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Production of *Escherichia coli* heat labile toxin (LT) B subunit in soybean seed and analysis of its immunogenicity as an oral vaccine

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Abstract

The B subunit of the heat labile toxin of enterotoxigenic *Escherichia coli* (LTB) was used as a model immunogen for production in soybean seed. LTB expression was directed to the endoplasmic reticulum (ER) of seed storage parenchyma cells for sequestration in de novo synthesized inert protein accretions derived from the ER. Pentameric LTB accumulated to 2.4% of the total seed protein at maturity and was stable in desiccated seed. LTB-soybean extracts administered orally to mice induced both systemic IgG and IgA, and mucosal IgA antibody responses, and was particularly efficacious when used in a parenteral prime-oral gavage boost immunization strategy. Sera from immunized mice blocked ligand binding in vitro and immunized mice exhibited partial protection against LT challenge. Moreover, soybean-expressed LTB stimulated the antibody response against a co-administered antigen by 500-fold. These results demonstrate the utility of soybean as an efficient production platform for vaccines that can be used for oral delivery.

Keywords: Microbial toxin; Plant-based vaccines; Transgenic soybean seed

1. Introduction

Effective needle-free immunization strategies are needed to accommodate large-scale vaccination programs and avoid injection-related risks. Such delivery systems have broad applications for the rapid immunization of humans, as well as production farm animals, and may be the only effective means for immunization of wild animal reservoirs to mitigate epizootic disease spread into humans and other animals. Vaccines administered at mucosal surfaces can induce local protection at the infection sites for many pathogens as well as induce systemic immunity. However, direct antigen delivery through an oral or nasal route generally leads to weak induction of immunity, if any, or to immunological tolerance [1]. To improve the efficacy of oral vaccination, antigens can be co-administered, or fused with a strong mucosal adjuvant [2–4]. Cholera toxin (CT) of *Vibrio cholerae* and heat labile toxin (LT) of enterotoxigenic *Escherichia coli* (ETEC) are well-studied examples of bacterial proteins with strong mucosal adjuvant activity [5–7]. LT is a hetero-oligomeric AB_5 type enterotoxin composed of a 27 kDa A subunit with toxic ADP ribosyl transferase activity and a stable noncovalent-linked pentamer of 11.6 kDa B subunits. ETEC infection and colonization of the small intestine, and the production of LT, causes acute diarrhea that can be fatal without intervention. The ADP-ribosylation of Gsα, catalyzed by the A subunit, triggers increased intracellular cAMP levels that induce chloride efflux and fluid loss from intoxicated cells lining the small intestine. The B subunit pentamer mediates holotoxin binding to ganglioside GM1 on intestinal epithelial cells, with lower affinity for GD1B, asialoGM1 and lactosylceramide gangliosides [8]. Oral vaccines against LT render protection against diarrhea associated with LT-producing *E. coli* [9,10]. LT is a potent immunogen whose adjuvant active dose is

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well below its immunogenic dose [11]. LT and detoxified mutants of LT trigger a stronger antibody response than LTB to co-administered antigens on a dose-for-dose basis. However, recombinant LTB is safely and commonly used as an adjuvant to stimulate antibody responses to co-administered protein antigens, and its GM1-binding function is essential for both immunogenicity and adjuvanticity [8]. As B subunit adhesion to the surface of a target cell is a prerequisite for entry of the A subunit, this protein–carbohydrate recognition event is a good target for vaccine action against the toxic effects of ETEC. LTB has also been used experimentally for the prevention and treatment of autoimmune diseases [12–14]. Importantly, LTB has been shown to protect against the development of oral tolerance to co-fed soluble vaccine proteins [3,15], a serious consideration in the food-based delivery of vaccines.

Transgenic plants offer the possibility to both produce and deliver an oral immunogen on a large-scale with low production costs and minimal purification or enrichment, and the potential exists for direct formulation of vaccines into animal feed and human consumables. The risks associated with human pathogen contamination and needle-based delivery are avoided. Vaccines made in crop plants, when administered orally, can elicit both systemic and mucosal immunity that is protective [16–18]. LTB, CTB and genetic fusions of immunogens to LTB and CTB have been successfully made in tobacco leaf, maize, tomato and potato [19–24], however, relatively low expression levels (grams B subunit per kilogram plant material) of biologically active material, not adequate for commercial development, have been achieved.

Soybean has great potential as a vaccine delivery platform because of its naturally high protein content, nutritional value and multiple product streams. Mature soy seed contains between 35 and 40% (w/w) protein, primarily the 7S glycinin and 11S β-conclycinin storage proteins, compared to 8–10% protein in maize and rice or 1–2% protein in leaf tissues (e.g. tobacco). Similar to other seed types, mature soybean seed has a low water content that confers storage stability on expressed proteins. Soybean-processing options include formulation into soy-based formulas or soymilk for infants, tofu and other soybean protein products for children and adults, as well as feed supplements for animals. Applications of the use of soybean for vaccine production, therefore, extend delivery to both humans and animals.

In this study, we have used a strategy for high-level protein accumulation that enhances protein stability in transgenic seed for the production of LTB in soybean seed. LTB was expressed under the control of a seed-specific promoter and the translation product was directed to inert de novo-produced protein accretions derived from the endoplasmic reticulum (ER). These bodies are analogous to protein bodies (PBs) or precursor protein vesicles (PPVs) [25–27]. Retention of heterologous proteins within the ER and ER-derived compartments mitigates posttranslational protein instability [26,27] and eliminates the potential for complex end-point plant-type glycosylation. As detailed herein, the level of accumulation of bacterial LTB achieved in soybean seed was substantially higher than previously shown in other crop plant expression systems that have been used to produce vaccines and other biopharmaceutical proteins [19,20,22–24,28–33]. Soybean-based LTB is produced with high fidelity as evidenced by the correct assembly of pentamers that actively bind ganglioside GM1, and is highly immunogenic, particularly when used in a prime-boost immunization strategy.

