REVIEW

Novel Transgenic Rice-Based Vaccines

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Abstract Oral vaccination can induce both systemic and mucosal antigen-specific immune responses. To control rampant mucosal infectious diseases, the development of new effective oral vaccines is needed. Plant-based vaccines are new candidates for oral vaccines, and have some advantages over the traditional vaccines in cost, safety, and scalability. Rice seeds are attractive for vaccine production because of their stability and resistance to digestion in the stomach. The efficacy of some rice-based vaccines for infectious, autoimmune, and other diseases has been already demonstrated in animal models. We reported the efficacy in mice, safety, and stability of a rice-based cholera toxin B subunit vaccine called MucoRice-CTB. To advance MucoRice-CTB for use in humans, we also examined its efficacy and safety in primates. The potential of transgenic rice production as a new mucosal vaccine delivery system is reviewed from the perspective of future development of effective oral vaccines.

Keywords Rice-based vaccine · MucoRice-CTB · Mucosal immunity · Oral vaccine

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Introduction

In 1798, Edward Jenner discovered that the injection of materials from cowpox protected against smallpox infection (Jenner 1798; Riedel 2005). Because dairymaids who had frequent contact with cows and had a high rate of cowpox infections were not infected with smallpox, Jenner hypothesized that cowpox infection protected against subsequent smallpox infections (Jenner 1798; Riedel 2005). As the Latin word for cow is *vacca* and cowpox is *vaccinia*, he called this new procedure *vaccination* (Jenner 1798). Strategies used against infectious diseases changed dramatically with the discovery of the "vaccine" by Jenner. From 1798 until now, many vaccines have played an extraordinary role in the control of numerous infectious diseases. Smallpox, which is contracted through contact and droplets, was completely eradicated by an injectable smallpox vaccine (World Health Organization 1979). However, multiple uncontrolled emerging and re-emerging infectious diseases remain throughout the world. In a survey of an estimated 8.795 million deaths in children younger than 5 years old worldwide in 2008, infectious diseases caused 68 % (5.970 million) with the largest percentages due to pneumonia (18 %) and diarrhea (15 %) (Black et al. 2010). Most pathogens that cause these respiratory and gastrointestinal infections invade the human body through mucosal interfaces by inhalation and ingestion. Therefore, the induction of protective mucosal immunity by vaccines is important for prevention of these uncontrolled mucosal infections at the site of invasion.

The traditional route of vaccine administration, subcutaneous injection, is used in vaccination against many infectious diseases. Injectable vaccines effectively induce systemic immune response, but often fail to induce sufficient mucosal immune response (Babiuk et al. 2008; Giri

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et al. 2005). In contrast, oral, intranasal, sublingual, intra-rectal, and intravaginal vaccination can induce not only systemic immune response, but also protective mucosal immune response (Agnello et al. 2013; Camero et al. 2007; Kong et al. 2013; Nochi et al. 2007a; Song et al. 2008). However, there are few mucosal vaccines approved for marketing worldwide. In 2013, oral vaccines targeted only two bacterial species (*Vibrio cholerae* and *Salmonella typhi*) and two viruses (rotavirus and poliovirus. Therefore, it is necessary to develop new effective oral vaccines against infectious and other diseases.

The surfaces of mucosal tissues, especially the gastrointestinal tract, are constantly exposed to a large amount of foreign materials, including potentially harmful and undesired agents, such as pathogens, and harmless or beneficial agents, such as foods and nonpathogenic microorganisms. Recognition and subsequent uptake of these foreign antigens is an essential function of the mucosal immune system. When vaccine or pathogen antigens are delivered into the human body via mucosal surfaces, these antigens are taken up by mucosal inductive sites, known as mucosa-associated lymphoid tissues (MALTs), which are present in the intestines and respiratory tracts. MALTs include gut-associated lymphoid tissues (GALTs) and nasopharynx-associated lymphoid tissues (NALTs) (Kunisawa et al. 2005). Both GALTs and NALTs are covered by follicle-associated epithelium consisting of a subset of differentiated microfold epithelial cells (M cells), columnar epithelial cells, and lymphoid cells, which play a central role in the initiation of mucosal immune responses. M cells are the primary cells responsible for sampling foreign antigens and presenting them to the MALTs system (Owen 1977). The basolateral membrane of M cells forms pockets containing T lymphocytes, B lymphocytes, and dendritic cells (Takahashi et al. 2009) (Fig. 1). Upon antigen transcytosis from the intestinal tract into M cells, antigens are presented to dendritic cells. Subsequently, antigen-specific IgA-committed B cells and T cells are induced locally and then migrate via the bloodstream to distant effector sites. Therefore, transcytosis of antigens into M cells and their presentation to dendritic cells are necessary for the effective induction of mucosal immunity and successful oral vaccination.
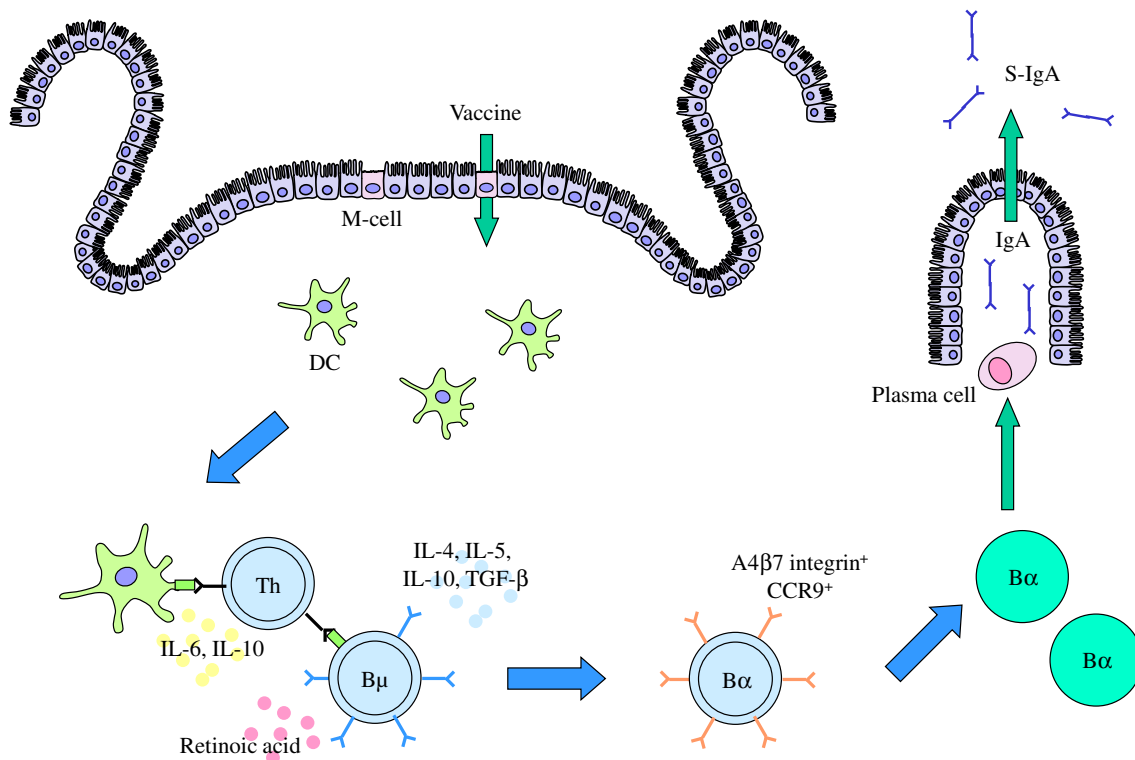

