Expression of a Synthetic Neutralizing Epitope of Porcine Epidemic Diarrhea Virus Fused With Synthetic B Subunit of *Escherichia coli* Heat Labile Enterotoxin in Rice Endosperm

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Abstract

Epitopes often require co-delivery with adjuvant and targeting proteins to enable recognition by the immune system, and this approach may also increase the efficacy of the antigen. In this study, we assess and describe the ability of transgenic rice plants to express a fusion protein consisting of the B-subunit of the *Escherichia coli* heat-labile enterotoxin (LTB) and a synthetic core-neutralizing epitope (COE) of porcine epidemic diarrhea virus (PEDV), inducing an enteric disease that is seen most predominantly in piglets. Both components of the fusion proteins were detected with Western blot analysis. The fusion protein was determined to assemble into pentamers, as was evidenced by its ability to bind to GM_1 gangliosides, and evidenced an average level of expression in a transgenic rice endosperm. This indicates that the expression system of the plant is capable of generating a sizable amount of antigen, possibly allowing for the successful development of an edible vaccine.

Index Entries: Edible vaccine; *Escherichia coli* heat labile toxin; fusion protein; endosperm-specific expression; porcine epidemic diarrhea virus.

1. Introduction

The potential of crop plants with regard to the production of biopharmaceuticals and other novel proteins is only beginning to be realized. Thus far efforts to generate recombinant proteins in plants have focused primarily on dicotyledonous plants, including the potato, tobacco, alfalfa, and *Arabidopsis* ***(1–4)***. However, these plants have also been associated with some disadvantages, especially in terms of the production and delivery of oral vaccines. Green leaf tissues harbor phenol, as well as a host of other potentially toxic compounds. They are also generally unpalatable, and any generated useful proteins must be purified and extracted before use. Although the yields of such proteins in cereal grains, such as wheat, rice, and maize, tend to be less abundant than in the green tissues of alfalfa and tobacco, the seed production characteristics inherent to these plants renders scaling up quite an easy proposition. Unlike proteins synthesized in vegetative plant tissues, seed storage proteins are compartmentalized in protein bodies, normally specialized vacuoles within the mature seeds. Another advantage associated with the production of functional proteins in cereal grains is a marked protein stability during long-term storage. scFV antibody levels in rice seeds have been shown to undergo no significant declines after 6 months of storage at room temperature ***(5)***. Chikwanda et al. ***(6)*** previously

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demonstrated that maize can be efficiently transformed to generate, accumulate, and store a fully assembled and functional candidate vaccine antigen against *Escherichia coli* heat-labile enterotoxin (LT) and cholera toxin (CT).

During endosperm development, the regulation of the transcription of genes encoding for storage proteins in specific expression patterns, along with high expression levels, make the promoters of seed storage proteins ideally suited to the control of target protein expression in cereal grains ***(7)***. HMW-GS promoters are currently the most powerful known endosperm-specific promoters and the sequence and efficiency of these promoters in transgenic plants have been studied and fairly well-characterized. These promoters are ideal candidates, and allow for a high level of tissue-specific transgene expression in cereals ***(8)***.

The use of transgenic plants has recently been suggested as an alternative for the production and delivery of vaccines. However, the model response to oral immunization remains a limiting factor with regard to the development of an effective plant-based vector ***(9)***. One carrier protein for subunit vaccines is the *E. coli* heat-labile enterotoxin (LT). Functional LTB is synthesized as monomers, which subsequently assemble into pentameric structures with a high degree of affinity for GM_1 gangliosides. LTB is a potent mucosal immunogen, which has been shown to elicit synthetic and mucosal responses after its application to mucosal surfaces ***(10)***. LT is comprised of one copy of the A-subunit, which exhibits adenosine 5′-diphosfate–ribosylation activity, and a homopentamer of B-subunits, which binds to the GM_1-gangliosides at the surfaces of eukaryotic cells. The homopentamer of LT consists of five identical 103-amino acid (11.6 kDa) B-peptides (LTB), which form a donut-shaped pentamer by noncovalent association ***(11)***.

