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Enhanced Expression of B-Subunit of *Escherichia coli* Heat-Labile Enterotoxin in Tobacco by Optimization of Coding Sequence

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Abstract

Escherichia coli heat-labile toxin (LT) is a potent mucosal immunogen and immunoadjuvant for coadministered antigens. We synthesized a gene encoding the B-subunit of LT (LTB) adapted to the coding sequence of tobacco plants and fused to the endoplasmic reticulum retention signal SEKDEL to enhance its level of expression in plants. The synthetic LTB gene was cloned into a plant expression vector adjacent to the CaMV 35S promoter and was introduced into tobacco by *Agrobacterium*-mediated transformation. The amount of LTB protein detected in transgenic tobacco leaves was 2.2% of the total soluble plant protein, which is approx 200-fold higher than in previous reports of native LTB gene expression in transgenic plants. Enzyme-linked immunosorbent assay indicated that plant-synthesized LTB protein bound specifically to G_{M1}-ganglioside, suggesting that the LTB subunits formed active pentamers.

Index Entries: Codon optimization; *Escherichia coli* heat-labile toxin; gene expression; *Nicotiana tabacum*; synthetic gene.

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) causes diarrheal diseases by producing one or more enterotoxins. Among children under the age of 5 in developing countries, ETEC is responsible for more than 650 million

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cases of diarrhea, resulting in about 800,000 deaths each year *(1)*. One of these enterotoxins is a heat-labile toxin (LT) that is related both immunologically and physicochemically to cholera toxin *(2,3)*. LT is composed of one copy of the A-subunit, which has adenosine 5'-diphosphate–ribosylation activity, and a homopentamer of B-subunits, which binds to G_{M1} gangliosides at the surface of eukaryotic cells. The homopentamer of LT consists of five identical 103 amino acid (11.6-kDa) B peptides (LTB) *(4)* that form a donut-shaped pentamer through noncovalent associations *(5)*. LTB is a potent mucosal immunogen and induces systemic and mucosal responses following administration to mucosal surfaces *(6,7)*. LTB also has strong adjuvant activity as a carrier protein for coadministered unrelated antigens *(8)*.

Oral delivery of vaccines is an attractive alternative to injection, owing largely to the low cost and ease of administration. In addition, plant-based vaccines are especially attractive because plants are free of human and animal diseases, thus reducing the costs of screening for viruses and bacterial toxins. In addition, the chances of acquiring mucosal immunity against infectious agents that enter the body across a mucosal surface are increased with oral vaccines *(9)*. Therefore, LTB has been expressed in tobacco *(10)*, potato *(11,12)*, and maize *(13,14)*. Although LTB protein expressed in plants produced oral immunization in mice *(11,13,14)* and in humans *(15)* when consumed as food, the expression levels were too low for large-scale oral administration, i.e., 0.01% of total soluble protein (TSP) in tobacco *(10)*, 0.19% of TSP in the potato tubers *(11)*, and 0.01% of TSP in the R_1 kernels of maize *(14)*. Expression levels need to be increased for practical use as an edible vaccine.

To increase the level of LTB expression in plants, several factors must be considered. First, because LTB is correctly assembled in plant cells, it is likely targeted via its cleavable amino-terminal signal peptide to the endoplasmic reticulum (ER), where the essential enzymes that catalyze folding and assembly reside. It has been shown previously that LTB accumulated in potato tubers to higher levels when it was fused to the ER retention signal SEKDEL and that oral immunization developed in mice that were fed the transgenic potatoes *(10)*. Second, the Kozak sequence is also important for increasing the expression level in eukaryotic cells. The optimal context consists of the sequence GCCACC in front of the start codon *(16)*. The purine (A or G) three bases before the AUG codon and the G immediately following it are the most critical nucleotides and can increase the efficiency of translation 10-fold *(17)*. Third, a high A + U bias in the corresponding mRNA can hamper a high level of gene expression in plants. For example, the *Bacillus thurigiensis cryIA(b)* toxin gene, a gene with a high A + U bias, required complete modification of the coding sequence to achieve strong expression in transgenic tomato and tobacco plants *(18)*. The synthetic *B. thurigiensis cryIA(b)* gene that had an optimized codon usage and lacked AUUUA sequences and potential plant polyadenylation sites produced up to 100-fold higher protein levels than the wild-type (WT) gene.

