Biotechnology and Bioprocess Engineering 16: 576-580 (2011)
DOI 10.1007/s12257-010-0195-8

RESEARCH PAPER

Highly Expressed Cholera Toxin B Subunit in the Fruit of a Transgenic Tomato (*Lycopersicon esculentum* L.)

Nguyen Hoang Loc, Le Thi Thinh, Moon-Sik Yang, and Tae-Geum Kim

Received: 15 June 2010 / Revised: 2 December 2010 / Accepted: 3 December 2010

Abstract The cholera toxin B subunit (CTB), a nontoxic molecule with potent biological properties, is a powerful mucosal and parenteral adjuvant that induces a strong immune response against co-administered or coupled antigens. A gene encoding CTB, which was modified based on the optimized codon usage in the plant, was synthesized and fused to the endoplasmic reticulum retention signal KDEL to enhance its expression level in plants. The synthetic CTB (sCTB) gene was introduced into a plant expression vector adjacent to the CaMV 35S promoter, and was transformed into tomato using an *Agrobacterium*-mediated transformation method. The integration of the sCTB gene into the genomic DNA of transgenic plants was confirmed by genomic DNA PCR amplification. The synthesis and assembly of CTB protein in transgenic plants was demonstrated through immunoblot analysis and G_{M1}-ELISA. The highest amount of CTB protein produced in transgenic tomatoes was approximately 0.9% of total soluble fruit protein which was 10-fold greater than the previously 0.081%. G_{M1}-ELISA indicated that plant-synthesized CTB protein bound specifically to G_{M1}-gangliosides, suggesting that the CTB subunits formed active pentamers.

Keywords: cholera toxin B subunit, edible vaccine, codon optimization, *Lycopersicon esculentum*

Nguyen Hoang Loc, Le Thi Thinh
Institute of Resources, Environment and Biotechnology, Hue University, Hue, Vietnam

Moon-Sik Yang, Tae-Geum Kim*
Department of Molecular Biology, Chonbuk National University, Jeonju 561-756, Korea
Tel: +82-63-270-4855; Fax: +82-63-270-4856
E-mail: tgkim@jbnu.ac.kr

Moon-Sik Yang
Jeonju Center, Korea Basic Science Institute, Jeonju 561-756, Korea

1. Introduction

Cholera is an epidemic diarrheal disease that continues to devastate many developing countries where socio-economic conditions are poor, sanitation and public hygiene systems are rudimentary, and safe drinking water is unavailable [1]. Cholera is caused by *Vibrio cholerae*, and disease severity is mediated by the potent action of the cholera toxin (CT), which stimulates the secretion of water and electrolytes into the intestine [2]. Cholera toxin is representative of adjuvants that efficiently induce mucosal immunity [3,4]. The generation of non-toxic CT derivatives that retain adjuvant activity could provide a safe alternative for the evaluation of these toxins as mucosal adjuvants in human and animals.

Cholera toxin is composed of distinct A and B subunits. The pentameric B subunit (CTB), which contains five identical polypeptides, targets the glycosphingolipid receptors on eukaryotic cell surfaces. Bacterial CTB has been expressed at total soluble protein levels of 0.02 and 0.04% in tomato leaves and fruits, respectively [5]. The low expression levels of foreign antigens in transgenic plants limit the development of effective plant-based vaccines and therefore, the achievement of high expression levels is crucial. The expression of vaccine components in plants has been accomplished by modifying a range of leader and polyadenylation signals [6], and by optimizing the codon usage for expression in plants [7-9]. Higher accumulation levels and enhanced protein stability have been achieved *via* the addition of an endoplasmic reticulum (ER) retention signal sequence, KDEL. The KDEL peptides perform essential functions that are related to protein folding and assembly in the ER [10].

In this study, we report the expression of modified CTB in tomato fruit. G_{M1}-ganglioside (galactosyl-N-acetylgalac-

tosamyl-sialyl-galactosylglucosyl ceramide) ELISA was performed to confirm that the recombinant CTB produced in transgenic fruit formed a functional pentameric structure.

