# Expression of *Escherichia coli* Heat-labile Enterotoxin B Subunit in Transgenic Tomato (*Solanum lycopersicum* L.) Fruit

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**Abstract**

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We report a feasibility study for expressing the LTB protein (*Escherichia coli* heat-labile enterotoxin B subunit) via *Agrobacterium*-mediated transformation of tomato (*Solanum lycopersicum* L.). We produced five regenerated plants obtained on the selection medium supplemented with an antibiotic. Stable integrations of the LTB gene into the genome of these plants were confirmed by Southern blot hybridization. Western blot analysis showed that only two of the five $T_0$ transgenic tomato plants expressed the pentameric LTB protein in the fruits. An enzyme-linked immunosorbent assay indicated that these two plants synthesized the LTB protein bound specifically to GM1 ganglioside, suggesting that the LTB subunits formed active pentamers. The LTB protein produced in tomatoes can be a potential candidate for inexpensive, safe, and effective plant-based vaccines.

**Keywords**: B subunit of *E. coli* heat-labile enterotoxin (LTB); LTB gene; *Solanum lycopersicum*; plant-based vaccine; tomato fruit; transgenesis

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of diarrhoea in children in developing countries and in travellers (Chen *et al*. 2011). The LTB (*E. coli* heat-labile enterotoxin B subunit) of ETEC is a potent mucosal immunogen and immunoadjuvant for co-administered antigens (Cheng *et al*. 1999; Ryan *et al*. 1999; Millar *et al*. 2001).

Many studies have indicated that LTB could be used as a potent adjuvant (Tochikubo & Yasuda 2000). LTB is highly resistant to proteolytic degradation and retains its pentameric quaternary structure in a pH as low as 2.0 (Rezaee *et al*. 2005). LTB is able to bind to GM1 ganglioside, a glycosphingolipid found ubiquitously on the cell membranes of mammals, and to other related receptors, such as GD1b-ganglioside, asialo-GM1, lactosylceramide and certain galactoproteins (Williams *et al*. 1999).

The tomato is one of the preferred plants for transformation for oral vaccines since its fruits are edible when fresh. Jani *et al*. (2002, 2004) and Loc *et al*. (2010b) successfully transferred the CTB (cholera toxin B subunit) gene controlled by the CaMV 35S promoter into tomato plants, and these transgenic plants expressed the CTB subunit in leaves and fruits, which could specifically bind to the GM1-ganglioside receptor, a special receptor for the CTB subunit.

The purpose of this work was to determine the expression of recombinant LTB in the fruit of transgenic tomatoes. The gene encoding LTB was introduced into tomato (*Solanum lycopersicum* L.) plants, and the LTB protein was found to have a pentameric form with the ability to bind GM1 ganglioside.

## MATERIAL AND METHODS

**Plant material**. Tomato (*Solanum lycopersicum* L. Cv. 311) seeds were germinated under sterile conditions on 1/2MS medium (Murashige & Skoog 1962) consisting of 30 g/l sucrose and 8 g/l agar. After ten days, cotyledons excised from *in vitro*-germinated

seedlings were cultured on MS medium consisting of 30 g/l sucrose and 8 g/l agar and supplemented with 2 mg/l zeatin, 0.1 mg/l indoleacetic acid and 0.1 mg/l thidiazuron for shoot regeneration. Regenerating shoots (3 cm in length) were rooted on MS medium supplemented with 0.5 mg/l indolebutyric acid. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 15 min. All cultures were incubated at 25 ± 2°C with a photoperiod of 10-h day light under the intensity of 35 μmol/$m^2$/s (Loc *et al*. 2010b).

**Construction of plant expression vector**. The plant expression vector used in this work, pMYO51, consisted of a synthetic LTB gene (414 bp) with a signal peptide, Kozak consensus sequence, and the ER retention signal (SEKDEL) controlled by the CaMV 35S promoter (Kang *et al*. 2004) (Figure 1). The pMYO51 vector was introduced into *Agrobacterium tumefaciens* strain LBA 4404 *via* the triparental mating method (Van Haute *et al*. 1983).

