

Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination

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Capable of inducing antigen-specific immune responses in both systemic and mucosal compartments without the use of syringe and needle, mucosal vaccination is considered ideal for the global control of infectious diseases. In this study, we developed a rice-based oral vaccine expressing cholera toxin B subunit (CTB) under the control of the endosperm-specific expression promoter 2.3-kb glutelin *GluB-1* with codon usage optimization for expression in rice seed. An average of 30 μ g of CTB per seed was stored in the protein bodies, which are storage organelles in rice. When mucosally fed, rice seeds expressing CTB were taken up by the M cells covering the Peyer's patches and induced CTB-specific serum IgG and mucosal IgA antibodies with neutralizing activity. When expressed in rice, CTB was protected from pepsin digestion *in vitro*. Rice-expressed CTB also remained stable and thus maintained immunogenicity at room temperature for >1.5 years, meaning that antigen-specific mucosal immune responses were induced at much lower doses than were necessary with purified recombinant CTB. Because they require neither refrigeration (cold-chain management) nor a needle, these rice-based mucosal vaccines offer a highly practical and cost-effective strategy for orally vaccinating large populations against mucosal infections, including those that may result from an act of bioterrorism.

mucosal immunity | protein body | oral vaccine | IgA | cholera toxin B subunit

The majority of emerging and reemerging infectious pathogens, including *Vibrio cholerae*, *Escherichia coli*, HIV, influenza virus, or coronavirus causing severe acute respiratory syndrome, invade and infect the host via the mucosal surfaces of the gastrointestinal, respiratory, and/or genitourinary tracts (1–3). Mucosal immunity forms a first line of defense by means of secretory IgA and cytotoxic T cells against epithelium-transmitted pathogens, and so it would seem important to develop vaccines that induce effective immune responses at mucosal barriers. Most current vaccines are administered by needle and syringe, generating effective antibody and cell-mediated responses in the systemic compartment, but not in mucosal sites (4). In contrast, mucosal vaccines administered either orally or nasally have been shown to be effective in inducing antigen-specific immune responses in both systemic and mucosal compartments (5–8). Because it elicits this two-layered protective immunity, mucosal vaccination is thought to be an ideal strategy for combating both emerging and reemerging infectious diseases (5–8). In fact, the Bill and Melinda Gates Foundation and the National Institutes of Health have proposed that mucosal vaccines be a focus of future vaccine development (9), a vision underlying the foundation of the Gates' research initiative, "Grand Challenges in Global Health" (9). Most traditional vaccines are not cost-effective because they cannot be stored at room temperature (RT), instead requiring that the

"cold chain" be preserved en route from vaccine manufacturer to the field of vaccination (i.e., that no gap be allowed in the refrigeration) (10). The cost of preserving that cold chain for currently used vaccines is estimated at between \$200 and \$300 million a year (10). Further, if inappropriately processed or disposed of, the needles and syringes used for the vaccination can pose the threats of environmental contamination and second-hand spread of infectious disease. Producing vaccine antigens in plants would offer many practical advantages (11, 12). First, it would be less expensive to produce vaccine antigens in plants than via industrial fermentation. Second, there is no need to take elaborate means to purify the vaccine if it is expressed in plant tissue. Third, the plant expression system minimizes risks arising from contamination. Collectively, these advantages make a plant-based subunit vaccine not only attractive, but also practical for the propagation of mucosal vaccine on the global scale (12).

As early as 1990, Curtiss and Cardineau expressed *Streptococcus mutans* surface protein antigen, the causative epitope for dental caries, in tobacco as a first step toward a potential plant-based mucosal vaccine (13). Since then, many vaccine antigen candidates, including bacterial diarrhea antigens, hepatitis B antigen, Norwalk virus antigen, and respiratory syncytial virus antigen, have been expressed in tobaccos or potatoes to demonstrate the feasibility of edible plant-based vaccines (14–21). However, these plant-based vaccines have remained a function of sophisticated bench-driven experiments and have not yet advanced to practical application. If such a vaccine is to be practicable for global immunization, it must be storable at RT for long periods, be protected from the harsh environment of the gastrointestinal tract, and target mucosal inductive tissues, including Peyer's patches (PPs) (8, 22).

We here introduce a rice-based oral vaccine possessing many practical advantages over most traditional or other plant-based oral vaccines. The rice-based oral vaccine is stable at RT for several years and is protected from digestive enzymes. When ingested, this vaccine induced antigen-specific antibodies with neutralizing activities. These results show that the rice-based oral vaccine offers a highly practical global strategy for cold-chain- and needle-free vaccination against infection.

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The authors declare no conflict of interest.

Abbreviations: CHO, Chinese hamster ovary; CT, cholera toxin; CTB, cholera toxin B subunit; LTb, heat-labile enterotoxin B subunit; PPs, Peyer's patches; PB, protein body; RT, room temperature.

