Expression of the B subunit of *E. coli* heat-labile enterotoxin in the chloroplasts of plants and its characterization

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Abstract

Transgenic chloroplasts have become attractive systems for heterologous gene expressions because of unique advantages. Here, we report a feasibility study for producing the nontoxic B subunit of *Escherichia coli* heat-labile enterotoxin (LTB) *via* chloroplast transformation of tobacco. Stable site-specific integration of the LTB gene into chloroplast genome was confirmed by PCR and genomic Southern blot analysis in transformed plants. Immunoblot analysis indicated that plant-derived LTB protein was oligomeric, and dissociated after boiling. Pentameric LTB molecules were the dominant molecular species in LTB isolated from transgenic tobacco leaf tissues. The amount of LTB protein detected in transplastomic tobacco leaf was approximately 2.5% of the total soluble plant protein, approximately 250-fold higher than in plants generated via nuclear transformation. The GM1–ELISA binding assay indicated that chloroplast-synthesized LTB protein bound to GM1-ganglioside receptors. LTB protein with biochemical properties identical to native LTB protein in the chloroplast of edible plants opens the way for inexpensive, safe, and effective plant-based edible vaccines for humans and animals.

Introduction

The expression and delivery of recombinant protein antigens as edible vaccines is attractive because of the numerous advantages that it offers, such as low production cost, easy administration and storage (Giddings et al., 2000). The chances of acquiring mucosal immunity against infectious agents that enter the body across a mucosal surface are also increased with edible vaccines (Tacket et al., 1998). However, the use of subunit vaccines for oral delivery has been resisted

because of the obvious likelihood of protein degradation in the gut. Recently, transgenic plants have been investigated as an alternative means of producing and delivering vaccines. Indeed, Norwalk virus capsid protein (Tacket et al., 2000), transmissible gastroenteritis coronavirus glycoprotein (Tuboly et al., 2000), hepatitis B surface protein (Kong et al., 2001), and foot and mouth disease virus VP1 (Dus Santos et al., 2002) have been successfully expressed in plants. The quantity of plant tissue constituting a vaccine dose must be of a practical size for consumption. However, the level of protein expression achieved in plants is as low as 0.01% and rarely exceeds 0.40% of the total soluble proteins (TSP) (Haq et al., 1995; Gomez et al., 1998;

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Richter et al., 2001; Mason et al., 2002). Thus, a high level of expression in plants is critical (Stoger et al., 2002). A dramatic increase in the recombinant protein yield can be achieved using chloroplast transformation, especially in tobacco, since a leaf cell contains as many as 100 chloroplasts with up to 100 plastid DNA copies each, for a total of approximately 10,000 genome copies per cell. The *Bt Cry*2Aa2 operon contributed 45.3% of the TSP (De Cosa et al., 2001) and CTB at 4.1% of TSP (Daniell et al., 2001) when they were expressed in tobacco leaf chloroplasts. In other plant chloroplasts, the *aadA* gene was expressed at 1% of TSP in tomato (Ruf et al., 2001) and 5% of GFP expressed in potato chloroplasts (Sidorov et al., 1999). Therefore, transgenic plastids are ideal expression factories for high-yield protein production (Bock, 2001), and it will be possible to express chloroplast transgenes in edible crops, for use as edible vaccines.

Transformation of the plastid genome (plastome) has several more advantages over nuclear transformation. Since plastids in most agronomically important plant species are maternally inherited, field management of transplastomic plants may be simpler, because pollen-mediated outcrossing is not a problem (Daniell et al., 1998; Scott & Wilkinson, 1999; Lutz et al., 2001). Additionally, multiple genes can be introduced and expressed by polycistronic operon expression in a single plastid transformation event (Staub & Maliga, 1995). Since the foreign gene is integrated by homologous recombination, the location of transgene insertion is predictable, and there are no position effects, as normally experienced with random insertion of transgenes in nuclear transformation (Svab et al., 1990). Lastly, gene silencing has not been reported in plastids; therefore, transgene expression is stable in the progeny of transplastomic plants.

Enterotoxigenic *E. coli* (ETEC) causes severe watery diarrhea due to the production of one or more enterotoxins. One of these is a high-molecular weight heat-labile toxin (LT), which is immunologically and physicochemically related to cholera toxin (CT) (Clements & Finkelstein, 1979; Clements et al., 1980). Each molecule has one 27 kDa A subunit, which has toxic ADP ribosyl transferase activity, and a pentamer of 11.6 kDa B (binding) subunits, which are noncovalently linked into a very stable doughnut-like structure. LT and CT bind *via* specific interactions of the B subunit pentamer with the GM1-ganglioside present on the intestinal epithelial cell surface. Both heat-labile enterotoxin (LTB) and CTB function as adjuvants for co-administered antigens (Dickinson,