**Tomato transformation**. Ten-day-old cotyledons were excised from *in vitro*-germinated seedlings and precultured on the shoot regeneration medium for 2 days. Then, we dropped the *Agro*-suspension onto the cotyledons (two drops onto each cotyledon) with a pipette (Loc *et al*. 2011). After 2 days of co-cultivation, cotyledons were cultured for 10 days on selection medium with 35 mg/l kanamycin (Km) and 200 mg/l cefotaxime (Cef), for 10 days on selection medium with 50 mg/l Km and 200 mg/l Cef, and for 4 weeks on selection medium with 100 mg/l Km and 200 mg/l Cef. After two weeks, the first shoots were regenerated. After 2 months, the *in vitro* shoots were transferred to a rooting medium without antibiotics.

**PCR analysis**. Genomic DNA was isolated from the leaves of $T_0$ transgenic and wild-type tomatoes using the universal and rapid salt-extraction method (Kang & Fawley 1997). PCR amplification was performed using specific primers for the LTB gene: forward 5'-GGATCCGCCACCATGGTGAAGGTGAAG-3' and reverse 5'-GGTACCTCATAGCTCAT-CTTTC-3'. PCR conditions were as follows: genomic denaturation at 95°C for 10 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s; then a final extension at 72°C for 10 min. PCR products were electrophoresed on 0.8% agarose gel and stained with ethidium bromide.

**Southern blot hybridization**. Southern hybridization was conducted as described by Sambrook and Russell (2001). Ten micrograms of genomic DNA were cut with *Kpn*I, separated by 0.8% agarose gel electrophoresis and then transferred to a Hybond-N+ membrane (BioRad, Hercules, USA). The blot was hybridized with a probe of synthetic LTB gene labelled with digoxigenin-dUTP (DIG high prime DNA labelling and detection starter kit I; Roche, Mannheim, Germany) at 46ºC overnight. After hybridization, blots were washed and incubated with antibody-conjugated digoxigenin and alkaline phosphatase (1:5000 v/v) at room temperature for 30 min. The BCIP/NBT (Roche, Mannheim, Germany) substrate is used for colour development.

**Western blot analysis**. The Western blot technique was performed as in Kang *et al*. (2003) with slight modifications. Proteins were extracted from the fruit tissue (about 0.5 g) of LTB transgenic tomatoes in 1 ml of extraction buffer (50mM HEPES, pH 7.5, 10mM potassium acetate, 5mM magnesium acetate, 1mM EDTA, 1mM dithiothreitol, and 2mM phenylmethanesulfonyl fluoride). Then, 20 micrograms of extract, which was determined by the Bradford protein assay (1976), were separated by 12% SDS-PAGE. Purified bacteria LTB (0.5 μg) were loaded as the positive control. The separated protein bands were transferred from the gel to a Hybond C membrane (BioRad, Hercules,

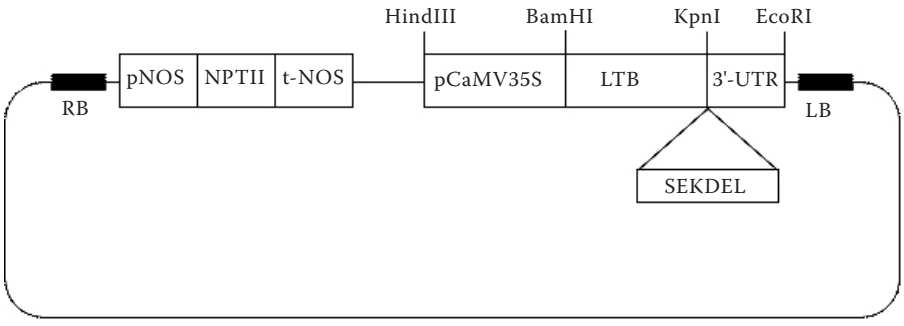


Figure 1. The structure of plant expression vector pMYO51 used for tomato transformation; an NPT II (neomycin phosphotransferase II) gene expression cassette was used for kanamycin (Km) selection of transgenic plants; LB and RB are the left and right borders of the T-DNA sequence, respectively; pNOS and t-NOS are the promoter and terminator of the nopaline synthase gene, respectively