See Commentary on page 10757.

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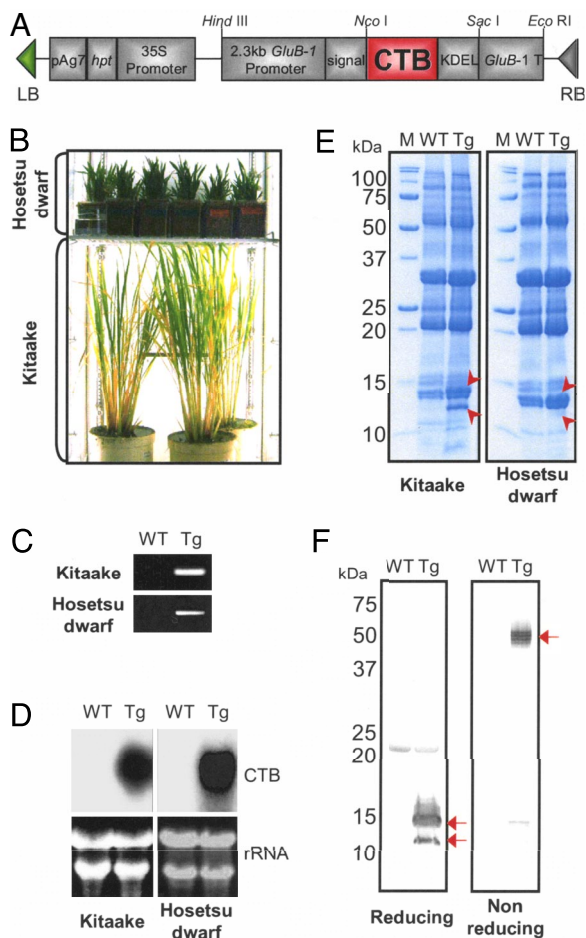


Fig. 1. Expression of CTB in transgenic rice. (A) T-DNA plasmid-inserted, codon-optimized CTB gene for rice seed, controlled by the rice seed storage protein glutelin 2.3-kb *GluB-1* promoter. The signal sequence of *GluB-1* and the retention signal to the endoplasmic reticulum coding KDEL are located at the N- and C-terminal regions, respectively. (B) The Kitaake and Hosetsu dwarf type rice strains expressed CTB in a closed chamber. (C) Integration of the CTB gene into the genomic DNA was confirmed by PCR. WT, Wild-type nontransgenic rice; Tg, CTB-expressed transgenic rice. (D) Northern blot analysis was performed for the confirmation of CTB mRNA expression. (E and F) SDS/PAGE and Western blot analysis revealed that high levels of CTB protein were expressed in rice. Arrowheads indicate 12- and 15-kDa forms of CTB (E). The CTB protein, composed of two fragments, forms a 55- to 65-kDa pentamer structure under nonreducing conditions. Arrows indicate monomeric (under reducing condition) and pentamer (under nonreducing condition) forms of CTB (F).

Results

Development of Rice-Based Mucosal Vaccine Expressing Cholera Toxin B Subunit (CTB) in Seeds. We purposely chose CTB as a prototype antigen to demonstrate both the capacity of the rice-based mucosal vaccine to induce systemic as well as mucosal immunity and to showcase the practicality of using the rice transgenic expression system. Once generated with binary vector (pGPTV-35S-HPT) (23), as described in Fig. 1A, codon-optimized CTB genes for rice seed were transfected into the rice plant [*Oryza sativa* L. cv Kitaake, a normal-sized rice (24); and Hosetsu, a dwarf type rice (25, 26), shown in Fig. 1B] by using *Agrobacterium tumefaciens*-mediated transformation (27). Hosetsu dwarf type rice, a naturally occurring gene mutant on the gibberellin biosynthesis pathway (25), is 20 cm in height and has a short life cycle (≈ 3 months). Genomic PCR analysis revealed that the CTB gene was integrated into the genomic DNA of both lines of rice plants (Fig. 1C). In addition, high levels of CTB-specific mRNA in the seeds of both lines of trans-