Here, we report the expression of an LTB gene in tobacco plants. Our goal was to increase the expression level of LTB in plants. Toward this

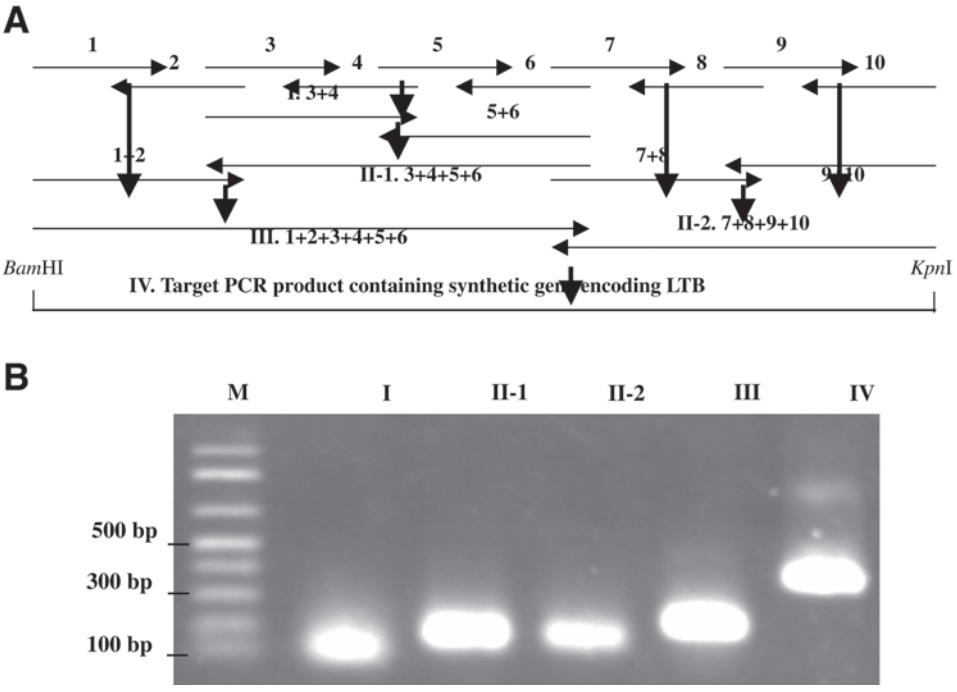

Fig. 1. Stepwise assembly to prepare 464-bp PCR product containing synthetic LTB gene. **(A)** PCR overlap extension strategy used for synthesizing optimized coding sequence of synthetic LTB gene using 10 long oligonucleotides. Each downward arrow represents one PCR reaction with the two templates and two primers above the arrow. **(B)** Agarose gel electrophoresis of products of separate reactions. M, 1.0-kb DNA ladder.

goal, we synthesized an LTB gene containing an adjusted coding sequence and Kozak and SEKDEL sequences and used it to construct a plant expression vector. Transgenic tobacco plants expressing the LTB gene were generated, and G_{M1}-binding analysis was performed to confirm that the expressed LTB had a functional pentameric structure.

Materials and Methods

Construction of Plant-Optimized Synthetic LTB Gene

The design of the synthetic LTB gene was based on the coding sequence of the *E. coli* heat-labile enterotoxin gene (GenBank Locus ABO11677). The synthetic gene has the same amino acid sequence as the *E. coli* gene except that the second codon was changed from AAT, which encodes Asn, to GTG, which encodes Val, to enhance gene expression with the Kozak sequence *(17)*. Potential mRNA processing and destabilizing motifs were removed, and the codon usage pattern for tobacco was taken from http://www.kazusa.or.jp/codon/. Ten oligonucleotides (57 nucleotides [nt] each) were synthesized by a commercial provider (Genotech, Korea). The gene was constructed by stepwise assembly (Fig. 1) carried out

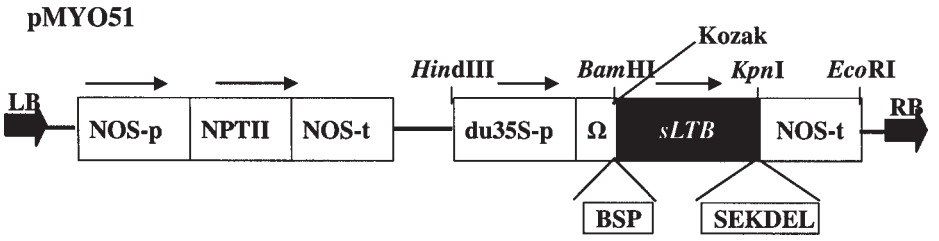

Fig. 2. Structure of binary vector pMYO51 for expression of LTB. LB, left T-DNA border; RB, right T-DNA border; NOS-p, nos promoter; NPTII, neomycin phosphotransferase gene; NOS-t, nos terminator; du35S-p, duplicated cauliflower mosaic virus 35S promoter; Ω, TMV omega-prime leader; BSP, bacterial signal peptide from LT; SEKDEL, ER-retention sequence motif; sLTB, synthetic LTB gene.