USA) using a Trans-blot® SD Semi-Dry Transfer Cell (BioRad) at 15 V for 30 min. Nonspecific antibody reactions were blocked by incubating the blot in 25 ml of 3% (w/v) bovine serum albumin (BSA) in TBST buffer (TBS + 0.05% Tween-20) with gentle agitation overnight at room temperature (RT). The blot was then incubated at RT for 2 h with gentle agitation in 10 ml of 1:2000 dilution of rabbit anti-LTB antiserum (Immunology Consultants Lab. Inc., Portland, USA) in TBST antibody dilution buffer containing 1.5% BSA and then washed three times with TBST buffer. Subsequently, the blot was incubated for 2 h at RT in a 1:5000 dilution of anti-rabbit IgG conjugated with alkaline phosphatase (Promega S3731, Madison, USA) in TBST buffer and washed three times with TBST buffer and once with TMN buffer. After washing, the colour was developed with BCIP/NBT in TMN buffer.

**LTB-GM1 binding assay**. The ability of LTB protein to bind to gangliosides was determined according to Kang *et al.* (2003). The microwell plate (Becton Dickinson Labware, Franklin Lakes, USA) was coated with 100 µl/well of 3 µg/ml GM1 ganglioside (Sigma-Aldrich, St. Louis, USA) in bicarbonate buffer, pH 9.6 at 4°C overnight. After three washes with PBST, the wells were blocked with 1% BSA in 0.01M PBS (300 µl/well) at 37°C for 2 h. The wells were washed three times with PBST and then incubated with the protein extract (100 µl/well) from the LTB transgenic tomato for 2 h at 37°C.

For the primary and secondary antibody treatments, the wells were incubated with a 1:5000 dilution of rabbit anti-LTB antibody (Immunology Consultants Lab. Inc., Portland, USA) (100 µl/well) in 0.01M PBS containing 0.5% BSA for 2 h at 37°C and washed four times with PBST. Subsequently, the wells were incubated with a 1:10000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma-Aldrich, St. Louis, USA) (100 µl/well) in 0.01M PBS containing 0.5% BSA for 2 h at 37°C and washed four times with PBST. Finally, the plate was incubated with 100 µl/well TMB substrates (Pharmingen 2606KC and 2607KC, San Diego, USA) for 30 min at RT in the dark. After incubation, the reaction was measured at an absorbance of 405 nm in an automated ELISA system (CODA Automated EIA Analyzer; BioRad, Hercules, USA). As a control, the wells were coated with 100 µl/well of 3.0 µg/ml BSA.

## RESULTS AND DISCUSSION

**Detection of transgene in transformed plants**. Five regenerated tomato plants from independent transformation events were grown under *in vivo* conditions (Figure 2). The presence of the LTB gene