2. Materials and Methods

2.1. Plant material

Tomato seeds (*Lycopersicon esculentum* L. cv. 311) were rinsed with 70% (v/v) ethanol for 30 sec, and subsequently surface-sterilized with 0.1% (w/v) $HgCl_2$ solution for 10 sec. After rinsing with sterile distilled water five times, sterilized seeds were germinated on ½ MS [11] medium consisting of 3% (w/v) sucrose and 0.8% agar, in an Erlenmeyer flask. Ten-days post germination, cotyledons excised from *in vitro*-germinated seedlings were cultured on MS medium consisting of 3% (w/v) sucrose, 0.8% agar, and supplemented with 2 mg/L zeatin, 0.1 mg/L indolylacetic acid, and 0.1 mg/L thidiazuron for shoot regeneration. Regenerated shoots (3 cm in length) were then excised from multiple-shoot clumps, and rooted on MS medium supplemented with 0.5 mg/L indolylbutyric acid. The pH of the medium was adjusted to 5.8, and then autoclaved at 121°C for 15 min. The cultures were incubated at 25 ± 2°C, under an intensity of 2,000 ~ 3,000 lux and with a photoperiod of 10-h.

2.2. Construction of the plant expression vector

The synthetic CTB (sCTB) gene was synthesized based on the optimized codon usage in the plant using overlap extension PCR [12]. The plant expression vector used in our study, pMYO53, consisted of a sCTB with its own signal peptide, as well as KDEL, under the control of a duplicated CaMV 35S (Cauliflower Mosaic Virus 35S RNA) promoter (Fig. 1).

2.3. Tomato transformation

The plasmid pMYO53 was introduced into the *Agrobacterium tumefaciens* strain LBA 4404 *via* tri-parental matting using the helper plasmid pRK2013 [13], and subsequently, the *Agrobacterium* was used to transform tomato. Cotyledons from 10-day-old seedlings were cut at the tip and base and middle pieces (approximately 0.7 × 0.3 cm) were pre-cultured for 48 h on shoot regeneration medium. The *Agrobacterium* suspension was dropped onto the cotyledons with a pipette (about 2 drops for each explant) and after 2 days of co-cultivation, cotyledons were cultured on shoot regeneration medium with 50 mg/L kanamycin (Km) and 200 mg/L cefotaxim (Cef) for 10 days, and then transferred to the same medium, but with only 100 mg/L Km. Shoots formed after 2 weeks of culture, and after approximately 2 months, shoots were excised and transferred to rooting medium without antibiotics. Rooted plantlets were acclimatized and transferred to pots for fruiting. Transgenic plantlets were checked for the presence of transgene(s) in leaf tissue using genomic DNA PCR amplification.

2.4. Genomic DNA isolation and PCR analysis

Total genomic DNA was extracted from leaf tissue of putative transgenic tomato using Kang and Fawley's method [14]. The presence of the sCTB gene was determined by genomic DNA PCR amplification analysis using a forward primer (5'-ATGGTGAAGCTCAAGTTCGG-3') and a reverse primer (5'-GTAGTTAGCCATGCTAATAG-3') specific for sCTB. The PCR amplification was performed as follows: A genomic denaturing at 95°C for 10 min; followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec; and a final extension step of 72°C for 10 min. The PCR products were separated by electrophoresis on 1% agarose gel and stained with ethidium bromide.

2.5. Immunoblot detection of CTB protein in transgenic tomato fruit

Total soluble protein was extracted from transgenic tomato plant fruit tissue, precipitated using acetone, and resuspended in 1 mL of extraction buffer (50 mM HEPES, pH 7.5, 10 mM potassium acetate, 5 mM magnesium acetate, 1 mM EDTA, 1 mM dithiothreitol, and 2 mM phenylmethanesulfonyl fluoride). An aliquot (120 μg) of total soluble protein, as determined by the Bradford protein assay (Bio-Rad, Hercules, CA, USA) [15], was obtained from fruit tissues of transgenic plants. It was then separated on sodium dodecylsulfate polyacrylamide gels (SDS-PAGE), consisting of stacking gels and separating gels with 5 and 12% (w/v) acrylamide, respectively, at 100 V for 2 ~ 3 h. Commercial bacterial CTB protein (1 μg) was also loaded as a positive control.

