RESEARCH

Expression of a Cholera Toxin B Subunit-Neutralizing Epitope of the Porcine Epidemic Diarrhea Virus Fusion Gene in Transgenic Lettuce (*Lactuca sativa* L.)

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Abstract Transgenic plants have been used as a safe and economic expression system for the production of edible vaccines. A synthetic cholera toxin B subunit gene (CTB) was fused with a synthetic neutralizing epitope gene of the porcine epidemic diarrhea virus (sCTB–sCOE), and the sCTB–sCOE fusion gene was introduced into a plant expression vector under the control of the ubiquitin promoter. This plant expression vector was transformed into lettuce (*Lactuca sativa* L.) using the *Agrobacterium*-mediated transformation method. Stable integration and transcriptional expression of the sCTB–sCOE fusion gene was confirmed using genomic DNA PCR analysis and northern blot analysis, respectively. The results of western blot analysis with anti-cholera toxin and anti-COE antibody showed the synthesis and assembly of CTB–COE fusion protein into oligomeric structures with pentameric sizing. The biological activity of CTB–COE fusion protein to its receptor, G_{M1}-ganglioside, in transgenic plants was confirmed via G_{M1}-ELISA with anti-cholera toxin and anti-COE antibody. Based on G_{M1}-ELISA, the expression level of CTB–COE fusion proteins reached 0.0065% of the total soluble protein in transgenic lettuce leaf tissues. Transgenic lettuce successfully expressing CTB–COE fusion protein will be tested to induce efficient immune responses against porcine epidemic diarrhea virus infection by administration with raw material.

Introduction

Porcine epidemic diarrhea virus (PEDV), an etiologic agent of diarrhea in pigs, is an enveloped and linear positive-sense ssRNA genome with a poly(A) tail virus. PEDV has been classified as a member of the *Coronaviridae* family [1, 2]. PED was first detected in Belgium [3] and the UK [4] in 1978, and outbreaks of this disease have been reported in many pig farming countries, leading to severe economic losses in Canada [5], Europe [6, 7], and Asia, including Japan [8], China [9], Korea [10], and more recently, Thailand [11]. The complete sequence of the entire genome of the strain CV777 was found to be 28,033 nucleotides in length, after excluding the poly(A) tail [12, 13]. The viral encoded-proteins from the PEDV genome contain the spike (S, 180–220 kDa), membrane (M, 27–32 kDa), and nucleocapsid (N, 55–58 kDa) proteins [14]. An important role of the spike protein is to enable the attachment of viral particles to receptors on the host cells and to support the subsequent penetration of viral particles into the cells via membrane fusion. The S glycoprotein also stimulates the induction of neutralizing antibodies in the host [12, 15]. Based on the sequence information for the neutralizing epitope of the transmissible gastroenteritis virus, the neutralizing epitope region of PEDV (CO-26K equivalent, COE gene) was identified within the spike protein gene [16]. The COE gene is, therefore, considered to be an important cloning and expression target in the development of the subunit vaccine.

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The use of transgenic plants which express antigen proteins is a promising strategy because of advantages relating to economy, scalability, safety, and the minimal risk of contamination with potential pathogens [17–20]. Plant-derived antigen proteins have delayed or prevented the onset of disease in animals and have proven to be safe and functional in human clinical trials [20]. However, it is commonly reported that most heterologous proteins accumulate in plant biomass at much lower levels than 1% of the total soluble protein [17, 21]. To improve the immunogenicity of the small amount of antigen protein expressed in transformed plants, plant-derived vaccines require efficient antigen delivery and adjuvant systems to present the appropriate antigens to the mucosal immune system [22].