Fig. 1 Overview of the mucosal immune system. Orally administered antigens are taken up by microfold epithelial cells (*M cells*) in the follicle-associated epithelium of Peyer's patch, and presented to helper T (*Th*) cells by dendritic cells (*DCs*). Interleukin (*IL*)-4, IL-5, IL-6, IL-10, and tumor growth factor (*TGF*)-β produced by Th cells induce B cell class switch from μ to α. Retinoic acid produced by DCs increases gut-homing receptors, such as α4β7-integrin and CC chemokine receptor (*CCR*)9 on antigen-primed Th cells and IgA-committed B cells. These antigen-primed Th cells and IgA-committed B cells migrate to the effector sites for terminal differentiation to IgA-producing plasma cells, and secrete secretory IgA (*S-IgA*)

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Table 1 Advantages and disadvantages of plant-based vaccines

		References
Advantages of plant-based vaccines		
Safety	Low risk of contamination with human pathogens during the vaccine preparation	Minor (2012), Wirz et al. (2012)
Cost	Low cost of production	Nochi et al. (2007a, b), Wirz et al. (2012)
	No purification requirement (rice-based vaccine)	
	Elimination of cost of syringes and needles (rice-based vaccine)	
Storage	No need for cold chain during transport and storage (rice-based vaccine)	Tokuhara et al. (2010)
Delivery	Resistance to enzymatic digestion in gastrointestinal tract (rice-based vaccine)	Nochi et al. (2007b), Tokuhara et al. (2010)
Scaling-up	Rapid scale-up of production	Streatfield and Howard (2003)
Disadvantages of plant-based vaccines		
Immune response	A risk of induction of oral tolerance after oral administration with plant-based vaccines.	Kirk and Webb (2005)
Allergen	A risk of contamination of allergenic proteins or plant-specific sugar chains	Goodman et al. (2008), van Ree (2002)

Transgenic Plant-Based Vaccines

In 1990, Curtiss and Cardineau expressed the *Streptococcus mutans* surface protein antigen A (SpaA), a causative pathogen for dental caries, in tobacco leaves (Curtiss and Cardineau 1990); this was an initial indication of a possibility of plant-based vaccines. The SpaA protein constituted approximately 0.02 % of the total leaf protein. Transgenic tobacco tissue added to the diet of mice induced a mucosal immune response to the SpaA protein, and binding of the induced IgG1 monoclonal antibody to the adhesion protein of *S. mutans* was demonstrated. The authors successfully filed for and received a US Patent in 1997 (Curtiss and Cardineau 1997). In 2006, the US Department of Agriculture approved the first plant-based vaccine, which was a subunit vaccine produced in tobacco that effectively protected chickens against the Newcastle disease virus (Vermij 2006).

Since 1990 researchers have developed several edible plant-based vaccines using bacterial antigens, such as the heat-labile toxin B subunit (LTB) from enterotoxigenic *Escherichia coli*, cholera toxin (CT) B subunit (CTB), and antigens from *Yersinia pestis* and viruses, such as hepatitis B virus, rotavirus, and Norwalk virus; these antigens were introduced into carrot, soybean, tomato, rice, potato, and tobacco (Jiang et al. 2007; Li et al. 2006; Moravec et al. 2007; Nochi et al. 2007b; Rosales-Mendoza et al. 2008; Santi et al. 2006; Thanavala et al. 2005; Zhang et al. 2006). Vaccines produced in plants have some advantages over traditional oral vaccines (which contain live, killed, or attenuated pathogens), including lower costs, a possibility of rapidly scaling-up production of the antigen protein, and no need for purification (Table 1). Seeds of many plants can be easily desiccated and used for antigen extraction.