The separated protein bands were electrophoretically transferred from the gel to a Hybond C membrane (Bio-

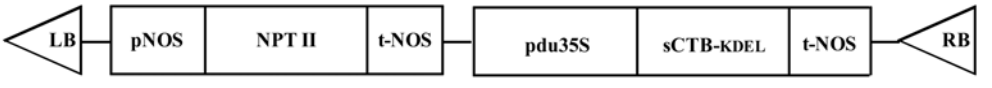

Fig. 1. Plant binary expression vector pMYO53. The synthetic cholera toxin B subunit (sCTB) fused with the ER retention signal sequence (KDEL) was synthesized and introduced into the plant expression vector under the control of the duplicated CaMV 35S promoter (pdu35S). Neomycin phosphotransferase (NPTII) was used as a seletion marker for transgenic plants. pNOS and t-NOS are the promoter and terminator of *nopaline synthase*. LB and RB represent the left border and right border of T-DNA, respectively.

rad) using a Trans-blot® SD semi-dry transfer cell (Bio-Rad) at 15 V for 30 min. Non-specific antibody reactions were blocked by incubating the membranes in 25 mL of 3% casein in TBST buffer (TBS plus 0.05% Tween-20), with gentle agitation overnight at room temperature (RT). Membranes were incubated for 2 h at RT with gentle agitation in a 1:5,000 dilution of rabbit anti-cholera toxin antibody (Sigma C-3062, St. Louis, MO, USA) in TBST antibody dilution buffer containing 1.5% casein, and then washed three times with TBST buffer. Membranes were then incubated for 2 h at RT in a 1:7,000 dilution of mouse anti-rabbit IgG conjugated with alkaline phosphatase (Sigma A-2556) in TBST buffer, and then washed three times with TBST buffer, and once with TMN buffer. After washing, the color was developed using BCIP/NBT alkaline phosphatase substrate (Sigma B-5655) in TMN buffer.

2.6. Quantification of CTB protein by ELISA

The expression level of CTB protein produced in the fruits of transgenic tomato plants was determined using quantitative ELISA analysis. Total soluble protein from transgenic plants was loaded into a 96-well microtiter plate with 100 μL/well of selected concentrations, and incubated overnight at 4°C. The plate was washed three times with PBST buffer and subsequently, the background was blocked by incubation in 1% BSA in PBS buffer (300 μL/well) at 37°C for 2 h. The wells were then washed three times with PBST buffer and the plate was incubated with a 1:3,000 dilution of rabbit anti-cholera toxin antibody (100 μL/well) in PBS containing 0.5% BSA at 37°C for 2 h, and then washed four times with PBST buffer. The wells were incubated with a 1:5,000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma G-7641) (100 μL/well) in PBS containing 0.5% BSA at 37°C for 2 h, prior to being washed four times with PBST buffer. Finally the plate was incubated with 100 μL/well TMB substrates (PharMingen 2606KC and 2607KC, Fallbrook, CA) for 30 min at RT in darkness to maximize the reaction rate. After incubation, the reaction was measured at 405 nm in an automated ELISA system (Bio-Rad).