2. Materials and methods

2.1. Seed-specific LTB expression cassette construction

A synthetic plant codon-optimized LTB gene [19,20] AAC60441, generously provided by A. Walmsley (Arizona Biodesign Institute) was modified by substitutions of the bacterial signal peptide with a 20 aa signal peptide from *A. thaliana* basic chitinase [34]. A 14 aa extension comprising the FLAG epitope and KDEL ER retention signal, and flanking Bsp120 restriction sites were introduced by PCR. The final sequence encoded a 137 aa protein of 15.5 kDa that yielded a 13.3 kDa LTB-FLAG protein after signal peptide cleavage. Following subcloning into pGEM T/A (Promega) for sequence verification, the Bsp120 LTB gene fragment was subcloned into the pGly vector, placing it under the control of soybean seed-specific glycinin promoter and terminator [35]. The final soybean transformation vector pGly::ER-LTB contained a hygromycin selection marker (kindly provided by N. Murai, Lousiana State University) under the control of potato ubiquitin 3 promoter and terminator [36].

2.2. Soybean transformation and screening of embryos

Glycine max [L.] Merrill cv. Jack was subjected to biolistic transformation and somatic embryogenesis as described by Schardl et al. [37] and Trick et al. [38], and regeneration of fertile plants was done according to Schmidt et al. [39]. Globular and cotyledonary-stage embryos were screened by PCR using LTB-specific primers prior to regeneration of individual lines. Cotyledonary-stage embryos were removed from liquid maturation media prior to the brief desiccation stage, frozen in liquid nitrogen, and ground with a mortar for Western blot analysis of expressed LTB [39].

2.3. Protein extraction of soybean embryos and seed

Protein extracts (in 50 mM Tris, pH 7.5, 5 mM EDTA, 100 mM NaCl) from somatic embryos or seed chips were heated at 65 °C for 10 min and normalized for protein concentration prior to SDS-PAGE and Western blot analysis. Membranes were blocked with 5% nonfat dry milk and probed with either mouse anti-FLAG monoclonal antibody M2 (Sigma, F3165, 1:5000 in PBST) or an anti-LTB rabbit polyclonal antibody (Immunology Consultants Laboratory; ICL, *E. coli* HLT, RECO-55G, 1:10,000), followed by alkaline

phosphatase-conjugated secondary antibody and substrate. For immunization, transgenic LTB-laden soybean seeds were ground in 5 vol. of PBS at 4 °C, the extracts were clarified by microcentrifugation at 20,000 × *g* for 5 min, and the total protein concentration was measured using the Bradford method [40]. The LTB concentration in soybean extracts was estimated by SDS-PAGE using purified tobacco mosaic virus coat protein (17.5 kDa) as the comparative protein standard.

2.4. Mass spectroscopy analysis

Total protein isolated from mature seeds according to a modified phenol method was subjected to two-dimensional IEF/SDS PAGE (pH 3–10, nonlinear) [41,42]. Gels were run in triplicate for LTB transgenic lines and the nontransformed control line. Image analysis was performed on Coomassie blue-stained gels following 2D IEF/SDS-PAGE separation using Phoretix 2D evolution software (Nonlinear Dynamics Ltd.; Newcastle, UK). In-gel trypsin digestion of protein spots was performed according to Gabelica et al. [43]. A QSTAR XL (Applied Biosystems, USA) hybrid quadrupole TOF MS/MS system was used for peptide sequence data acquisition. The peptide electrospray tandem mass spectra were processed using Analyst QS software (ABI, USA) and searched against the Prosite database using Mascot (Version 1.9). A MALDI-TOF Voyager-DE STR (Applied Biosystems) mass spectrometer was used to analyze tryptic digests from SDS-PAGE gels.

2.5. Size exclusion chromatography

Homozygous transgenic seeds were ground in cold extraction buffer (20 mM Na phosphate, pH 7.0, 0.2 M NaCl, 5 mM EDTA) and the protein concentration was adjusted to 1 mg/ml. Two milliliters of clarified, filtered soybean extract were loaded onto a calibrated gel filtration column (Akta Explorer FPLC, HiLoad Superdex 200 16/60, GE Healthcare). Fractions were collected and the presence of LTB was detected by Western blotting.

2.6. Analysis of ganglioside GM1-binding

LTB in transgenic soybean embryos and seed was examined for ligand-binding activity using a GM1 ELISA [20,23]. Plates were pre-coated with Type III GM_1 gangliosides (Sigma G2375, 1.5 μg per well in sodium bicarbonate binding buffer, pH 9.6) and then blocked for 1 h at room temperature with 5% (w/v) nonfat dried milk (BioRad). Plates were washed between steps with phosphate-buffered saline with 0.05% Tween 20 [PBST]. The wells were then incubated overnight at 4 °C with extracts from transgenic soybean embryos or seeds. Plates were incubated with rabbit anti-LTB antibody (IgG fraction, 1:10,000) at 37 °C for 1 h. Binding was detected with an alkaline phosphatase-conjugated goat anti-rabbit antibody [IgG (H+L), 1:4000 (KPL)], followed by addition of *p*-nitrophenol phosphate. Absorbance at 405 nm was measured using a Molecular Devices SpectroMax Plus spectrophotometer.