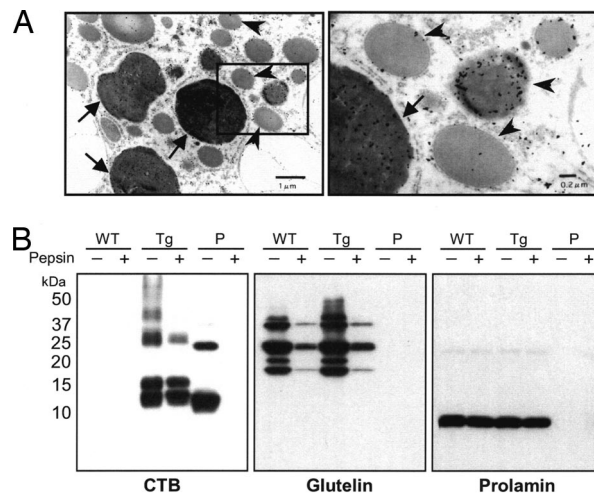


Fig. 2. Localization and digestive enzyme resistivity of rice-expressed CTB in PBs. (A) Data obtained through immunoelectron microscopic analysis with anti-CTB antibody. A positive signal was obtained with 20 nm of gold particles. CTB expressed in rice are stored in PB-I (arrowheads) and PB-II (arrows). (B) Pepsin digestion was carried out under the conditions described in *Materials and Methods*. Approximately 90% of glutelins but not 13k prolamins were digested *in vitro*, whereas $\approx 75\%$ of rice-based CTB but not purified rCTB remained intact.

genic but not nontransgenic wild-type (WT) rice were expressed at 15 days after flowering (Fig. 1D). When we examined the accumulation of CTB protein in the transgenic rice seed by SDS/PAGE and Western blot analysis with anti-CTB polyclonal antibody, two bands (12 kDa and 15 kDa) were detected under denaturing conditions (Fig. 1E and F). Using densitometry analysis with rCTB as a standard, we found that expression levels of CTB reached an average of 30 μ g per seed in the Kitaake strain, representing 2.1% of the total seed protein (0.15% of seed weight). The expression level of CTB in Hosetsu dwarf type rice was lower (average of 5 μ g per seed) than that in Kitaake rice. Furthermore, Western blot analysis under nonreducing conditions revealed that CTB expressed in rice formed a pentamer with 55 to 65 kDa (Fig. 1F), indicating that most of the CTB expressed in rice seed is considered to be a functionally native form possessing the ability to bind to the GM1 ganglioside, known to be expressed at the apical surface of the intestinal epithelium and to be a receptor for CTB (28).

CTB Expressed in Protein Bodies (PBs) of Rice Seed Is Resistant to Gastrointestinal Harsh Environment. In addition to being easy to produce and administer, an effective oral vaccine would also have to have a built-in safeguard against digestion, particularly against the harsh acidic environments found in the stomach. The starchy endosperm in rice contains two types of protein storage organs, PB-I and PB-II, which are distinguished by their shape, density, and protein composition (29). The main storage proteins for PB-I are the alcohol-soluble prolamins (e.g., 13k prolamin) and the water-soluble glutelins (e.g., glutelin B1) (29, 30). Because they are water-soluble, the glutelins (PB-II) are more vulnerable to digestion in the gastrointestinal tract than are prolamins (PB-I). Immunoelectron microscopic analysis reveals that CTB is localized not only on the surface of PB-I, but also on the inside of PB-II (Fig. 2A). To examine the ability of the CTB accumulated in the rice PB to withstand protease digestion in the stomach, total seed proteins were subjected to pepsin treatment. Western blot analysis (Fig. 2B) revealed that the signal intensity of the 13k prolamin was not significantly changed by the pepsin treatment, whereas $\approx 90\%$ of the glutelins were digested by pepsin under these conditions. In addition, $\approx 75\%$ of the CTB accumulated in rice seed remained intact after pepsin treatment (Fig. 2B). These findings suggest that most