The porcine epidemic diarrhea virus (PEDV) has been identified as belonging to the Coronaviridea, and has been shown to induce acute enteritis in pigs. The mortality rate of PEDV-infected piglets is as high as 90% ***(12)***. Since the first reports of PEDV in Belgium and the United Kingdom, the appearance of this disease has also been reported in a host of swine-raising countries, most notably Europe and Korea ***(13)***. The development of edible vaccines that can be delivered directly to mucous membranes would represent a safe method for the induction of modulated systemic immune response, and this would also circumvent any injection-associated hazards. The neutralizing epitope of PEDV was identified on the basis of sequence information for the neutralizing epitope of the transmissible gastroenteritis virus (TGEV). The identified neutralizing epitope of PEDV (COE) was then expressed in the tobacco plant through Agrobacterium-mediated transformation combined with a viral vector system ***(14)***. We attempted to develop a successful plant-based vaccine by the coupling of a synthetic PEDV epitope to synthetic LTB. In this study, we induced the expression of the LTB–COE fusion protein in rice seed endosperm, followed by a GM_1 binding assay to confirm that the recombinant synthetic LTB–COE had formed a functional pentameric structure.

2. Methods

2.1. Cloning

The enzymes, *Xba*I and *Kpn*I were used to excise the 1000-bp fragment from the plant expression vector designated pMYV210, which is located downstream of the ubiquitin promoter and upstream of the nopalin synthase (NOS) terminator ***(15)***. The pMYV210 plasmid harbors synthetic LTB fused to a synthetic COE gene. This fragment was cloned in front of the wheat Bx17 HMW-GS promoter, and fused with the rice *actin* first intron in a plant expression vector harboring the *htp* selection marker gene (*see* **Fig. 1**). The resultant plasmid was then sequenced to ensure correct orientation and the fidelity of the ligation junction. DNA for rice transformation was acquired with an Intron Biotechnology plasmid prep kit, operated in accordance with the manufacturer's instructions.

2.2. Rice Transformation

Embryogenic rice callus (*Oryza sativa* L.) was transformed by microprojectile bombardment, according to the method described by

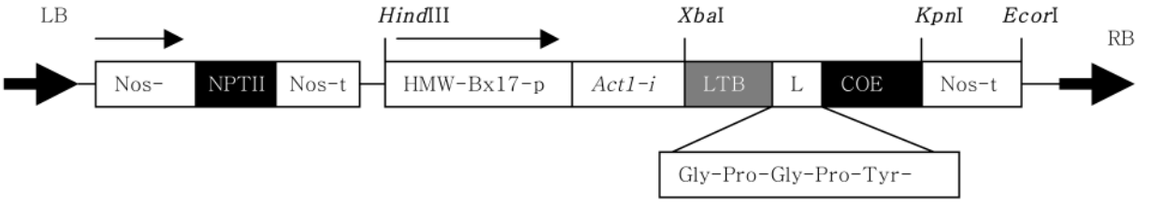

Fig. 1. Schematic representation of the binary plant expression vector pMYV210 fused with Bx17 HMW endosperm-specific promoter. LT: left T-DNA border; RB: right T-DNA border; NOS-p: nos promoter; http: hygromycin phosphotransferase; NOS-t: nos terminator; HMW-Bx17-p: endosperm-specific promoter; actin1-i: rice actin first intron; L: peptide liner; COE: synthetic COE gene fused with SEKDEL.

Lemaux et al. ***(16)***. Four days after the bombardment, the tissues were transferred to N6SEh medium containing hygromycin B-selective agent, then grown at 25°C under dim light conditions ***(17)***. The tissues were subcultured at 3- to 4-week intervals on the same medium, containing 50 mg/L of hygromycin B. When a sufficient quantity of rapidly growing materials was available, the tissues were retransferred to a Murashige & Skoog (MS) based regeneration medium, which contained 0.5 mg/L of α-naftaleneacetic acid (NAA) ***(18)***, and 2 mg/L of benzyl amino purine (BAP). After 4 weeks, the regenerated shoots were again transferred into fresh MS medium containing 50 mg/L hygromycin B. When the shoots reached the top of the box, the plantlets were transferred to soil.