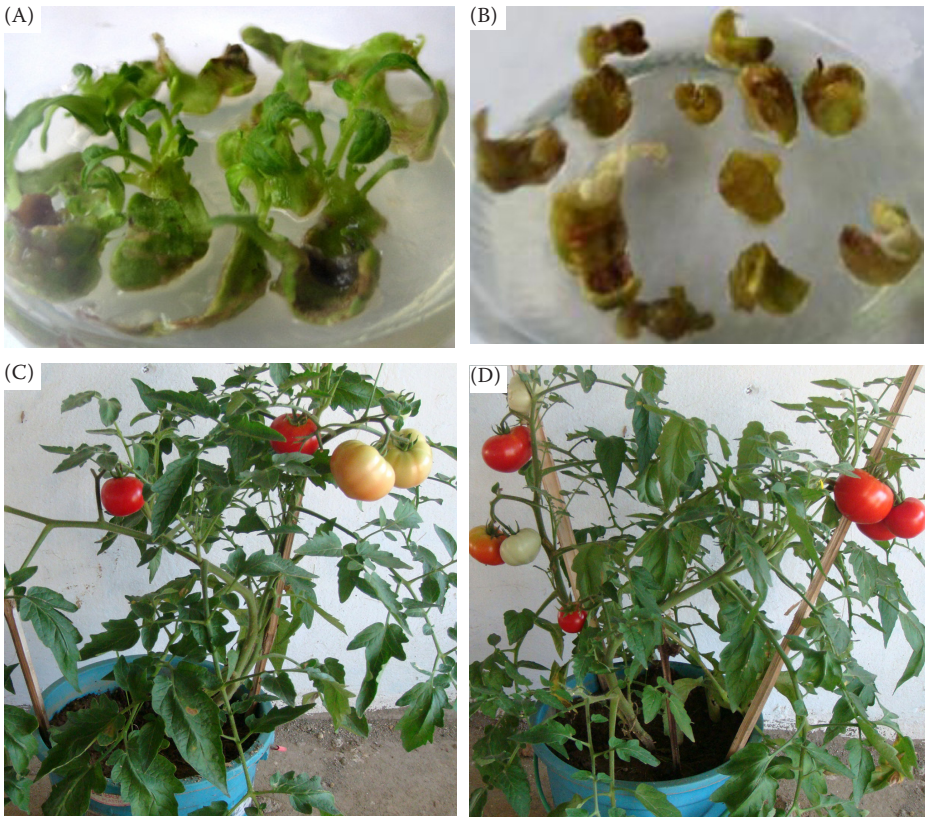


Figure 2. *In vitro* shoot regeneration from an LTB-transformed cotyledon disc on selection medium containing 100 mg/l kanamycin (A), non-transformed cotyledon disc on selection medium containing 100 mg/l kanamycin (B), LTB-transgenic plant (C) and wild-type plant (D) were grown in the pot

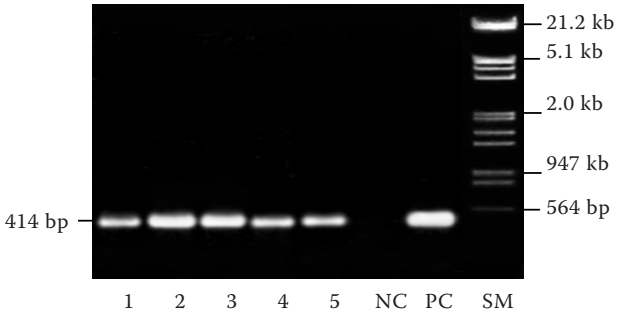


Figure 3. Genomic DNA PCR amplification to confirm the LTB gene in transgenic tomato plants SM – DNA size marker (λ DNA/*Eco*RI+*Hin*dIII), PC – pMYO51 vector, NC – non-transgenic tomato plant, lanes 1–5 – LTB transgenic tomato plants

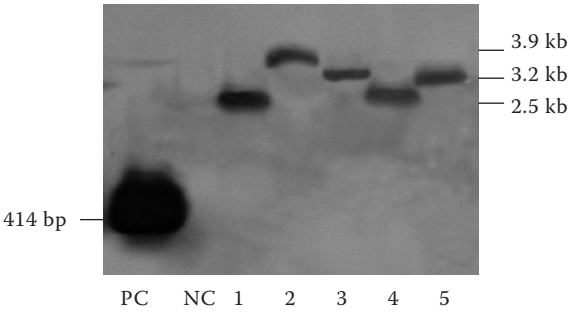


Figure 4. Southern blot analysis; PC – LTB gene; NC – wild-type tomato plant; lanes 1–5 – LTB transgenic tomato plants

in the genomic DNA was determined by PCR analysis. All transformed tomato plants were confirmed to have the exogenous LTB gene of an expected size of 414 bp (Figure 3). Southern hybridization was also conducted and showed the integration of the LTB gene into the genome of these plants. The results of Southern blot showed that the transgenic plants contained only one copy of the LTB gene with the hybridization signals of approximately 2.5–3.9 kb, reflecting the presence of the LTB gene in their genome (Figure 4). Some studies showed many copies of the transferred gene present in the genome of plants, e.g. *ctyIIa3* gene in watercress (Jin *et al*. 1999) and LTB gene in maize (Chikwamba *et al*. 2002). However, Jiang *et al*. (2007) found only one copy of the CTB gene in the tomato genome.