2.7. G_{M1}-ganglioside binding assay

The receptor binding ability of CTB protein produced in transgenic plants was confirmed by the G_{M1}-ganglioside enzyme-linked assay (G_{M1}-ELISA) [16]. A microtiter plate was coated with 100 μL per well of monosialoganglioside G_{M1} (3.0 μg/mL) (Sigma G-7641) in bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$), with a pH of 9.6 at 4°C overnight. After three washes with PBST, the plate was blocked with 1% BSA in PBS buffer. The plate was then washed three times with PBST buffer, and then G_{M1}-ganglioside-coated plates were incubated with various concentrations of total soluble protein from transgenic and wild-type plants in PBS buffer (100 μL/well) at 37°C for 2 h. The primary and secondary antibody treatments were as described previously. As a control, a microtiter plate was coated with 100 μL/well BSA (3.0 μg/mL).

3. Results and Discussion

3.1. Transformation of sCTB gene and PCR analysis

The cholera toxin B subunit gene was previously expressed in transgenic tomato fruit with 0.04 and 0.081% of total soluble protein [5,17]. In this study, the synthetic CTB (sCTB), which was modified based on the optimized codon usage in a plant, was introduced into tomato to improve the protein expression level. Six plants resulting from independent transformation events were selected and maintained under *in vitro* conditions. The presence of the sCTB gene in transgenic plants was confirmed by genomic DNA PCR amplification, followed by gel electrophoresis of the PCR products (Fig. 2). The expected size (414 bp) of each PCR product was amplified (lanes 1-6) in all transformed plants and a PCR product with the same size was obtained in the pMYO53 vector as a positive control (PC). No PCR products were detected in wild-type tomato plants (NC).

3.2. Immunoblot analysis of plant-synthesized CTB protein

Total soluble protein (TSP) was extracted from fruit tissues of the transgenic tomato plants (#1 - #6). Commercial bacterial CTB (positive control) was used to detect antibody-specific protein in the transgenic tomatoes and an immunoblot analysis of fruit tissues of transgenic plants revealed an oligomeric CTB protein with a molecular weight of approximately 50 kDa (#1, #2, and #5). Wild-type tomato fruit did not cross-react with the anti-cholera toxin antibody and therefore, the specific signal band corresponding to CTB protein was not detected on the membrane (Fig. 3A). The oligomeric CTB protein dis-

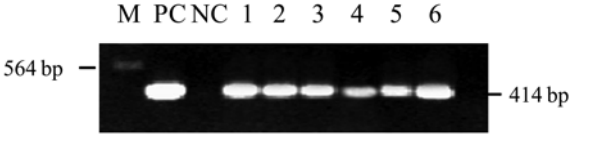

Fig. 2. Genomic DNA PCR amplification analysis for detection of the sCTB gene in the transgenic plant genomes. The genomic DNA of transgenic plants was isolated and used as a template for PCR products to detect the sCTB gene. The PCR products were separated on a 1.0% agarose gel. Lane M, the DNA size marker (λ/*Hind*III); lane PC, the pMYO53 plasmid used as a positive control for PCR; lane NC, the wild-type tomato plant genomic DNA; lanes 1 ~ 6, the transgenic tomato plant genomic DNA used as PCR templates.

sociated into monomers of 15 kDa when the transgenic fruit homogenates were boiled for 10 min (Fig. 3B) and both the multimeric and monomeric forms of the plant-derived CTB had slightly higher molecular weights than the bacterial CTB (50 kDa versus 40 kDa for the pentamers, and 15 kDa versus 12 kDa for the monomers, respectively). This discrepancy was presumably due either to the six extra amino acids that were added at the C-terminus for ER retention (SEKDEL) [18] or to the post translational modification of the plant produced CTB, especially N-glycosylation. An N-glycosylation on the CTB domain and an ER retention signal attached at the C-terminus of the fusion protein has been reported [19]. Our result was similar to that obtained from expression systems in other plants such as tobacco [12] and lettuce [20].

3.3. Quantification of CTB protein in transgenic fruits

An ELISA analysis was performed to measure the expression level of CTB protein in the fruit tissue of transgenic tomato plants (#1, #2, and #5). The percentage of CTB protein in each plant was calculated from TSP used in three replicates of the assay. According to this method, the concentrations of TSP loaded in the microtiter plate wells yielded CTB protein levels of approximately 0.89, 0.77, and 0.08% of the TSP in the fruit tissues of the respective transgenic plants (Fig. 4). Results of earlier experiments [5,17] revealed that the expression level of CTB in tomato fruit was less than 0.081%. Some reports have also shown high levels of sCTB in plants such as lettuce [20], rice [21], and tobacco [22] or in microorganisms such as *Saccharomyces cerevisia*e [23].

