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Production of a Heat-labile Enterotoxin B Subunit-porcine Epidemic Diarrhea Virus-neutralizing Epitope Fusion Protein in Transgenic Lettuce (*Lactuca sativa*)

Nguyen-Xuan Huy, Young-Sook Kim, Sang-Chel Jun, Zhewu Jin, Seung-Moon Park, Moon-Sik Yang, and Tae-Geum Kim*

Division of Biological Sciences and the Research Center for Bioactive Materials, Chonbuk National University, Jeonju 561-756, Korea

Abstract Plant-based vaccines have been produced in transgenic plants including tobacco, potatoes, corn, and rice. However, these plants are not suitable for administration without cooking. To overcome this obstacle, a fusion gene encoding the synthetic enterotoxigenic *Escherichia coli* heat-labile enterotoxin B subunit genetically fused with a synthetic neutralizing epitope of porcine epidemic diarrhea virus (sLTB-sCOE) was introduced into lettuce cells (*Lactuca sativa*) by *Agrobacterium*-mediated transformation methods. The integration and expression of the sLTB-sCOE fusion gene was confirmed in transgenic lettuce by genomic DNA PCR amplification and Northern blot analysis, respectively. Synthesis and assembly of the LTB-COE fusion protein into oligomeric structures with pentamer size were observed in transgenic plant extracts by Western blot analysis with anti-LTB or anti-COE antibodies. The binding of plant-produced LTB-COE to intestinal epithelial cell membrane glycolipid receptors was confirmed by G_{M1}-ganglioside enzyme-linked immunosorbent assay (G_{M1}-ELISA). Based on the ELISA results, LTB-COE fusion protein made up about 0.026~0.048% of the total soluble protein in the transgenic lettuce leaf tissues. The synthesis and assembly of LTB-COE monomers into biologically active oligomers in transgenic lettuce leaf tissues demonstrates the feasibility of using uncooked edible plant-based vaccines for mucosal immunization.

Keywords: enterotoxigenic Escherichia coli *heat-labile enterotoxin B subunit*, *edible vaccine*, *lettuce, mucosal immunization, PEDV*

INTRODUCTION

Porcine epidemic diarrhea virus (PEDV) has been identified as belonging to the Coronaviridae family and causes acute enteritis in pigs. The mortality rate of piglets infected by PEDV is as high as 90% [1]. Since the initial identification of PED in Belgium and the United Kingdom in 1978, outbreaks of the disease have also been reported in Korea and Europe [2-5]. Most vaccines are given parenterally. The development of edible vaccines delivered to mucosal surfaces could provide a safe method for inducing modulated systemic immune responses and preventing injection-related hazards [6]. The passive mucosal immune responses from feeders might be an effective way of protecting piglets against PEDV infection, because the digestive track infection typically occurs in 1~2 week-old suckling pigs [1]. The neutralizing epitope of PEDV was identified based on the sequence information for the neutralizing epitope of the transmissible gastroenteritis virus (TGEV) [7] and oral administration of the transgenic plants expressing the neutralizing epitope induced effective protective immune responses against PEDV infection in mice [8].

Transgenic plants and plant cell suspension cultures have been increasingly used as vehicles for the production of edible vaccines for protection against a wide variety of infectious and autoimmune diseases [9-15]. Plant-based vaccines are inexpensive to produce, easy to store, safe from contamination by animal pathogens, and lack the risk

***Corresponding author**
Tel: +82-63-270-4855 Fax: +82-63-270-4856
e-mail: tgkim@chonbuk.ac.kr

of needle-associated injury and disease spread. In 1990, the World Health Organization (WHO) launched the Children's Vaccine Initiative, establishing goals for the development of mucosal vaccines that are safe, inexpensive, easily (orally) administrated, widely accessible for distribution, and capable of being stored without refrigeration [16]. Plant-based vaccines are realistic mucosal vaccine candidates for these purposes. However, low and no immune responses are obstacles for the use of transgenic plants as vaccines, which are due to the low expression levels of vaccine antigens representing only 0.001~0.3% of the total soluble protein in transgenic plants [17]. The expression of antigens in plants could be improved by using a strong promoter, chloroplast transformation [18], plant-based codon optimization [19], agroinfiltration [20], and magnification methods [21]. A useful, alternative strategy is to use LTB or CTB as carriers and adjuvants to stimulate immune responses to the antigen or to increase uptake of the antigen molecules into the mucosal immune system [22].