Seeds are also suitable for long-term preservation of proteins without the need for the cold chain (Nochi et al. 2007b). The requirement for the cold chain is a major burden for vaccination in developing countries because of high costs, which are estimated to reach 200–300 million dollars per year worldwide (Das 2004). Rice is one of the most important food crops in the world, feeding over half of the global population (Khush 2005). Rice is also well studied, and a highly accurate complete sequence of the rice genome is available and has been already analyzed (International Rice Genome Sequence Project 2005). Therefore, rice is an attractive candidate for the development of new effective plant-based vaccines.

Rice-Based Vaccines for Allergic and Autoimmune Diseases

Rice-based vaccines for allergic and autoimmune diseases are summarized in Table 2. Takagi et al. (2005b) reported that oral immunization with transgenic rice seeds producing T-cell epitope peptides specific for pollen allergens suppressed allergen-specific helper T (Th)2-mediated IgE responses and clinical symptoms of pollinosis in mice. These results demonstrated for the first time a new strategy of oral immunization with rice products. In Japan, Japanese cedar pollen is a major cause of pollinosis, with symptoms of allergic diseases, such as rhinitis and conjunctivitis (Yasueda et al. 1983). Takagi and his colleagues targeted two major allergens isolated from pollen, Cry j 1 and Cry j 2, and multiple T-cell epitope sequences identified from these proteins (Yoshitomi et al. 2002) for their rice-based vaccine (Takagi et al. 2005b). T-cell epitope peptides of Cry j 1 and Cry j 2 were fused with the subunits of soybean

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Table 2 Rice-based vaccines for the allergic diseases and the autoimmune diseases

Target disease	Target antigen	Expression level	Functional evaluation	References
Cedar pollinosis	T-cell epitopes of cedar pollen (Cry j 1 and Cry j 2) with soybean glycinin	0.5 % of total seed protein	Suppression of allergen-specific IgE response and clinical symptom	Takagi et al. (2005b)
Cedar pollinosis	T-cell epitopes of cedar pollen (7Crp) with glutelin	60 μg/grain	Suppression of allergen-specific T-cell proliferative response and serum IgE	Takagi et al. (2005a, 2006)
Cedar pollinosis	T-cell epitopes of cedar pollen (Cry j 1 and Cry j 2) with CTB	35 μg/grain	Suppression of allergen-specific IgE response and clinical symptoms	Takagi et al. (2008)
Cedar pollinosis	T-cell epitopes of cedar pollen (Cry j 1) with glutelin	15 % of total seed protein	–	Yang et al. (2007a)
Cedar pollinosis	Modified T-cell epitopes of cedar pollen (Cry j 1 and Cry j 2)	0.5–1.3 mg/g rice seed	Suppression of allergen-specific T-cell, IgE and IgG and clinical symptoms	Wakasa et al. (2013)
House dust mite allergy	House dust mite allergen (Der p 1)	58 μg/grain	Inhibition of T-cell proliferative response, allergen-specific IgE and infiltration of eosinophils into the airway	Suzuki et al. (2009, 2011), Yang et al. (2008)
Rheumatoid arthritis	Type II collagen	1 μg/seed	Suppression of specific IgG2a response	Hashizume et al. (2008)

glycinin A1aB1b; the fusion protein accumulated at a level of 0.5 % of total protein in transgenic rice seeds (Takagi et al. 2005b). Glycinin, a major soybean storage protein produced in transgenic rice under the control of rice glutelin GluB-1 promoter, colocalized with glutelins in the protein bodies (PB) (Katsube et al. 1999). To increase the antigenicity of oral vaccines, a more efficient mucosal carrier was needed. CTB was considered to be a candidate for such a carrier (Sun et al. 1994), because it is not toxic (Finkelstein and LoSpalluto 1969) and is required for binding to the specific receptor expressed in the intestine (Heyningen 1974). Therefore, the next effort was aimed to produce Cry j 1 and Cry j 2 fused with CTB in transgenic rice seeds (CTB-3Crp) (Takagi et al. 2008). Oral immunization with this new anti-allergy vaccine, CTB-3Crp, suppressed allergen-specific IgE responses and pollen-induced clinical symptoms at 50-fold lower doses than T-cell epitope peptides fused with rice glutelin acidic subunit (Takagi et al. 2008). A separated study reported the efficacy of vaccination against house dust mite allergy (Yang et al. 2008). A major allergen in house dust is derived from mites of the genus *Dermatophagoides* (Tovey et al. 1981). The investigators expressed the allergen of *D. pteronyssinus* feces, Der p 1, fused with the KDEL sequence, a signal for retention in the endoplasmic reticulum (ER), in rice seeds (Yang et al. 2008). C-terminal KDEL-tagged Der p 1 accumulated in the ER-derived PB-I at 58 μg/grain. Oral immunization with this protein suppressed T-cell proliferative responses in vitro, serum

allergen-specific IgE and IgG responses, and accumulation of eosinophils and lymphocytes in airways in vivo (Suzuki et al. 2009, 2011).