2.3 PCR Analysis of Transgenic Plants

Plant DNA was extracted from the leaf tissues of the transgenic plants, in accordance with the method described by Kang and Fawley ***(19)***. The presence of synthetic LTB fused to the PEDV gene was verified by the results of polymerase chain reaction (PCR) analysis. The 1000-bp fragment was amplified using the following primers: reverse, 5′-GTCACGGTACCTCATAGCCATCTTCTCAGAATAAACATCTGTGATTCC-3′; and forward, 5′-GCGGCCGCTCTAAGAGATCCGCCACCATGGTGAAGGTGAAGTGCTACG-3′. Genomic DNA samples (100 ng) obtained from the transgenic and wild-type (WT) plants, along with 20 ng of plasmid, were used as templates for the detection of the LTB–PEDV gene. The PCR reactions were conducted in a total volume of 50 µL, consisting of 100 ng of leaf genomic DNA, 10× PCR buffer, 200 µ*M* dNTP mix, 40 p*M* primers, and 2 U of *Taq* polymerase. The reaction conditions were as follows: initial PCR activation (95°C, 3 min) was followed by 30 amplification cycles (denaturing, 94°C, 30 s; annealing, 55°C, 30 s, and extension, 72°C, 30 s) and a 5-min final extension step at 72°C.

2.4. Reverse Transcriptase–PCR Analysis

Total RNA was extracted from mature rice seeds of transgenic plants harboring the sLTB gene and WT plants, in accordance with the method established by Z. Li and H. Trick ***(20)***. Total RNA samples were treated with DNase I enzyme, and cDNA synthesis was conducted using an Intron Biotechnology Power cDNA Synthesis Kit with oligo T primers. The PCR reaction was conducted using the following primers: bx17promF (5′-CTACGAATCAATTCACTGCTGACAGTTCAC-3′) and fLTB-F (5′-GCGGCCGCTCTAAGATCCGCCACCATGGTGAAGGTGAAGTGCTACG-3′).

2.5. Immunoblot Analysis

Individual mature dried rice endosperms (100 mg) frozen in liquid nitrogen were ground to a fine powder, using a mortar and pestle. Of the extraction buffer (200 m*M* Tris-HCl, pH 8.0, 100 m*M* EDTA, 14 m*M* 2-mercaptoethanol, 1 m*M* phenylmethylsulfonyl fluoride, and 0.05% Tween 20), 400 µL was added to the rice powder, soaked in a vortex shaker for 10 min at room temperature, and finally centrifuged for 15 min on a bench top centrifuge at maximum speed, to

remove any insoluble debris. Protein concentrations were determined as total soluble proteins (TSP) with Bradford assays with BioRad protein dye concentrate, using a standard curve derived from bovine serum albumin (BSA) ***(21)***.

Of TSP from the rice endosperm, 20 mg was then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, Hercules, CA). The extracted protein samples were either boiled for 10 min, or loaded directly onto the gel with no heat treatment. The separated proteins were transferred to Hybond C membranes (Promega, USA) using a minitransblot apparatus (BioRad) at 130 mA for 2 h. To prevent nonspecific antibody reactions, the membranes were blocked overnight with 10% nonfat milk powder in TBST buffer (*tris*-buffered saline with 0.05% Tween-20) at room temperature. The membranes were incubated with a 1:3000 dilution of rabbit anti-LTB antiserum (Immunology Consultants, OR) or a 1:300 dilution of mouse anti-COE antiserum in TBST containing 5% nonfat dry milk, and than washed three times in TBST buffer. The membranes were then incubated for an additional 2 h with a 1:7000 dilution of antirabbit IgG conjugated with alkaline phosphatase (Promega S3731) or with a 1:5000 dilution of antimouse IgG conjugated with alkaline phosphatase (Promega S3732) in TBST buffer. The membranes were washed three times in TBST buffer and once in TMN buffer (100 m*M* Tris at pH 9.5, 5 m*M* $MgCl_2$, 100 m*M* NaCl). Color was developed using BCIP/NBT (USB) in TMN buffer.