2.7. Electron microscopy

Immunolocalization of LTB in late maturation cotyledons (200 mg fresh weight) of control and transgenic plants was accomplished by high-pressure freezing late maturation cotyledons [44] using a Baltec HPM 010 (Balzers, Liechtenstein). Immunolabeling of thin sections was accomplished using anti-LTB polyclonal antibody (diluted 1:50) and 10 nm anti-rabbit colloidal gold (Ted Pella Inc., Tustin, CA) as primary and secondary reagents, respectively. Grids were contrasted with uranyl acetate and examined under a Zeiss Leo 912AB (Zeiss SMT) electron microscope.

2.8. Parenteral and oral immunization of mice

Inbred female C57BL/6J mice (Jackson Laboratory) were immunized either subcutaneously or orally with soluble protein extracts from LTB transgenic soybean seed or nontransgenic cv. Jack seed. Mice were fasted for 12 h, but allowed water ad libitum prior to oral immunization by gavage using a ball-tip feeding needle (Popper & Sons, Inc., New Hyde Park, NY). Five mice were used per group. Group 1 was immunized subcutaneously (s.c.) with soybean extract (500 ng LTB in complete Freund's adjuvant), followed by secondary s.c. immunization after 14 days. Group 2 was primed with 500 ng soybean LTB by s.c. immunization, then followed by immunization at weekly intervals by oral gavage (equivalent to 25 μg soybean LTB in 150 μl extract). Group 3 was immunized by oral gavage (25 μg soybean LTB in 150 μl extract) at weekly intervals. Control mice were vaccinated by mock s.c. primary immunization followed by oral gavage or by oral gavage alone with a soluble protein extract made from nontransgenic soybean seed.

2.9. Serum and fecal sample preparation

Blood samples were taken from immunized mice by tail bleed at days 0, 10, 21, 35 and 60 for analysis of Ig class-specific systemic LTB-specific antibodies. Equal volumes of serum from individual mice for each experimental group were pooled for determination of anti-LTB antibody titer. Fecal pellets, collected from groups of mice once weekly, were lyophilized for 48 h and weighed. PBS containing 0.2 mg/ml trypsin inhibitor, 0.05% sodium azide (w/v), and a cocktail of protease inhibitors (Roche) was added at 10 μl/mg of dry fecal material. Samples were soaked in buffer overnight at 4 °C, microcentrifuged at 20,000 × *g* for 10 min, and the supernatants analyzed for sIgA levels indicative of a secretory mucosal immune response.

2.10. Determination of anti-LTB antibodies

Antibody titers were determined in serum and fecal samples from immunized mice by use of the GM1 ganglioside

ELISA. Microtiter plates pre-coated with mixed bovine type III gangliosides were incubated with 1.0 μg per well of purified LTB (John Clements, Tulane University) at 37 °C for 1 h, and then washed and blocked. Serial diluted serum or fecal samples were added and the plates incubated for 1 h at 37 °C. Anti-LTB levels were determined following incubation with goat anti-serum against mouse IgG (Sigma A3688) or IgA (Sigma A4937) conjugated to alkaline phosphatase diluted 1:5000 in 1% (w/v) milk in PBS-Tween at 37 °C for 1 h, followed by *p*-nitrophenol phosphate (*p*NPP, Sigma N7653). Endpoint titer is defined as the reciprocal of the highest dilution of pooled serum samples or fecal extracts to give an optical absorbance (OD 405 nm) greater or equal to twice the control. Cross-reactivity of anti-LTB antibody with CTB was determined by binding CTB (Sigma) at 1 μg per well in place of LTB.

2.11. Antiserum-mediated inhibition of LTB and CTB binding to GM1-gangliosides

Pooled sera (100 μl) from mice immunized with extracts from LTB-laden soybean seed or nontransgenic soybean seed were mixed with 2.5 ng of purified bacterial LTB or CTB and pre-incubated at 37 °C for 1 h. GM1-bound plates were prepared as described. After washing with PBS, sera/LTB mixtures were added to wells and the plate incubated at 4 °C overnight. Unbound LTB or CTB was measured by ELISA. After blocking the plate, B subunit binding was detected using rabbit anti-LTB antibody (1:5000) or anti-CT antibody (1:5000 dilution, Sigma C3602), and goat anti-rabbit IgG conjugated with alkaline phosphatase (1:50,000; Southern Biotech) as primary and secondary reagents, respectively. After addition of pNPP substrate, absorbance at 405 nm was determined. No competition for LTB binding between mouse and rabbit anti-LTB antibodies was detected.

2.12. Patent mouse assay

Following oral LTB immunization, protection against toxin challenge was determined using the patent mouse assay [45,46]. Challenge of immunized mice was performed on day 64. Briefly, mice were fasted for 12 h and challenged by oral gavage with 200 μl of 0.9% saline (pH 7.2) containing 25 μg purified LT (kindly provided by John Clements), or saline alone, using five mice per group. Intragastric delivery was performed using a ball-tip feeding needle. Water was available ad libitum. Three hours after toxin administration, mice were euthanized by CO_2 inhalation. After applying hemostats to both ends of the intestinal tract, the gut was removed from duodenum to anus. The weight of the gut, with fat pads and the mesentery carefully removed, was taken separately from weight of the remaining body. The gut/carcass ratio was used to assess the extent of toxin-induced water influx into the gut. A dose–response analysis was initially performed on C57BL/6J mice with LT doses ranging from 10 to 100 μg. The data were statistically evaluated by variance analysis (ANOVA) and comparison of means (one tailed Student's *t*-test) was determined at a significance level $p < 0.05$.