Cholera toxin consists of one A subunit with enzymatic activity and five B subunits with binding activities to the host cells [23–25]. The cholera toxin binds to a lipid raft via G_{M1}-ganglioside for the internalization and activation of the toxin. The G_{M1}–cholera toxin complex in the lipid raft transports the holotoxin (A/B_5 subunits toxin) into the ER via the Golgi [26]. Therefore, cholera toxin B subunit (CTB) was used as a powerful mucosal adjuvant and mucosal carrier molecule. The immunogenicities of antigens can be improved when coupled to CTB as a carrier and adjuvant due to both the increased uptake of coupled antigen across the mucosal barrier and the more efficient presentation of coupled antigens, not only by dendritic cells and macrophages, but also by naïve B cells [22]. Cholera toxin B subunit (CTB), produced by *Vibrio cholera* and *Escherichia coli* heat-labile enterotoxin (LTB), have been widely used as a model adjuvant and carrier protein for mucosal immunization when conjugated with the desired antigen [27–29]. Since the CTB subunit was first chosen as a carrier protein in 1984 [30], CTB fusion proteins with insulin [31], insulin/GAD [32], and the full-length rotavirus NSP4 [33] were expressed in transgenic potato plants, with the B chain of human insulin expressed in transgenic tobacco plants [34]. More recently, *Ascaris suum* As14 was expressed in transgenic rice seeds [35] and was verified to have the correct biological assembly. In previous studies, the synthetic COE gene (sCOE) of PEDV, which was synthesized based on the optimal codon usage in a plant, was expressed in tobacco plants [36–38], and the synthetic LTB–sCOE fusion gene was expressed in transgenic tobacco plants [39], rice seeds [40], and lettuce plants [41]. Lettuce is a commercially important crop belonging to the Asteraceae family. The raw leaves of this crop are consumed by humans, and the time to obtain an edible product is only weeks, compared to the months needed for crops such as tomato or potato [42]. Therefore, the lettuce plant may be a good choice for the expression of heterologous protein in an edible vaccine. In this study, the synthetic COE gene of PEDV was fused with the synthetic CTB gene (sCTB–sCOE), and the sCTB–sCOE fusion gene was expressed in the lettuce plant. The successful expression of biologically functional CTB–COE fusion protein in lettuce plants could prove to be an alternative approach for oral vaccines for protection against PEDV infection.

Materials and Methods

Construction of the Plant Expression Vector

The synthetic CTB (sCTB) gene was amplified from pMYO52 [43], which contains the synthetic CTB, using the PCR amplification method with an sCTB gene-specific primer set. The forward primer was 5′-GGA TCC GCC ACC ATG GTG AAG-3′ and the reverse primer was 5′-TAC GTA GGG CCC GGG CCC GTT AGC CAT GCT-3′. The forward primer included the Kozak sequence (GCCACC) [44] in the front of the start codon. The underlined sequences of the reverse primer contained the flexible hinge sequence (GPGP), which was located between the CTB and COE peptides. Convenient *Bam*HI restriction sites at the 5′-end and *Sna*BI at the 3′-end of the sequence were included for subsequent subcloning. The PCR amplification conditions consisted of a genomic denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension step of 72°C for 5 min. The PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI). The correct sequence of sCTB was bidirectionally confirmed using DNA sequence analysis and the plasmid-designed pMYV510. The *Eco*RI-digested fragment from pMYV510 containing the sCTB gene was introduced into the same sites of the pCR2.1-Topo vector (Invitrogen, Carlsbad, CA) to yield pMYV511. The plasmid pMYV206 [37], containing the synthetic COE gene (sCOE) with the endoplasmic reticulum retention signal sequence (SEKDEL), was digested with *Sna*BI and *Kpn*I, and was subcloned into the same sites of the plasmid pMYV511, yielding pMYV512. The *Bam*HI and *Kpn*I-digested fragment of pMYV512 was inserted into the same sites of the plant expression vector between the ubiquitin promoter and the nopaline synthase (NOS) terminator, creating pMYV514 (Fig. 1). The plasmid pMYV514 was transformed into the *Agrobacterium tumefaciens* strain LBA4404 using the tri-parental mating method [45].

Lettuce Transformation

Lettuce seeds (*Lactuca sativa* L.) were rinsed in 70% ethanol for 1 min, washed three times with distilled water, and then surface-sterilized in 2% sodium hypochlorite with a few drops of Tween 20 for 10 min. Surface-sterilized seeds were washed five times with distilled water and germinated
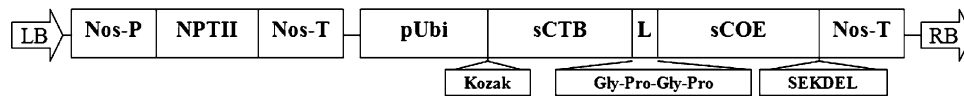
Fig. 1 Schematic representation of the plant expression vector. The expression of the synthetic CTB (sCTB) and synthetic COE (sCOE) genes of the PEDV fusion gene (sCTB–sCOE) were driven under the control of the ubiquitin promoter (pUbi). LB and RB are the *left* border and *right* border of T-DNA, respectively. L is the linker peptide (Gly-Pro-Gly-Pro) for hinge flexibility. Nos-P and Nos-T are the promoter and terminator of the nopaline synthase gene from *Agrobacterium tumefaciens*, respectively. Kozak is the Kozak consensus sequence. SEKDEL is an ER retention signal. NPTII is the neomycin phosphotransferase II gene for kanamycin selection of transgenic plants