2.6. Quantification of sLTB-sCOE by ELISA

The sLTB–COE protein levels in the rice endosperms were determined by quantitative enzyme-linked immunosorbent assay (ELISA). Between each step, the 96-well plates were washed repeatedly with PBST buffer. The plates were coated with total soluble proteins, diluted in bicarbonate buffer (15 m*M* Na_2CO_3, 35 m*M* $NaCO_3$, pH 9.6), after which the plates were incubated overnight at 4°C. The wells were then blocked by 2 h of incubation with 3% BSA in PBST at 37°C. The plates were incubated for an additional 2 h with a 1:5000 dilution of rabbit anti-LTB serum (Immunology Consultant Lab, OR) in 1% BSA/PBS at 37°C. After washing, the plates were incubated for another 2 h with a 1:10,000 dilution of goat antirabbit horseradish peroxide-conjugated IgG (Promega G-7641) containing 1% BSA, at 37°C. Finally, the plates were developed with the addition of AB buffer for 30 min at room temperature, in darkness. A dilution series of recombinant LT-B standard and nontransgenic rice was incubated in this assay. All measurements were performed in triplicate, and analysis of variance was carried out using the statistical analysis program Excel (Microsoft Corp., USA).

2.7. GM_1 Ganglioside-Binding Assay

Between each step, the 96-well plates were washed repeatedly with PBST buffer. The plates were coated with GM_1 ganglioside overnight at 4°C, then blocked for 1 h with 3% BSA in PBS buffer, at 37°C. Control wells were coated with 3 µg/µL of BSA. Of total soluble protein from the transgenic rice endosperm and the WT plants, 1 µg were added to each of the wells and incubated for 2 h at 37°C. The remainder of the procedure proceeded as described in **Subheading 2.6.**.

3. Results and Discussion

3.1. Genetic Modification of Rice

The synthetic LTB-fused COE gene was inserted in the expression cassette (*see* **Fig. 1**). The plasmid harbored the HMW Bx17 endosperm-specific promoter upstream with the rice actin first intron (Act1), and hygromycin resistance for the selection of stable transformants. The plasmid (designated pMYVN343-V209) was introduced into the rice genome using the Biolistic method. The bombardment of the rice calli with DNA-coated gold particles was followed by hygromycin-containing medium selection. The transformation of cultivar *Oryza sativa* L finally generated four independent transgenic lines, each harboring the LTB–COE gene construct. To confirm the stable integration of the synthetic LTB–COE gene into the transformant chromosomes, genomic DNA was isolated from the leaves of the four independent rice plants. All of the plants evidenced the presence of a 850-bp band, suggesting that the synthetic LTB–COE gene