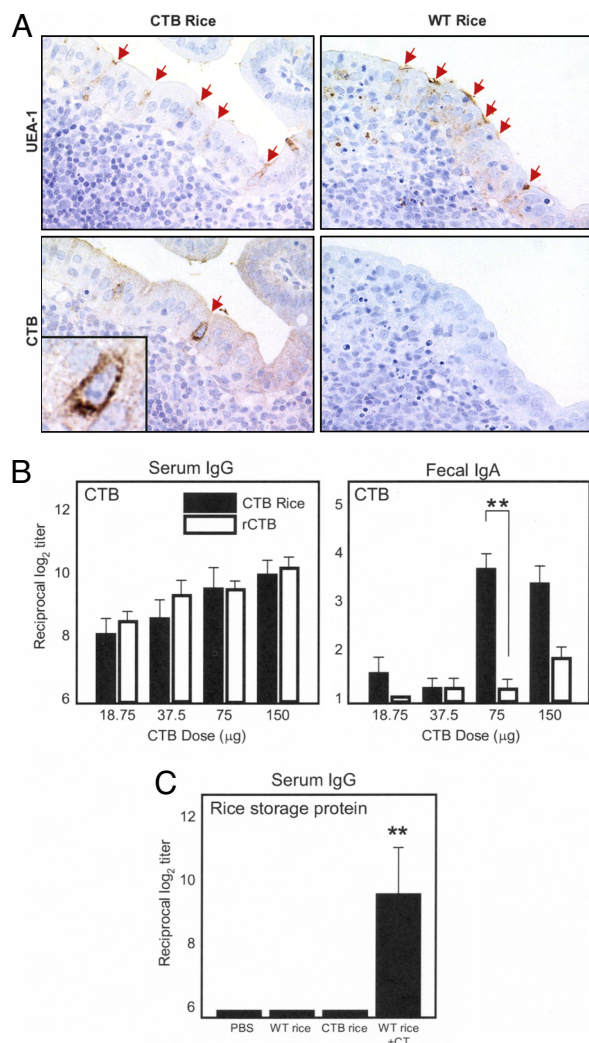


Fig. 3. Effective uptake of rice-expressed CTB by M cells for the induction of antigen-specific immune responses. (A) Rice-expressed CTB was administered into an intestinal loop containing PPs. Thirty minutes after the inoculation, the brisk CTBs were taken up by UEA-1-positive M cells (arrow), but not columnar epithelial cells. (B) When mice were orally immunized with rice-expressed CTB, purified rCTB, or nontransgenic rice dissolved in water or water alone as controls, equal levels of CTB-specific serum IgG responses were induced in mice immunized with rice-expressed CTB or purified rCTB, but not in mice receiving WT rice or water alone. In contrast, CTB-specific fecal IgA responses were also induced in mice immunized with a small amount (50 mg of seed powder containing 75 μ g of CTB) of rice-expressed CTB, but not with an identical dose of purified rCTB. **, $P < 0.01$, CTB rice vs. rCTB. (C) Rice-expressed CTB did not induce rice storage protein-specific serum IgG responses. **, $P < 0.01$, WT rice plus CT vs. CTB rice.

of the CTB expressed in transgenic rice seed can be protected from the harsh conditions of the gastrointestinal tract. To characterize the mucosal immunogenicity of the rice-based oral vaccine in more detail, we opted to use the Kitaake CTB system for the remainder of the study.

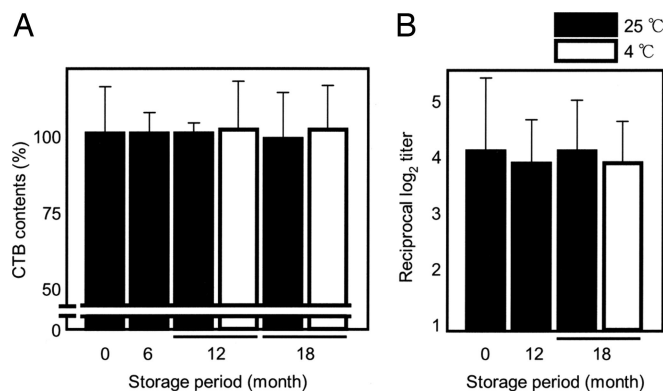


Fig. 4. Temperature stability of rice-expressed CTB. (A) One thousand rice seeds expressing CTB were preserved in a 500-ml sealed bottle for >1.5 years at 4°C as well as at RT (25°C). The content of CTB in preserved rice was not changed compared to that in freshly harvested rice ($29 \pm 4 \mu\text{g}$ per seed). (B) Mice were orally immunized with preserved rice-expressed CTB (50 mg of seed powder containing $75 \mu\text{g}$ of CTB) as described in Fig. 3. The preserved rice induced CTB-specific mucosal IgA responses that were comparable to those observed for freshly harvested rice.

next orally immunized mice with the seed powder of rice-expressed CTB or purified rCTB. Rice-expressed CTB induced CTB-specific serum IgG and fecal IgA antibodies (Fig. 3B). CTB-specific fecal IgA responses were also induced in mice immunized with a low dose of rice-expressed CTB (e.g., 50 mg of rice powder containing 75 μ g CTB), whereas the same dose of purified rCTB induced no or very low levels of antigen-specific IgA responses (Fig. 3B). Furthermore, it should be emphasized that rice-expressed CTB induced no rice storage protein-specific immune responses (Fig. 3C). These findings demonstrated that the rice-based mucosal vaccine is an effective delivery vehicle for the induction of antigen-specific mucosal IgA responses.

Rice-Based Mucosal Vaccine Maintained Immunogenicity for More than 1.5 Years at RT. Inasmuch as our results provide supportive evidence for the protective advantage of rice-based mucosal vaccine, which includes stability in the harsh condition of the gastrointestinal tract (Fig. 2B), it was logical to examine whether the rice-based vaccine preserved at RT (25°C) for an extended period maintained its stability and mucosal immunogenicity. To this end, rice-based mucosal vaccine was preserved for 0.5, 1.0, or 1.5 years at either RT (25°C) or 4°C. Densitometry analysis revealed that the antigen in rice seed remained stable at RT for >1.5 years (Fig. 4A). Furthermore, the rice preserved at RT for 1.5 years induced the same level of CTB-specific fecal IgA responses as freshly harvested rice (Fig. 4B). These data suggest that the rice-based mucosal vaccine is more stable than the purified antigen of the subunit vaccine, as well as more effective for induction of IgA-committed mucosal immune responses.