2.13. Demonstration of adjuvanticity of soy LTB

Groups of three C57BL/6J mice were immunized intraperitoneally with 5 μg purified bacterial FimHt alone or in combination with 5 μg equivalent of soybean seed LTB, or with nontransgenic seed extract in a volume of 50 μl normal saline. Ten days after primary immunization, mice received a second dose of FimHt with transgenic LTB or nontransgenic seed extract. Twenty-one days after initial immunization, blood samples were taken from each group and pooled for determination of serum anti-FimHt and anti-LTB antibody titers. Anti-LTB IgG was assessed as previously described. Anti-FimHt IgG was determined by ELISA using purified recombinant bacterial FimHt (kindly provided by Scott Hultgren, Washington University School of Medicine).

3. Results

3.1. LTB expression in transgenic seed

Soybean somatic embryos were transformed with an LTB gene whose expression was specific to the cotyledons as regulated by the soybean glycinin promoter. The heterologous protein was directed to the endoplasmic reticulum (ER). Cotyledonary embryos and T_1 seed from transgenic plants regenerated therefrom were analyzed by PCR using LTB-specific primers to detect the presence of the transgene and by Western blotting to assess LTB accumulation. Five independent LTB transgenic lines were generated. Line 157, described herein, showed the highest level of LTB accumulation, and was propagated for the collection of homozygous T_2 seed. A minimum of 20 T_2 seeds per plant were re-tested for LTB expression by dot blot analysis. Soybean seeds appeared unaffected by LTB transgene expression and no obvious phenotypic changes were observed in any of the events or in T_2 homozygous plants (line 157).

Recombinant LTB accumulated in soybean seed to substantial levels as evident by a band of the predicted molecular mass revealed by SDS-PAGE and Coomassie staining (Fig. 1A). Both rabbit antisera against LT or mouse anti-FLAG M2 antibody recognized a protein of approximately 14 kDa on a Western blot of SDS-PAGE-resolved extracts heated for 10 min at 65 °C prior to electrophoresis (Fig. 1B) and a protein of approximately 60 kDa in non-heated samples (not shown). The monoclonal antibody against the FLAG epitope tag did not cross-react with any other soybean proteins, hence it was primarily used for immunoanalysis.

Densitometric analysis of total soluble soybean protein extracts following 2D gel separation in triplicate indicated that LTB accumulation was 1.8–2.4% of the total protein (in soybean, total protein and total soluble protein are nearly equivalent). This is in agreement with densitometric analy-

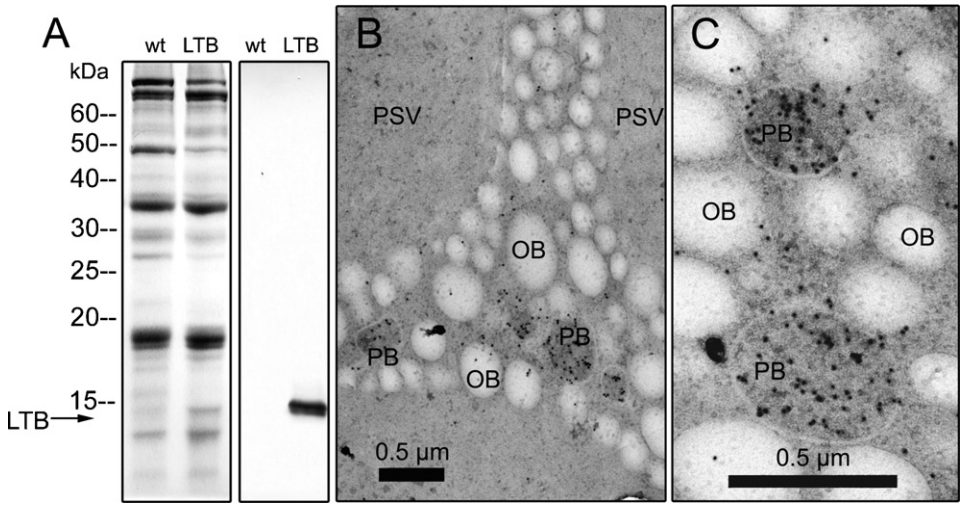

Fig. 1. Analysis of LTB accumulation in transgenic soybean seeds. (A) Extracts from mature seeds were separated by SDS-PAGE and the proteins stained with Coomassie blue (left) or immunoblotted and detected using an anti-FLAG MAb (right). LTB is indicated by the arrow. (B and C) High pressure frozen thin sections of mid-mature seeds were probed with anti-LTB antibody and colloidal gold (10 nm particles). LTB was localized to electron dense protein bodies in transgenic seed that were absent in control nontransgenic seed. PSV, protein storage vacuole; OB, oil body; PB, protein body. Scale bars represent 0.5 μm.

sis of soluble protein extracts resolved on Coomassie-stained SDS gels, measured against a calibration curve, that indicated LTB comprised between 2.5 and 3.5% of the soluble seed protein. The ratio of LTB to total soybean protein did not change throughout seed maturation, and no loss of LTB protein was observed upon storage of dry seeds for at least a 3-month period. The ranges given reflect the observed variation between individual transgenic seed samples.

The subcellular localization of LTB within seed parenchyma cells was determined by transmission electron microscopy of ultra-thin sections from high-pressure frozen cotyledons following immunogold labeling with anti-LTB or anti-FLAG antibodies (Fig. 1B and C). The accumulated LTB was primarily localized to dense cytoplasmic protein accretions apparently derived from the ER as indicated by characteristic ribosomes bound to the limiting membrane. These organelles, termed protein bodies (PBs) or protein precursor vesicles (PPVs) depending upon their protein content, were not observed in nontransgenic seed indicating that LTB expression and accumulation induced the de novo formation of sequestering PBs. When the numbers of gold particles were assessed in over 50 PBs ranging from 200 to 500 nm in diameter, an average of 14.72 ± 4.17 particles per body was observed with a greater number of particles seen in larger, more mature protein bodies.