in a 25-µL volume of a polymerase chain reaction (PCR) mixture containing 10 pmol of oligonucleotide, 0.5 µ*M* dNTP, standard pfu DNA polymerase buffer, and 0.6 U of pfu Turbo DNA Polymerase (Stratagene). Conditions for the assembly reactions were as follows: initial denaturation at 94°C for 3 min followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 7 min. For stepwise assembly, five reactions were carried out in the first round of PCR. For the second round, 1 µL of the previously amplified PCR product was used as the template. The Kozak sequence, GCCACC, was introduced in front of the start codon of the synthetic LTB coding sequence using a suitable primer. Restriction enzyme sites for *Bam*HI and *Kpn*I were introduced into the 5' and 3' ends of the synthetic LTB gene, respectively, to facilitate further subcloning steps. The final product was amplified using ExTaq polymerase (Takara, Japan) and cloned into the pGem T-Easy vector (Promega, Madison, WI), yielding plasmid pMYO50. To ensure that the gene had been amplified without introducing nucleotide errors, both strands were sequenced using primers specific for the T7 and SP6 promoters.

Construction of a Binary Plant Expression Vector

The synthetic LTB gene fragment was removed from pMYO50 by digestion with *Bam*HI and *Kpn*I and was inserted into the plant expression vector pMY27 *(19)* downstream of the CaMV 35S promoter and the TMV omega-prime leader (the transcriptional and translational enhancer) and upstream of the nopaline synthase (NOS) terminator, yielding pMYO51 (Fig. 2). The ligation reaction mixture was used to transform *E. coli* strain TOP10 (Invitrogen, Carlsbad, CA), and kanamycin-resistant colonies were isolated after overnight incubation at 37°C. Native LTB gene was expressed in the *E. coli* M15 cell, and the recombinant LTB protein was purified using a QIAexpress System (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Plant Transformation

Nicotiana tabacum L. cv TI560 plants were cultured under a 16-h light/8-h dark photoperiod at 25°C on Murashige and Skoog (MS) medium (Sigma, St. Louis, MO) supplemented with 0.7% Bacto-agar and 3% sucrose. The binary plant expression vector pMYO51 was transformed into *Agrobacterium tumefaciens* LBA 4404 along with the helper plasmid pRK2013 using the triparental mating method. The *Agrobacterium* clone that contained the synthetic LTB gene was propagated under selection pressure and used to infect tobacco plants. The tobacco leaves were cut into 0.5- to 1-cm^2 sections and placed for 10 min in a culture dish that contained a suspension of $2–5 \times 10^9$ cells/mL of the *A. tumefaciens* LBA4404 strain that harbored the synthetic LTB gene. The leaf sections were then blotted on sterile filter paper and cocultivated on MS medium supplemented with 0.1 mg/L of α-naphthaleneacetic acid (NAA) and 1.0 mg/L of 6-benzylaminopurine (BAP) for 48 h at 28°C in the dark. After cocultivation, the explants were transferred into MS medium supplemented with 0.1 mg/L of NAA, 1 mg/L of BAP, 300 mg/L of kanamycin, and 500 mg/L of cefotaxime. The explants were transferred into fresh medium every 2 wk. The developed shoots were transferred into hormone-free MS medium containing 300 mg/L of kanamycin and 500 mg/L of cefotaxime to induce root formation.

Detection of LTB Gene in Transgenic Plants

Plant DNA was extracted from the leaf tissues of transgenic plants according to the method described by Kang and Fawley *(20)*. The presence of the LTB gene was confirmed by PCR analysis. Forward and reverse primers (i.e., primers no. 1 and no. 10 in Fig. 1A, respectively), were used in PCR reactions carried out as described in Construction of Plant-Optimized Synthetic LTB Gene to determine the presence of the synthetic LTB gene. Genomic DNA samples (100 ng) from the transgenic and WT plants, along with 20 ng of plasmid pMYO51 containing the synthetic LTB gene, were used as templates for detection of the LTB gene.