A concept of rice-based vaccine was further extended to the control of autoimmune diseases. Hashizume et al. (2008) produced a new vaccine against type II collagen (CII), a major factor involved in the development of human rheumatoid arthritis (Trentham et al. 1977, 1978). CII expressed vaccine was produced by a fusion of four tandem repeats of a CII peptide containing a human T-cell epitope with rice glutelin (gluA-4XCII$_{250-270}$) in transgenic rice seeds. Feeding mice with the transgenic rice seeds delayed serum CII-specific IgG2a response against injection of bovine CII (Hashizume et al. 2008).

Rice-Based Vaccines for Alzheimer's Disease

Alzheimer's disease (AD) is thought to be caused by deposition of amyloid β protein (Aβ) in the cerebral cortex (Carlson 2003; Hardy and Higgins 1992). Vaccination by injection of Aβ peptide is a potential therapy for AD in both animal models (Bard et al. 2000; Janus et al. 2000; Morgan et al. 2000; Schenk et al. 1999) and humans (Check 2002; Holmes et al. 2008). Not only injectable but also mucosal vaccines, including plant-based ones, have effects on AD in animal models (Ishii-Katsuno et al. 2010; Kim et al. 2003, 2005). Nojima and his coworkers introduced the Aβ gene fused with the green fluorescent protein

Table 3 Rice-based vaccines for the infectious disease

Target pathogen	Target antigen	Expression level	Functional evaluation	References
Vibrio cholera	CTB subunit	2.1 % of total seed protein	Production of neutralizing antibody against CT and protection from challenging with CT	Kurokawa et al. (2013), Nochi et al. (2007b, 2009), Tokuhara et al. (2010), Yuki et al. (2009, 2013)
Clostridium botulinum	Heavy chain of botulinum type A neurotoxin	100 μg per seed	Production of neutralizing antibody against BoNT/A and protection from challenging with BoNT/A	Yuki et al. (2012)
Helicobacter pylori	UreB	Unknown	–	Gu et al. (2006)
IBDV	VP2 of IBDV	0.678–4.521 % of total seed protein	Production of neutralizing antibody against IBDV and protection from challenging with virulent IBDV	Wu et al. (2007)
NDV	Fusion protein of NDV	0.25–0.55 μg in 200 μg of total leaf and seed	Production of specific antibody	Yang et al. (2007b)
CMV	Glycoprotein B of CMV	–	–	Tackaberry et al. (2008)
HBV	HBV surface protein and presurface one region	31.5 ng/g dry weight grain	Production of specific antibody	Qian et al. (2008)
Chlamydophila psittaci	MOMP of *C. psittaci*	0.0079–0.0096 % of seed protein	Production of specific antibody and protection from challenging with virulent *C. psittaci*	Zhang et al. (2008, 2009, 2013)
Ascaris suum	16-kDa protein of *A. suum*	50 μg/g seed	Production of specific antibody and protection from challenging with *A. suum* eggs	Matsumoto et al. (2009)
JEV	Envelope protein of JEV	1.1–1.9 μg/mg of total soluble protein	Produciton of specific neutralizing antibody	Wang et al. (2009)
FMDV	Capsid precursor polypeptide of FMDV	0.6–1.3 μg/mg of total soluble protein	Produciton of specific neutralizing antibody and protection from challenging with FMDV	Wang et al. (2012)
Escherichia coli	Heat-LTB of *E. coli*	0.086 μg/mg of lyophilized rice callus	Production of specific antibody and inhibition of function of LTB	Kim et al. (2010b)
PEDV	Neutralizing epitope region (COE) of PEDV	0.083 % of total soluble protein	Production of specific antibody and antibody-secreting cells systemically	Huy et al. (2012)

CTB cholera toxin (CT) B subunit, *IBDV* infectious bursal disease virus, *BoNT/A* botulinum toxin type A, *UreB* urease subunit B, *NDV* Newcastle disease virus, *CMV* cytomegalovirus, *HBV* hepatitis B virus, *MOMP* major outer membrane protein, *JEV* Japanese encephalitis virus, *FMDV* foot-and-mouth disease virus, *LTB* labile toxin B subunit, *PEDV* porcine epidemic diarrhea virus, *COE* core neutralizing epitope

(GFP) gene into *O. sativa* L. cv. Hayayuki by *Agrobacterium*-mediated transformation, and also demonstrated that subcutaneous injection of Aβ fusion protein expressed in transgenic rice improved memory in Tg2576 mice, an animal model of AD (Nojima et al. 2011a, b; Yoshida et al. 2011). Nojima et al. (2011b) injected subcutaneously AD model mice with the fusion protein mixed with CTB as a carrier. This induced anti-Aβ antibody production, reduced Aβ content in both serum and brain, and improved Y-maze test results (which reflect memory). GFP fluorescence was useful to distinguish the transgenic and wild-type seeds.

Rice-Based Vaccines for Infectious Diseases

Rice-based vaccines for infectious diseases are summarized in Table 3. Gu et al. (2006) successfully produced the *Helicobacter pylori* urease subunit B (UreB) recombinant protein in transgenic rice. *Helicobacter pylori* infects the stomach, and is the main cause of gastritis, peptic ulceration, and gastric cancer (Marshall and Warren 1984; Parsonnet et al. 1991). The urease activity of *H. pylori* neutralizes stomach acidity, thus enabling *H. pylori* to colonize the stomach (Sidebotham and Baron 1990).

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UreB is thought to be a new target for *H. pylori* infection treatment, and some investigators achieved successful vaccination against UreB (Fujii et al. 2004; Yang et al. 2005). Gu et al. (2006) introduced recombinant UreB into transgenic rice by *Agrobacterium*-mediated transformation. Though their idea seems attractive for the development of rice-based oral vaccine against *H. pylori* infection, the authors did not report the efficacy of vaccination in vivo.