**Detection of LTB protein in fruits of transgenic plants**. Fruits of five transgenic tomato plants were used for LTB protein characterization. The total soluble proteins (TSPs) were extracted from fruits of the individual transgenic tomato plants. Western blot analysis of these fruits detected a pentameric LTB protein with a molecular weight of about 45 kDa (Figure 5A). This result is similar to those obtained from different plant expression systems, such as those of tobacco (Kang *et al*. 2003), lettuce (Kim *et al*. 2007), *Peperomia pellucida* (Loc *et al*. 2010a), and watercress (Loc *et al*. 2011). However, there were only fruits of two transgenic plants expressing the LTB protein (#1 and #3). Western blot analysis of leaves from the above five transgenic plants revealed that three of them express the LTB protein (#1, #2 and #3) (data not shown). The oligomeric LTB protein of two plants, #1 and #3, dissociated into monomers of 11.6 kDa when the TSP extracts were boiled for 10 min (Figure 5B). The other transgenic plants and wild-type fruit did not cross-react with the LTB antibody.

ELISA was used to estimate the amount of LTB expressed in fruit tissues of two transgenic tomato plants (#1 and #3). The percentage of LTB protein in each fruit was calculated from the TSP used in three replicates of the assay. The results showed that the concentrations of TSP loaded in the microtitre plate wells yielded LTB protein levels of 1.04% (#1) and 1.19% (#3) of TSPs in the fruit of transgenic tomato plants (Loc *et al*. 2012). Based on the results of the

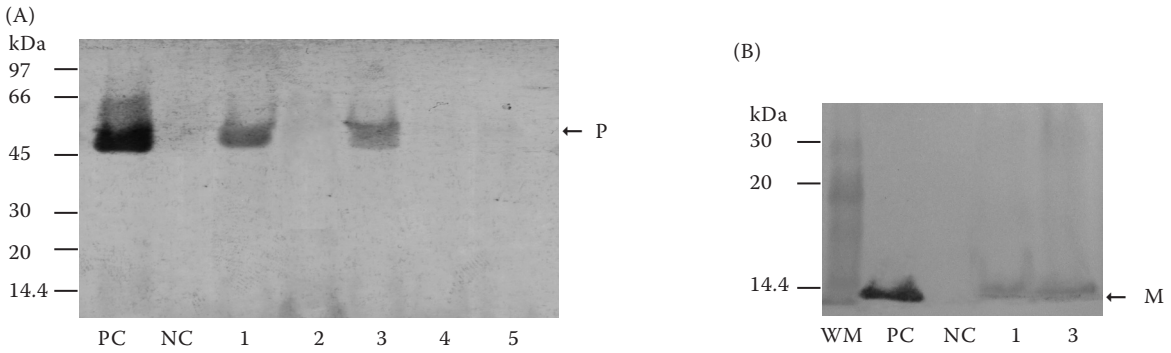


Figure 5. Western blot analysis of LTB protein in fruits of transgenic tomato plants under the unboiled (A) and boiled (B) conditions; WM – protein weight marker (14.4–97 kDa); PC – purified bacteria LTB protein; NC – wild-type tomato plant; lanes 1–5 (A), 1 and 3 (B) – LTB transgenic plants; P – pentameric form; M – monomeric form

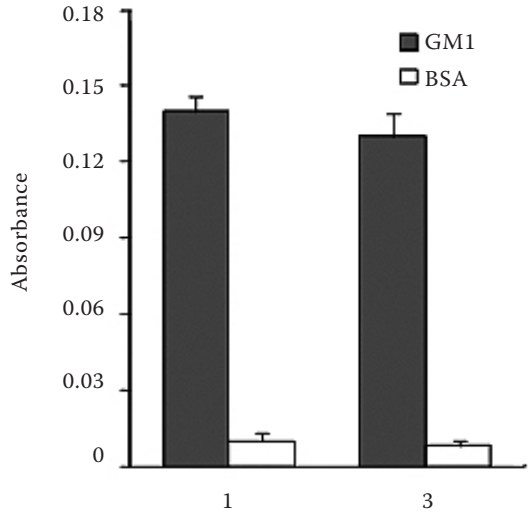


Figure 6. GM1-ELISA analysis of LTB pentamer formation in fruit tissues of two transgenic tomato; columns 1 and 3 – LTB transgenic plants (mean ± SE, calculated from three repetitions); GM1 – ganglioside; BSA – bovine serum albumin