Northern Blot Analysis

Total RNA was extracted from the leaves of transgenic and WT plants using Trizol (Invitrogen) according to the manufacturer's instructions. Samples of total RNA were denatured with formaldehyde and formamide and were fractionated in a 1.5% agarose gel with 3-(*N*-morpholino) propanesulfonic acid/acetate/EDTA buffer. The RNA was blotted onto Hybond-N+ membranes (Amersham Pharmacia Biotech, UK). Prehybridization was performed at 60°C for 1 to 2 h in 6X SSPE, 0.5% sodium dodecyl sulfate (SDS), 5X Denhardt's solution, and 100 µg/L of salmon sperm DNA. The blots were hybridized overnight at 60°C in a buffer (pH 7.4) containing 1 m*M* EDTA, 250 m*M* $Na_2HPO_4 \cdot 7H_2O$, 1% hydrolyzed casein, and 7% SDS with random-primed, ^{32}P-labeled probes made from synthetic LTB coding

sequences. The membranes were washed and exposed to Kodak XAR film as described previously *(21)*.

Immunoblot Detection of CTB Protein in Transformed Tobacco Tissues

TSP was extracted from leaf tissues (~0.5 g fresh weight) of transgenic tobacco plants by grinding in liquid nitrogen with a mortar and pestle. One milliliter of extraction buffer (200 m*M* Tris-Cl [pH 8.0], 100 m*M* NaCl, 400 m*M* sucrose, 10 m*M* EDTA, 14 m*M* 2-mercaptoethanol, 1 m*M* phenylmethylsulfonyl fluoride, 0.05% Tween-20) was added, and the homogenate was centrifuged at 13,000*g* for 15 min at 4°C. The supernatant (5–10 µL) containing 20 µg of TSP was used for further analysis. Extracted proteins, along with prestained molecular weight markers, were resolved by 15% SDS-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA) and transferred onto Hybond C membranes (Promega) in transfer buffer (50 m*M* Tris [pH 8.3], 40 m*M* glycine, 0.04% SDS, 20% methanol) using a minitransblot apparatus (Bio-Rad) at 130 mA for 2 h. To prevent nonspecific antibody reactions, the membranes were blocked with 5% nonfat milk powder in TBST buffer (Tris-buffered saline with 0.05% Tween-20) at room temperature overnight. The membranes were incubated with a 1:3000 dilution of rabbit anti-LTB antiserum (Immunology Consultants, OR) in TBST antibody dilution buffer containing 2.5% nonfat dry milk and then washed three times with TBST buffer. The membranes were incubated for 2 h with a 1:7000 dilution of anti–rabbit IgG conjugated with alkaline phosphatase (Promega S3731) in TBST buffer. The membranes were washed three times with TBST buffer and once with TMN buffer (100 m*M* Tris [pH 9.5], 5 m*M* $MgCl_2$, 100 m*M* NaCl). Color was developed using BCIP/NBT (USB) in TMN buffer.

Quantification of LTB Protein Levels in Transgenic Tobacco Tissues

The LTB expression level in transgenic tobacco plants was determined by enzyme-linked immunosorbent assay (ELISA). The wells of ELISA plates were coated with TSP of WT and transgenic plants, and the plates were incubated overnight at 4°C. The plates were washed three times with washing buffer PBST (phosphate-buffered saline [PBS] containing 0.05% Tween-20), blocked with 1% (w/v) bovine serum albumin (BSA), then washed three times with PBST. The plates were incubated with a 1:5000 dilution of rabbit anti-LTB antiserum containing 0.5% BSA for 2 h at 37°C and washed three times with PBST. Next, the plates were incubated with a 1:10,000 dilution of goat anti–rabbit IgG conjugated with horseradish peroxidase (Promega G-7641) containing 0.5% BSA and washed three times with TBST buffer. The plates were developed with TMB substrates (Pharmingen 2606KC and 2607KC) for 30 min at room temperature in the dark. Reaction products were detected at 405 nm and quantified by comparison with known amounts of bacterial LTB-antibody complex. The results are expressed as the LTB protein level.

G_{M1}-Ganglioside-Binding Assay

G_{M1}-ELISA was performed to determine the affinity of the G_{M1}-ganglioside receptor for the plant-derived LTB protein. Ninety-six-well microtiter plates were coated with monosialoganglioside-G_{M1} (Sigma G-7641) by incubating with 100 µL/well of G_{M1} (3 µg/mL) in bicarbonate buffer (15 m*M* Na_2CO_3, 35 m*M* $NaHCO_3$, pH 9.6) at 4°C overnight. Control wells were coated with 100 µL/well of BSA (3.0 µg/mL). The plates were washed three times with PBST buffer, and the wells were blocked with 1% BSA in 0.01 *M* PBS for 2 h at 37°C followed by three washes with PBST. The plates were incubated with increasing concentrations of TSP from the leaves of transgenic and WT plants in bicarbonate buffer (100 µL/well) for 2 h at 37°C. The remaining procedure was as described in the preceding section.