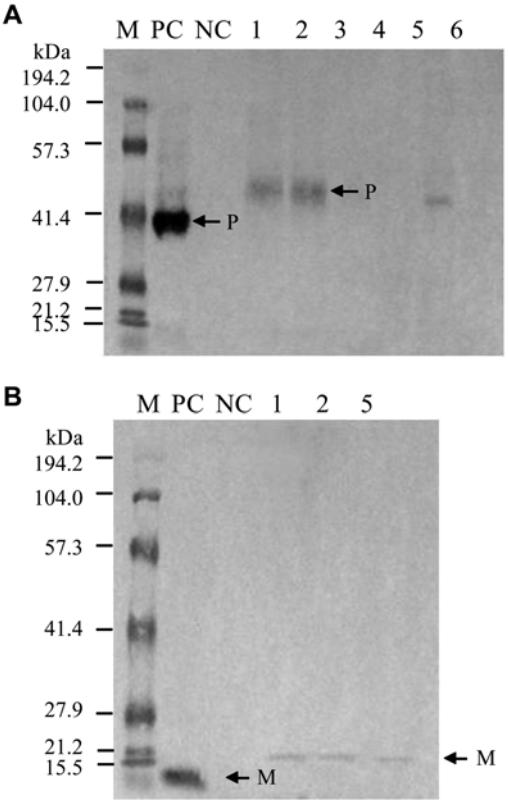

Fig. 3. Western blot analysis of CTB protein in transgenic tomato plants. The protein extracts of fruit tissues from transgenic plants were analyzed for expression of CTB protein using the anti-cholera toxin antibody under unboiled (A) and boiled (B) conditions. Lane M, the protein weight marker; lane PC, commercial bacterial CTB as a positive control; lane NC, protein extract from a wild-type plant; lanes 1 ~ 5, protein extracts from transgenic plants expressing CTB protein.

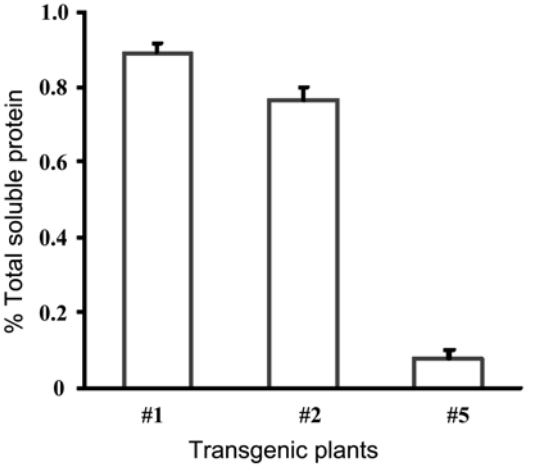

Fig. 4. ELISA quantification of CTB protein produced in transgenic tomato fruit tissues. Plant protein extracts from fruit tissues of transgenic plants and known amounts of commercial bacterial CTB were used to measure the CTB protein expression level. Transgenic plants #1, #2, and #5, which showed a positive signal in western blot analysis, were used to measure the amounts of CTB protein in transgenic tomato fruit tissues. Error bars represent the standard deviations.