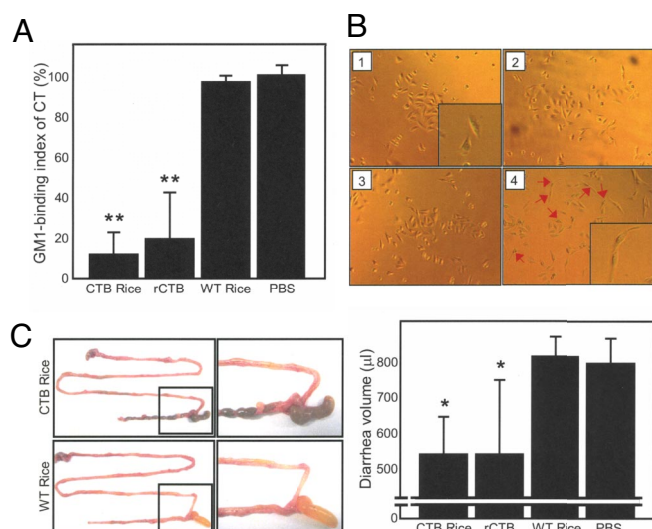


Fig. 5. Induction of protective immunity against CT by rice-expressed CTB. (A) The neutralizing index calculated with OD₄₅₀ obtained by GM1-ELISA. The serum of mice immunized with rice-expressed CTB or purified rCTB, but not with nontransgenic rice or PBS, completely blocked the binding of CT to coated GM1-ganglioside. **, $P < 0.01$, CTB rice or rCTB vs. PBS. (B) The elongation assay with CHO cells revealed a morphology similar to normal cells (1) when the cells were stimulated with CT pretreated with serum from mice immunized with rice-expressed CTB (2) or purified rCTB (3), but a marked elongation when stimulated by CT pretreated with the serum from mice immunized with nontransgenic rice, PBS, or nontreated CT (4) as shown by the arrows. (C) In contrast to mice receiving WT rice or PBS, mice orally vaccinated with rice-expressed CTB showed no symptoms of diarrhea and a low level of intestinal water. *, $P < 0.05$, CTB rice or rCTB vs. PBS.

serum of mice immunized with WT rice showed a massive elongation of CHO cells (Fig. 5B) similar to that induced by the native form of CT. Most important, when orally challenged with CT, the mice vaccinated with rice-expressed CTB showed no clinical sign of diarrhea (Fig. 5C), whereas those fed the WT rice or PBS developed severe diarrhea. However, some mice immunized with purified rCTB suffered from diarrhea (Fig. 5C). Consistent with these findings, the volume of intestinal water in mice immunized with rice-expressed CTB was significantly lower after challenge with CT than in mice receiving WT rice or PBS (Fig. 5C). These data directly demonstrate that oral vaccination with rice-expressed CTB could offer a high degree of protection against CT challenge.

Discussion

In this study, we have developed a physically and chemically stable and immunologically effective vaccine antigen-expressing transgenic rice seed that can withstand the harsh environment of the gastrointestinal tract and induce protective immunity against mucosal infections. The use of transgenic rice for vaccine production offers several benefits over other plants for vaccine production. For the implementation of global vaccination strategy, a well designed oral vaccine system should satisfy the following criteria: (i) produce sufficient quantities of inserted antigen for the immunization (33), (ii) preserve the expressed antigen for a long time at RT (9, 34), (iii) induce protective immunity (8, 34), (iv) protect from enzymatic digestion in gastrointestinal tract (8), and (v) effectively deliver the inserted antigen to mucosal inductive tissues, including antigen-sampling M cells (8, 35). Although several plants have currently been used for the creation of an “edible vaccine,” seed crops such as soybean, maize, wheat, or rice seem to be the most suitable plants for fulfillment of the previous requirements. It was recently shown that a soybean-based oral vaccine expressing heat-labile

toxin B subunit (LTB) of *E. coli* induced antigen-specific IgG and IgA responses (36). Although maize also has been used for the expression of LTB (20), a biological nature of long-distance pollen scattering is the major environmental concern (37). Further, the difficulty of transforming the inserted gene by use of the wheat vector system unfortunately disqualified its suitability for the oral vaccine development. In contrast, rice self-fertilizes, and thus its pollen is considered to fry within only 10 m (37). In addition, rice plants have unique features in the storage of protein using two systems of PB-I and PB-II (29), which are suitable for accumulation of vaccine antigen. Furthermore, rice is the only crop that full of genome sequences was elucidated, and thus it easily applied the genetic information for the creation of gene-manipulated product (38). It is expected that this 430-Mb genome information contributes to the development of useful transgenic rice (38).