3.2. Molecular and functional characterization of soy LTB

To determine whether the basic chitinase signal peptide at the N-terminus of the LTB was properly cleaved, the protein band corresponding to LTB was excised from a Coomassie-stained gel, trypsin-digested and subjected to MALDI-TOF mass spectrometry. Four peptides covering in total 43% of the recombinant LT-B protein were identified in the resulting spectrum. Among these, two peptides of molecular masses 1567.71 and 1496.68 Da were identified that corresponded to the N-terminal peptide APQSITELCSEYR with, and without, the cysteine modified with an acrylamide adduct, respectively. These results indicated that correct processing of the signal peptide had occurred.

The proportion of soybean-expressed LTB present as a bioactive pentamer was determined by Superdex200 FPLC gel exclusion chromatography of seed extracts. Individual fractions were analyzed by Western blot using anti-FLAG antibody after briefly boiling in SDS-PAGE sample buffer (not shown). LTB was present exclusively in three fractions corresponding to proteins of 42–93 kDa, with the strongest signal being at about 63 kDa, which correlates well with an expected molecular mass of 66.4 kDa for the LTB pentamer. These fractions showed positive GM1 binding. LTB protein was not detectable in fractions corresponding to the size of a monomeric form.

Soybean LTB was tested for biological activity by examining its ability to bind ganglioside GM1. GM1 binding is dependent upon proper B subunit protein folding and pentamer assembly, as well as the formation of intramolecular disulfide bonds. Extracts from both soybean cotyledonary-stage embryos and mature seed showed dose-dependent GM1 binding similar to that with purified B subunit from LT or CT (Fig. 2). No GM1 binding activity was detected in control nontransgenic extracts.

3.3. Immunogenicity of soy LTB

Three different mouse immunization regimens, including prime-boost and gavage strategies, were used to investigate the ability of soy LTB to elicit serum and intestinal IgG/IgA

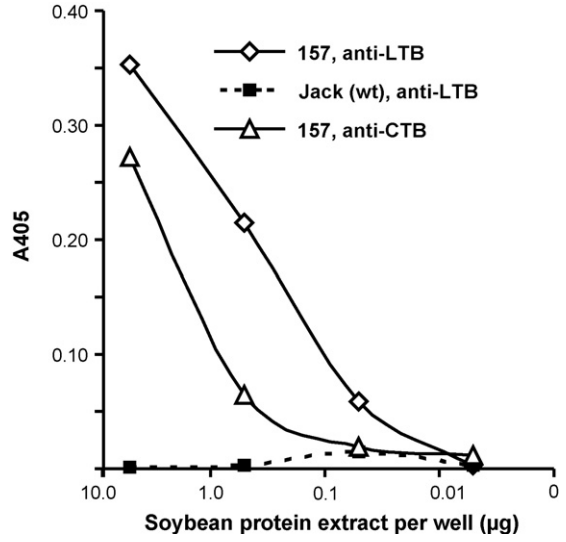

Fig. 2. Binding of LTB in soybean extracts to ganglioside GM1 as determined by ELISA. Seed extracts from LTB transgenic (line 157) and control (Jack) soybean were bound in serial dilutions to GM1-coated plate and probed with both anti-LTB and anti-CTB antibodies.

antibody responses. Significant anti-LTB titers were detected in pooled sera and fecal samples following soy LTB administration. Antibody class switching from IgM to IgG occurred at about 13 days following primary subcutaneous immunization (not shown). As expected, serum anti-LTB IgG titers were highest with parenteral subcutaneous immunization. At day 35 (14 days after secondary boost), the endpoint titer was determined to be 1:32,000, reflecting significant immunological recall (Fig. 3). High levels of serum anti-LTB IgG were also observed at day 35 using a single primary subcutaneous immunization, followed by a set of 2 gavages (prime-boost

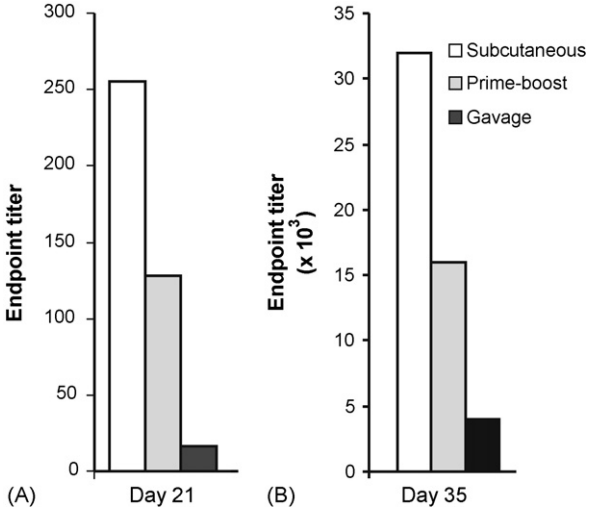

Fig. 3. ELISA determination of anti-LTB IgG in pooled ($n = 5$) serum samples taken at day 21 (A) and day 35 (B) from mice undergoing parenteral and oral immunization. The sera were serially diluted and assayed using a GM1-binding ELISA. Endpoint titer represents the highest dilution giving an OD405 at least twice that of the nontransgenic background level.