Results and Discussion

Design of Synthetic LTB Gene

To achieve high expression levels of heterologous genes in plants, it is often essential to optimize the coding sequence to mimic that of highly expressed plant genes *(22–24)*. Several causes of poor expression of foreign genes in plants have been reported, such as mRNA instability *(25)*, premature polyadenylation *(26)*, abnormal splicing *(27)*, and improper codon usage *(18)*. Therefore, DNA sequences that might contribute to RNA instability in plants, such as the ATTTA sequence, and the potential polyadenylation signal sequence, AATAAA, were eliminated from the synthetic gene (Fig. 3). The synthetic gene encoding the mature LTB was constructed based on the codon usage of highly expressed nuclear-encoded genes of *N. tabacum (28)*. The amino acid sequence of synthetic LTB was the same as that of the native LTB except at position 2: the AAT triplet for Asn was replaced with the GTG triplet for Val (Fig. 3) because the placement of G immediately following the AUG initiation codon and the Kozak sequence, GCCACC, was reported to increase the efficiency of translation 10-fold *(17)*. The signal peptide at the N-terminus should be removed during processing and maturation of the LTB protein, and, thus, the amino acid change at position 2 should not affect the mature LTB protein expressed in the plant cell *(10)*. Codons rarely used in plants, such as XCG and XUA (X denotes U, C, A, or G), were avoided in the synthetic LTB (Fig. 3). It was reported that rare mRNA codons tend to form higher-order secondary structures, which might require additional time for ribosomal movement through the critical region *(29,30)*.

The ratio of XXG/C was increased from 36% in WT LTB to 72% in the synthetic LTB, which is characteristic of highly expressed nuclear-encoded plant genes *(28)*. Furthermore, the G/C ratio was increased from 37 to 47% to avoid a high A/U ratio, which has been reported to be associated with low mRNA stability, and thus low levels of protein expression, in higher plants *(31)*. The nucleotide sequence that encodes the ER retention signal

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S                                                          GGATCCGCCACC
                                                            BamHI Kozak

N ATG AAT AAA GTA AAA TGT TAT GTT TTA TTT ACG GCG TTA CTA TCC  45
S ATG GTG AAG GTG AAG TGC TAC GTT TTG TTC ACC GCT TTG CTC TCC
  Met Asn Lys Val Lys Cys  Tyr Val Leu Phe Thr Ala Leu Leu Ser

N TCT CTA TGT GCA TAC GGA GCT CCC CAG TCT ATT ACA GAA CTA TGT  90
S TCT CTC TGC GCT TAC GGA GCT CCA CAG TCC ATT ACA GAG CTC TGC
 Ser Leu Cys Ala Tyr Gly  Ala Pro Gln Ser Ile Thr Glu Leu Cys

N TCG GAA TAT CGC AAC ACA CAA ATA TAT ACG ATA AAT GAC AAG ATA 135
S TCC GAG TAC AGG AAC ACA CAA ATC TAC ACC ATC AAC GAT AAG ATC
  Ser Glu Tyr Arg Asn Thr Gln Ile Tyr Thr Ile Asn Asp Lys Ile

N CTA TCA TAT ACG GAA TCG ATG GCA GGT AAA AGA GAA ATG GTT ATC 180
S CTC TCC TAC ACC GAG TCC ATG GCT GGA AAG AGG GAG ATG GTT ATC
  Leu Ser Tyr Thr Glu Ser Met Ala Gly Lys Arg Glu Met Val Ile

N ATT ACA TTT AAG AGC GGC GCA ACA TTT CAG GTC GAA GTC CCG GGC 225
S ATT ACA TTC AAG AGC GGA GCT ACA TTC CAG GTG GAG GTG CCA GGA
 Ile Thr Phe Lys Ser Gly Ala Thr Phe Gln  Val Glu Val Pro Gly

N AGT CAA CAT ATA GAC TCC CAA AAA AAA GCC ATT GAA AGG ATG AAG 270
S AGC CAA CAT ATT GAT TCC CAG AAG AAG GCT ATT GAG AGG ATG AAG
 Ser Gln His Ile Asp Ser Gln Lys Lys Ala Ile  Glu Arg Met Lys

