolerance

Oral tolerance is the most common response of the host to the environment. Development of oral tolerance is an important natural mechanism whereby the host becomes tolerant to ingested food proteins and other antigens. This induction of immune tolerance can be provided by direct mucosal exposure to antigens. Therefore, the development of mucosal tolerance through oral immunization against pollen antigens could be an essential and powerful tool for inhibiting allergic reactions, including IgE-mediated hypersensitivities such as type I allergic disease [18,19].

Oral tolerance in mice occurs after either administration of a single high dose of antigen (10–500 mg) or repeated exposure to lower doses (1–5 mg) [20]. These high- and low-dose tolerances are mediated by separate and distinct mechanisms [20]. Clonal deletion and anergy, which can be elicited by large doses of antigen (high-dose tolerance), are characterized by the absence of antigen-specific T-cell proliferation and decreased interleukin (IL)-2 production, resulting in oral tolerance [20,21]. High-dose oral tolerance-induced deletion can be mediated by high-affinity T-cell receptor (TCR) crosslinking or by inhibitory ligands CD95 through CD95, leading to apoptosis. Anergy occurs when incomplete activation signals are sent through TCR interactions between factors such as CD80 or CD86 on antigen-presenting cells with CD28 on T cells (low-affinity interaction) or when a lack of costimulation molecules occurs during activity. However, low-dose tolerance is mediated by the active suppression of immune responses by T cells [19,20]. It is controlled by regulatory T cells that can be divided into three subsets, such as CD4⁺CD25⁺ natural regulatory T cells, Th3 cells, and Tr1 cells [19,20]. CD4⁺CD25⁺ regulatory T cells express high levels of cytotoxic T lymphocyte antigen 4 (CTLA4) molecules and the immunosuppressive cytokines IL-10 and transforming growth factor beta (TGF- β). TGF- β -producing Th3 cells have also been implicated in low-dose tolerance [19,20]. Active suppression of the immune responses is therefore achieved

through cognate interactions and soluble (IL-10, TGF- β) or cell-surface-associated suppressive cytokines.

Rice seed-based edible vaccines as an effective treatment for cedar pollen allergies

Allergen-specific immunotherapy using intact native allergens has been used successfully to treat allergic diseases, but this type of therapy has been associated with an increased risk for systemic anaphylaxis mediated by allergen-specific IgE. To avoid these side effects, peptide immunotherapy using dominant T-cell epitopes was proposed as a treatment alternative [21,22]. Peptide immunotherapy has been mainly performed through systemic injection, intranasal application, or oral administration in animal models. These treatments result in a reduction in allergen-specific T-cell proliferation and IgE levels, and in changes in the pattern of cytokine release by Th2 cells. Shifts in the immunologic balance from allergen-specific Th2 to Th1 or Th0 cytokine, or down-regulation of the cytokine response released from Th1 and Th2 cells have been observed after treatment with a peptide vaccine [23].

As a new immunotherapy based on the principle of peptide immunotherapy, rice seeds that accumulate major T-cell epitopes derived from pollen allergens were used as a vehicle to deliver mucosal tolerogens. To confirm the oral tolerogenic efficacy of a rice seed-based edible vaccine, transgenic rice seed containing only the BALB/c mice major T-cell epitope peptides Cry j 1 p277–290 and Cry j 2 p246–59 was created [24]. Because these short T-cell epitopes could not be expressed directly, they were introduced into variable regions of the soybean seed storage protein glycinin (A1aB1b) as a fusion protein (Fig. 1). The epitopes are thus expressed as part of a stable storage protein. After the endosperm-specific 2.3-kb glutelin *GluB-1* promoter and the 0.6Kb *GluB-1* terminator were ligated to this fusion protein gene, the entire expression vector was transferred into the rice genome through *Agrobacterium*-mediated transformation. The modified glycinin-containing major mouse T-cell epitopes were thus specifically expressed in the endosperm of transgenic rice seeds under the control of the rice major seed storage protein glutelin *GluB-1* promoter [24]. The modified glycinin accumulated at a level of 7 μ g per grain (0.5% of total seed protein) in mature rice seeds. When 10 transgenic rice seeds (70 μ g modified glycinin; 2 μ g total epitope dose) were fed daily to mice for 4 weeks, the mice had far less of an immune reaction to pollen in a systemic challenge than mice fed nontransgenic rice controls, indicating that the rice seed-based vaccine induced oral immune tolerance. Treated mice showed not only significant reductions in the specific CD⁺4 T-cell proliferative reaction but also reduced allergen-specific IgE and IgG levels and production of IL-4, IL-5, and IL-13 associated with allergen-specific Th2 cells; histamine release from mast cells was also inhibited by feeding with the transgenic rice seeds [24]. The clinical symptoms of allergy