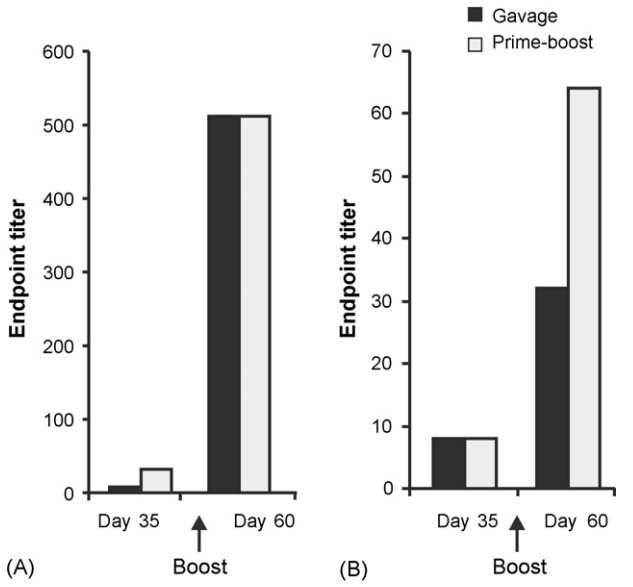

Fig. 4. ELISA determination of serum (A) and fecal (B) anti-LTB IgA in mice immunized either in a parenteral prime-oral boost or oral gavage regimen with extracts from mature transgenic soybean seed. Equal volume samples were pooled from five mice per group and serial dilutions were analyzed using a GM1-binding ELISA. Endpoint titer represents the highest dilution giving an OD405 at least twice that of the nontransgenic background level.

strategy) with an endpoint titer of 1:16,000. Serum anti-LTB IgG levels also increased following weekly gavages with an endpoint titer of 1:4000 when assayed at day 35. Serum anti-IgA titers following soy LTB administration via gavage and prime-boost were 1:4 and 1:32, respectively, at day 35 (Fig. 4A). When mice were boosted by soy LTB gavage at day 48, and serum anti-LTB IgA titers analyzed at day 60, endpoint titers for mice undergoing prime-boost or gavage immunization were both approximately 1:512. Pooled fecal samples from immunized groups of mice showed significant anti-LTB IgA antibody titers (Fig. 4B). At day 35, fecal IgA titers from prime-boost and gavaged mice were both 1:8. Following a gavage boost at day 48, and analysis at day 60, mucosal IgA titers rose to 1:64 and 1:32, respectively. These results demonstrated that a higher anti-LTB IgG and IgA induction potential could be achieved using a prime-boost immunization regimen, as opposed to immunization by gavage only, for the delivery of soy LTB.

Pooled sera from soy LTB-immunized mice blocked the binding of LTB to ganglioside GM1 in vitro by 96% and binding of CTB by about 78% (not shown). Serum from mice immunized with nontrangenic soybean extract blocked LTB binding by only 3%. Both cholera toxin and heat labile toxin have an AB_5-type multimeric structure, with essentially identical A subunits and 80% sequence identity in their B subunits. Therefore, it is hypothesized that during ETEC infection, anti-LTB antibodies made in response to soy LTB immunization will inhibit LT holotoxin binding to intestinal epithelial cells and be partially cross-protective against CT effects.

3.4. Reduction of LT-induced intestinal fluid influx in soy LTB-immunized mice

The protective efficacy of the induced humoral immune responses was examined in orally-immunized mice challenged with LT by use of the patent-mouse assay that provides a measure of the extent of toxin-induced water influx into a nonligated intestine. A preliminary dose–response analysis performed with LT doses ranging from 10 to 100 μg indicated that a linear dose-dependent response was obtained with up to 50 μg of administered toxin from a baseline level of fluid accumulation or G/C ratio of 0.082–0.083 (Fig. 5A). Challenge of LTB or mock-immunized mice was performed on day 64 by intragastric administration of a 25 μg dose of purified bacterial LT (or saline) to animals. The mean gut-to-carcass (G/C) ratio was determined for each immunization group. Reduced enterotoxicity was observed in mice orally immunized with soy LTB (Fig. 5B). Thus, partial protection against LT challenge was achieved in mice orally vaccinated with raw non-purified soluble protein extracts from LTB-transgenic soybean seed. No statistical differences were observed in baseline G/C ratios within the negative control groups (saline-challenged transgenic soy versus nontransgenic soy or buffer-immunized mice). The differences observed between mice orally vaccinated with LTB transgenic soybean and the unvaccinated control group were statistically significant at the 99% confidence level ($p = 0.00131$).

3.5. Adjuvanticity of soy LTB

Mice were given primary and secondary immunizations with bacterial FimHt alone or in combination with soybean

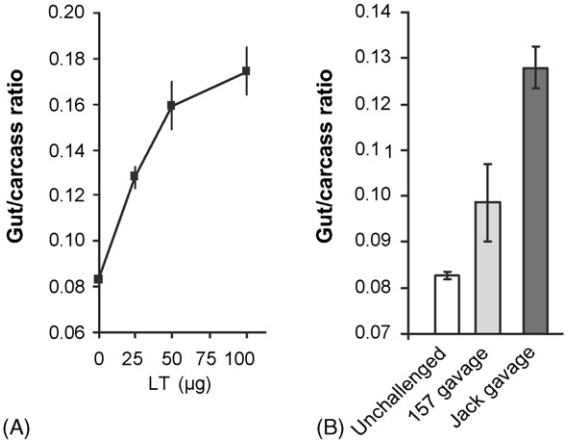

Fig. 5. Patent mouse assay for evaluation of enterotoxicity following LT challenge. (A) Dose response curve for LT. The indicated amounts of LT were delivered intragastrically and mice were dissected after 3 h for determination of the gut/carcass ratio. For 0 and 25 μg of LT, $n = 5$; for 50 and 100 μg, $n = 3$. (B) Response of mice orally immunized with extracts from either LTB transgenic (line 157) or nontransgenic (Jack) soybean seed and then challenged orally with 25 μg LT holotoxin. The data represent the means from five animals in each group with standard deviations shown.