Cholera toxin and enterotoxigenic *E. coli* (ETEC) heat-labile enterotoxin are representatives of the heteromultimeric AB toxins produced by a number of bacterial pathogens. They are the most effective enterocyte-targeting molecules [23]. The LTB has been shown to function as an effective carrier and adjuvant for genetically-linked foreign proteins [24,25]. The LTB binds to a broader receptor population in mammalian cells compared with that of the cholera toxin B subunit, which binds only to ceramide-galactose sugar receptor molecules, such as G_{M1} ganglioside [26]. Thus, the LTB has the capacity to function as a potent mucosal adjuvant when co-administered to mice intranasally with hen egg lysozyme [27].

The successful expression of LTB-COE fusion protein had been reported in tobacco and rice [28,29], although these plants are not suitable to be orally administered uncooked. Requests have been made for antigen expression in an uncooked, edible plant. In this study, the capacity of a transgenic lettuce plant to produce a fusion protein, which consists of LTB and COE of PEDV and to form the assembly of LTB-COE fusion protein into a pentameric structure was investigated.

MATERIALS AND METHODS

Construction of the Plant Expression Vector

The synthetic LTB (sLTB) and synthetic neutralizing epitope of PEDV (sCOE) were synthesized based on plant-optimized codon usage by using an overlap PCR strategy, constructed into plant expression vectors and introduced into tobacco in our previous experiments [28]. The plant expression vector pMYV210, which has been transformed into tobacco, was used in this study and consists of a sLTB-sCOE fusion gene, a signal peptide of LTB, and the ER retention signal (SEKDEL), under the control of an ubiquitin promoter [30].

Plant Transformation of Lettuce

Seeds of lettuce plants, *L. sativa,* were germinated under sterile conditions in Magenta GA-7 culture boxes (Sigma Chemical, Co., St. Louis, MO, USA) on Murashige and Skoog (MS) basal media [31] containing 3.0% sucrose and 0.2% Gelrite at 25ºC. The cotyledon explants were transformed by incubation for 15 min with *A. tumefaciens* harboring pMYV210 [32]. After blotting the explants onto sterile filter paper, they were transferred to MS basal solid medium, pH 5.7, containing plant growth regulators, 0.1 μg/mL 2-naphthaleneacetic acids (NAA) and 0.5 μg/mL 6-benzylamino purine (BA), and incubated in the dark for 2 days at 25ºC. For selection of transgenic plant cells and for counter selection against continued *Agrobacterium* growth, the explants were transferred to MS solid medium containing kanamycin (100 μg/mL) and cefotaxime (300 μg/mL). Transgenic plant cells were allowed to be regenerated into calli on the selective medium for 2~3 weeks. After 3~6 weeks of further incubation in the light room, regenerated shoots were excised from the calli, and transferred to MS basal solid medium with antibiotics and without growth regulators to stimulate root formation. The putative transgenic lettuce plantlets formed roots in 3~6 weeks. The plantlets were transferred to the greenhouse to mature.

Detection of the sLTB-sCOE Fusion Gene in Transgenic Plants

Genomic DNA was isolated from leaf tissues of non-transgenic and transgenic lettuce using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The concentration of genomic DNA was measured at 260 nm in a UV spectrophotometer. The presence of the sLTB-sCOE fusion gene in transgenic lettuce genomic DNA (400 ng) was determined by PCR analysis using the primer set specific for sLTB-sCOE fusion gene; the forward primer was 5′-GGATCCG-CCACCATGGTGAAGGTGAAG-3′ and the reverse primer was 5′-GGTACCTCATAGCTCATCTTTC-3′. Amplification used a program of denaturing at 94ºC for 10 min followed by 30 cycles of 94ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 30 sec, and a final extension step of 72ºC for 10 min. The PCR products were separated by electrophoresis in a 1.0% agarose gel.

Northern Blot Analysis

Total RNA was isolated from leaf tissues of non-transgenic and transgenic lettuce using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), and was separated by electrophoresis through agarose gel containing formaldehyde [33]. The separated RNA was then transferred to a Hybond N^+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membrane was hybridized with a 32P-labeled sLTB-sCOE probe using Prime-a-Gene labeling system (Promega, Madison, WI) at 65ºC in a hybridization incubator (FINEPCR Combi-H, Seoul, Korea). The membrane was washed twice with 2x SSC and 0.1% SDS, and then washed twice again

with 2x SSC and 1% SDS for 15 min at 65ºC. Hybridized bands were detected by autoradiography using X-ray film (Fuji Photo Film Co., Tokyo, Japan).