in Magenta GA-7 culture boxes (Sigma, St. Louis, MO) containing MSO medium [46] with 3.0% sucrose and 0.2% Gelrite at 25°C for 5 days. The *Agrobacterium* harboring the sCTB–sCOE fusion gene was propagated in LB medium containing 50 μg/ml kanamycin and 50 μg/ml rifampicin, and was used to infect lettuce cotyledons via incubation for 15 min. The explants were blotted on sterile filter paper, transferred to MS co-culture medium containing plant growth regulators including 0.1 μg/ml 2-naphthaleneacetic acids (NAA) and 0.5 μg/ml 6-benzyl-amino purine (BA), and finally incubated in the dark for 5 days at 25°C. After co-cultivation, the explants were transferred to MS selection medium containing 100 μg/ml kanamycin and 300 μg/ml cefotaxime for 4–6 weeks for shoot induction under 16 h of light per day. The developed shoots were excised and transferred into a hormone-free MS medium with antibiotics to induce root formation. The plantlets were transferred to the greenhouse for maturation.

PCR Analysis of Transgenic Lettuce Plants

Genomic DNA was isolated from wild-type and putative transgenic lettuce leaf tissues using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA). The concentration of genomic DNA was measured at 260 nm in a UV spectrophotometer. PCR amplification was carried out to detect the presence of the sCTB–sCOE fusion gene in the genomic DNA of transgenic plants with the primer set specific for the sCTB–sCOE fusion gene; the forward primer of CTB and the reverse primer specific for sCOE is 5′-GGT ACC TCA TAG CTC ATC TTT CTC AG-3′. Samples were first heated to 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min, before a 5 min final extension at 72°C. PCR products were analyzed using 1.0% agarose gel electrophoresis, followed by staining in sterile distilled water containing 1 μg/L ethidium bromide. In addition, transgenic lettuce genomic DNA extracts were subjected to PCR analysis with primers (5′-GCA TTC TGC TGG CGC TG-3′ and 5′-GGA ACG TCA GTG GAG-3′) specific for the plasmid region outside the T-DNA portion to address the possibility that PCR products can come from the presence of contaminating *Agrobacterium* plasmid DNA.

Northern Blot Analysis

Total RNA was extracted from leaf tissues of wild-type and transgenic lettuce plants using Trizol Reagent (Invitrogen) according to the supplier's protocol. The RNA samples (30 μg) were separated by electrophoresis on a 1.2% formaldehyde-containing agarose gel [47] and were capillary-blotted onto a Hybond N+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was hybridized with a ^{32}P-labeled random-primed (Promega) sCTB–sCOE probe at 65°C in buffer solution (pH 7.4, 1 mM EDTA, 250 mM $Na_2HPO_4 \cdot 7H_2O$, 1% hydrolyzed casein, and 7% SDS) in a Hybridization Incubator (Finemould Precision Ind. Co., Seoul, Korea). The membrane was washed twice with 2× SSC plus 0.1% SDS and twice again with 2× SSC plus 1% SDS for 15 min at 65°C. Hybridized bands were detected using autoradiography with X-ray film (Fuji Photo Film Co. HR-G30, Tokyo, Japan).

Immunoblot Detection

Total soluble protein (TSP) was extracted from leaf tissues of wild-type and transgenic lettuce plants via grinding in liquid nitrogen with a mortar and pestle. The homogenate was suspended with extraction buffer [48] (1:2 w/v) (200 mM Tris–HCl, 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) and centrifuged at 13,000×g for 15 min at 4°C to remove insoluble cell debris. The protein concentration was measured using the Bradford protein assay (Bio-Rad, Hercule, CA). An aliquot of supernatant containing 50 μg of TSP was separated by 10% (unboiled condition) or 12% (boiled condition) sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad) at 120 V for 2–3 h in Tris–glycine buffer (25 mM Tris–HCl, 250 mM glycine, pH 8.3, and 0.1% SDS). The separated protein bands were transferred onto Hybond C membranes (Amersham Pharmacia Biotech) in transfer buffer (50 mM Tris–HCl, 40 mM glycine, and 20% methanol) using a Mini-Transblot Apparatus (Bio-Rad) for 2 h at 130 mA. To prevent non-specific antibody reactions, the membranes were

blocked with 10% non-fat milk powder in TBST buffer (Tris-buffered saline with 0.05% Tween 20) with gentle agitation (20 rpm) on a rotary shaker. The membranes were incubated with a 1:5,000 dilution of rabbit anti-cholera toxin antibody (Sigma C-3062) or mouse anti-COE antibody in TBST antibody dilution buffer containing 3.0% non-fat dry milk and were then washed three times with TBST buffer. The membrane was incubated for 2 h with a 1:5,000 dilution of anti-rabbit IgG conjugated with alkaline phosphatase (Promega S3731) or a 1:7,000 dilution of anti-mouse IgG conjugated with alkaline phosphatase (Promega S372B) in TBST buffer. The membranes were washed twice with TBST buffer and once with TMN buffer (100 mM Tris, pH 9.5, 5 mM $MgCl_2$, and 100 mM NaCl). Color was developed using BCIP/NBT (USB, Cleveland, OH) in TMN buffer.