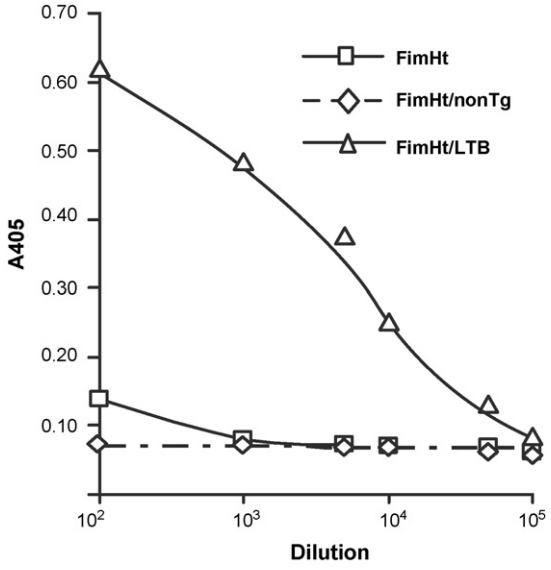

Fig. 6. Soybean-expressed LTB stimulates antibody production against the pathogenic *E. coli* adhesin FimHt. The adjuvanticity of soy LTB was tested by immunizing groups of mice with 5 μg purified bacterial FimHt alone, or in combination with seed extract containing the equivalent of 5 μg LTB or with nontransgenic seed extract. Anti-FimHt and anti-LTB antibody levels in pooled sera were analyzed by ELISA and endpoint titers were determined. Absorbance values represent the means of triplicate samples.

LTB or nontransgenic soybean seed extract. Anti-FimHt IgG endpoint titers in pooled sera after immunization with FimHt alone or with FimHt plus LTB were 1:100 and 1:50,000, respectively (Fig. 6). The anti-LTB IgG endpoint titer was determined to be 1:100,000 (not shown). Nontransgenic seed extract had no effect on anti-FimHt IgG production. Under these experimental conditions, the co-administration of bacterial FimHt with soy LTB enhanced the anti-FimHt IgG antibody response by 500-fold.

4. Discussion

The B subunit of the heat labile toxin, LT, a diarrhea-inducing enterotoxin produced by certain strains of pathogenic *E. coli*, was used as a test antigen for expression in transgenic soybean. Its requirement for oligomer formation for receptor-binding, coupled with its well-established capacity to induce robust local and systemic antibody responses upon oral delivery, made LTB an ideal immunogen for test production in soybean seed. In this study, the pentameric assembly of LTB was produced with high fidelity in soybean seed. Soy LTB was biochemically stable, functionally active and highly immunogenic.

Different food and non-food crops have been used as platforms for the production of pharmaceutical proteins [16–18]. Soybean seed, with its high protein content, can be reconfigured to produce high levels of recombinant heterologous protein for more efficaceous delivery of an oral vaccine. Soybean seed, once desiccated, can be easily stored and trans-

ported. Seed can also be processed as meal or flour, and therefore, rendered nonviable for end-users while remaining a source of active vaccine protein that can be packaged/labeled for ready-use. This latter strategy addresses biosafety concerns and reduces the risk for a biopharmaceutical-producing crop to become an inadvertent food source, while at the same time providing a valuable venue for large-scale oral immunization. A soymilk-based LTB formulation could be envisioned as an anti-diarrheal oral therapy to protect mucosal surfaces against pathogenic effects. Soybean is a self-pollinating crop with no relatives in the western hemisphere capable of partnering for outcrossing, which reduces the risk of unintentional cross-pollination.

In this study, LTB expression was regulated using the strong seed-specific promoter of the major soybean storage protein, glycinin. Foreign proteins synthesized by seeds are often post-translationally unstable, in part due to their trafficking and storage destinations [26,47]. However, following synthesis, proteins can be retained or retarded in the ER by the addition of ER retention sequences for increased stabilization [48]. Soybean LTB expression was induced in a manner that allowed ER retention by use of signal peptide and the KDEL motif. For proteins that require chaperone assistance, oligomer formation, disulfide bond formation and/or co-translational glycosylation, ER synthesis is required for the production of active heterologous protein products. Soy LTB maintained its native protein subunit conformation, formed stable oligomers, and was biologically active. As a general platform for heterologous protein production using the same strategy, expression levels similar to those of LTB have been achieved for another foreign protein of 30 kDa (Schmidt and Herman, in preparation), and work to express the FimHt bacterial adhesin vaccine protein and a single chain antibody are underway.

The approach used in this study to generate high levels of a model biopharmaceutical protein was based upon the storage of ER-synthesized proteins in de novo formed inert protein accretions that confer the capacity to accumulate transgene products in transgenic soybean seeds (Schmidt and Herman, in preparation). The overexpression and accumulation of bacterial LTB to high levels in soybean seed has proven that a vaccine immunogen can be sequestered within the ER and ER-derived seed protein bodies or precursor protein vesicles. These protein accretions may have enhanced immunogenicity, as the enveloped antigenic protein aggregates may be more slowly dispersed as an oral-delivered vaccine. This concept is similar to the use of immunogen-harboring liposomes, micelles, and immunostimulating complexes (ISCOMS) for vaccine delivery. The retarded dispersal due to biological microencapsulation may allow greater immunogen penetration into the gastrointestinal tract, and thus increase the potential for eliciting a more potent gut-associated immune response.

