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Synthesis and assembly of *Escherichia coli* heat-labile enterotoxin B subunit in transgenic lettuce (*Lactuca sativa*)

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Abstract

Escherichia coli heat-labile enterotoxin B subunit (LTB) strongly induces immune responses and can be used as an adjuvant for co-administered antigens. Synthetic LTB (sLTB) based on optimal codon usage by plants was introduced into lettuce cells (*Lactuca sativa*) by *Agrobacterium tumefaciens*-mediated transformation methods. The sLTB gene was detected in the genomic DNA of transgenic lettuce leaf cells by PCR DNA amplification. Synthesis and assembly of the sLTB protein into oligomeric structures of pentameric size was observed in transgenic plant extracts using Western blot analysis. The binding of sLTB pentamers to intestinal epithelial cell membrane glycolipid receptors was confirmed by G_{M1}-ganglioside enzyme-linked immunosorbent assay (G_{M1}-ELISA). Based on the results of ELISA, sLTB protein comprised approximately 1.0–2.0% of total soluble protein in transgenic lettuce leaf tissues. The synthesis and assembly of sLTB monomers into biologically active oligomers in transgenic lettuce leaf tissues demonstrates the feasibility of the use of edible plant-based vaccines consumed in the form of raw plant materials to induce mucosal immunity.

Keywords: *Escherichia coli* heat-labile enterotoxin B subunit; Edible vaccine; Lettuce; Mucosal immunization

Enterotoxigenic *Escherichia coli* and *Vibrio cholera* are the leading causes of diarrhea in infants and travelers in developing countries. The diseases that these bacteria cause can be extremely debilitating and may be fatal in the absence of treatment [1,2]. The heat-labile toxin (LT)[1] of enterotoxigenic *E. coli* and the cholera toxin (CT) of *V. cholera* are representative of the heteromultimeric AB toxins produced by a number of bacterial pathogens; these toxins are the most effective enterocyte-targeting molecules [3]. The *E. coli* heat-labile enterotoxin B subunit (LTB) and cholera toxin B subunit (CTB) were shown to function effective carriers and adjuvants for genetically linked foreign proteins [4,5] and were found be immunogenic in mice [6,7] and humans [8] when expressed in plants. Although LTB and CTB showed only 20% difference in their nucleotide and amino acid sequences, they can be distinguished by their individual biochemical and immunological properties. The LTB binds to a broader receptor population on mammalian cells than CTB, which binds only to ceramide-galactose sugar receptor molecules such as G_{M1} ganglioside [9]. Thus, the LTB has shown a higher capacity to function as a potent mucosal adjuvant than CTB when co-administered to mice intranasally with hen-egg lysozyme [10].

Throughout the last decade, genetically engineered plants have been increasingly used as vehicles for edible vaccines that protect against a wide variety of human infectious and autoimmune diseases [11–17]. The most realistic mucosal plant-based vaccine candidates will be inexpensive to produce, easy to store, safe from contamination of animal pathogens, and without the risk of needle-associated injury and disease spread. However, the expression level of vaccine

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[1] *Abbreviations used:* LT, heat-labile toxin; CT, cholera toxin; TSP, total soluble protein.

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protein antigens in genomically-based transgenic plants is only 0.001–0.3% of total soluble protein [18], which potentially limits the extent of the immune response. To generate greater levels of immunity, alternative strategies must be developed, such as the use of adjuvants to stimulate immune responses to the antigen or the targeting of available antigen molecules to the mucosal immune system. When LTB was expressed in transgenic potatoes and administered to mice, it stimulated production of LTB-specific serum and mucosal antibody responses to a substantial extent, providing partial protection against LT [13]. Although it has been reported that LTB and CTB have been produced successfully in tobacco and potato plants, these plants are not suitable to be administered by consumption in the raw form. Expression of the antigen in an edible uncooked plant is desirable. In this study, the capacity of a transgenic lettuce plant to synthesize sLTB and to assemble the sLTB protein into a pentameric structure was investigated.

Materials and methods

Construction of plant expression vector

The sLTB was synthesized based on optimized codon usage of a plant using overlap PCR strategy [19]. The plant expression vector used in this study, pMYO51, consists of a synthetic LTB, a signal peptide, and the ER retention signal (SEKDEL), under the control of Cauliflower Mosaic Virus 35S promoter (CaMV 35S). The plant expression vector was introduced into *Agrobacterium tumefaciens* strain LBA4404 by the tri-parental mating method [20], and the presence of plasmid DNA in the transformed *Agrobacterium* cells was confirmed by restriction endonuclease digestion and agarose-gel electrophoresis prior to transformation of lettuce [19].

Plant transformation of lettuce

Seeds of lettuce plants of the species, *Lactuca sativa*, were germinated under sterile conditions in Magenta GA-7 culture boxes (Sigma, St. Louis, MO) on Murashige and Skoog (MS) basal medium [21] containing 3.0% sucrose and 0.2% Gelrite at 25 °C. The cotyledon explants were transformed by incubation for 15 min with *A. tumefaciens* harboring pMYO51 [22]. After blotting the explants on sterile filter paper, they were transferred to MS basal solid medium (pH 5.7) containing plant growth regulators, 0.1 μg/ml naphthaleneacetic acid (NAA), and 0.5 μg/ml 6-benzylamino purine (BA), and were incubated in the dark for 2 d at 25 °C. 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 regenerate into calli on the selective medium for 2–3 weeks. After 3–6 weeks of further incubation in a 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 for maturation.

Detection of the sLTB gene in transgenic plants

Genomic DNA was isolated from non-transgenic and 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. The presence of the sLTB gene in transgenic lettuce genomic DNA (400 ng) was determined by PCR analysis using the primer set specific for the sLTB gene; the forward primer was 5′-GGATCCGCCACCATGGTGAAGGTG AAG-3′ and the reverse primer was 5′-GGTACCTCATA GCTCATCTTTC-3′. Amplification was performed by a process of denaturing at 94 °C for 10 min, followed by 30 cycles of 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 10 min. The PCR products were separated by electrophoresis in a 1.0% agarose gel.

Northern blot analysis

Total RNA was isolated from non-transgenic and transgenic lettuce leaf tissues using Trizol Reagent (Invitrogen 15596-026, Carlsbad, CA) and was separated by electrophoresis through agarose gel containing formaldehyde [23]. The separated RNA was then transferred to Hybond N^+ membrane (Amersham Pharmacia Biotech RPN82B, Piscataway, NJ). The membrane was hybridized with a [^{32}P]-labeled sLTB probe using Prime-a-Gene labeling system (Promega U1100, Madison, WI) at 65 °C in a Hybridization Incubator (FINEPCR Combi-H, Seoul, Korea). The membrane was washed twice with 2 × SSC and 0.1% SDS, and was then washed two more times with 2 × SSC and 1% SDS for 15 min at 65 °C. Hybridized bands were detected by autoradiography using X-ray film (Fuji HR-G30, Tokyo, Japan).

Detection of sLTB protein in transgenic lettuce tissues

Transgenic lettuce leaf tissues were analyzed for the presence of sLTB protein using immunoblot detection methods. Transgenic leaf tissues were homogenized by grinding in a mortar and pestle while liquid nitrogen was added 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, 0.05% Tween-20). Tissue homogenates were centrifuged at 17,000*g* in a Beckman GS-15R centrifuge for 15 min at 4 °C 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 12% sodium dodecylsulfate polyacrylamide gel electrophoresis