G_{M1}-Ganglioside Binding Assay

The microtiter plates were coated with 100 μl per well of monosialoganglioside-G_{M1} (Sigma) (3.0 μg/ml) in bicarbonate buffer (15 mM Na_2CO_3, 25 mM $NaHCO_3$, pH 9.6) covered with plastic wrap and incubated at 4°C overnight. The wells were washed three times with PBST buffer (PBS plus 0.05% Tween 20), blocked by the addition of 300 μl per well of 1% BSA in PBS buffer, and incubated at 37°C for 2 h, followed by three washes with PBST buffer. The wells were loaded with soluble protein extracts from the leaf tissues of transformed and wild-type lettuce, along with commercial bacterial CTB (Sigma), for 2 h at 37°C. The plates were washed three times with PBST buffer and then incubated with 100 μl per well of a 1:5,000 dilution of rabbit anti-cholera toxin antibody or mouse anti-COE antibody in PBS antibody dilution buffer containing 0.1% BSA for 2 h at 37°C. The plates were washed three times with PBST buffer and loaded with 100 μl per well of a 1:7,000 dilution of anti-rabbit IgG or a 1:10,000 dilution of anti-mouse IgG conjugated with alkaline phosphatase (Promega S372B) for 2 h at 37°C and washed three times with PBST buffer. The plates were developed with the addition of 100 μl per well of alkaline phosphatase buffer [10% (v/v) diethanol amine, 0.1% $MgCl_2$, 0.02% sodium azide, pH 9.8] plus one tablet of phosphate substrate (Sigma S0942-100TAB) for 30 min at room temperature in the dark. The plates were read at a 405 nm wavelength in an ELISA reader (Packard Instrument, Meriden, CA). The amount of biologically active CTB–COE fusion protein expressed in the leaf tissues of transgenic lettuce plants was quantified by comparison with known amounts of the bacterial CTB–antibody complex. All experiments were performed in triplicate, and analysis of variance was carried out using the statistical analysis program Excel (Microsoft, USA).

Results

Construction of Plant Expression Vector and Lettuce Transformation

The sCTB gene containing the Kozak sequence [43] in the front of the start codon was fused with the sCOE gene containing an ER retention signal (SEKDEL) at the C-terminus [37]. The flexible hinge sequence (Gly-Pro-Gly-Pro) was used to incorporate a degree of flexibility between the CTB and COE proteins. The expression of the sCTB–sCOE fusion gene was under the control of the ubiquitin promoter. The neomycin phosphotransferase II (NPTII) gene provides kanamycin resistance as a selection marker in transgenic plants. The plant expression vector, pMYV514, which contained the sCTB–sCOE fusion gene (Fig. 1), was transformed into lettuce using the *Agrobacterium*-mediated transformation method. Four to six weeks after transformation, kanamycin-resistant shoots were selected and transferred to MS basal medium containing antibiotics but not phyto-hormones. Eleven independent putative transgenic plants formed roots 4–6 weeks later.

PCR Analysis of Transgenic Lettuce Plants

Genomic DNA was isolated from 11 putative selected transgenic and wild-type lettuce plants using the DNeasy Plant Mini Kit. PCR analysis was performed to confirm the presence of the sCTB–sCOE fusion gene in the genomic DNA of transgenic lettuce plants. As expected, the PCR product (850 bp) corresponding to the sCTB–sCOE fusion gene was detected in the genomic DNA of all 11 transgenic plants (Fig. 2a). No band was amplified from wild-type plants. This PCR result showed the stable integration of the sCTB–sCOE fusion gene into the chromosomes of transgenic plants using an *Agrobacterium*-meditated transformation method. PCR amplification with primers specific to the plasmid region excluding T-DNA showed a 530 bp fragment from pMYV514 but did not detect any corresponding band from the genomic DNA of transformed lettuce (Fig. 2b).