Detection of LTB-COE Fusion Protein in Transgenic Lettuce

Transgenic lettuce leaf tissues were analyzed for the detection of LTB-COE fusion protein using immunoblot detection methods with antibodies against LTB or COE. Transgenic leaf tissues were homogenized by grinding in a mortar and pestle with liquid nitrogen and extracted with extraction buffer (1:1 w/v) (200 Mm, Tris-Cl; pH, 8.0; 100 mM, NaCl; 400 mM, sucrose; 10 mM, EDTA; 14 mM, 2-mercaptoethanol; 1 mM, phenylmethylsulfonyl fluoride; and 0.05%, Tween-20). Tissue homogenates were centrifuged at 17,000 × g in a Beckman GS-15R centrifuge for 15 min at 4ºC to remove insoluble cell debris. An aliquot of the supernatant containing 100 μg of total soluble protein, as determined by the Bradford protein assay (Bio-Rad, Inc., Hercules, CA), was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 1.5 to 2 h in Tris-glycine buffer (25 mM Tris-Cl, 250 mM glycine, pH 8.3, and 0.1% SDS). The separated proteins were transferred from the gel to a Hybond C membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using a Mini Trans Blot electrophoretic transfer cell (Bio-Rad) for 2 h at 130 mA in the transfer buffer (50 mM Tris, 40 mM glycine, 0.04% SDS, 20% methanol, and pH 8.3). Nonspecific antibody binding was blocked by incubation of the membrane in 20 mL of 5% non-fat dry milk in a TBS buffer (20 mM Tris-Cl, pH 7.5, and 500 mM NaCl) for 1 h with gentle agitation on a rotary shaker (40 rpm), followed by washing in a TBS buffer for 5 min. The membrane was incubated overnight at room temperature with gentle agitation in a 1:2,000 dilution of rabbit anti-LTB antiserum (Immunology Consultants Lab Inc., USA) or mouse anti-COE monoclonal antibody in TBST antibody dilution buffer (TBS with 0.05% Tween-20 and 1% non-fat dry milk) followed by 3 washes in a TBST buffer (TBS with 0.05% Tween-20). The membrane was incubated for 1 h at room temperature with gentle agitation in a 1:7,000 dilution of anti-rabbit IgG or anti-mouse IgG conjugated with alkaline phosphatase (Promega) in an antibody dilution buffer. The membrane was washed twice in a TBST buffer and once in a TMN (100 mM Tris, pH 9.5, 5 mM $MgCl_2$, and 100 mM NaCl) buffer, as before. After washing, the color was developed with BCIP/NBT (USB, Cleveland, OH) in TMN buffer.

G_{M1}-ganglioside Binding Assay

The expression levels of plant-expressed LTB-COE fusion protein and their affinity for G_{M1}-ganglioside receptor were determined by G_{M1}-ELISA [34]. The microtiter plate (Becton Dickinson Labware, USA) was coated with 100 μL per well of monosialoganglioside G_{M1} (3.0 μg/mL) (Sigma) dissolved in bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, and pH 9.6), covered with Saran wrap and incubated at 4ºC overnight. The wells were washed 3 times with PBST and blocked by adding 300 μL/well of 1% BSA in PBS and incubated at 37ºC for 2 h, followed by washing 3 times with PBST. The wells were loaded with centrifuged transgenic lettuce leaf extracts (5 μg of protein) with serial dilutions (100 μL per well) and purified bacterial LTB (5 ng of protein) as a standard curve, and incubated overnight at 4ºC. The wells were washed 3 times with PBST and loaded with 100 μL per well of a 1:8,000 dilution of rabbit anti-LTB primary antibodies and incubated for 2 h 37ºC, followed by washing the wells 3 times with PBST. The plate was then incubated with 100 μL per well of secondary antibody, a 1:20,000 dilution of alkaline phosphatase-conjugated anti-rabbit IgG (Sigma) for 2 h at 37ºC and washed 3 times with 300 μL of PBST per well. The plate was finally incubated with 100 μL per well of TMB substrates L (PharMingen, USA) for 30 min at room temperature. The plate was measured at a 405 nm wavelength in an ELISA reader (Packard Instrument, USA). The affinity of plant-expressed LTB-COE fusion protein for G_{M1}-ganglioside receptor was detected and the amount of LTB-COE fusion protein synthesized in the transgenic plant was estimated based on the known amount of purified bacterial LTB.