1996; Elson, 1996) as well as immunogens (Clements & Finkelstein, 1978; Gilligan et al., 1983). When recombinant LTB (rLTB) was expressed in *Bacillus brevis* and administered intranasally to mice with diphtheria toxoid (D_T), it substantially stimulated production of D_T-specific serum IgG antibody and induced moderate D_T-specific mucosal IgA antibody responses in the nasal cavity and lung, suggesting that purified rLTB is a promising immuno-adjuvant for mucosal immunizations (Kozuka et al., 2000). It has been shown that recombinant multimers of the B subunits of LT and CT can be produced successfully in potato and that they are immunogenic when taken orally by animals (Mason et al., 1998; Lauterslager et al., 2001) and humans (Tacket et al., 1998). However, LTB formed less than 0.01 and 0.19% of TSP when expressed in the nucleus of tobacco (Haq et al., 1995) and potato (Mason et al., 2001), respectively. High expression of LTB in plants is necessary for its use as an edible vaccine. This paper reports the efficient high expression of LTB in tobacco chloroplasts and its binding to GM1-ganglioside in order to investigate the functional pentameric structure.

Materials and methods

Plant material

Seeds of *Nicotiana tabacum* cv TI560 were surface-sterilized with 70% ethanol for 3 min followed by 15 min in 10% sodium hypochlorite. Seeds were then washed five times in sterile water before being placed in Petri dishes containing MS salts ($4.6 \, g \, l^{-1}$) supplemented with sucrose ($30 \, g \, l^{-1}$) and bactoagar ($7.5 \, g \, l^{-1}$) at pH 5.7, and grown in an environmentally controlled culture room at 25°C on a photoperiod of 16 h light/8 h dark photoperiod.

Construction of plastid expression vector

The plasmid, pLD-CtV2, was originally constructed for stable plastid transformation in tobacco (De Cosa et al., 2001). LTB was prepared from genomic DNA of *E. coli* strain 1032, which was amplified by PCR using primers designed to introduce a ribosome binding site (GGAGG) eight bases upstream from the start codon in the native LTB from which the signal peptide coding sequence was removed. The forward (5′-

GGGGAATTCAGGAGGTTCAGTCATGGCTCCCC AGTCTATTACAGAAC-3′) and reverse (5′-GGGGG TACCCTAGTTTTCCATACTGATTGC-3′) primers contained *Eco*RI and *Xba*I sites, respectively. The denaturation, annealing and extension reactions were performed at 94°C for 1 min, 55°C for 1 min, and at 72°C for 1 min, respectively. The reaction was repeated for a total of 30 cycles in a volume of 50 μl. The PCR product was cloned into TA cloning vector pGemTeasy (Promega) and the *Eco*RI and *Xba*I fragment was ligated into the same sites of pLD-CtV2. The resulting plasmid was named pMY-CH-LTB. The LTB gene was also expressed in *E. coli* M15, and the rLTB protein was purified using QIAexpress System (Qiagen) according to the manufacturer's instructions.

Plastid transformation

The leaves of tobacco grown aseptically on hormone free MS agar medium described in the section 'Plant Material' were cut and placed on filter paper on top of the MS agar medium before bombardment. The tobacco leaves were then bombarded with gold (1 μm) or tungsten (0.7 μm) particles using a Biolistic PDS-1000/He Particle Delivery System (BioRad) following the manufacturer's instructions and placed in a culture room at 25°C. Two to 3 days after bombardment, the leaves were cut into about 0.5 cm^2 pieces and placed on RMOP selection medium (Daniell, 1997) containing 500 mg l^{-1} spectinomycin. The shoots generated from the selection medium after 5–6 weeks were cut into smaller pieces (~0.2 cm^2) and placed on selection medium.

PCR and Southern blot analyses

Total genomic DNA was extracted by the method of Kang and Fawley (1997). The primer pairs used to amplify the LTB fragment from genomic DNA were 1F (5′-AAAACCCGTCCTCAGTTCGGATTGC-3′) and 1R (5′-CCGCGTTGTTTCATCAAGCCTTA CG-3′), 2F (5′-CTGTAGAAGTCACCATTGTTG TGC-3′) and 2R (5′-TGACTGCCCACCTGAGAGC GGACA-3′), 3F (5′-TCCTCAGTTCGGATTGCAG GC-3′) and 3R (5′-GGGGGTACCCTAGTTTTCCA TACTGATTGC-3′), and 4F (5′-GGGGAATTCAGG AGGTTCAGTCATGGCTCCCCAGTCTATTACAGA AC-3′) and 4R (5′-TATGCCATCCTAAGGTGCTG C-3′). PCR was performed with DNA isolated from transformed and wild-type plant leaves using con-

ditions as described in the section 'Construction of chloroplast expression vector'.

Southern blot analysis was performed as described in Sambrook and Russell (2001). Aliquots (10 μg) of total genomic DNA from the leaves of transformed and wild-type plants were digested with *Bgl*II and electrophoresed on a 0.8% agarose gel, which was then transferred to Hybond-N+ membrane (Amersham). The blot was hybridized with a ^{32}P-labeled random-primed (Promega) probe at 65°C in a Hybridization Incubator (Finemould Precision Ind. Co., Korea). After hybridization overnight, blots were washed and exposed to X-ray film.