To show the unique features and feasibility of rice-based mucosal vaccine, we purposely used CTB as a vaccine antigen because CTB has been immunologically well characterized and extensively used for the analysis of antigen-specific immune response in both mucosal and systemic compartments. One of the major limitations of plant-based vaccines is the achievement of a high expression of inserted vaccine antigen that is sufficient to induce protective immunity (33). To achieve high expression and accumulation of inserted vaccine antigen in rice seed, an endosperm-specific expression promoter gene, 2.3-kb *GluB-1*, followed by an endoplasmic reticulum retention signal peptide, KDEL, was used for the expression of CTB (Fig. 1A). By optimizing the codon usage of CTB for expression in rice, the accumulation level of CTB was achieved at $\approx 2.1\%$ of total seed protein (0.15% of seed weight) (Fig. 1E and F). Although CTB has been expressed in the potato, the level of expression was $\approx 0.3\%$ of total protein (0.002% of fresh weight) in potato tubers (17); $\approx 4\%$ of total leaf protein (0.5% of leaf weight) was achieved in tobacco leaves by using a chloroplast expression system (35). However, the tobacco leaf is not applicable in the practical sense for oral vaccination. Thus, the use of the rice transgenic system allowed the efficient expression of inserted vaccine antigen, although we cannot directly compare the level of the inserted antigen expression to other previously published plant-based vaccine systems.

The SDS/PAGE and Western blot analyses with anti-CTB polyclonal antibody showed two protein species with 12 kDa, which was almost the same as that of authentic CTB (39), and 15 kDa were detected (Fig. 1E and F), suggesting that part of CTB expressed in rice seed might contain a full or partial *GluB-1* signal peptide at the N terminus. The SDS/PAGE under nonreducing conditions and subsequent Western blot analyses showed that the molecular mass of two protein species was shifted to 55–65 kDa (Fig. 1F). Because these two protein species were recognized by anti-CTB polyclonal antibody and possessed a molecular weight comparable to a pentameric structure under natural condition, most rice-expressed CTBs were considered to be a functionally native pentameric form for the induction of an antigen-specific immune response.

The tolerableness of inserted vaccine antigen in rice seed against the harsh digestive tract environment was also attributed by the site of protein accumulation in the rice seed. In general, rice starchy endosperm cells contain two types of protein storage organelles (PB-I and PB-II) with a different shape, density, and protein composition (29). PB-I and PB-II mainly contain prolamins (e.g., 13k prolamin) and glutelins (e.g., glutelin B1) as storage proteins, which are defined as alcohol- and water-soluble proteins, respectively (29, 30). Thus, glutelins (PB-II) are considered to be more digestible and sensitive than prolamins (PB-I) in the gastrointestinal tract. The immunoelectron microscopic analysis showed that CTB accumulation occurred in PB-I and PB-II of the endosperm cells of the rice seed (Fig. 2A). Thus, the direction of inserted protein expression (e.g., CTB) in PB-I seems to be responsible for the tract of resistance against digestive enzyme activity, and thus allows for

the effectiveness in the induction of antigen-specific immune response by minimum dose of oral antigen. To support this view, an *in vitro* pepsin digestion study showed that most of prolamin and $\approx 75\%$ of the CTB accumulated in the rice seed were protected from the pepsin treatment, whereas most of the glutelin in the rice seed and all purified rCTB were digested (Fig. 2B). In contrast, LTB expressed in maize kernel seems to be less resistant to peptic degradation when compared to CTB in the rice seed because LTB protein was accumulated mainly in the starch granules of transgenic maize kernels (40). These findings indicated that accumulation of vaccine antigen in PB-I would provide physicochemical stability against digestive enzymatic effects. Taken together, the rice-based vaccine was stable and more effective than the purified subunit vaccine, as well as other plant-based vaccines, in the harsh environment of the gastrointestinal tract for the induction of protective immunity.

As described above, in the gastrointestinal tract, the antigens ingested from the luminal site are taken up by PPs via antigen-sampling cells known as M cells for initiation of antigen-specific T helper cells and IgA-committed B cells (41). Targeting vaccine antigen delivery to M cells should be a goal in mucosal vaccine development. Intestinal loop assay with rice-expressed CTB demonstrated that CTB were taken up by M cells (Fig. 3A). According to many biodegradable microsphere studies, the $<10\text{-}\mu\text{m}$ microspheres have been shown to be efficient delivery vehicles into antigen-sampling M cells in PPs (42). The diameters of rice PB-I and PB-II range in size between 1 to 2 μm and 3 to 4 μm , respectively (43). In addition, PB-I, but not PB-II, accumulated most of its CTB at the surface, perhaps further enhancing antigen uptake by M cells. Taken together, these results demonstrated that rice-expressed CTBs were not only effectively taken up by M cells located in the follicle-associated epithelium of PPs, but also could serve as effective carriers of mucosal vaccines to intestinal inductive tissues such as the PPs.