N GAC ACA TTA AGA ATC ACA TAT CTG ACC GAG ACC AAA ATT GAT AAA 315
S GAT ACA TTG AGG ATC ACA TAC CTC ACC GAG ACC AAG ATT GAT AAG
 Asp Thr Leu Arg Ile Thr Tyr Leu Thr Glu  Thr Lys Ile Asp Lys

N TTA TGT GTA TGG AAT AAT AAA ACC CCC AAT TCA ATT GCG GCA ATC 360
S TTG TGC GTG TGG AAC AAC AAG ACC CCA AAC TCC ATT GCT GCT ATC
 Leu Cys Val Trp Asn Asn Lys Thr Pro Asn Ser Ile  Ala Ala Ile

N AGT ATG GAA AAC TAG
S AGC ATG GAG AAC TAC TCT GAG AAA GAT GAG CTA TGA GGTACC
 Ser Met Glu Asn ***   S   E    K   D    E    L  ***   KpnI
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Fig. 3. Sequence comparison between native bacterial LTB gene (N) and plant-optimized coding sequence of synthetic LTB gene (S). The nucleotides shown in bold in the S sequence were changed from those of the N sequence. The potential polyadenylation sequence, AATAAA, and instability elements, ATTTA, are double underlined. The Kozak sequence, GCCACC, is enclosed in a square.

(SEKDEL) was fused in-frame with the 3' end of the LTB open reading frame. Finally, restriction enzyme sites for *Bam*HI and *Kpn*I were introduced at the 5' and 3' ends of the synthetic LTB gene, respectively, to provide convenient restriction sites for cloning into plant expression vectors.

PCR Detection of LTB Fusion Gene in Transgenic Tobacco Plants

PCR analysis was performed to confirm the presence of the synthetic LTB gene in the genomic DNA of transgenic tobacco plant leaves. As expected, a 414-bp PCR product was obtained from the genomic DNA of all 12 transgenic plants (Fig. 4). No band was amplified from WT plants.

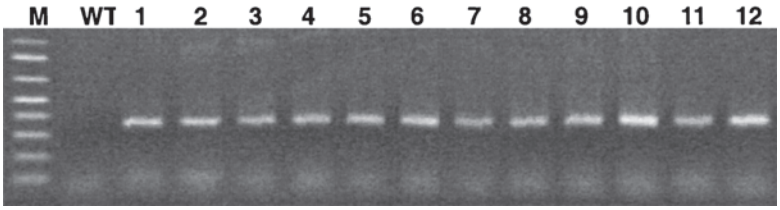

Fig. 4. Detection of synthetic LTB gene in transgenic tobacco plants. Genomic DNA was isolated from transgenic and WT leaf tissues, and PCR amplification was performed. The PCR products of the synthetic LTB gene were separated using 1.2% agarose. M, 1-kb DNA ladder; WT, PCR product with DNA template from WT plant; lanes 1–12, PCR products with DNA template from independent transgenic lines.

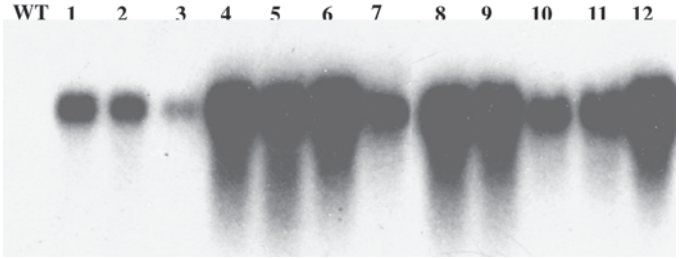

Fig. 5. Detection of synthetic LTB mRNA in transgenic tobacco plants. Total RNA (20 μg) from WT and transformed tobacco plants was separated on a 1.2% formaldehyde agarose gel, transferred to a membrane, and hybridized with a ^{32}P-labeled synthetic LTB probe. WT, wild-type plant; lanes 1–12, transgenic plant lines.

This confirmed the stable insertion of the synthetic LTB gene into tobacco chromosomes using *Agrobacterium*-mediated transformation.

Northern Blot Analysis

Northern blot analysis using a synthetic LTB gene probe was performed on total RNA from the leaves of transformed plants to measure the expression level of LTB mRNA. All of the transformed plants showed positive signals for synthetic LTB, but there were variations in the signal intensities among the transformed plants (Fig. 5). High levels of transcripts were found in transgenic lines nos. 6 and 12. Therefore, these two lines were used for Western blot, ELISA, and G_{M1}-binding analyses.