Immunoblot analysis

Leaf samples (~0.5 g) from transplastomic and wild-type tobacco plants grown in a greenhouse were ground in liquid nitrogen 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 (100 μg) of TSP, as determined by Bradford protein assay (Sigma), from transformed and wild-type plants were loaded on 15% SDS–PAGE gels before electrophoresis. Purified LTB expressed in *E. coli* M15 cells was also loaded in the range of 0.5–4 μg. The separated protein bands were transferred from the gel to Hybond C membranes (Promega) using a Mini Trans Blot^{TM} electrophoretic transfer cell (BioRad). Nonspecific antibody reactions were blocked by incubating the membranes in 25 ml of 5% nonfat dry milk in TBST buffer (TBS with 0.05% Tween-20) with gentle agitation overnight. The membrane was incubated for 2 h at room temperature with gentle agitation in 10 ml of 1:2000 dilution of rabbit anti-LTB antiserum (Immunology Consultants Lab., Inc., OR) in TBST antibody dilution buffer containing 2.5% nonfat dry milk and then washed three times with TBST buffer. The membrane was incubated for 2 h in 1:7000 dilution of anti-rabbit IgG conjugated with alkaline phosphatase (Promega S3731) in TBST buffer and washed three times with TBST buffer, and once with TMN buffer. After washing, the color was developed with BCIP/NBT (USB) in TMN buffer.

Quantification of LTB protein level by ELISA

LTB protein levels expressed in transgenic tobacco plants were determined using a quantitative ELISA assay. The leaves of transplastomic and wild-type
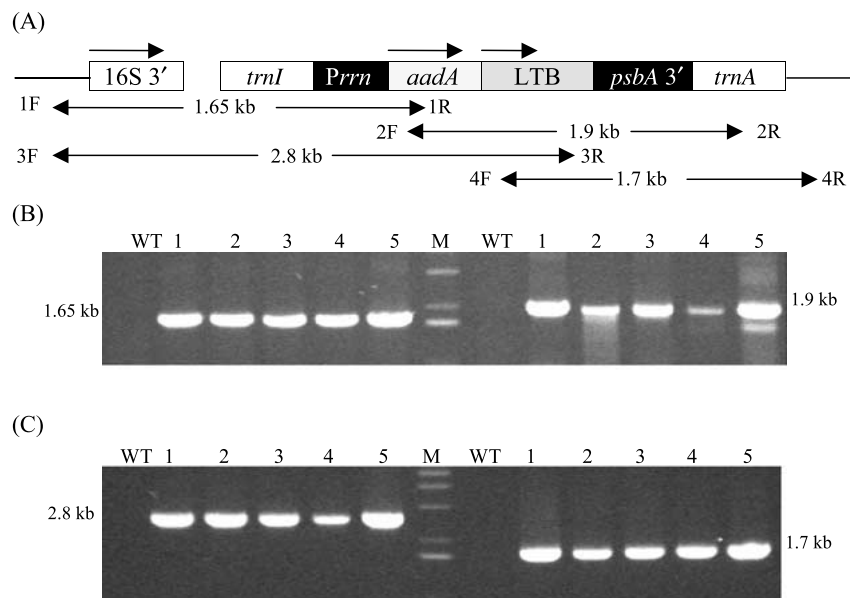
(A)

(B)

(C)

Figure 1. PCR analysis for wild-type and chloroplast transformants. (A) Chloroplast genome of transplastomic tobacco plant. Primer 1F anneals to native chloroplast DNA and 1R *aadA* producing a 1.65 kb fragment, 2F *aadA* and 2R *trnA* producing a 1.9 kb fragment, 3F native chloroplast and 3R LTB producing a 2.8 kb fragment, and 4F LTB and 4R native chloroplast producing a 1.7 kb fragment. (B) PCR products amplified from genomic DNA of wild-type and transgenic plants. WT, wild-type plant; 1–5, transgenic plants; M, 1 kb DNA ladder.