Oral immunization with rice-expressed CTB induced CTB-specific serum IgG and mucosal IgA responses (Fig. 3B). A low dose of rice-expressed CTB (e.g., 50 mg of rice powder containing 75 μg CTB) sufficiently induced CTB-specific mucosal IgA responses, whereas the same contents of purified rCTB induced no or low levels of antigen-specific IgA responses (Fig. 3B). The differences in required dosage may be because of the physicochemical stability exhibited by rice-based mucosal vaccines and their ability to withstand digestive effects. Although oral immunization with rice-expressed CTB can induce CTB-specific immune responses, it did not induce any rice storage protein-specific immune responses (Fig. 3C), suggesting that rice-expressed CTB did not show adjuvant activity for rice protein. Furthermore, Southern blot analysis confirmed the genetic stability of the CTB-transgenic rice (data not shown); CTB expressed in the rice seed could be preserved for >1.5 years not only at 4°C , but also at RT, without any degradation (Fig. 4A), and the rice preserved for 1.5 years at RT induced an equal level of CTB-specific fecal IgA responses as freshly harvested rice (Fig. 4B). Our results provided further evidence for a significant potential benefit of rice-based mucosal vaccine for the development of stable vaccine with immunogenicity. Thus, a rice-based mucosal vaccine can be introduced as a first cold-chain-free vaccine.

Finally, we showed the protective effect induced by oral immunization with a rice-based mucosal vaccine. CT binds its receptor GM1-ganglioside, which is ubiquitously expressed in intestinal epithelium, and causes severe diarrhea (39). Our results demonstrated that the serum from mice immunized with rice-expressed CTB completely blocked the binding of CT to GM1-ganglioside (Fig. 5A) and also inhibited the elongation of CHO cells caused by CT (Fig. 5B). Most important, mice immunized with rice-expressed CTB showed no clinical sign of diarrhea after orally challenged with CT, whereas some mice immunized with purified rCTB suffered from diarrhea (Fig. 5C) perhaps because the level of antigen-specific mucosal IgA was lower in the purified rCTB-fed group than

in the group receiving rice-expressed CTB. Therefore, we conclude that the rice-based mucosal vaccine would be effective for the induction of protective immunity compared to other types of mucosal vaccine.

In summary, we have developed a rice-based oral vaccine that offers significant advantages over currently available vaccines. In the rice-based vaccine, the vaccine antigen, CTB, accumulated in the PBs of starchy endosperm cells, from which they were taken up by M cells for the induction of antigen-specific mucosal immune responses with neutralizing activity. In addition, the rice-based CTB vaccine remained stable and maintained immunogenicity at RT for >1.5 years and was protected from pepsin digestion *in vitro*. Taken together, these findings suggest that a rice-based oral vaccine would be a most effective and highly practical vaccine regimen against infectious diseases, whether naturally occurring or stemming from acts of bioterrorism. Given its cost effectiveness and ease of administration, it would be a vaccine whose benefits could be fully enjoyed in developing countries, where the need is often the greatest.

Materials and Methods

DNA Constructions and Transformation of Rice Plants. The CTB gene of *V. cholerae* was modified to a suitable form for rice seed and inserted into a binary vector (pGPTV-35S-HPT) (23). The resulting plasmid (Fig. 1A) was transformed in two lines of rice plants, *Oryza sativa* L. cv Kitaake (24) and Hosetsu (25, 26), using an *Agrobacterium*-mediated method described previously (27).

DNA and RNA Analyses. Using the cetyltrimethylammonium bromide extraction method, we extracted genomic DNA from the leaf tissues of transgenic rice and analyzed the integration of the CTB gene in genomic DNA using PCR (23). The expression of mRNA encoding CTB in the rice seed was analyzed by Northern blot as previously described (23). Briefly, total RNA (30 μg) extracted from the developing seeds of rice plants using the phenol/chloroform extraction method was separated on a 1.0% (wt/vol) formaldehyde/agarose gel and transferred to Hybond N⁺ membranes (GE Healthcare, Piscataway, NJ). The amplified CTB gene was used as a hybridization probe after labeling with [$\alpha\text{-}^{32}\text{P}$] dCTP (GE Healthcare).