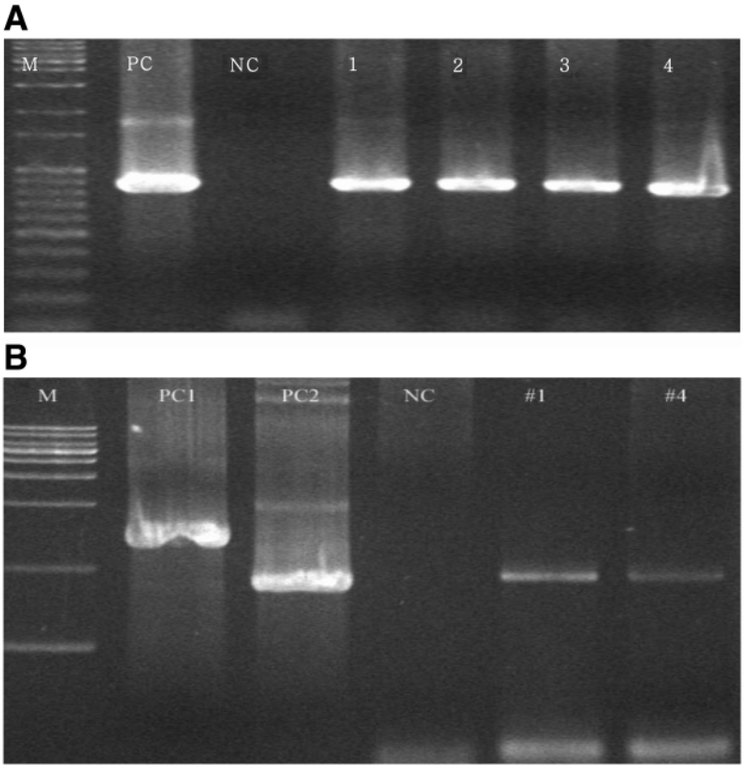

Fig. 2. Characterization of transgenic rice plants. **(A)** PCR analysis of transformed and wild-type plants conducted by amplification of the LTB-COE gene. M: 1-kb DNA ladder; PC: PCR product generated from DNA template of pMYV210 plasmid; NC: PCR product generated from the DNA template of the wild-type plant; lanes 1–4: PCR products generated from the DNA templates of independent transgenic lines. **(B)** Expression of LTB-COE mRNA using RT-PCR. PC1 and PC2: PCR products of plasmid amplification containing Bx17 promoter-intron-LTB-COE gene and Bx17 promoter-LTB-COE gene (intronless), respectively; #1 and #4: PCR products with cDNA templates of independent transgenic lines.

had been successfully integrated into the chromosomal DNA of the transgenic plants (*see* **Fig. 2A**).

3.2. RT-PCR

Four plants were regenerated and grown to maturity in a greenhouse. Because an endosperm-specific promoter was used, the transgene was expected only to express in the transgenic rice endosperms. Two of the four regenerated transgenic plants were found to be fertile. The seeds were harvested after 4 wk of anthesis, and the total RNA was extracted from both the WT and transgenic plants. LTB–COE mRNA expression levels were evaluated by RT-PCR analysis (*see* **Fig. 2B**). Both of the transgenic rice plants evidenced positive sLTB–COE signals, but there was some degree of variation inherent to the signal items between the two lines. Higher transcription levels were detected in transgenic line number 1. The two lines were used in Western blotting, ELISA, and GM_1-binding analysis. The addition of an intron between the promoter and coding regions resulted in improvements in gene expression, both the dicots and monocots ***(22,23)***. We used the rice Act1 first intron fused to the promoter of Bx17 HMW glutenin subunit. The relatively highly observed levels of expression may have been the result of the presence of an intron that elicited high gene transcription levels. The RT-PCR results indicated that the LTB–COE gene had been transcribed in the rice endosperms of the transgenic plants.