Northern Blot Analysis

Northern blot analysis was conducted with a [^{32}P]-labeled sCTB–sCOE probe to measure the expression of the sCTB–sCOE fusion gene in the transgenic lettuce plants. Nine out of eleven transgenic lettuce plants showed a positive signal for sCTB–sCOE mRNA, and there were variations in the expression levels of the sCTB–sCOE fusion gene among the transformed plants (Fig. 3). High levels of sCTB–sCOE mRNA were found in transgenic
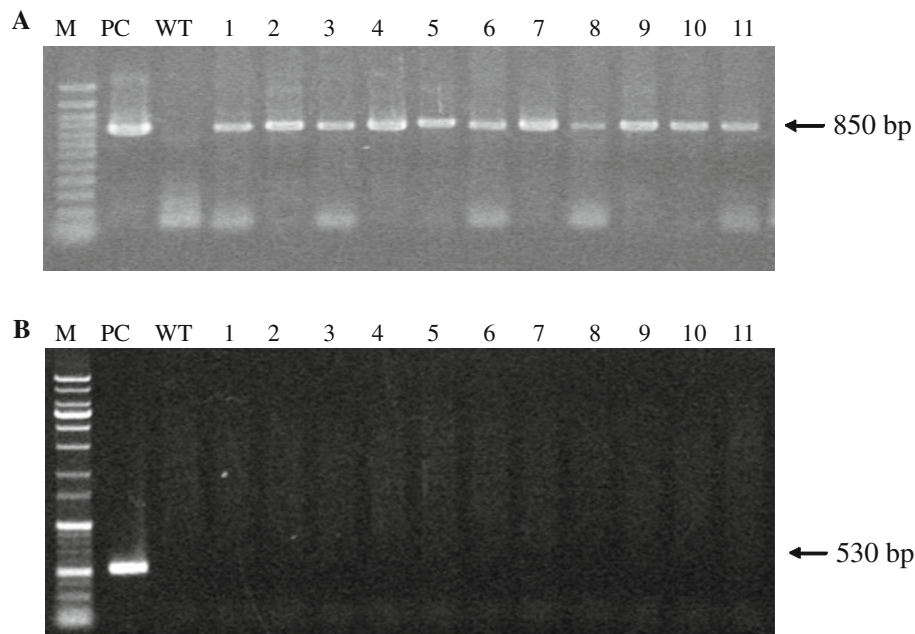
Fig. 2 Genomic DNA PCR analysis used to detect the sCTB–sCOE fusion gene in transgenic lettuce plants. The genomic DNA from transgenic and wild-type plants was conducted to confirm the integration of the sCTB–sCOE fusion gene into the chromosome of transgenic lettuce plants (**a**). PCR amplification with primers specific for the plasmid region excluding the T-DNA region (immediately downstream of the T-DNA *right* border) showed no *Agrobacterium* contamination (**b**). *Lane M* is a 100-bp DNA ladder (ELPIS-Biotech. Inc., Seoul, Korea). *Lane PC* is pMYV514 template DNA used as a positive control for PCR. *Lane WT* is genomic DNA of wild-type lettuce plants used as a negative control. *Lanes 1–11* are genomic DNA extracted from transgenic lettuce plants used as a template for PCR
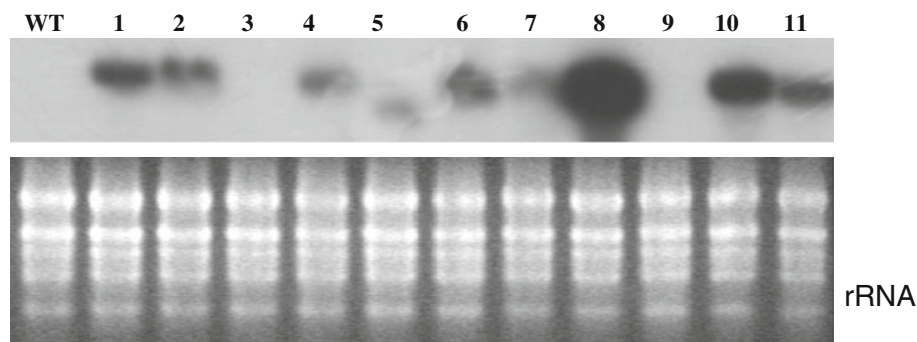

Fig. 3 Northern blot analysis to confirm the presence of sCTB–sCOE transcripts in transgenic lettuce plants. The total RNA was obtained to estimate the transcripts of the sCTB–sCOE fusion gene using the ^{32}P-labeled sCTB–sCOE probe in transgenic lettuce plants. *Lane WT* is total RNA from a wild-type lettuce plant. *Lanes 1–11* are total RNA from transgenic lettuce plants. Transgenic lettuce plants #1, #8, and #10 showed strong positive signals

lines #1, #8, and #10, and these three transgenic lines were selected for further analysis.