Protein Analyses. Total seed protein was extracted from seeds as described previously (23). Briefly, seeds of rice plants were ground to a fine powder using a Multibeads shaker (Yasui Kikai, Osaka, Japan) and extracted in 2% (wt/vol) SDS, 8 M urea, 5% (wt/vol) β -mercaptoethanol, 50 mM Tris-HCl (pH 6.8), and 20% (wt/vol) glycerol before being separated by SDS/PAGE with a 15% to 25% gradient polyacrylamide gel (Daiichi Pure Chemical, Tokyo, Japan). The gel was subsequently transferred to Hybond-P PVDF membranes (GE Healthcare) for Western blot analysis with 5 $\mu\text{g}/\text{ml}$ rabbit anti-CTB antibody prepared in our laboratory. Accumulation levels of CTB were determined by densitometry analysis of Western blot against a standard curve generated with the use of rCTB expressed in *Bacillus brevis* and purified by using immobilized galactose (Pierce, Rockford, IL) in our laboratory (44). Using immunoelectron microscopic analysis, the distribution of CTB expressed in rice seed was analyzed. Briefly, rice seeds (at 12–15 days after flowering) were fixed with 4% paraformaldehyde (Wako, Osaka, Japan) and 0.1% glutaraldehyde (Nacalai Tesque, Kyoto, Japan) and embedded in LR White (London Resin, Hampshire, U.K.). Ultrathin sections (150 nm) were stained with 5 $\mu\text{g}/\text{ml}$ rabbit anti-CTB antibody, followed by gold particle (20 nm)-conjugated goat anti-rabbit IgG (E.Y. Labs, San Mateo, CA) diluted to 1:10. The sections were then stained with 4% uranyl acetate and examined by using a transmission electron microscope (JEM100S; JEOL, Tokyo, Japan).

Pepsin Treatment. Seed powder (10 mg containing 15 μ g of CTB) and purified rCTB (15 μ g) were incubated with 0.5 mg/ml pepsin (2,500–3,500 units per mg protein; Sigma–Aldrich, St Louis, MO) in 0.1 ml of 0.1 M sodium acetate buffer (pH 1.7) with gentle rocking for 1 h at 37°C. After neutralization, the degradation of CTB, glutelin, or prolamin was analyzed by Western blot with 5 μ g/ml rabbit anti-CTB, anti-glutelin GluB-1, or anti-13k prolamin antibodies prepared in our laboratory, respectively.

Uptake of CTB by M cells. A rice-expressed CTB or a nontransgenic rice was administered into an intestinal loop ($\approx$ 1 cm) containing PPs of mice anesthetized by using 2 mg of ketamine (Sigma–Aldrich) per mouse. Thirty minutes after inoculation, the tissues were removed and fixed in tissue fixative (Genostaff, Tokyo, Japan) overnight at 4°C and then embedded in paraffin. Several mirror sections (5 μ m) were subjected to immunohistochemistry with 5 μ g/ml anti-CTB antibody and biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and to lectin histochemistry with 20 μ g/ml biotinylated UEA-1 (Vector Laboratories). Both sections were finally incubated with SAB-PO (Nichirei, Tokyo, Japan) and visualized for the distribution of CTB and the localization of M cells by 3,3'-diaminobenzidine tetrahydrochloride (Dojin Laboratories, Kumamoto, Japan).

Oral Immunization. One immunization study was carried out by using 6-week-old C57BL/6J and BALB/c mice (CLEA, Tokyo, Japan). On six occasions at 2-week intervals, mice (six mice per group) were orally immunized with 12.5, 25, 50, or 100 mg of CTB-transgenic rice, with a corresponding dose of purified rCTB (18.75, 37.5, 75, or 150 μ g) or with either 100 mg of nontransgenic rice dissolved in water or water alone as controls. For examination of the adjuvant effect of rice-expressed CTB, 100 mg of CTB-transgenic or WT rice was orally immunized with or without 10 μ g of CT (List Biological Laboratories, Campbell, CA). One week after the final immunization, serum and fecal extracts were collected, and CTB or rice storage protein-specific Ig responses were measured by ELISA with 5 μ g/ml rCTB or 20

μ g/ml rice-storage protein extracted with 0.01% Triton X-100 as described previously (45).

Neutralizing Assay. Serial-diluted serum collected from immunized mice were treated with 50 ng/ml of CT and subjected to GM1-ELISA as previously described with some modifications (17). Briefly, 5 μ g/ml of monosialoganglioside GM1 (Sigma–Aldrich)-coated 96-well plates (Thermo, Milford, MA) were incubated with CT that had been treated first with serum from immunized mice and then with an HRP-conjugated anti-CTB antibody prepared in our laboratory. The color was developed with the addition of TMB substrate (Moss, Pasadena, MD), and absorbance was measured at 450 nm. In addition, a CHO cell (ATCC, CCL-61) assay (32) was performed by using serum treated with 50 ng/ml CT. After 14 h of stimulation in 5% CO₂ in a humidified incubator at 37°C, morphological changes were observed under a microscope. In addition, we performed an *in vivo* challenge experiment with CT. The vaccinated mice were orally challenged with 20 μ g of CT. After 14 h, clinical symptoms of diarrhea were observed, and the volume of intestinal water was measured.

Data Analysis. Data are expressed as the mean $\pm$ SD. All analyses for statistically significant differences were performed with Tukey's *t* test, with *P* values of <0.01 and <0.05 considered significant.

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