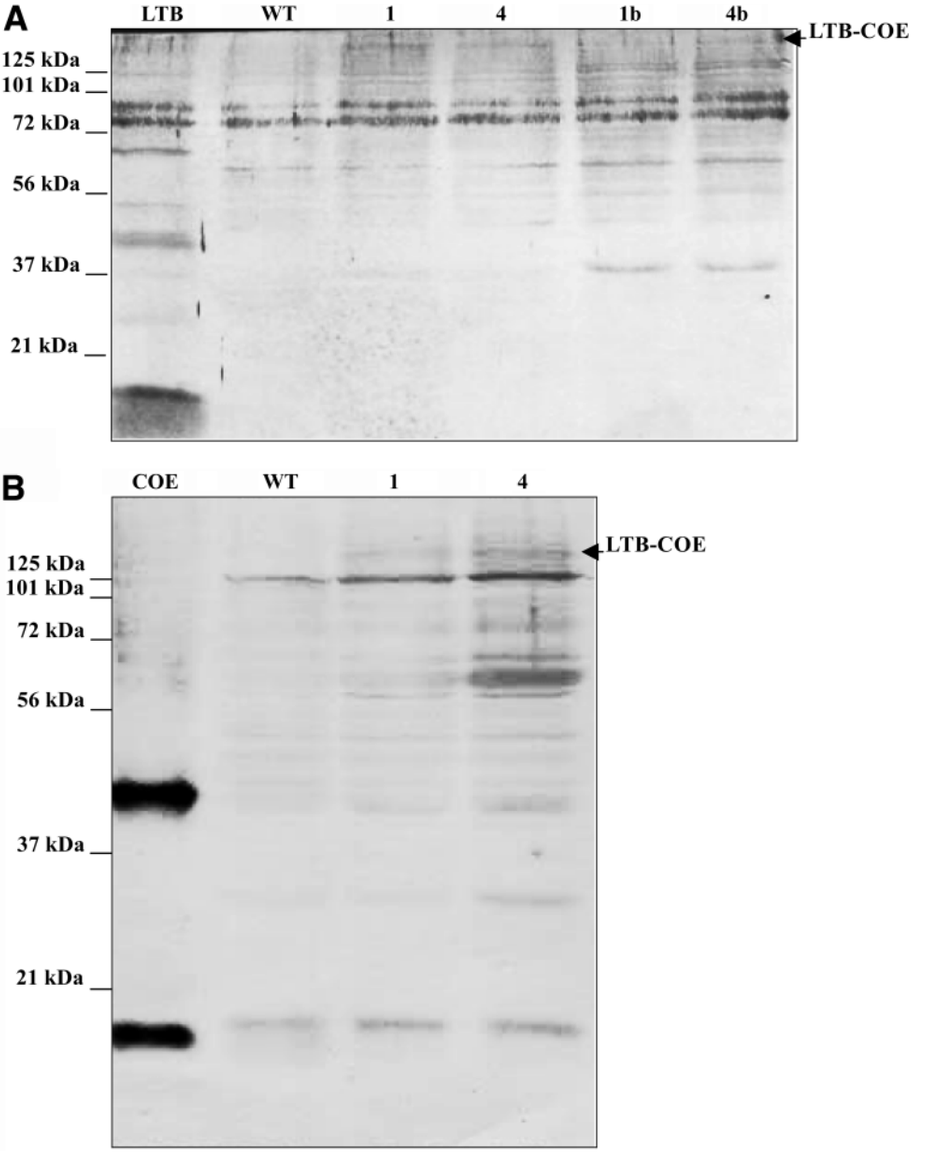

Fig. 3. Western blot analysis of transgenic plants using a rabbit anti-LTB antiserum **(A)** and mouse anti-COE antiserum **(B)**. **(A)** LTB: 0.5 μg of purified bacterial LTB monomer; WT: unboiled total protein (15 μg) from wild-type plant; lanes 1, 4: unboiled total protein (15 μg) from two transgenic plant lines; lanes 1b, 4b: boiled total protein (15 μg) from two transgenic rice lines. **(B)** COE: 0.2 μg of purified bacterial COE; WT: unboiled total protein (15 μg) from wild-type plant; lanes 1, 4: unboiled total protein (15 μg) from two transgenic plant lines.

3.3. Immunoblot Analysis of Synthetic LTB–COE Protein and ELISA Analysis

The two transformed rice plants (lines 1 and 4) were analyzed with regard to the presence of the LTB–COE protein by immunoblot analysis, using the matured rice endosperms of the transgenic plants. To determine the level of recombinant LTB–COE production, the total proteins were extracted, separated with 15% and 10 % SDS-PAGE, and transferred to nitrocellulose membranes.

The two rice lines were then assessed with regard to the presence of the COE protein by immunoblot analysis using purified bacterial COE, and analyzed for the presence of the LTB protein using purified bacterial LTB as a positive control. In the anti-LTB Western blot analyses, a 12-kDa protein was observed in the bacterial control, as a LTB monomer (*see* **Fig. 3**). The transgenic rice lines contained LTB–COE fusion proteins of between 50 and >130 kDa. These may have been multimers of the fusion protein. The oligomeric fusion proteins were dissociated into 35-kDa monomers by boiling the protein homogenates for 10 min. However, boiling of transgenic plant homogenates was not completely dissociated. It is assumed that oligomeric structure of LTB was too strong to be dissociated by 10 min of boiling. To detect more complete monomeric structure in rice, it may be helpful to use another method such as acid-induced dissociation.

The ELISA was conducted to acquire quantitative estimates of LTB protein levels in the endosperms of the transgenic rice plants. The quantities of LTB–COE fused protein were determined by comparisons with known amounts of purified bacterial LTB protein, and were expressed as a percentage of the total soluble proteins in the sample (%LTB). The optimal concentrations of soluble protein in the wells of the ELISA plates evidenced LTB protein levels of approximately 1.3% of TSP in the transgenic rice endosperms (*see* **Fig. 4A**).