Immunoblot Detection

Based on the northern blot analysis, transgenic plant lines #1, #8, and #10 were selected to characterize the CTB–COE fusion protein. The CTB–COE fusion protein was detected in both boiled and unboiled samples of the leaf tissues of transgenic lettuce with anti-cholera toxin antibody and anti-COE antibody. No signal band corresponding to CTB–COE fusion protein was detected in unboiled or boiled protein extracts of wild-type lettuce. In the unboiled condition, assembly of CTB–COE fusion protein into oligomeric structures resembling pentamers and with a molecular weight of around 170 kDa was detected with both anti-cholera toxin and anti-COE antibody, while the bacterial CTB positive control was observed at around 45 kDa. A 16 kDa band was observed in the bacterial COE protein. When transgenic protein

Fig. 4 Detection of CTB–COE fusion protein in leaf tissues of transgenic lettuce. The total soluble protein (TSP) from leaf tissues of transgenic lettuce plants was obtained to detect CTB–COE fusion protein after boiling for 5 min (**c**, **d**) or unboiled (**a**, **b**), with anti-cholera toxin antibody (**a**, **c**) or anti-COE antibody (**b**, **d**) as the primary antibody. *Lane M* is a prestained protein ladder (Fermentas, Glen Burnie, MD). *Lane bCTB* is commercial bacterial CTB. *Lane bCOE* is COE protein purified in *E. coli*. *Lane WT* is protein extract from wild-type lettuce leaves as a negative control. *Lanes #1, #8*, and *#10* are protein extracts from leaf tissues of transgenic lettuce plants. *P* and *M* indicate pentamer and monomer of the CTB–COE fusion protein, respectively
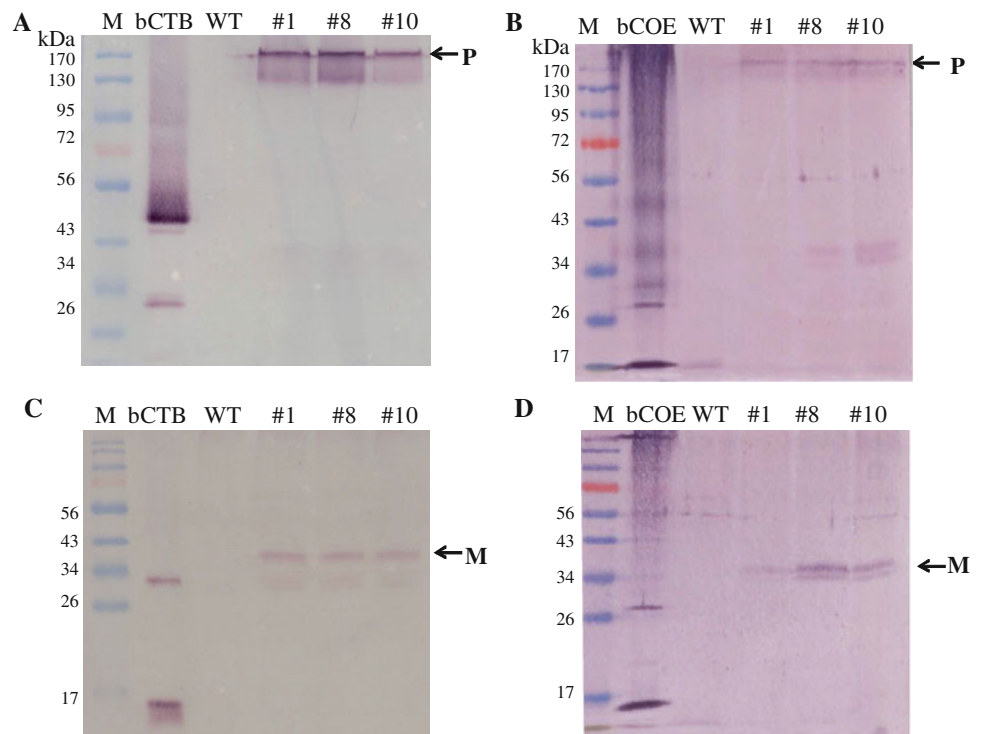
extracts were boiled for 5 min, the oligomeric fusion protein was dissociated into 36 kDa monomers with both anti-cholera toxin and anti-COE antibody, and the monomer of the CTB protein was detected around 12 kDa (Fig. 4).

Biding of CTB–COE Fusion Protein to G_{M1}-Ganglioside

The biological function of the CTB–COE fusion protein produced in leaf tissues of transgenic lettuce was determined using G_{M1}-ELISA. The G_{M1}-ganglioside binding capacity of CTB–COE fusion protein in the protein extracts of transgenic leaf was detected with anti-cholera toxin antibody and anti-COE antibody (Fig. 5a, b) as the primary antibody. The affinity of CTB–COE fusion protein for G_{M1}-ganglioside with anti-cholera toxin antibody was stronger than that of anti-COE antibody. These results indicated that the monomeric CTB–COE fusion proteins were properly assembled into a biologically functional form, and that COE protein was conserved in the fusion structure.