plants were ground in liquid nitrogen and homogenized in bicarbonate buffer, pH 9.6 (15 mM Na_2CO_3, 35 mM $NaHCO_3$), and the concentration was determined using Bradford Reagent (Sigma). TSPs from transplastomic and wild-type plants were loaded into a 96-well microtiter plate (Becton) with 100 μl per well of selected concentrations and incubated overnight at 4°C. The plate was washed three times with washing buffer PBST. The background was blocked by incubation in 1% bovine serum albumin (BSA) in PBS (300 μl per well) at 37°C for 2 h, and then the wells were washed three times with PBST. The plate was incubated with a 1:5000 dilution of rabbit anti-LTB antibody (Immunology Consultants Lab., Inc., OR) (100 μl per well) in 0.01 M PBS containing 0.5% BSA for 2 h at 37°C, and washed four times with PBST. The wells were incubated with 1:10000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma G-7641) (100 μl per well) in 0.01 M PBS containing 0.5% BSA for 2 h at 37°C, and washed four times with PBST. The plate was finally incubated with 100 μl per well TMB substrates (Pharmingen 2606KC and 2607KC) for 30 min at room temperature in the dark to maximize the reaction rate. After incubation, the reaction was measured at 405 nm in an ELISA reader (Packard, CT).
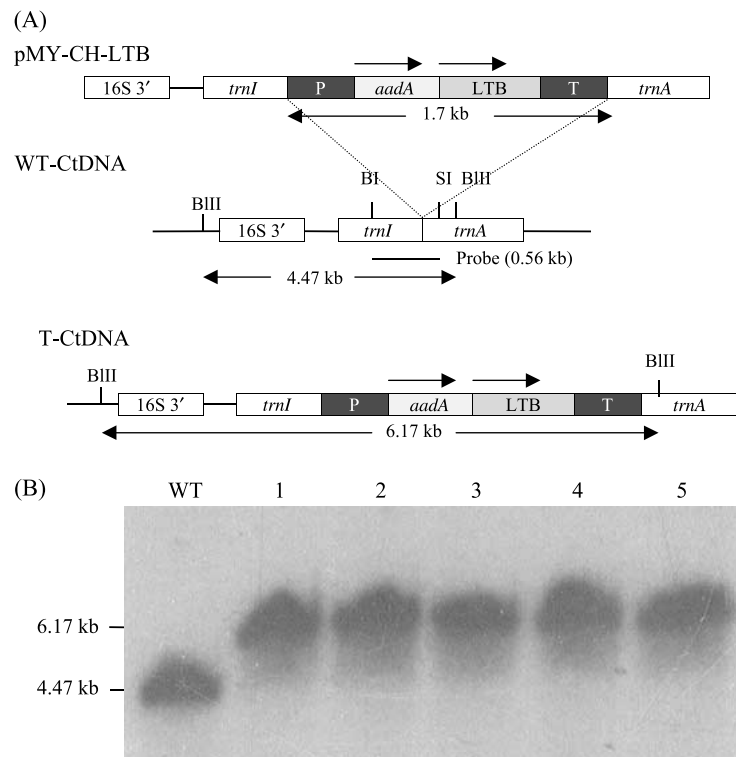
LTB–GM1 binding assay

To determine the ability of LTB to bind to gangliosides, a microtiter plate was coated with monosialoganglioside GM1 (Sigma) by incubating the plate with 100 μl per well GM1 (3.0 μg ml^{-1}) in bicarbonate buffer, pH 9.6 at 4°C overnight. As a control, plates were coated with 100 μl per well BSA (3.0 μg ml^{-1}). After three washes with PBST, the plate was blocked with 1% BSA in 0.01 M PBS. The plate was washed three times with PBST, and then the GM1 coated plates were incubated with various concentrations of TSP from transplastomic and wild-type plants in PBS (100 μl per well) for 2 h at 37°C. The primary and secondary antibody treatments were as described above.

Results

Construction of plastid expression vector

The LTB gene was inserted into a plastid expression cassette. The plasmid pMY-CH-LTB consists of a plastid rRNA operon promoter (P*rrn*) with a ribosome binding site (GGAGG), the aminoglycoside adenylyltransferase (*aadA*) gene to provide spectinomycin and

Figure 2. (A) Map of chloroplast targeting region in wild-type (WT-CtDNA) and transplastomic plant (T-CtDNA). Arrows indicate direction of transcription. Map position of the probe (0.56 kb) is marked by a heavy line; the wild-type (4.47 kb) and transgenic (6.17 kb) fragments generated by *Bgl*II (BIII) digestion are marked by thin lines. P, 16S rRNA promoter; T, *psbA* 3′-UTR. (B) Southern blot analysis to confirm LTB gene integration into the tobacco chloroplast genome by homologous recombination. Genomic DNA of wild-type (WT) and transgenic plants (1–5) were digested by *Bgl*II and hybridized with the probe, 0.56 kb fragment of *trnI* and *trnA* digested with *Bam*HI (BI) and *Sac*I (SI), generating a 4.47 kb for wild-type and a 6.17 kb fragments for transgenic plants.

streptomycin resistance for selecting stable transformants, and the plastid *psbA* gene 3′-untranslated region for stabilizing the mRNA.

Plastid transformation and selection of transplastomic lines

The plasmid pMY-CH-LTB was introduced into chloroplast genome using the biolistic process. Bombardment of tobacco leaves with DNA-coated tungsten particles was followed by selection on spectinomycin-containing medium (Svab & Maliga, 1993). Multiple plastid transformation experiments were attempted and selection for spectinomycin resistance eventually yielded cells with uniformly transformed plastid genome populations, which were then regenerated into plants. A second round of selection on spectinomycin-containing medium was performed for transformed plants to produce homoplastomic plants.