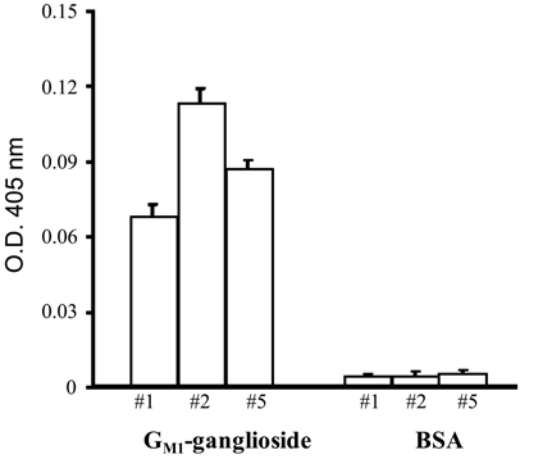

Fig. 5. G_{M1}-ELISA analysis of CTB pentamer formation in transgenic tomato fruit tissues. The binding affinity to the receptor of plant-produced CTB protein was measured using G_{M1}-ELISA in transgenic plants. Transgenic plants #1, #2, and #5, which showed high expression of CTB in western blot analysis, were used to test the binding affinity for G_{M1}-ganglioside. Error bars represent the standard deviations.

3.4. Binding assay of CTB protein to G_{M1}-ganglioside

To study the oligomerization of CTB protein produced in a transgenic plant, its binding ability to the G_{M1}-ganglioside receptor was tested using G_{M1}-ganglioside ELISA. G_{M1}-ganglioside is the receptor for biologically active CTB protein *in vivo*, and a pentameric structure is required for appreciable receptor binding. In the G_{M1}-ELISA binding assays, CTB protein produced in transgenic plants demonstrated a strong afnity for G_{M1}-ganglioside, but not for BSA (Fig. 5). Based on the results from G_{M1}-ELISA, the CTB protein expressed in the two transgenic plants (#1 and #2) was very similar. The strong relative binding efcacy of plant-produced CTB for G_{M1}-ganglioside indicated that a plant-derived CTB subunit interacts with G_{M1}.ganglioside.

4. Conclusion

A modified CTB gene based on the optimized codon usage of the plant was introduced into tomato fruits to increase the protein expression level. The highest expression level was evaluated as 0.89% of total soluble protein, which is 10-fold greater than the previously reported 0.081% [17]. In future experiments, transgenic plants will be fed to animals to examine the immunogenicity of tomato-derived CTB *via* oral administration.

Acknowledgements

This research was supported by a grant from the Higher Education Project of the Vietnam Ministry of Education and Training (2008-2011) and by a grant from the Technology Development Program for Agriculture and Forestry, Ministry for Agriculture, Forestry and Fisheries, Republic of Korea.