G_{M1}-ELISA was employed to evaluate the expression levels of the assembled CTB–COE fusion protein in leaf tissues of transgenic lettuce plants. The amount of assembled CTB–COE fusion protein produced in transgenic lettuce leaves was calculated via comparison with a known amount of bacterial CTB and was expressed as a percentage of total soluble protein (TSP) extracted from transgenic

lettuce plants. The amount of plant-produced CTB–COE fusion protein was found to be between 0.003 and 0.0065% of TSP (Fig. 5c). The strongest expression of CTB–COE fusion protein was found in transgenic plant line #8, corresponding with the results of the northern blot analysis.

Discussion

Porcine epidemic diarrhea virus causes acute enteritis in pigs. The death rate of infected-piglets is over 95%, leading to significant economic losses in swine husbandry [3, 37]. Hence, it is important to develop an effective vaccination to protect against PEDV infection. The neutralizing epitope region (COE) was identified within the spike protein that was capable of inducing neutralizing antibodies against the PEDV [16]. Although transgenic plants expressing small amounts of COE antigen (8–20 µg antigen/g wet weight) elicited an effective immune response against PEDV infection, improvement of the antigen protein amount was suggested to optimize the immune response [36]. In order to achieve high expression levels of a heterologous gene in plants, it is useful to optimize the coding sequence to mimic that of highly expressed plant genes and to eliminate any mRNA-destabilizing motifs. The synthetic COE gene, which was modified based on the optimized codon usage in plant, showed an approximately fivefold higher expression than that of the native gene in a transgenic plant [38]. It has
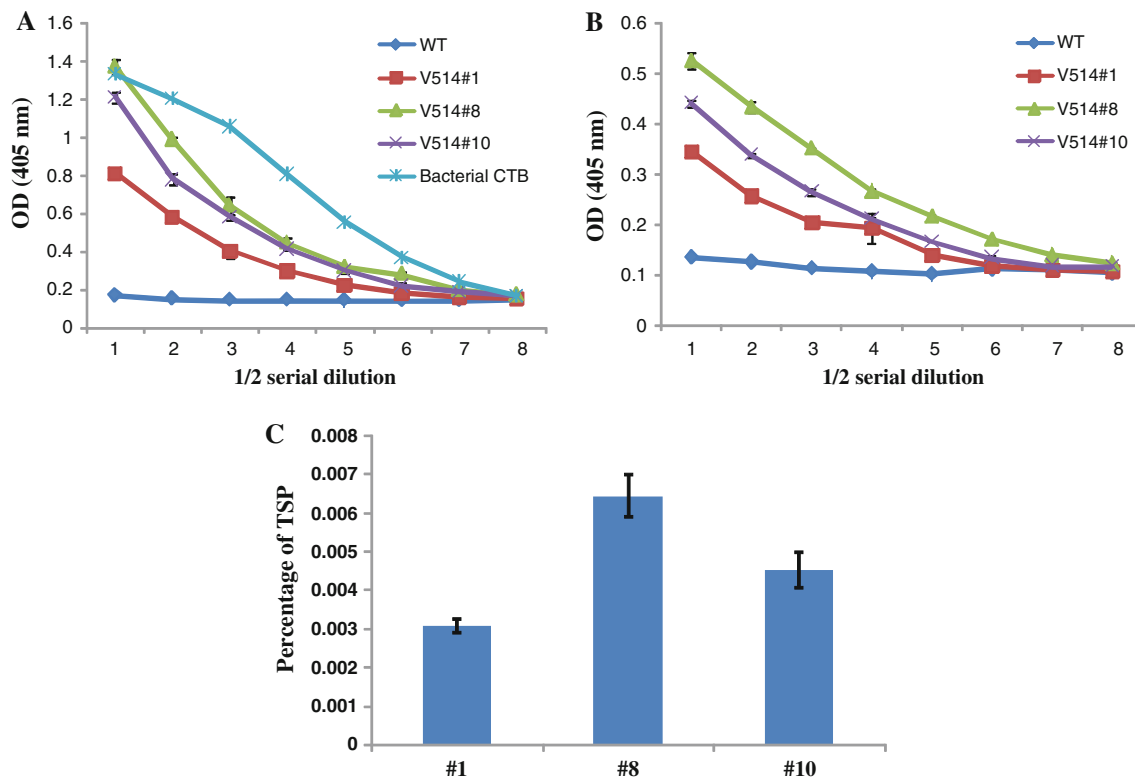
Fig. 5 G_{M1}-ELISA of CTB–COE fusion protein produced in transgenic plants. The biological activity of CTB–COE fusion protein with the intestinal membrane G_{M1}-ganglioside receptor was confirmed with anti-cholera toxin antibody (**a**) and anti-COE antibody (**b**). The expression levels of CTB–COE fusion protein were estimated in leaf tissues of transgenic lettuce plants (**c**)