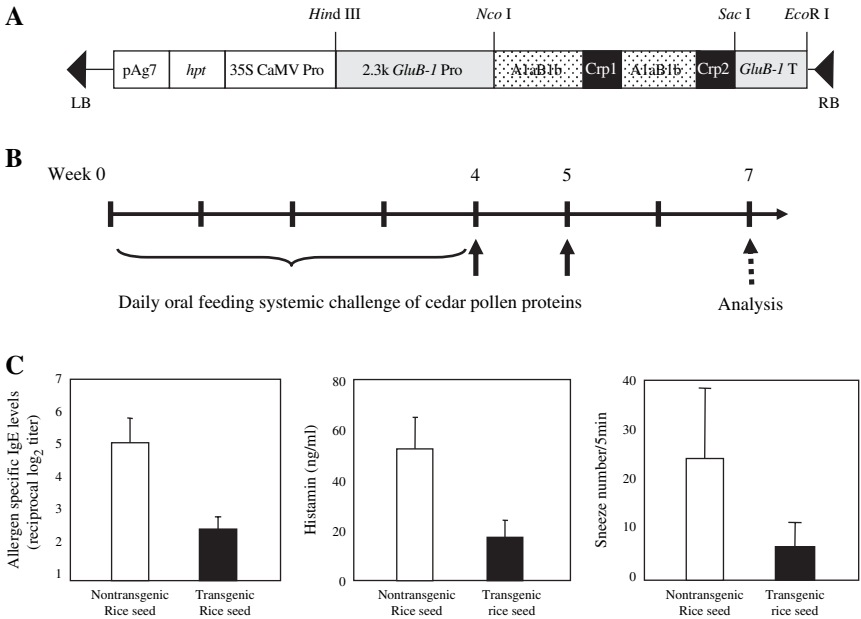


Fig. 1. Oral immune suppression induced by feeding transgenic rice seeds accumulating A1aB1b containing major mouse T-cell epitopes derived from Cry j 1 and Cry j 2 allergens. (A) Schematic presentation of the binary vector used for expression of the modified A1aB1b in transgenic rice seed. (B) Feeding schedule and challenge with cedar pollen allergens. (C) Inhibition of allergen-specific serum IgE, serum histamine and the number of sneezes by oral administration of transgenic rice seeds compared with nontransgenic rice seeds. 35S CaMV Pro, cauliflower mosaic virus 35S promoter; *GluB-1* Pro, rice glutelin *GluB-1* promoter; *hpt*, hygromycin phosphotransferase gene; LB, left border; pAg7, agropine synthase polyadenylation signal sequence; RB, right border.

(sneezing) were also suppressed when assayed in an experimental sneezing mouse model [24]. These results showed that oral immune tolerance was induced by administering transgenic rice seeds containing the major T-cell epitopes.

A human version of the transgenic rice seed-based edible vaccine against Japanese cedar pollinosis was generated based on the feasibility of an oral peptide immunotherapy strategy against pollen allergy using rice seed-based vaccines in mice. An artificial hybrid peptide 7Crp gene encoding a 96 amino acid protein of seven-linked human dominant T-cell epitope peptides derived from Cry j 1 and Cry j 2 allergens was synthesized using codons optimized for expression in rice seed [25]. To enhance accumulation of the 7Crp peptide, nucleotide sequences for the N-terminal GluB-1 signal peptide and C-terminal KDER ER retention signal were ligated to 7Crp. The 7Crp peptide was specifically accumulated in mature seeds at a maximum level of approximately 60 μ g per grain (4% of total seed protein) under the control of the

2.3-kb glutelin *GluB-1* promoter in transgenic rice plants generated by *Agrobacterium*-mediated transformation (Fig. 2). Like glutelin, the 7Crp peptide is coordinately synthesized during seed maturation, and is specifically accumulated in the endosperm, whereas leaf, stem, and embryo tissues have no signal [25]. Intracellular localization was examined using immunoelectron microscopy. 7Crp was predominantly deposited in protein bodies in the rice endosperm despite the attachment of the KDEL ER retention signal [25].