Fig. 1. Plant expression vector pMYV210. Genes located within the T-DNA sequence flanked by the right and left borders (RB and LB) include the synthetic LTB (sLTB) and the synthetic COE (sCOE) gene of PEDV, under the control of ubiquitin promoter (pUbi) and an NPTII (neomycin phosphotransferase II) expression cassette for kanamycin selection of transgenic plants. The NOS-P and NOS-T are promoter and terminator, respectively, from the *A. tumefaciens* nopaline synthase gene. Kozak is the Kozak sequence. L is a peptide linker (GPGPYV). SEKDEL is an ER retention signal peptide.

RESULTS AND DISCUSSION

Detection of the sLTB-sCOE Fusion Gene in Transgenic Lettuce Plants

In a previous experiment, the sLTB-sCOE fusion gene was modified based on plant-optimized codons and expressed in tobacco plants [28]. The sLTB-sCOE fusion gene contains the Kozak sequence (GCCACC) [35] in front of start codon and an ER retention signal (SEKDEL) in the c-terminus of sCOE, and under the control of ubiquitin promoter in plant expression vector, pMYV210 (Fig. 1). It was reported that the CTB signal peptide and the linkage of an ER retention signal to the c-terminus of the fusion protein may help sequester the fusion protein in the ER, and result in the facilitated CTB assembly and the increased expression levels of fusion protein in transgenic plants [11]. Plant ex-

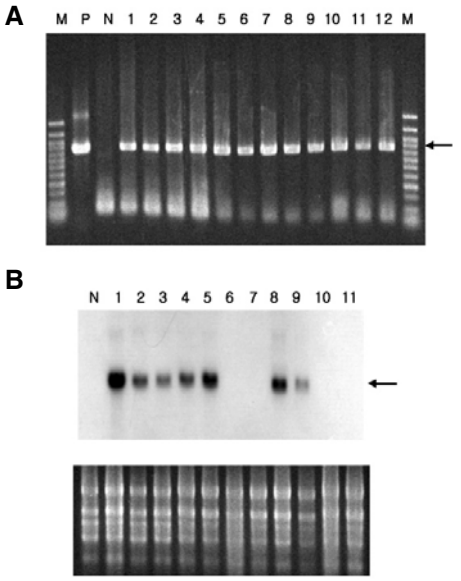

Fig. 2. (A) PCR analysis to detect the sLTB-sCOE fusion gene and (B) northern blot analysis to detect the sLTB-sCOE transcripts in transgenic lettuce. (A) Genomic DNA (400 ng) isolated from transgenic lettuce leaf tissues was used to demonstrate the presence of the sLTB-sCOE fusion gene in the plant using PCR amplification with primers specific for the sLTB-sCOE fusion gene. Lane M, is a 100 bp DNA Mass Ladder Marker (ELPIS-Biotech Inc, Korea); lane P, is pMYV210 template DNA used as a positive control for PCR; lane N, is non-transgenic plant genomic DNA used as a negative control; and lanes, 1-12 are transgenic plant genomic DNA used as PCR templates. (B) The total RNA extracts in the transgenic leaf tissues were used to detect the sLTB-sCOE transcripts. Lane N is total RNA extracts from non-transgenic plants used as a negative control; lanes 1-11 are total RNA extracts from transgenic plants. The lower picture shows the Et-Br stained gel showing total RNA. Arrows indicate sLTB-sCOE gene and sLTB-sCOE mRNA, respectively.