ELISA and Western blot, 1 g of fruit tissue (fresh weight) from the transgenic tomato plants contained approximately 14–16 μg of recombinant plant-synthesized LTB protein. In other study, we found that the amount of LTB protein of *in vitro* transgenic tomato leaves was approximately 0.8–1.0% of TSPs (Nhi *et al*. 2010). Walmsley *et al*. (2003) found that the LTB protein of transgenic tomato fruit was assembled into pentamers. It showed an average expression level of 37.8 μg/g in freeze-dried transgenic tissues.

**Binding assay of LTB protein to GM1-ganglioside**. GM1-ganglioside has been shown to be the receptor for biologically active LTB protein, and the LTB pentameric structure is required for appreciable receptor binding. In the GM1-ELISA binding assays, LTB protein produced in fruits of two transgenic tomato plants (#1 and #3) demonstrated a strong relative affinity to GM1-ganglioside but not to BSA (Figure 6). Based on the absorbance measurement used to determine GM1 binding, the LTB protein expressed in the fruits of two transgenic tomato plants was similar: 0.14 in #1 and 0.13 in #3. Other reports have also found the equivalent ability of LTB for GM1 such as in the *Peperomia pellucida* plant (0.14–0.15) (Loc *et al*. 2010a) and *watercress plant (*0.08-0.24) (Loc *el al*. 2011). Moravec *et al*. (2007) showed a stronger binding ability of LTB protein from transgenic soybean seed to GM1-ganglioside (approximately 0.35). The strong relative binding efficacy of plant-produced LTB for GM1 indicates that the plant-derived LTB subunit can interact with GM1.

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## References

Bradford M.M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Analytical Biochemistry, **72**: 248–254.

Chen S., Wang X., Cheng Z. (2011): Construction of PVX virus-expression vector to express enterotoxin fusion gene *LTB-ST* and transformation into tobacco. African Journal of Biotechnolology, **10**: 1022–1028.

Cheng E., Cardenas-Freytag L., Clements J.D. (1999): The role of cAMP in mucosal adjuvanticity of *Escherichia coli* heat-labile enterotoxin (LT). Vaccine, **18**: 38–49.

Chikwamba R., Mcmurray J., Shou H., Frame B., Pegg S.E., Scott P., Manson H., Wang K. (2002): Expression of a synthetic *E.coli* heat-labile enterotoxin B subunit (LT-B) in maize. Molecular Breeding, **10**: 253–265.

Jani D., Meena L.S., Rizwan-Ul-Haq Q.M., Singh Y., Sharma A.K., Tyagi A.K. (2002): Expression of cholera toxin B subunit in transgenic tomato plants. Transgenic Research, **11**: 47–54.

Jani D., Singh N.K., Bhattacharya S., Meena L.S., Singh Y., Upadhyay S.N., Sharma A.K., Tyagi A.K. (2004): Studies on the immunogenic potential of plant-expressed cholera toxin B subunit. Plant Cell Reports, **22**: 471–477.

Jiang X.L., He Z.M., Peng Z.Q., Qi Y., Chen Q., Yu S.Y. (2007): Cholera toxin B protein in transgenic tomato fruit induces systemic immune response in mice. Transgenic Research, **16**: 169–175.

Jin R.G., Liu Y.B., Tabashnik B.E., Borthakur D. (1999): Tissue culture and *Agrobacterium*-mediated transformation of watercress. Plant Cell, Tissue and Organ Culture, **58**: 171–176.

Kang T.J., Fawley M.W. (1997): Variable (CA/GT)n simple sequence repeat DNA in the alga *Chlamydomonas*. Plant Molecular Biology, **35**: 943–948.

Kang T.J., Han S.C., Jang M.O., Kang K.H., Jang Y.S., Yang M.S. (2004): Enhanced expression of B subunit of *Escherichia coli* heat-labile enterotoxin in tobacco by optimization of coding sequence. Applied Biochemistry and Biotechnology, **117**: 175–187.