Transgenic rice seeds (560 µg total dose of 7Crp) were administered orally to B10.S mice for 32 days and then the mice were nasally challenged with intact Cry j 1 allergen. B10.S mice recognize only one epitope (Cry j 1 p211–225), but the T-cell proliferative responses and serum IgE levels against Cry j 1 were depressed compared with control mice fed nontransgenic rice seeds, thus indicating the potential usefulness of the transgenic 7Crp seeds for controlling pollen allergy in humans [25]. T-cell proliferative activity (ie, immunogenicity) is retained even after boiling 7Crp rice for 20 minutes at 100°C or autoclaving for 20 minutes, indicating that oral immune tolerance would be effective with steamed or cooked rice. This observation suggests that rice seed-based edible vaccine could be provided in a form familiar to Japanese consumers, and thus may be a practical

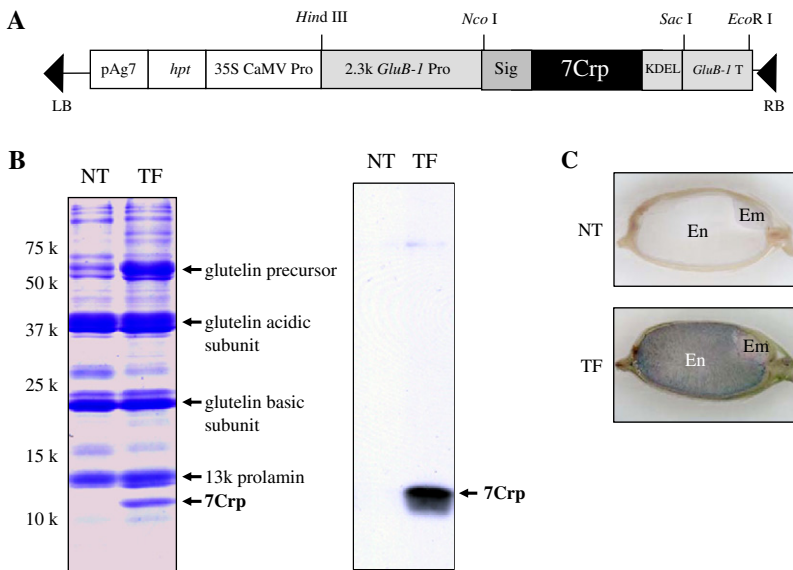


Fig. 2. Expression of 7Crp epitope peptide in transgenic rice seed. (A) Schematic of the construct used for expression of 7Crp in transgenic rice seed. (B) Accumulation of 7Crp in transgenic rice seed. Coomassie brilliant blue-stained SDS-PAGE of total proteins extracted from the seeds of nontransgenic (NT) and transgenic (TF) rice. Expression was confirmed by Western blotting using specific anti-7Crp serum. (C) In situ immunolocalization of 7Crp in transgenic seed. En, endosperm; Em, embryo.

treatment for cedar pollinosis. An effective human dose of 7Crp for inducing oral tolerance to Japanese cedar pollen was extrapolated from the effective dose (2 μ g) for mice and the mean relative body weights of mice (30 g) and humans (60 kg). The consumption of approximately 12 g of 7Crp rice (at approximately 50 μ g per 20 mg of grain) per day should be sufficient. Japanese citizens eat approximately 100 g of rice per day as a staple food, and therefore 7Crp seed can be used to supplement the daily diet through mixing with normal rice.

The food safety of 7Crp seeds, or of any subsequently developed rice seeds containing immunoactive constituents, must be carefully examined before use. Regulatory standards based on substantial equivalence have been used to determine the food safety of transgenic crops. With this method, a comparative assessment of the toxicology and nutrient composition is made between the newly developed transgenic food and a conventional counterpart. When several macro- and micronutrients were compared between the cognate parental nontransgenic and transgenic 7Crp seeds, the amino acid, lipid, and mineral compositions were essentially identical.

Rice is a new vehicle for production and delivery of edible vaccines

Artificial T-cell epitope peptides used as tolerogens have been produced in recombinant bacteria or yeasts and then extracted and purified for peptide immunotherapy. This production must be performed at a facility that has pharmacologic technology, and the purification process is very costly. In contrast, peptide vaccines produced in the edible parts of crops makes large-scale production efficient, accessible, and responsive to demand, because direct delivery is achievable without the need for purification or partition into effective doses. These advantages of plant-based production offer a simpler and cheaper method of vaccine mass production.