been shown that the expression of foreign proteins has been enhanced by the C-terminal fusion of the ER retention signal SEKDEL, since the SEKDEL motif is expected to sequester the protein and aid in protein assembly in the ER [43]. Strong or tissue-specific promoters are believed to increase the antigen expression level. The ubiquitin promoter is a useful, strong, and constitutive promoter for high gene expression in tobacco plants compared to Cauliflower Mosaic Virus 35S and the rice actin promoter [49]. Due to the high expression of vaccine antigen in transgenic plants, it is important to decrease the feeding amount during immunization because using a large amount of plant materials due to the low expression levels in transgenic plants often results in pain.

The use of ligands to deliver target antigens into mucosal immune systems is an alternative to overcome the low immune response in a plant-edible vaccine. In this experiment, the synthetic CTB gene was used to fuse with the synthetic COE gene, and the CTB fusion gene was transformed into lettuce plants using a *Agrobacterium*-mediated transformation method. The approximately 170 kDa pentameric CTB–COE fusion protein was confirmed using western blot analysis with anti-cholera toxin and anti-COE antibody, indicating that the plant-produced

CTB–COE fusion protein contained CTB and COE protein. The binding activity of functional CTB–COE fusion protein for its biological receptor, G_{M1}-ganglioside, was confirmed using G_{M1}-ELISA with anti-cholera toxin and anti-COE antibody. Therefore, these results indicated that the COE protein of CTB–COE fusion protein did not prevent the formation of CTB pentamer and that both components of the fusion protein retained antigenicity. It suggested that the plant-produced CTB–COE fusion proteins could be efficiently taken up into the mucosal immune system, where they could induce a strong immune response against PEDV infection.

The positive detectable signal for the sCTB–sCOE fusion gene in nine transgenic lettuce plants was detected in northern blot analysis, but no detectable signal was found in the two transgenic lettuce plants (Fig. 3). These different transcript levels of the sCTB–sCOE fusion gene depended on the different incorporation sites of the target gene in the chromosome of different plants, a phenomenon referred to as the "position effect" [50]. Recently, it was reported that the expression of transgenes in transgenic plants was controlled by RNA silencing [51]. It was observed that the CTB–COE fusion protein monomer was detected around 36 kDa under the boiled condition. This

molecular weight is greater than the calculated molecular weight of the CTB–COE fusion protein (30.4 kDa), based on amino acid sequences (Fig. 4). This difference in migration may be due to two glycosylation sites within the CTB and two glycosylation sites within the COE.

Transgenic lettuces transformed with COE alone were constructed in this study but its expression was not detected via western blot analysis (data not shown). Although COE alone was not expressed in transgenic lettuce, the COE fused with CTB was expressed in transgenic lettuce. The fusion with proteins which were highly expressed and stable, has been used to improve the expression level of protein which were hardly expressed in transgenic plants, known as "fusion partner" [52]. The CTB has been expressed with high level and stable in transgenic plants [53, 54]. The expression level of CTB in transgenic lettuce was reported high level with 0.24% of total soluble protein compared to that of CTB–COE fusion protein (0.0065% of TSP) [55]. The low expression level of CTB–COE fusion protein compared to CTB alone may be due to the low expression of COE gene. The low expression of antigen gene in transgenic plants was problem to induce efficiently immune responses. In previous experiment, we reported that LTB (1.7 µg; 0.12% of TSP) expressed in transgenic rice callus elicited antigen-specific immune responses in mouse. Based on this result, it is expected that the increase of feeding amount and frequency of transgenic plants expressing CTB–COE fusion protein can induce immune responses. The stably expression of CTB–COE fusion protein was detected in second generation.

In conclusion, transgenic lettuce plants expressing the synthetic cholera toxin B subunit-synthetic COE of the PEDV fusion protein were constructed to increase their uptake into the mucosal immune system and to improve the immune response. The successful expression of functional CTB–COE fusion protein was confirmed using western blot analysis and G_{M1}-ELISA. In future experiments, transgenic lettuce plants expressing functional CTB–COE fusion protein will be tested to induce an immune response against PEDV infection by mucosal immunization.

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