References

1. Kaper, J. B., J. G. Morris, and M. Levine (1995) Cholera. *Clin. Microbiol. Rev.* 8: 48-86.
2. Field, M., M. C. Rao, and E. B. Chang (1989) Intestinal electrolyte transport and diarrheal disease. *N. Engl. J. Med.* 321: 800-806.
3. Elson, C. O. (1989) Cholera toxin and its subunits as potential oral adjuvants. *Curr. Top. Microbiol. Immunol.* 146: 29-33.
4. McGhee, J. R., J. Mestecky, M. T. Dertzbaugh, J. H. Eldridge, M. Hirasawa, and H. Kiyono (1992) The mucosal immune system: From fundamental concepts to vaccine development. *Vaccine.* 10: 75-88.
5. Jani, D., L. S. Meena, Q. M. Rizwan-ul-Haq, Y. Singh, A. K. Sharma, and A. K. Tyagi (2002) Expression of cholera toxin B subunit in transgenic tomato plants. *Transgenic Res.* 11: 47-54.
6. Richter, L. J., Y. Thanavala, C. J. Arntzen, and H. S. Mason (2000) Production of hepatitis B surface antigen in transgenic plants for oral immunization. *Nat. Biotechnol.* 18: 1167-1171.
7. Mason, H. S., T. A. Haq, J. D. Clements, and C. J. Arntzen (1998) Edible vaccine protects mice against *Escherichia coli* heat-labile enterotoxin (LT): Potatoes expressing a synthetic LT-B gene. *Vaccine* 16: 1336-1343.
8. Tuboly, T., W. Yu, A. Bailey, S. Degrandis, S. Du, L. Erickson, and E. Nagy (2000) Immunogenicity of porcine transmissible gastroenteritis virus spike protein expressed in plants. *Vaccine* 18: 2023-2028.
9. Streatfield, S. J., J. M. Jilka, E. E. Hood, D. D. Turner, M. P. Bailey, J. M.Mayor, S. L. Woodard, K. K. Beifuss, M. E. Horn, D. E. Delaney, I. R. Tizard, and J. A. Howard (2001) Plant-based vaccines: Unique advantages. *Vaccine* 19: 2742-2748.
10. Pelham, H. R. (1988) Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. *EMBO J.* 7: 913-918.
11. Murashige, T. and F. Skoog (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant* 15: 473-497.
12. Kang, T. J., N. H. Loc, M. O. Jang, and M. S. Yang (2004) Modification of the cholera toxin B subunit coding sequence to enhance expression in plants. *Mol. Breeding* 13: 143-153.
13. Van Haute, E., H. Joos, M. Maes, G. Warren, M. Van Montagu, and J. Schell (1983) Intergeneric transfer and exchange recombination of restriction fragments cloned in pBR322: A novel strategy for the reversed genetics of the Ti plasmids of *Agrobacterium tumefaciens*. *EMBO J.* 2: 411-417.
14. Kang, T. J. and M. W. Fawley (1997) Variable (CA/GT)n simple sequence repeat DNA in the alga *Chlamydomonas*. *Plant Mol. Biol.* 35: 943-948.
15. Bradford, M. M. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal. Biochem.* 72: 248-254.
16. Huy, N. X., Y. S. Kim, S. C. Jun, Z. Jin, S. M. Park, M. S. Yang, and T. G. Kim (2009) Production of a heat-labile enterotoxin B subunit-porcine epidemic diarrhea virus-neutralizing epiyope fusion protein intransgenic lettuce (Lactuca sativa). *Biotechnol. Bioproc. Eng.* 14: 731-737.
17. Jiang, X. J., Z. M. He, Z. Q. Peng, Y. Qi, Q. Chen, and S. Y. Yu (2007) Cholera toxin B protein in transgenic tomato fruit induces systemic immune response in mice. *Transgenic Res.* 16: 169-175.
18. Arakawa, T., D. K. Chong, J. L. Merritt, and W. H. Langridge (1997) Expression of cholera toxin B subunit oligomers in transgenic potato plants. *Transgenic Res.* 6: 403-413.
19. Matoba, N., H. Kajiura, I. Cherni, J. D. Doran, M. Bomsel, K. Fujiyama, and T. S. Mor (2009) Biochemical and immunological characterization of the plant-derived candidate human immunodeficiency virus type 1 mucosal vaccine CTB-MPR. *Plant Biotechnol. J.* 7: 129-145.
20. Kim, Y. S., B. G. Kim, T. G. Kim, T. J. Kang, and M. S. Yang (2006) Expression of a cholera toxin B subunit in transgenic lettuce (*Lactuca sativa* L.) using *Agrobacterium*-mediated transformation system. *Plant Cell Tiss. Org. Cult.* 87: 203-210.
21. Oszvald, M., T. J. Kang, S. Tomoskozi, B. Jenes, T. G. Kim, Y. S. Cha, L. Tamas, and M. S. Yang (2008) Expression of cholera toxin B subunit in transgenic rice endosperm. *Mol. Biotechnol.* 40: 261-268.
22. Kang, T. J., B. G. Kim, J. Y. Yang, and M. S. Yang (2007) Expression of a synthetic cholera toxin B subunit in tobacco using ubiquitin promoter and *bar* gene as a selectable marker. *Mol. Biotechnol.* 32: 93-100.
23. Lim, J. G. and H. S. Jin (2008) Heterologous expression of cholera toxin B subunit in *Saccharomyces cerevisiae*. *Biotechnol. Bioproc. Eng.* 13: 598-605.