An effective rice-based vaccine for a nonhuman infectious disease was reported in 2007 (Wu et al. 2007). They targeted the infectious bursal disease virus (IBDV), which causes an immunosuppressive disease of chickens mainly by attacking an important lymphoid organ, the bursa of Fabricius (Mahgoub et al. 2012). Chickens immunized with a major IBDV protein, VP-2b, and its precursor VP-2a produced virus-neutralizing antibodies that passively protected them from IBDV infection (Fahey et al. 1989). Wu et al. (2007) cloned the VP2 cDNA from IBDV strain ZJ2000 into the binary expression vector pCambia1301-Gt1, and produced the recombinant VP2 protein in transgenic rice seeds. Oral immunization with this transgenic rice successfully induced production of neutralizing antibodies against IBDV in chickens, and protected against a highly virulent IBDV strain, BC6/85.

Another effective rice-based vaccine for birds is the Fusion (F) glycoprotein of Newcastle disease virus (NDV) (Yang et al. 2007b). Newcastle disease is a life-threatening disease with mortality rates of 0.8–1.7 per 1,000 bird-days for adult village chickens (Henning et al. 2008). The F protein is a major surface NDV glycoprotein and the principal antigen that elicits a protective immune response (Seal et al. 2000). Yang et al. (2007b) cloned the DNA encoding the NDV F protein into the binary vectors p13GN or p13UN, and transformed rice by the *Agrobacterium*-mediated method. They successfully elicited specific antibodies in mice immunized intraperitoneally with transgenic rice plant extracts together with Freund's adjuvant.

Tackaberry et al. (2008) reported the expression of glycoprotein B of human cytomegalovirus (CMV) in transgenic rice seeds and suggested a new possibility for prevention of CMV infection, although the efficacy of vaccination in vivo was not reported. After the initial infection, CMV establishes lifelong latency in human cells, especially cells of the myeloid lineage (Gandhi and Khanna 2004). Newborn babies infected during pregnancy, and immunocompromised patients often develop uncontrolled replication of CMV. Vaccination is one of the potential strategies, and some clinical trials have been reported, including Phase II trials in pregnant women (Pass et al. 2009) and in transplant recipients (Griffiths et al. 2011). These trials used recombinant CMV glycoprotein B together with MF59 adjuvant with varying degrees of success.

High incidence rates of CMV infections in developing countries (Manicklal et al. 2013) require a reduction in the cost of vaccination against CMV. Thus, if Tackaberry and coworkers' rice-based vaccine against CMV is effective in humans, it may provide a new strategy for the control of CMV infections.

The rates of hepatitis B virus (HBV) infections are also high in developing countries, especially in Asia and Africa (Franco et al. 2012), and a cheap cold chain-free vaccine against HBV is needed. The available HBV vaccine consists of the HBV surface antigen (HBsAg) synthesized in yeast (Valenzuela et al. 1982). After the release of HBsAg vaccine on the market and initiation of the universal HBV vaccination program, the incidence of hepatocellular carcinoma greatly decreased (Chang et al. 1997). However, 11.9 % of vaccinated subjects have no or inadequate levels of antibodies to HBsAg (Roome et al. 1993). Therefore, to reduce costs and improve the effectiveness, the development of a new rice-based vaccine against HBV is needed. Qian et al. (2008) introduced the modified HBsAg gene *SS*1 encoding the hepatocyte receptor-binding presurface 1 region (preS1) fused to the HBV surface (S) protein into rice seeds. Immune responses against both S and preS1 epitopes were successfully induced in mice immunized intraperitoneally with the recombinant protein derived from transgenic rice seeds.

Zhang et al. (2008, 2009) reported the effectiveness of a transgenic rice vaccine against *Chlamydophila psittaci*. Chlamydosis caused by *C. psittaci* occurs mainly in birds, but also rarely in mammals (Harkinezhad et al. 2009b). The major outer membrane protein (MOMP) of *C. psittaci* is important for maintaining the structural rigidity of the infectious form of this pathogen, and for its attachment to the host cells (Harkinezhad et al. 2009a; Vanrompay et al. 1995). Zhang et al. (2008) fused the MOMP gene of *C. psittaci* with the LTB gene and introduced the fusion gene into rice. *Escherichia coli* LTB, one of the two subunits of *E. coli* heat-labile toxin (LT), that binds to the surface of the host cells, promotes the maturation of and antigen presentation by dendritic cells, and promotes the antigen-specific cytotoxic T lymphocyte responses (Fu et al. 2009; Petrovska et al. 2003). Therefore, LTB may act as an adjuvant, making the resultant protein highly immunogenic. Oral immunization of mice with MOMP fused to LTB produced in transgenic rice seeds induced not only MOMP-specific immune responses (serum IgG, IgA, and some cytokines such as an IFN-γ), but also partial (approximately 50 %) protection against a lethal challenge with virulent *C. psittaci*. Recently, Zhang et al. (2013) also reported that their rice-based vaccine improved some arterial blood gas measurements in mice challenged with *C. psittaci*, suggesting protection from lung damage caused by *C. psittaci*.