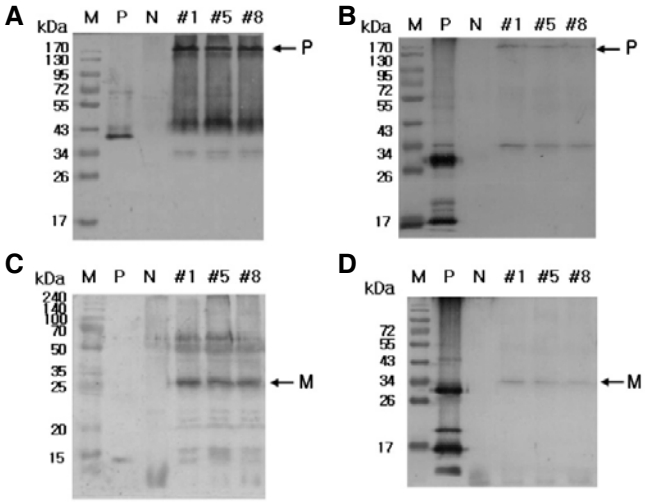

Fig. 3. Immunoblot detection of LTB-COE fusion protein in transgenic lettuce leaf tissues. The leaf tissue extracts from the transgenic lettuce plants after boiling (C and D) for 5 min to ensure protein denaturation or left unboiled (A and B) were analyzed for expression of the LTB-COE fusion protein using anti-LTB (A and C) or anti-COE (B and D) antibody as the primary antibody. Lane M, contains molecular weight markers; lane P, is LTB protein purified in *E. coli* (A and C) and lane P, is purified bacterial COE (B and D); lane N, is protein extracts of non-transgenic lettuce leaf tissues (100 μg protein per lane) as a negative control; and lanes, no.1, 5, and 8 are protein extracts of transgenic plant leaf tissues (100 μg protein per lane) showing high expressions of sLTB-sCOE fusion genes in Northern blot analysis. Arrows M and P indicate the bands of monomer and pentamer structures of the LTB-COE fusion protein, respectively.

pression vector, pMYV210 was transformed into lettuce (*L. sativa*) by *Agrobacterium*-mediated transformation methods. Twelve independently-derived, putative transgenic kanamycin-resistant lettuce plants formed roots 4~6 weeks after the transgenic shoots were transferred to MS basal medium containing antibiotic kanamycin (100 μg/mL) without phytoregulators. A DNA fragment corresponding in size (850 bp) to the sLTB-sCOE fusion gene was amplified by genomic DNA PCR with sLTB-sCOE specific primers in all of the transgenic lettuce plants. No DNA bands corresponding to the sLTB-sCOE fusion gene were detected in the non-transgenic lettuce (Fig. 2A).

Northern Blot Analysis

The transcription of sLTB-sCOE fusion gene was detected using Northern blot analysis conducted with a 32P-labeled sLTB-sCOE probe in the total RNA of the transgenic lettuce leaf tissues. Seven out of 11 transgenic lettuce plants showed a positive signal for sLTB-sCOE, but no signal was found in 4 transgenic and non-transgenic lettuces (Fig. 2B). Although genomic DNA PCR analysis showed the bands of sLTB-sCOE fusion genes in all of the transgenic plants, 4 out of 11 transgenic plants showed undetectable mRNA bands of sLTB-sCOE in Northern blot analysis. These different expression levels of sLTB-sCOE fusion gene among the transgenic plants are due to the different incorporation sites of the target gene in the chromosomes of individual plants, known as the 'position effect' [36,37]. The three transgenic plants showing high expression of sLTB-sCOE fusion gene (no.1, 5, and 8) were selected for further analysis.

Detection of LTB-COE Fusion Proteins in Transgenic Plants

Based on the results from the Northern blot analysis, 3 transgenic plants showing high level of mRNA expression in transgenic leaf tissues (no.1, 5, and 8) were selected for use