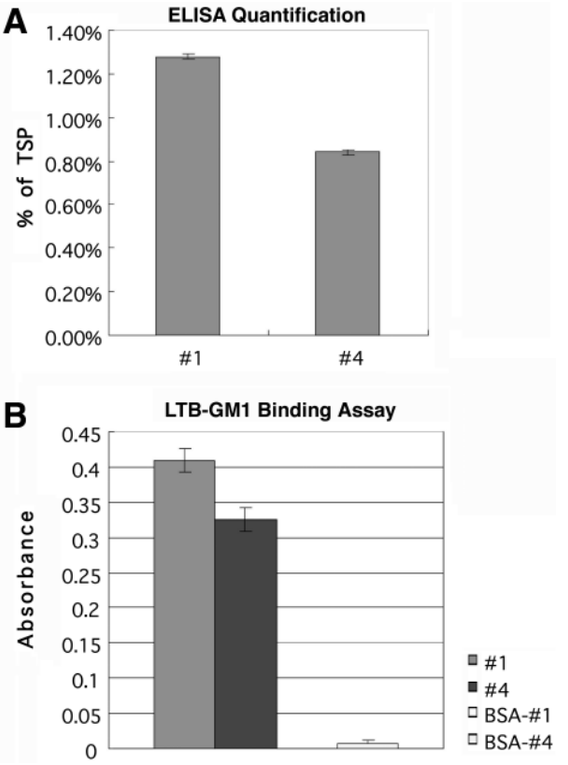

Fig. 4. **(A)** ELISA detection of plant-synthesized LTB protein levels in the endosperms of transgenic rice plants. Nos. 1 and 4: two transgenic plant lines. **(B)** GM1-binding assay of plant-derived LTB. ELISA assay was conducted by coating the plates with either GM_1-monosialoglanglioside or BSA as receptor molecules.

3.4. Binding Assay of LTB Protein to GM_1 Receptor

The ability of the B-subunits to bind to ganglioside was assessed using 96-well plates that were coated with GM_1 gangliosides. In this assay, the rice endosperm-produced LTB–COE evidenced a profound affinity for the GM_1 ganglioside, but not for BSA (*see* **Fig. 4B**). On the basis of the absorbance measurements, the expression of the LTB–COE protein was found to be similar between the two transgenic lines assessed. The ability of the plant-derived LTB–COE to bind to GM_1 ganglioside suggests that the specific protein residues that form the GM_1-binding sites, as well as the oligosaccharide moiety of GM_1 ganglioside, are conserved and function in a cooperative manner *(24,25)*. This apparent cooperation affirms the notion that monomeric B-subunits accumulate within the lumen of the endoplasmic reticulum (ER) of plant cells. This indicates that the expression of LTB-fused COE exerted no inhibitory effects on the formation of LTB pentamers, and that both components of the fusion protein exhibit adaptable antigenicity.

4. Conclusion

Rice is one of the world's most important foods, and is consumed daily by at least 60% of the world's population, particularly in Asian countries, where population continues to increase.

Rice constitutes approx 20% of the total dietary calories consumed by the population of the world ***(26)***. It is the principal food resource in many different regions of Asia, Africa, and South America. As a protein source, milled rice harbors the lowest protein content (ca. 5%) among the major cereal species; moreover, these specific proteins are not readily digestible by humans or other monogastric animals. However, the overall amino acid composition of rice proteins is significantly more balanced than in the proteins of other cereals, primarily because of the relatively higher levels of lysine in rice.