Matsumoto et al. (2009) reported a new rice-based vaccine against parasitic infections, especially roundworm *Ascaris suum*. *Ascaris suum* infects pigs (but not humans), resulting in a decrease in daily weight gain and an increase in feed-to-gain ratio, resulting in economic losses (Stewart and Hale 1988). Matsumoto et al. (2009) produced a 16-kDa protein of *A. suum* (As16) fused with CTB in transgenic rice seeds, and demonstrated that oral immunization with the chimeric protein together with mucosal CT adjuvant induced As16-specific serum IgG antibodies and reduced the number of larvae in the lungs after infection with *A. suum* embryonated eggs. Human roundworm, *Ascaris lumbricoides*, has a protein identical to *A. suum* As16 (Tsuji et al. 2003). Therefore, the recombinant As16 vaccine may also prevent parasitic infections in humans.

It is estimated that 30,000–50,000 cases of Japanese encephalitis occur each year, causing 10,000–15,000 deaths (Tsai 2000). Foot-and-mouth disease causes a contagious vesicular disease of cloven-hoofed animals (Jamal and Belsham 2013). Recently, rice-based vaccines against Japanese encephalitis virus (JEV) and foot-and-mouth disease virus (FMDV) were reported (Wang et al. 2009, 2012). The authors produced the envelope (E) protein of JEV and the precursor polypeptide (P1) of FMDV in transgenic rice leaves. The E protein of JEV is the largest structural protein in this virus; it is the major target for the humoral immune response, and is important for viral entry into human cells (Solomon 2003). The P1 protein of FMDV is thought to be an important B-cell and T-cell epitopes (Bittle et al. 1982; Rodriguez et al. 1994; Saiz et al. 1994). Intraperitoneal immunization with recombinant E protein produced in transgenic rice plants induced JEV-specific neutralizing antibodies, and oral immunization also induced mucosal IgA and IgG antibodies. Immunization with recombinant P1 produced in transgenic rice had similar results, but also led to partial virus clearance in immunized mice challenged with FMDV (Wang et al. 2012).

Rice-based vaccines against *E. coli* and porcine epidemic diarrhea virus (PEDV) were also developed (Huy et al. 2012; Kim et al. 2010b). Enteropathic *E. coli* is a major pathogen associated with persistent diarrhea in children in developing countries (Abba et al. 2009). As mentioned above, *E. coli* LTB binds to the host cells and is sometimes used as an adjuvant in vaccination. Kim et al. (2010b) inserted the LTB gene into the rice genome, and examined the recombinant protein derived from callus (but not seeds) by western blot analysis and GM1 ganglioside enzyme-linked immunosorbent assay (GM1 ELISA) (Svennerholm and Holmgren 1978); the latter assay uses the ganglioside GM1 as a specific sorbent for LT. Oral immunization with rice-based LTB vaccine elicited LTB-specific serum IgG and fecal IgA. Serum from immunized

mice suppressed binding of LTB to the GM1 ganglioside receptor, suggesting that serum IgG inhibited the biological function of LTB.

Kim and his coworkers developed a rice-based M cell-targeting ligand fusion protein vaccine to control PEDV infections (Huy et al. 2012). PEDV causes acute diarrhea, vomiting, and dehydration with 90–95 % mortality in pigs (Stevenson et al. 2013). The neutralizing epitope of PEDV, encoded by the core neutralizing epitope (COE) gene, was fused with the M cell-targeting peptide ligand Co1, which was expected to enhance the uptake of the fused antigen by M cells in Peyer's patch (Kim et al. 2010a). Mice orally immunized with the extracts of transgenic rice calli produced COE-specific serum IgG and fecal IgA antibodies; among lymphocytes isolated from the spleen, COE-specific IgG- and IgA-secreting cells were also activated, suggesting that efficient systemic and mucosal immune responses can be elicited using this approach.

We were among the first laboratories to develop an immunologically effective rice-based mucosal vaccine for an infectious disease (Nochi et al. 2007b), which is discussed in the following section.

Recent Progress for the Development of MucoRice-CTB

According to a report from the World Health Organization (2012), 58 countries reported a total of 589,854 cholera cases caused by *V. cholerae*, including 7,816 deaths (i.e., a fatality rate of 1.3 %). A higher incidence of cholera with higher mortality was observed in the developing countries. After *V. cholerae* is ingested, most bacteria are killed by gastric acid in the stomach; surviving organisms colonize the small intestine and produce CT (Harris et al. 2012). CT, the enterotoxin of *V. cholerae*, consists of a dimeric A subunit (CTA) and five identical B subunits, CTB. The larger of the CTA polypeptides, A1, is toxic. CTB binds tightly to GM1 ganglioside, which is abundant in the intestinal brush border membrane, and is not toxic (King and Van Heyningen 1973). Our group chose CTB as a vaccine antigen because it has been well characterized immunologically and extensively used for the analysis of antigen-specific mucosal and systemic immune responses. The CTB gene of *V. cholerae* was optimized for translation in rice and inserted into the binary vector pGPTV-35S-HPT; the resultant plasmid was transformed into *O. sativa* L. cv. Kitaake or Nipponbare by the *Agrobacterium*-mediated method. Recombinant CTB content reached an average of 30 μg per transgenic rice seed, representing 2.1 % of the total seed protein (0.15 % of seed weight). Immunoelectron microscopic analysis and western blot analysis indicated that CTB accumulated in rice PB-I and

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PB-II and was protected from pepsin digestion. In contrast, purified recombinant CTB (produced in bacteria, not in rice) was almost completely digested by pepsin treatment. Therefore, rice-based vaccine is an effective delivery vehicle for oral immunization. In addition to resistance of recombinant rice protein to gastric acid, we confirmed the uptake of orally administered CTB from M cells using immunohistochemistry with an anti-CTB antibody and *Ulex europaeus* agglutinin, which is a known marker for murine M cells (Clark et al. 1993). These findings demonstrate that MucoRice-CTB can be considered as a next-generation plant-based effective oral vaccine that can deliver vaccine antigen to the inductive site.