Kim T.G., Kim M.Y., Kim B.G., Kang T.J., Kim Y.S., Jang Y.S., Arntzen C.J., Yang M.S. (2007): Synthesis and assembly of *Escherichia coli* heat-labile enterotoxin B subunit in transgenic lettuce (*Lactuca sativa*). Protein Expression and Purification, **51**: 22–27.

Loc N.H., Bach N.H., Kim T.G., Yang M.S. (2010a): Tissue culture and expression of *Escherichia coli* heat-labile

enterotoxin B subunit in transgenic *Peperomia pellucida*. Protein Expression and Purification, **72**: 82–86.

Loc N.H., Thinh L.T., Kim T.G., Yang M.S. (2010b): High expression of cholera toxin B subunit in transgenic tomato (*Lycopersicon esculentum* L.) fruits. Biotechnology and Bioprocess Engineering, **16**: 576–580.

Loc N.H., Song N.V., Tien N.Q.D., Minh T.T., Nga P.T.Q., Kim T.G., Yang M.S. (2011): Expression of the *Escherichia coli* heat-labile enterotoxin B subunit in transgenic watercress (*Nasturtium officinale* L.). Plant Cell, Tissue and Organ Culture, **105**: 39–45.

Loc N.H., Long D.T., Nhi P.Y., Hong H.T.K., Kim T.G., Yang M.S. (2012): Preliminary evaluation of transgenic tomato plants expressing *Escherichia coli* heat-labile toxin B subunit grown under *in vivo* condition. Annals of Biological Research, **3**: 2070–2073.

Millar D.G., Hirst T.R., Snider D.P. (2001): *Escherichia coli* heat-labile enterotoxin B subunit is a more potent mucosal adjuvant than its closely related homologue, the B subunit of cholera toxin. Infection and Immunity, **69**: 3476–3482.

Murashige T., Skoog F. (1962): A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum, **15**: 473–497.

Moravec T., Schmidt M.A., Herman E.M., Woodford-Thomas T. (2007): Production of *Escherichia coli* heat labile toxin (LT) B subunit in soybean seed and analysis of its immunogenicity as an oral vaccine. Vaccine, **25**: 1647–1657.

Nhi P.Y., Kim T.G., Yang M.S., Loc N.H. (2010): Expression of *Escherichia coli* heat-labile enterotoxin B subunit in transgenic tomato (*Lycopersicon esculentum* L.). Vietnam Journal of Biotechnology, **8**: 1279–1285.

Ryan E.T., Crean T.I., John M., Butterton J.R., Clements J.D., Calderwood S.B. (1999): *In vivo* expression and immunoadjuvancy of a mutant of heat-labile enterotoxin of *Escherichia coli* in vaccine and vector strains of *Vibrio cholerae*. Infection and Immunity, **67**:1694–1701.

Sambrook J., Russell D.W. (2001): Molecular Cloning: A Laboratory Manual. 3rd Ed. Cold Spring Harbor Laboratory, New York.

Tochikubo K., Yasuda Y. (2000): Principles of mucosal immunity and development of mucosal vaccines using cholera toxin B subunit and its related adjuvants. Recent Research Developments in Microbiology, **4**: 387–405.

Van Haute E., Joos H., Maes M., Warren G., Van Montagu M., Schell J. (1983): Intergeneric transfer and exchange recombinant of restriction fragments cloned in pBR322: A novel strategy for the reversed genetics of the Ti plasmids of *Agrobacterium tumefaciens*. The EMBO Journal, **2**: 411–417.

Walmsley A.M., Alvarez M.L., Jin Y., Kirk D.D., Lee S.M., Pinkhasov J., Rigano M.M., Arntzen C.J., Mason H.S. (2003): Expression of the B subunit of *Escherichia coli* heat-labile enterotoxin as a fusion protein in transgenic tomato. Plant Cell Reports, **21**: 1020–1026.

Williams N.A., Hirst T.R., Nashar T.O. (1999): Immune modulation by the cholera-like enterotoxins: from adjuvant to therapeutic. Immunology Today, **20**: 95–101.

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