Foreign gene integration into chloroplast genome was primarily determined by PCR screening of chloroplast transformants (Figure 1). Primer sets 1F–1R, 3F–3R, and 4F–4R were designed to confirm incorporation of the foreign gene into the chloroplast genome at the directed site by homologous recombination. The rationale for this strategy is that there can be no PCR product unless there is site-specific integration, because one primer binds to the native chloroplast genome adjacent to the point of integration and the second primer to the *aadA* or LTB gene. In addition, the primer set 2F–2R was used to confirm the integration of the LTB gene in tandem with the *aadA* gene. Therefore, the PCR products in the DNA of all five independent plants using different primer sets (1.65 kb (1F–1R), 2.8 kb (3F–3R), and 1.7 kb (4F–4R) fragments) confirmed site-specific integration in the chloroplast genome. As expected, there was no PCR product in the wild-type tobacco DNA.
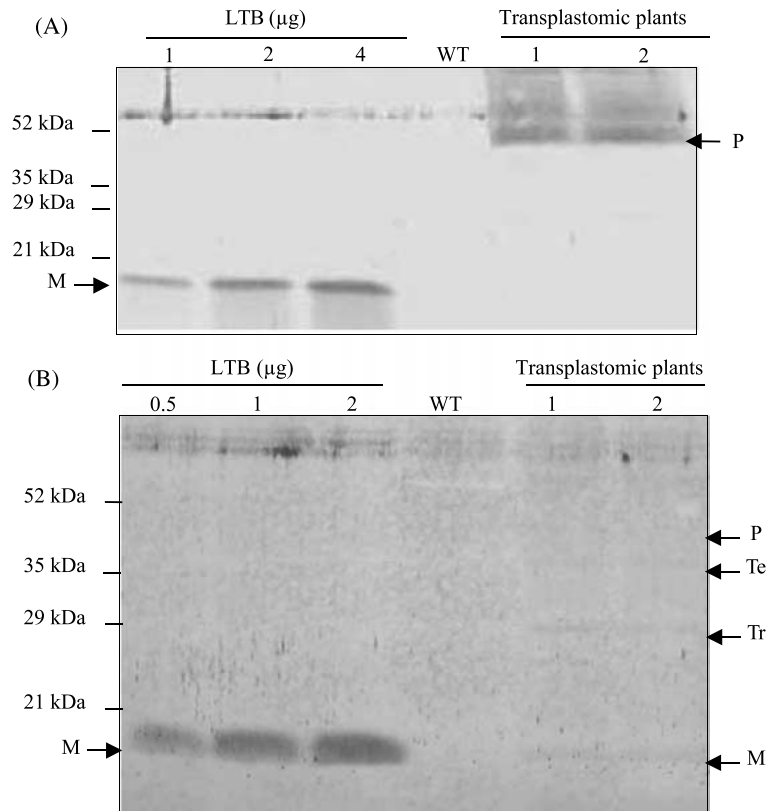
Figure 3. Immunoblot analysis of chloroplast LTB protein. Leaf proteins were loaded directly or boiled for 5 min before 15% SDS–PAGE. (A) Unboiled TSPs (100 μg) from two transplastomic plant lines (lines 2 and 5) and wild-type plant along with 1, 2, and 4 μg of purified bacterial protein were separated on 15% SDS–PAGE. (B) Boiled TSPs (100 μg) from two transplastomic plant lines (lines 2 and 5) and wild-type plant along with 0.5, 1, and 2 μg of purified bacterial protein were separated on 15% SDS–PAGE. P, pentamer; M, monomer; Te, tetramer; Tr, trimer.

The transformed tobacco lines showing site-specific integration according to the PCR product were further analyzed using Southern blot hybridization to verify site-specific integration and to establish copy number. In the chloroplast genome, there are *Bgl*II sites upstream from the 16S rRNA gene and downstream from the *trnA* region, which generate a 4.47 kb fragment when digested with *Bgl*II. A transformed chloroplast genome has a foreign gene cassette inserted between the *trnI* and *trnA* regions, which increases the size of the *Bgl*II fragment to 6.17 kb. Total DNA from each clone and wild-type tobacco were digested with *Bgl*II, and the blot was hybridized with a *trnI–trnA* probe (0.81 kb) labeled with ^{32}P-dCTP using a DNA labeling system (Prime-a-Gene Labeling System, Promega). The tissue transformed with pMY-CH-LTB showed the expected 6.17 kb fragments, reflecting the presence of the transgene (Figure 2). The 4.47 kb fragment was found in the wild-type plant, which does not contain a transgene. In transgenic plants, the 4.47 kb fragment was not found, suggest-

ing that homoplastomy has been achieved. From this result, it is assumed that genetic stability of transplastomic plants requires homoplastomy (Bock et al., 2001).

Immunoblot analysis of chloroplast-synthesized LTB protein

The two randomly selected transformed plants (lines 2 and 5) were analyzed for the presence of LTB protein by immunoblot analysis with purified bacterial LTB (Figure 3). Immunoblot analysis of tobacco plants transformed with the LTB gene revealed an oligomeric LTB protein with molecular weight of 45 kDa (Figure 3(A)). However, when B subunits were boiled for 10 min, they dissociated into tetramers, trimers, and monomers with molecular weight of 12 kDa similar to the bacteria-derived LTB (Figure 3(B)). That is consistent with the previous report on the LTB expressed in the nuclear transformation of maize (Chikwamba et al., 2002). As expected the level

of LTB protein accumulation did not differ significantly in the two transplastomic plants because of site-specific integration into the chloroplast genome.