At full seed maturity (200–250 mg, dry weight), LTB accumulated to about 2 mg per seed and total accumulation did not change upon seed desiccation. LTB protein was, therefore, stable during seed maturation and with subsequent storage, and would presumably not be degraded in harvested seed under transport conditions. Based upon percentage of seed weight, this level of LTB achieved is much greater than the accumulation levels of LTB or other biopharmaceutical proteins achieved in comparable seed crop protein production platforms such as maize or rice seed. Recently, other researchers have produced pharmaceutical products in soybean. The major K99 fimbrial antigen of enterotoxigenic *E. coli* infecting cattle was produced under the control of the constitutive cauliflower mosaic virus 35S promoter with the resulting plants showing general tissue expression at a 0.5% total soluble protein (TSP) level [49]. Similarly, basic fibroblast growth factor (bFGF) expressed under the 35S promoter accumulated to 0.04% TSP in seeds [50]. When bFGF was expressed under a glycinin promoter, the yield increased to 0.8% TSP in seed, and with the addition of a 19 aa signal peptide from glycinin to the bFGF product, the final yield in the seed averaged about 1.2% TSP. The latter results underscore the importance of using a strong seed-specific promoter and signal peptide in the design of soybean transgenic constructs. As evidenced in our current work, even more enhanced production levels can be achieved, exceeding 2% TSP, when the heterologous protein is both targeted and retained within the ER and ER-derived protein bodies. Assuming a yield of 250 seeds per plant, it is estimated that using an elite LTB-expressing soybean line, about 1 kg of LTB protein can be generated from 2000 plants, a capacity that is easily accommodated under high biosafety level containment conditions such as in a production greenhouse. This production space is far less in area than required for other production plant species (e.g. maize, rice, tobacco).

The feasibility of using transgenic soybean seed for the oral delivery of vaccines in a food material has been demonstrated using a mouse model. Immunization of mice with LTB transgenic soybean extracts elicited robust systemic anti-LTB IgG and IgA antibody responses, as well as significant levels of intestinal anti-LTB IgA. The serum anti-LTB IgG titer from mice immunized by parenteral primary immunization followed by a series of oral gavage boosts was approximately four-fold higher than in mice immunized by oral gavage only. Likewise, serum anti-LTB IgA titers rose more rapidly over the 35-day experimental period in mice undergoing prime-boost immunization than oral gavage. Following a final oral boost at day 48, serum IgA titers in both cases rose almost equivalently when measured at day 60, and significantly exceeded IgA levels elicited by parenteral immunization alone. These results demonstrate that systemic IgA responses were enhanced by oral mucosal immunization. Importantly, the fecal anti-LTB IgA titer in mice immunized by prime-boost was twice as high as that in mice immunized solely by gavage following the final boost at day 48. A comparison of the antibody responses in parenterally-immunized mice, mice immunized using a prime-boost regime, and mice immunized solely by oral gavage indicated that a more opti-

mal balance of systemic IgG/IgA immunity, and mucosal sIgA immunity was achieved using a parenteral prime-oral gavage boost strategy. These results are in accordance with a study using a parenteral prime-oral boost strategy for potato LTB [51]. Importantly, the protective potential of soy LTB was demonstrated by the fact that antiserum from LTB-immunized mice inhibited both LTB and CTB binding to ganglioside GM1 in vitro. The elicitation of an anti-LTB antibody response that is protective against ETEC-induced disease, as well as cross-reactive against CT-induced disease symptomology is feasible. In addition, partial protection against fluid accumulation in the gut was achieved following LT challenge of mice orally-immunized with soy LTB.

Soy LTB was useful in potentiating immunity when co-administered with another unrelated vaccine antigen, bacterial FimHt. When used for parenteral vaccination with Freund's adjuvant, FimHt, a naturally-occurring truncated form of the uropathogenic *E. coli* (UPEC) pilus-associated FimH bacterial adhesin, is effective in reducing pathogen colonization of the bladder mucosa and infection in a murine cystitis model, and may be useful in the prevention or treatment of recurrent and acute UPEC infections of the urogenital tract [52,53]. Under the experimental conditions used, parenteral co-immunization of bacterial FimHt with soy LTB enhanced the serum anti-FimHt IgG antibody response by 500-fold. The ability of soy LTB to function as a mucosal adjuvant following nasal or oral administration is now under investigation, as is its use as a vaccine carrier to target immunogens to gut-associated lymphoid tissues.

It is acknowledged that soybean contains approximately 15 proteins that are recognized by IgE antibodies from soy-sensitive individuals, and therefore, the potential for allergic sensitization must be considered if soybean is to be safely used as a platform for oral vaccine delivery to humans. P34 (Gly m Bd 30k) is the major allergen in soybean [54] and nulls of this immunodominant allergen have recently been identified [55]. The LTB-expressing line 157 is currently being introgressed into the P34 null soybean genetic background.

Progressive research over the past decade, and current and future pre-clinical and clinical studies, will ultimately prove whether plant-based vaccines are an efficaceous alternative to traditionally-made vaccines. In this light, a clinical study has recently been carried out using recombinant LTB delivered in transgenic maize [56]. In addition, a new plant-based vaccine against Shiga toxin type 2 (Stx2) has recently been described [57]. The potential, therefore, exists for the development of numerous therapies to combat *E. coli*-induced pathologies, and other infectious diseases. It is anticipated that the work described herein will guide the development of a valuable soy-based oral immunization strategy with its low cost per unit vaccine production capability and suitability for processing into oral delivery materials that will be broadly useful for disease prevention in animals, and eventually, in humans.

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