In Northern blot analysis, the signal from synthetic LTB transcripts was much stronger than that from native LTB transcripts (data not shown). This was probably owing to the absence of mRNA-destabilizing sequences and polyadenylation signals in the synthetic gene. The high levels of protein expression may have been owing to high levels of gene transcription. Perhaps the best-known mRNA destabilizing element in higher eukaryotes is the A/U-rich destabilizing element that is found in the 3'-untranslated regions of many labile mammalian mRNAs *(32)*.

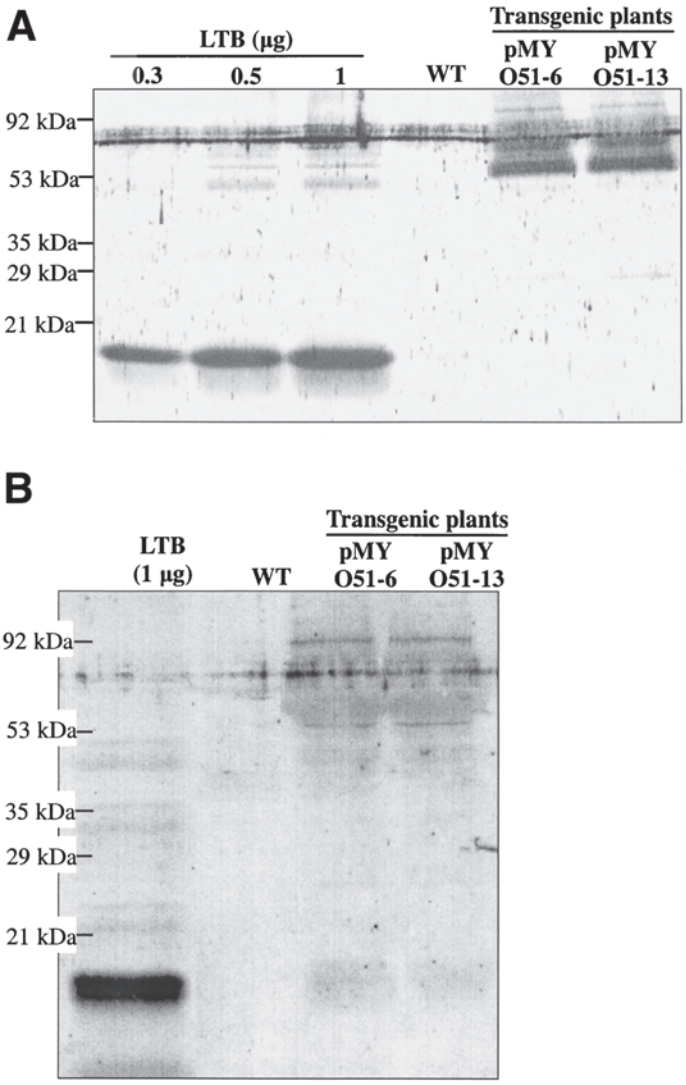

Fig. 6. Western blot analysis of transgenic plants using rabbit anti-LTB antiserum. **(A)** Lanes 1–3: 0.3, 0.5, and 1 μg of purified bacterial LTB monomer, respectively; lane 4: WT, unboiled total protein (15 μg) from WT plant; lanes 5 and 6: unboiled total protein (15 μg) from two transgenic plant lines. **(B)** Lane 1: 1 μg of purified bacterial LTB monomer; lane 2: 1 μg of purified bacterial LTB monomer; lanes 3 and 4: boiled total protein (15 μg) from two transgenic plant lines.

Immunoblot Analysis of Plant-Synthesized LTB Protein

Immunoblot analysis was performed to detect the presence of the LTB subunits in the transgenic plant lines nos. 6 and 12, which were selected based on the results of Northern blotting. Antibodies revealed that the plant-produced LTB formed a 55-kDa oligomeric structure (Fig. 6A). When the pentameric LTB samples were boiled for 5 min, the multimeric structure dissociated into 12-kDa monomers (Fig. 6B). Nontransformed plants