For the immunogenicity of MucoRice-CTB, mice were initially employed for oral vaccination studies. CTB-specific serum IgG and fecal IgA antibodies were detected in mice orally immunized with MucoRice-CTB, indicating that oral immunization with MucoRice-CTB could induce not only systemic but also mucosal immune response. To confirm the CT-neutralizing activity of serum IgG antibody after immunization with MucoRice-CTB, serum samples were subjected to the commonly used GM1-ELISA (Svennerholm and Holmgren 1978) and an elongation assay with Chinese hamster ovary (CHO) cells (Guerrant et al. 1974). In GM1-ELISA analysis, serum samples mixed with CT were incubated in GM1-coated plates and then evaluated by ELISA with an anti-CTB antibody. GM1-ELISA revealed that serum from mice orally immunized with MucoRice-CTB inhibited CT binding to GM1 ganglioside. The CHO elongation assay revealed no morphological changes in CHO cells incubated with CT pretreated with serum from mice orally vaccinated with MucoRice-CTB, suggesting that serum antibodies from these mice successfully neutralized CT. In addition to these in vitro experiments, an in vivo experiment showed that mice immunized with MucoRice-CTB showed no clinical signs of diarrhea when orally challenged with CT. The finding further demonstrated that oral MucoRice-CTB induced gut IgA antibodies possessed neutralizing activities against CT.

In addition to these advantages on induction of protective immunity, MucoRice-CTB has another advantage of long-term storage without refrigerator. In our experiments, oral immunization with MucoRice-CTB stored at room temperature for more than 3 years induced CTB-specific serum IgG and fecal IgA at the same level as freshly harvested MucoRice-CTB. This feature of MucoRice-CTB means that it has a great advantage of being able to be stored even in developing countries in the absence of cold chain.

We also clarified several advantageous points that may affect the future use of MucoRice-CTB in humans. Because the duration of protective immunity is an important issue for vaccination, we examined the time course of CTB-specific serum IgG and fecal IgA antibody titers after oral immunization with MucoRice-CTB (Tokuhara et al. 2010). Although the levels of CTB-specific serum IgG remained high during the 6 months after oral immunization (repeated four times) with MucoRice-CTB, fecal IgA gradually decreased and the IgA titers at six months were half the level shortly after vaccination. However, partial protection against CT challenge remained at 6 months, and a single oral booster vaccination with MucoRice-CTB restored CTB-specific fecal IgA to the same levels as shortly after vaccination. Whether Muco-Rice-CTB can also induce cross-protective immunity against LT is another important issue, because, as mentioned above, enteropathic *E. coli* is commonly found in developing countries in children (up to 63 %) with persistent diarrhea (Abba et al. 2009). Oral immunization with MucoRice-CTB reduced the incidence of LT-producing enteropathic *E. coli*-induced diarrhea (Tokuhara et al. 2010). In addition, before using MucoRice-CTB in human clinical studies, it is necessary to examine its immunogenicity and safety in nonhuman primates. We examined whether vaccination with MucoRice-CTB would induce a mucosal immune response to CTB in cynomolgus macaques (*Macaca fascicularis*) (Nochi et al. 2009). As expected, the levels of CTB-specific serum IgG in macaques orally immunized with MucoRice-CTB increased, and the IgG antibodies showed toxin-neutralizing activity in GM1-ELISA. No allergic immune responses or other adverse effects were noticed.

To rule out the possibility of an unexpected increase in rice allergen protein production in MucoRice-CTB, we compared the levels of allergen proteins in MucoRice-CTB and wild-type rice using two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) and shotgun MS/MS proteomics (Kurokawa et al. 2013). 2D-DIGE is a protein labeling and separation technique that uses optical fluorescence detection of differentially labeled proteins separated on the same gel (Timms and Cramer 2008). We found that the amount of the major rice allergens, members of the α-amylase/trypsin inhibitor-like protein family (Adachi et al. 1993; Nakamura and Matsuda 1996), was lower in MucoRice-CTB than in wild-type rice, Nipponbare. We also performed shotgun MS/MS proteomics, which is a label-free quantification method (Lee and Koh 2011), and found that the amount of IgE-binding allergen proteins was similar or lower in MucoRice-CTB than in wild-type rice. These findings further demonstrated that allergen aspect of concern for MucoRice-CTB is minimized to none.

To advance the MucoRice production system further, our attempt was extended to express antigens with higher molecular weight than CTB (monomer, 11 kDa). Among