ELISA quantification of LTB expression

An ELISA assay was used to estimate the LTB protein level in the leaf tissues of transplastomic plant lines 2 and 5. The percentage of LTB protein in each plant was calculated from the total soluble leaf protein used in three replicates of the assay. According to this method, the concentrations of soluble protein loaded in the microtiter plate wells yielded LTB

protein levels equal to 2.3 and 2.5% of TSPs in the respective transplastomic plant lines (Figure 4(A)).

Binding assay of LTB protein to GM1 receptor

The ability of the B subunits to bind to gangliosides was examined using 96-well plates coated with GM1-gangliosides. In the GM1–ELISA binding assays, chloroplast-produced LTB protein demonstrated a strong affinity for GM1-ganglioside, but not for BSA (Figure 4(B)). Based on the absorbance measurement used to determine GM1 binding, the LTB protein expression by the two transplastomic plant lines was also very similar. The strong binding efficiencies of plant LTB for GM1 indicate that plant-derived LTB subunit binding to GM1 is cooperative (Schon & Freire, 1989; Merritt et al., 1994).
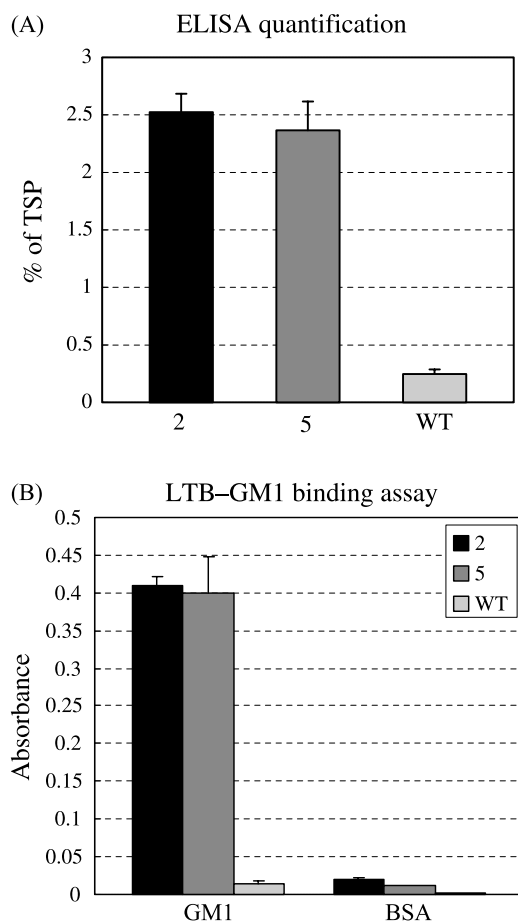
Discussion

Following the success of Haq et al. (1995) in expressing LTB protein in potato, Mason et al. (1998) fed the transformed potato plants to mice, and found that the LTB in potato tubers stimulated strong serum and mucosal antibody responses against LTB and provided partial protection against oral challenge with LT. Therefore, they demonstrated that the LTB expressed in plants has the potential to be used as an edible vaccine. The production of oligomeric LTB protein in edible plants may induce mucosal and systemic antibodies in mammals at levels sufficient to provide protective immunity against an ETEC challenge after feeding on transgenic plant tissues. It has been reported that the production of LTB in transgenic potato tubers at the level of 0.01% of TSP is sufficient to elicit both systemic and mucosal antibody production in mice, demonstrating the *in vitro* toxin-neutralization capability of oral immunization (Haq et al., 1995). However, it may be desirable to increase antigen expression levels to create more effective plant-based oral vaccines for larger animals and humans.

Chloroplasts offer an ideal compartment for the overproduction of foreign proteins. An additional significant advantage of using chloroplasts is their potential to process eukaryotic proteins, including folding and the formation of disulfide bridges. The CT B subunit gene was expressed up to 4.1% tobacco TSP (Daniell et al., 2001), which is 410-fold greater expression than that of the native LTB gene expressed in the nuclear genome. The chloroplast-synthesized CTB bound to the intestinal membrane GM1-ganglioside

Figure 4. (A) ELISA for the determination of LTB protein level in the leaf of transplastomic plants. WT, wild-type plant; 2 and 5, two transplastomic plants (lines 2 and 5, respectively). (B) GM1 binding ability of chloroplast-synthesized LTB. Plate coated with GM1 and BSA was incubated with TSP of wild-type and two transplastomic plants. The absorbance was measured at 405 nm.

receptor, indicating correct folding and disulfide bond formation of CTB pentamers. Another protein that has been expressed in tobacco chloroplasts is human somatotropin (hST), which is used clinically; hST contributed more than 7% of TSP and was expressed in a biologically active, disulfide-bonded form in tobacco chloroplasts (Staub et al., 2000).