The use of particular cereals for the production of vaccines has several potential advantages over classic methods. Transgenic plants normally express only a small antigenic portion of a pathogen or toxin, thereby avoiding the risks of infection toxicity and reducing the potential for adverse reactions to occur ***(27,28)***. Furthermore, thus far, no known human or animal pathogens are capable of infecting plants without the risk of viral or prion contamination. The synthesis of foreign proteins in transgenic crops relies on many technologies already used in food production protocols, including sowing, harvesting, storage, transport, and plant material processing protocols. Thus, large-scale vaccine production using these methods is relatively economical. In addition, expression of a protein within the natural storage tissues of plants guarantees protein stability, thus reducing the costs associated with storage ***(29)***. Another advantage of these techniques is that when target proteins are expressed in commonly consumed food plants, oral administration is possible, which thereby facilitates the development of edible vaccines. Despite the attractiveness of mucosal vaccination, mucosally administered proteins tend not to be immunogenic ***(30,31)***, and require a greater quantity of antigen than does parental vaccination. Because the mucosal surfaces represent the body's first line of defense against many transmissible diseases, edible plant-based vaccines might potentially be used alone, or in conjunction with other vaccination routes ***(32,33)***.

Because of its ganglioside-binding properties, LTB is capable of bringing conjugated antigens into close contact with the mucosal system, thereby rendering an oral vaccination approach more efficient. Transgenic rice containing LTB fused to the PEDV protein then functions as a carrier protein and adjuvant, as well as a vaccine. Because of the use of an endosperm-specific promoter, protein expression was observed only in the endosperms of the transgenic rice seeds in this study. The storage of antigens in protein bodies is expected to protect the proteins from enzymatic degradation, as well as from the hostile acidic gastric environment ***(34)***. Furthermore, this will allow the protein to be slowly released.

The primary objective of this study was to introduce the synthetic LTB-fused COE protein gene into transgenic rice endosperms and express it through a particle bombardment transformation system. The transgenic rice described in this report will be administered to mice by gastric gavage in the near future to assess the effectiveness of the rice-produced vaccine.

Acknowledgments

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Rice constitutes approx 20% of the total dietary calories consumed by the population of the world ***(26)***. It is the principal food resource in many different regions of Asia, Africa, and South America. As a protein source, milled rice harbors the lowest protein content (ca. 5%) among the major cereal species; moreover, these specific proteins are not readily digestible by humans or other monogastric animals. However, the overall amino acid composition of rice proteins is significantly more balanced than in the proteins of other cereals, primarily because of the relatively higher levels of lysine in rice.

The use of particular cereals for the production of vaccines has several potential advantages over classic methods. Transgenic plants normally express only a small antigenic portion of a pathogen or toxin, thereby avoiding the risks of infection toxicity and reducing the potential for adverse reactions to occur ***(27,28)***. Furthermore, thus far, no known human or animal pathogens are capable of infecting plants without the risk of viral or prion contamination. The synthesis of foreign proteins in transgenic crops relies on many technologies already used in food production protocols, including sowing, harvesting, storage, transport, and plant material processing protocols. Thus, large-scale vaccine production using these methods is relatively economical. In addition, expression of a protein within the natural storage tissues of plants guarantees protein stability, thus reducing the costs associated with storage ***(29)***. Another advantage of these techniques is that when target proteins are expressed in commonly consumed food plants, oral administration is possible, which thereby facilitates the development of edible vaccines. Despite the attractiveness of mucosal vaccination, mucosally administered proteins tend not to be immunogenic ***(30,31)***, and require a greater quantity of antigen than does parental vaccination. Because the mucosal surfaces represent the body's first line of defense against many transmissible diseases, edible plant-based vaccines might potentially be used alone, or in conjunction with other vaccination routes ***(32,33)***.

Because of its ganglioside-binding properties, LTB is capable of bringing conjugated antigens into close contact with the mucosal system, thereby rendering an oral vaccination approach more efficient. Transgenic rice containing LTB fused to the PEDV protein then functions as a carrier protein and adjuvant, as well as a vaccine. Because of the use of an endosperm-specific promoter, protein expression was observed only in the endosperms of the transgenic rice seeds in this study. The storage of antigens in protein bodies is expected to protect the proteins from enzymatic degradation, as well as from the hostile acidic gastric environment ***(34)***. Furthermore, this will allow the protein to be slowly released.

The primary objective of this study was to introduce the synthetic LTB-fused COE protein gene into transgenic rice endosperms and express it through a particle bombardment transformation system. The transgenic rice described in this report will be administered to mice by gastric gavage in the near future to assess the effectiveness of the rice-produced vaccine.

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