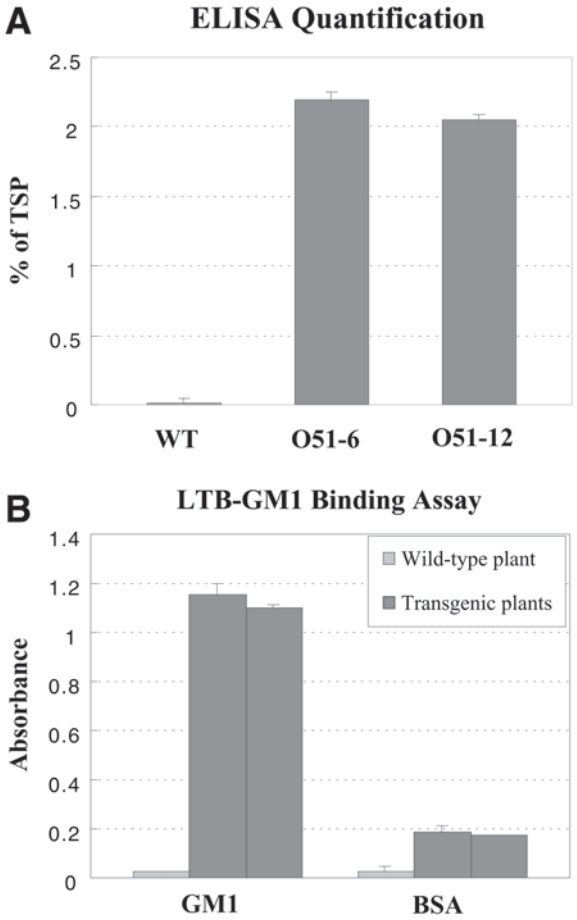

Fig. 7. **(A)** ELISA detection of plant-synthesized LTB levels in leaves of transgenic plants. W, WT plant; O53-6 and O53-12, two transgenic plant lines. **(B)** G_{M1} binding of bacterial LTB and plant LTB. The ELISA assay was performed by coating the plates with either G_{M1}-monosialoganglioside or BSA as receptor molecules.

showed no crossreactivity with the LTB antibody. The apparent molecular weight of the pentamer was less than expected, probably because the globular shape of the oligomeric structure allows greater mobility through the gel.

ELISA Quantification of LTB Expression

ELISA was performed to obtain quantitative estimates of LTB protein levels in the leaf tissues of two transgenic tobacco plant lines, pMYO51-6 and pMYO51-12. The amounts of LTB protein in the transgenic plants were quantified by comparison with known amounts of purified bacterial LTB protein and expressed as a percentage of total soluble plant protein (% LTB) in the sample. Optimal concentrations of soluble protein loaded in the wells of the microtiter plates revealed LTB protein levels of approx 2.2% of TSP in transgenic tobacco leaf tissues (Fig. 7A), which is more than 200-fold

higher than previously reported for tobacco plants expressing the native LTB gene *(10)*. Based on the results of the ELISA and immunoblot assay, 1 g of tobacco leaf tissue (fresh weight) contained 100–150 µg of recombinant LTB protein.

G_{M1} Receptor-Binding Assay

The biologic functions of LTB, such as binding to the G_{M1}-ganglioside cell receptor, are dependent on the formation of a pentamer configuration of LTB subunits. To test for an active pentamer formation by the LTB subunits produced in plants, ELISA plates were coated with the G_{M1} receptor; authentic LTB was used as a positive control. In the G_{M1}-ELISA-binding assay, G_{M1}-ganglioside demonstrated a strong affinity for the plant-produced LTB. No affinity to the BSA control was observed (Fig. 7B). These results clearly show that the plant-produced LTB protein is present in the plant cells in the active pentameric form *(33)*.

The results of the present study show that the LTB gene can be modified for enhanced expression in tobacco plants. Plant-optimized synthetic genes encoding LTB were constructed and expressed in potato *(11)* and maize *(13)*. The LTB expression level in potato tubers was 0.19% of total protein when the synthetic gene did not contain a Kozak sequence or SEKDEL *(11)*. The LTB expression level in the maize R_1 kernel was 0.01% of total protein with the CaMV 35S promoter and 0.07% of total protein with the gamma zein promoter when the synthetic LTB contained the SEKDEL, but not the Kozak, sequence. The LTB gene synthesized in the present study had a higher expression level (2.2% of TSP) than the genes reported previously, although different plants were used. This suggests that the Kozak and SEKDEL sequences as well as coding sequence optimization increase the accumulation of LTB in transgenic tobacco plants.

LTB produced in potato and maize has been shown to be immunogenic in mice *(11,13,14)* and humans *(15)*. This protein is an ideal model for an oral vaccine, and its adjuvant properties make it attractive for coexpression or coadministration with other plant-synthesized or conventional vaccines. The highly expressed synthetic LTB gene reported here may be introduced into palatable edible plants, such as banana, carrot, tomato, or lettuce, for the purpose of creating carrier proteins and adjuvants, as well as for a cholera vaccine. In our laboratory, studies are currently under way to express the synthetic LTB gene in lettuce and carrot plants.

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