The 2.5% LTB protein of TSP that was observed is approximately 250-fold greater expression than that of LTB *via* nuclear genome integration (Haq et al., 1995). Moreover, the expression level may be underestimated, because the band larger than 45 kDa seen in the Western blot analysis (Figure 3(A)) results from aggregates of oligomers in plant extracts (Daniell et al., 2001). The chloroplast-synthesized LTB showed a strong affinity for GM1-ganglioside, suggesting that the LTB conserved the antigenic sites for binding and proper folding of pentameric LTB structure. This proves the feasibility of using plastids to express pharmaceutically useful proteins. The LTB expression in the tobacco chloroplasts did not affect growth rates, flowering or seed setting, and we could not morphologically distinguish between the transplastomic and wild-type plants in the greenhouse. This result agrees with the previous report (Daniell et al., 2001) in which transplastomic tobacco plants expressing CTB gene had no pleiotropic effects compared to wild-type plants.

Food plants would be more practical alternatives for the production of heat-labile recombinant protein antigens like LTB or CTB, and the ability of palatable food plant species that can be consumed without cooking, such as bananas, tomatoes, lettuce, carrots, and avocados, to produce vaccine antigens must be evaluated. In tomato, foreign protein accumulation was found not only in the chloroplasts of green leaves, but also in the chromoplasts of tomato fruits (Ruf et al., 2001). This is the first example of protein expression in the plastids of an edible plant part, and provides the promise of high-level expression of edible vaccines. We are currently attempting to express LTB in the plastids of lettuce and carrot plants.

The development of edible transgenic plants with high expression levels of multimeric LTB and CTB, which can function both as an immunogen and a carrier peptide for other antigen epitopes, will move us closer to a low-cost, convenient, effective and safe strategy for preventing infectious and autoimmune diseases in humans, especially in regions of the developing world where the resources of modern medical technology are largely unavailable.

References

Bock R (2001) Transgenic plastids in basic research and plant biotechnology. *J Mol Biol* **312**: 425–438.

Chikwamba R, Cunnick J, Hathaway D, McMurray J, Mason H and Wang K (2002) A functional antigen in a practical crop: LT-B producing maize protects mice against *Escherichia coli* heat labile enterotoxin (LT) and cholera toxin (CT). *Transgenic Res* **11**: 479–493.

Clements JD and Finkelstein RA (1978) Immunological cross-reactivity between a heat-labile enterotoxin(s) of *Escherichia coli* and subunits of *Vibrio chorelae* enterotoxin. *Infect Immun* **21**: 1036–1039.

Clements JD and Finkelstein RA (1979) Isolation and characterization of homogeneous heat-labile enterotoxins with high specific activity from *Escherichia coli* cultures. *Infect Immun* **24**: 760–769.

Clements JD, Yancey RJ and Finkelstein RA (1980) Properties of homogeneous heat-labile enterotoxin from *Escherichia coli*. *Infect Immun* **29**: 91–97.

Corneille S, Lutz K, Svab Z and Maliga P (2001) Efficient elimination of selectable marker genes from the plastid genome by the CRE-*lox* site-specific recombination system. *Plant J* **27**: 171–178.

Daniell H (1997) Recombinant gene expression protocols. In: Tuan R (ed), Methods in Molecular Biology. Vol. 62 (pp. 47–58) Humana Press, Totowa, NJ.

Daniell H, Datta R, Varma S, Gray S and Lee SB (1998) Containment of herbicide resistance through genetic engineering of the chloroplast genome. *Nat Biotechnol* **16**: 345–348.

Daniell H, Lee SB, Panchal T and Wiebe PO (2001) Expression of the native cholera toxin B subunit gene and assembly as functional oligomers in transgenic tobacco chloroplasts. *J Mol Biol* **311**: 1001–1009.

De Cosa B, Moar W, Lee SB, Miller M and Danielle H (2001) Overexpression of the *Bt cry2*Aa2 operon in chloroplasts leads to formation of insecticidal crystals. *Nat Biotechnol* **19**: 71–74.

Dickinson BL (1996) Use of *Escherichia coli* heat-labile enterotoxin as an oral adjuvant. In: Kiyono H, Ogra PL and McGhee JR (eds), Mucosal Vaccines. (pp. 73–87) Academic Press, San Diego, CA.

Dus Santos MJ, Wigdorovitz A, Trono K, Rios RD, Franzone PM, Gil F et al. (2002) A novel methodology to develop a foot and mouth disease virus (FMDV) peptide-based vaccine in transgenic plants. *Vaccine* **20**: 1141–1147.

Elson CO (1996) Cholera toxin as a mucosal adjuvant. In: Kiyono H, Ogra PL and McGhee JR (ed), Mucosal Vaccines. (pp. 59–72) Academic Press, New York, NY.

Giddings G, Allison G, Brooks D and Carter A (2000) Transgenic plants as factories for biopharmaceuticals. *Nat Biotechnol* **18**: 1151–1155.

Gilligan PH, Brown JC and Robertson DC (1983) Immunological relationships between cholera toxin and *Escherichia coli* heat-labile enterotoxin. *Infect Immun* **42**: 683–691.