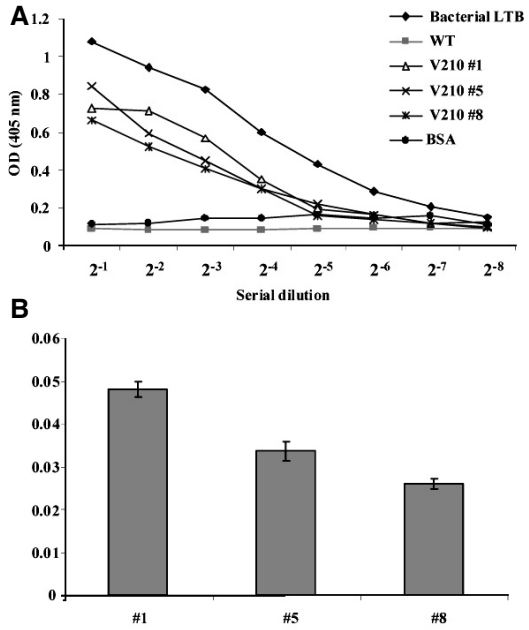

Fig. 4. G_{M1}-ELISA measurement of LTB-COE fusion protein in transgenic lettuce leaf tissues. (A) The binding affinity to the receptor and (B) the amount of plant-produced LTB-COE fusion protein were measured using G_{M1}-ELISA in transgenic plants. Plant protein extracts from transgenic plants and known amounts of purified bacterial LTB were used to measure the expression levels of LTB-COE fusion proteins in transgenic plants. Transgenic plants no.1, 5, and 8, which showed high expression of LTB-COE in Northern blot and Western blot analyses, were used to measure the amounts of LTB-COE fusion protein in the transgenic lettuce leaves tissues. Error bars represent the standard deviations.

in the detection of the LTB-COE fusion proteins by immunoblot methods. The LTB-COE fusion proteins were detected both in boiled and unboiled samples of transgenic lettuce leaf tissue extracts with anti-LTB antibody (Figs. 4A and 4C). No signal band corresponding to the LTB-COE fusion proteins were detected in boiled or unboiled non-transgenic plant protein extracts. The LTB-COE fusion proteins were also detected both in boiled and unboiled samples of transgenic lettuce leaf tissue protein extracts with anti-COE antibody (Figs. 4B and 4D). The bands corresponding to LTB-COE fusion proteins into oligomeric structures resembling native pentamers were detected both with antibodies against LTB and COE in unboiled transgenic lettuce leaf protein extracts (Figs. 4A and 4B). Several bands and smear bands shown in the immunoblot analysis with antibodies against LTB indicate that assembly and aggregates of LTB-COE fusion proteins might be dependant on high-level expression of LTB-COE fusion proteins in transgenic plant leaf tissues. This result coincides with that of different plant expression systems, including lettuce and tobacco [32,38].

G_{M1}-ganglioside Binding Assay

The biological functions of the LTB-COE fusion protein, such as binding to the G_{M1}-ganglioside receptor, which is an intestinal epithelial cell membrane glycolipid receptor, are dependent on forming a pentamer structure of LTB-COE monomers. The G_{M1}-ELISA demonstrated that the LTB-COE fusion protein produced in transgenic lettuce showed a strong affinity to G_{M1}-ganglioside. Non-transgenic plant protein extracts and BSA negative controls showed no affinity to G_{M1}-ganglioside (Fig. 5A). Assembly of LTB-COE monomers into oligomeric (pentamer) structures is essential for epithelial cell binding and uptake into mucosal immune systems. Therefore, the performance of LTB carrier and adjuvant functions in animals immunized with the LTB-antigen fusion proteins is contingent on pentamer assembly. Both immunoblot analysis and G_{M1}-ELISA experiments demonstrated that LTB-COE fusion proteins assembled into biologically active pentamer structures in transgenic lettuce.

The amounts of LTB-COE fusion protein produced in the transgenic lettuce leaf tissues were measured against known amounts of purified bacterial LTB protein and were expressed as a percentage of the total soluble protein (TSP) extracted from the transgenic lettuce plants (% LTB-COE). The amount of LTB-COE fusion protein ranged from 0.026~0.048% of TSP (Fig. 5B). Although it is difficult to compare the exact expression levels of target proteins between different laboratories, the production of LTB in the transgenic potato tubers at the level of 0.01% of TSP was sufficient to elicit both systemic and mucosal immune responses in mice [11]. Thus, it is expected that the LTB-COE fusion protein produced in transgenic lettuce may be enough to elicit a significant immune response in mice and other animals.

CONCLUSION

In this study, we developed an edible lettuce plant producing detectable amounts of enterotoxigenic *E. coli* enterotoxin B subunit-porcine epidemic diarrhea virus-neutralizing epitope fusion protein and its assembly into biologically active forms was confirmed by G_{M1}-ELISA. The amount of LTB-COE fusion protein was found to range between 0.026 ~0.048% of TSP in transgenic lettuce leaf tissues. This edible transgenic lettuce would also be a useful system to express other antigen proteins for mucosal immunization by oral consumption. The ability of plant-producing LTB-COE fusion protein to generate both immunogenicity and adjuvanticity will be the subject of analysis in future animal mucosal immunization experiments.

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