Pharmaceutical proteins produced in transgenic plants generally have not been targeted to specific tissues, but have been expressed broadly in the vegetative tissues such as leaves, roots, fruits, and tubers or seeds using CaMV 35S or maize ubiquitin-1 constitutive promoters. Expression and accumulation of these proteins is in most cases limited to less than 1% of total protein [26,27]. If edible vaccines are to be clinically effective, sufficient levels of the active peptides must be produced and accumulated in the food-use tissues. Low levels of expression can be overcome by increasing promoter activity or by changing the target tissue, subcellular location, plant species, genetic background, codon use of the transgene, post-translational signal sequences, or other factors [28]. Transgene products expressed in leaves, tubers, and roots are not highly accumulated irrespective of high levels of mRNA transcripts.

Seed is the ideal plant production site because it is a natural storage organ that accumulates storage proteins, starch, and lipids, offering ample

storage space for foreign recombinant proteins [29]. Seed-specific promoters with high activity have been reported in rice, maize, common bean, and wheat [29], and compartment-specific promoters were recently developed in rice [30]. The β -glucuronidase reporter gene can be highly expressed in the rice aleurone, subaleurone, and inner starchy endosperm under the control of the 10-kd prolamin, glutelin *GluB* subfamily, and 26-kd globulin promoters, respectively [30]. The tissues and subcellular compartments suitable for accumulation may differ on a case-by-case basis depending on the tolerogen.

As a production system, rice seed has several advantages over other cereal crops, including easier storage and processing, greater biomass (yield per unit area), and the fact that it is essentially self-crossing and can be grown at a lower cost to the producer. In addition, a transformation system has been established and the whole genome has been sequenced. The 7Crp epitope peptide is stably accumulated only in the seed endosperm tissue of transgenic rice, even though high transcript levels were observed in seed embryos, leaves, stems, and roots. Accumulation levels can also be increased by changing the genetic background of a host. For example, a low storage protein mutant could provide additional storage capacity for introduced gene products [31,32]. Two- to threefold differences have been reported in accumulation levels among hosts with different genetic backgrounds. Furthermore, recombinant proteins expressed in seed are stable for more than a year even if stored at room temperature. The combination of peptide stability without refrigeration, high accumulation levels, and targeting of the product to specific edible tissues indicates that seed is by far the best organ to serve as a natural bioreactor.

Vaccine proteins accumulated in the seed are more resistant than conventional purified protein vaccines to enzymatic digestion in the gastrointestinal tract. Therefore, much smaller amounts of edible vaccine can be delivered as a seed storage protein and still effectively induce oral tolerance. To be effective, antigens must be delivered through M cells in Peyer's patches and intestinal epithelial cells in gut-associated lymphoid tissue (GALT) to induce immune tolerance. Efficacy at lower concentrations may be caused by the concentration of antigens through packaging in the plant cell wall and further concentrated by compartmentalization in specialized protein body organelles.

Another approach may include enhancing the efficacy of delivery to the mucosal immune system using mucosal adjuvants [20,33]. The cholera toxin B-subunit (CTB) is a candidate adjuvant because it has been implicated in the induction of specific mucosal immune responses. Expression of T-cell epitopes as a fusion protein with CTB may induce immune tolerance at a much smaller concentration because of binding of CTB to M cells through the polysaccharide GM-1 ganglioside as CTB receptor on the intestinal epithelium. This process would result in site-specific delivery and presentation of the active peptide to the GALT. CTB may also increase the molar

concentration of the antigen through pentamerization, resulting in a further increase in antigen concentration at the GALT [33].

Bringing a rice seed-based edible vaccine to market

Many hurdles must be cleared before transgenic rice seed edible vaccines can be brought to market. Several ecologic risks associated with transgene escape during cultivation and transport remain unresolved with genetically altered crops in general [34]. Cross-contamination of seeds during the distribution process (product contamination) and out-crossing with neighboring nontransgenic rice (genetic hybridization) are also unresolved concerns. These risks, however, can be controlled through regulatory measures. Biopharmaceutical plants may need to be grown in a contained environment, with either physical or geographic isolation. An easily recognizable marker may be introduced to discriminate between the edible vaccine rice and normal nontransgenic rice, such as anthocyanins or other natural plant pigments, which would essentially color-code the engineered seeds. On the other hand, incorporating selectable marker genes that confer resistance to antibiotics would need to be eliminated during commercialization. Marker-free transgenic rice plants harboring the 7Crp gene have already been established using only the multi-auto-transformation (MAT) vector system.

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