Gomez N, Carrillo C, Salinas J, Parra F, Borca MV and Escribano JM (1998) Expression of immunogenic glycoprotein S polypeptides from transmissible gastroenteritis coronavirus in transgenic plants. *Virology* **249**: 352–358.

Haq TA, Mason HS, Clements JD and Arntzen CJ (1995) Oral immunization with a recombinant bacterial antigen produced in transgenic plants. *Science* **268**: 714–716.

Kang TJ and Fawley MW (1997) Variable (CA/GT)n simple sequence repeat DNA in the alga *Chlamydomonas*. *Plant Mol Biol* **35**: 943–948.

Kong Q, Richter L, Yang YF, Arntzen CJ, Mason HS and Thanavala Y (2001) Oral immunization with hepatitis B surface antigen expressed in transgenic plants. *Proc Natl Acad Sci USA* **98**: 11539–11544.

Kozuka S, Yasuda Y, Isaka M, Masaki N, Taniguchi T, Matano K et al. (2000) Efficient extracellular production of recombinant *Escherichia coli* heat-labile enterotoxin B subunit by using the expression/secretion system of *Bacillus brevis* and its mucosal immunoadjuvanticity. *Vaccine* **18**: 1730–1737.

Lauterslager TG, Florack DE, van der Wal TJ, Molthoff JW, Langeveld JP, Bosch D et al. (2001) Oral immunisation of naive and primed animals with transgenic potato tubers expressing LT-B. *Vaccine* **19**: 2749–2755.

Lutz KA, Knapp JE and Maliga P (2001) Expression of bar in the plastid genome confers herbicide resistance. *Plant Physiol* **125**: 1585–1590.

Mason HS, Haq TA, Clements JD and Arntzen CJ (1998) Edible vaccine protects mice against *Escherichia coli* heat-labile enterotoxin (LT): potatoes expressing a synthetic LT-B gene. *Vaccine* **16**: 1336–1343.

Mason HS, Warzecha H, Mor T and Arntzen CJ (2002) Edible plant vaccines: applications for prophylactic and therapeutic molecular medicine. *Trends Mol Med* **8**: 324–329.

Merritt EA, Sixma TK, Kalk KH, van Zanten BA and Hol WG (1994) Galactose-binding site in *Escherichia coli* heat-labile enterotoxin (LT) and cholera toxin (CT). *Mol Microbiol* **13**: 745–753.

Ruf S, Hermann M, Berger IJ, Carrer H and Bock R (2001) Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. *Nat Biotechnol* **19**: 870–875.

Schon A and Freire E (1989) Thermodynamics of intersubunit interactions in cholera toxin upon binding to the oligosaccharide portion of its cell surface receptor, ganglioside GM1. *Biochemistry* **28**: 5019–5024.

Sambrook J and Russell DW (2001) Molecular Cloning: A Laboratory Manual. 3rd edn. Cold Spring Harbor Laboratory, Plainview, NY.

Scott SE and Wilkinson MJ (1999) Low probability of chloroplast movement from oilseed rape (*Brassica napus*) into wild *Brassica rapa*. *Nat Biotechnol* **17**: 390–392.

Sidorov VA, Kasten D, Pang SZ, Hajdukiewicz PTJ, Staub JM and Nehra NS (1999) Stable chloroplast transformation in potato: use of green fluorescent protein as a plastid marker. *Plant J* **19**: 209–216.

Staub JM and Maliga P (1995) Expression of a chimeric *uidA* gene indicates that polycistronic mRNAs are efficiently translated in tobacco plastids. *Plant J* **7**: 845–848.

Staub JM, Garcia B, Graves J, Hajdukiewicz PT, Hunter P, Nehra N et al. (2000) High-yield production of a human therapeutic protein in tobacco chloroplasts. *Nat Biotechnol* **18**: 333–338.

Stoger E, Sack M, Fischer R and Christou P (2002) Plantibodies: applications, advantages and bottlenecks. *Curr Opin Biotechnol* **13**: 161–166.

Svab Z and Maliga P (1993) High-frequency plastid transformation in tobacco by selection for a chimeric *aadA* gene. *Proc Natl Acad Sci USA* **90**: 913–917.

Svab Z, Hajdukiewicz P and Maliga P (1990) Stable transformation of plastids in higher plants. *Proc Natl Acad Sci USA* **87**: 8526–8530.

Tacket CO, Mason HS, Losonsky G, Clements JD, Levine MM and Arntzen CJ (1998) Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. *Nat Med* **4**: 607–609.

Tacket CO, Mason HS, Losonsky G, Estes MK, Levine MM and Arntzen CJ (2000) Human immune responses to a novel Norwalk virus vaccine delivered in transgenic potatoes. *J Infect Dis* **182**: 302–305.

Tuboly T, Yu W, Bailey A, Degrandis S, Du S, Erickson L and Nagy E (2000) Immunogenicity of porcine transmissible gastroenteritis virus spike protein expressed in plants. *Vaccine* **18**: 2023–2028.