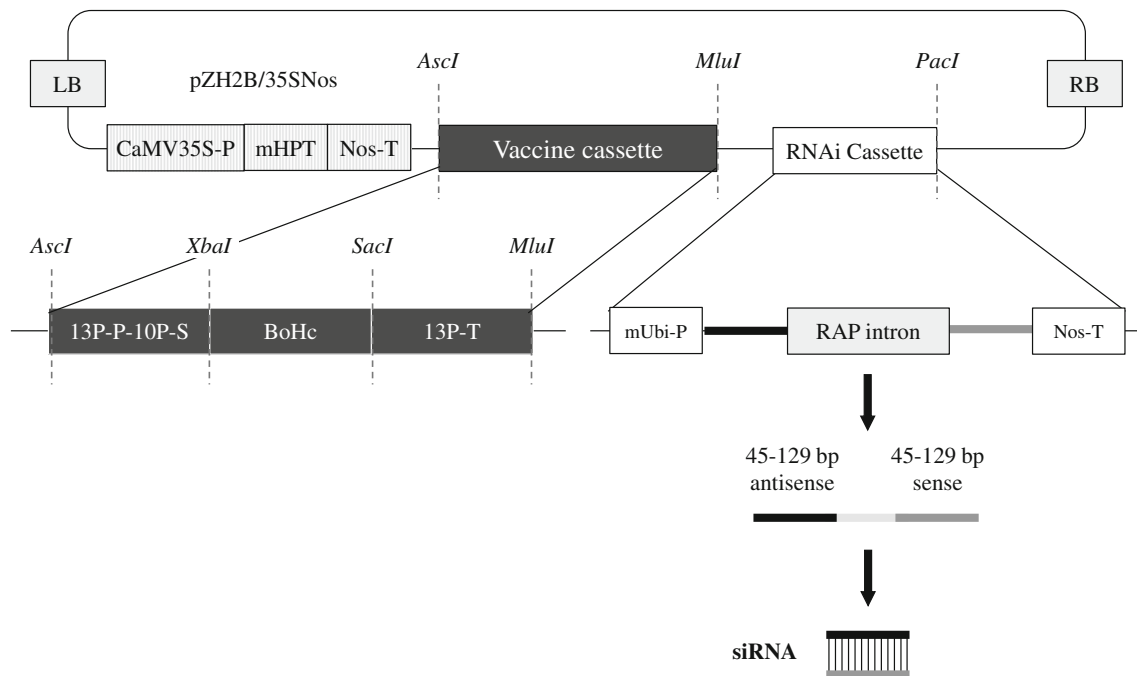## Tandem T-DNA vector for BoHc Expression in Rice (MucoRice-BoHc)

Fig. 2 T-DNA vector for RNAi-mediated suppression of rice storage proteins and overexpression of BoHc in rice seeds. A tandem T-DNA plasmid vector contains a BoHc overexpression cassette (vaccine cassette) with the sequence encoding the BoHc antigen under the control of the promoter of rice 13-kDa prolamin, and an RNAi cassette, including antisense sequence (45–129 bp) specific for endogenous rice storage protein, for suppression of major endogenous proteins, 13-kDa prolamin and glutelin, under the control of the ubiquitin promoter. *LB* T-DNA left border, *CaMV35S-P* cauliflower mosaic virus 35S promoter, *mHPT* mutant hygromycin phosphotransferase, *Nos-T* nos terminator, *13P-P* 13-kDa prolamin promoter, *10P-S* signal sequence of 10-kDa prolamin, *BoHc* 45-kDa fragment of C-terminal half of the heavy chain of botulinum neurotoxin type A, *13P-T* 13-kDa prolamin terminator, *mUbi-P* ubiquitin promoter, *RAP intron* rice aspartic protease intron, *RB* T-DNA right border. Restriction endonuclease sites are *italicized*

pathogens and toxins that can be absorbed from the mucosal surface, we chose a nontoxic 45-kDa fragment of the C-terminal half of the heavy chain of botulinum neurotoxin type A (BoHc) and successfully obtained MucoRice-BoHc, which induced protective immunity against BoHc in mice (Yuki et al. 2012). To achieve higher levels of production of this antigen by suppressing the endogenous rice proteins, we attempted to suppress 13-kDa prolamin and glutelin A by RNAi. Suppression of production of these proteins substantially improved the BoHc accumulation (100 μg/seed) in comparison with seeds without RNAi (10 μg/seed) (Fig. 2).

In transgenic rice seeds, CTB is *N*-glycosylated at Asn32 with a plant-specific *N*-glycan carrying β1,2-xylose or α1,3-fucose residues (Kajiura et al. 2013). These residues strongly contribute to IgE binding to glycoallergens (van Ree et al. 2000). Therefore, we substituted Asn with Gln in a CTB glycosylation sites to prevent plant-specific glycosylation of CTB (MucoRice-CTB/Q) (Yuki et al. 2013). We confirmed that in macaques the resultant MucoRice-CTB/Q vaccine induced similar levels of CTB-specific systemic IgG and mucosal IgA antibodies with toxin-neutralizing activity as the original MucoRice-CTB. As a result of the removal of the plant *N*-glycan, MucoRice-CTB/Q is expected to be a safer mucosal vaccine of equivalent immunogenicity with the original one.

Conclusion

Mucosal vaccination, such as oral, intranasal, sublingual, intrarectal, and intravaginal immunization, can induce not only systemic immune response, but also protective mucosal immune response. Development of new delivery system that effectively induces transcytosis of antigens into M cells and their presentation to dendritic cells are necessary for the successful mucosal vaccination. Plant-based vaccine is one of potential candidates for new mucosal vaccine because of their advantages in stability, safety, and scalability. The plant-based expression systems are thought to be a very safe system because they have low risk of contamination with human pathogens during the vaccine

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preparation. Especially, rice has some advantages over other plants in terms of vaccine production. CTB-antigens produced in rice seeds remain stable for at least 3 years without cold chain, are protected from digestion in the stomach, and are efficiently taken up by M cells. These findings suggest rice-based vaccines are shelf-stable and effective antigen-delivery system for induction mucosal immune responses. In addition to protective effect of rice-based vaccines against infectious diseases, immunization with allergens or self-antigens produced in rice seed could also prevent allergic diseases or autoimmune diseases by the induction of immune tolerance in animal models. Therefore, the rice-based vaccine is a new candidate for efficient, safe and cost-effective vaccine.

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