Plant Cell Rep (2012) 31:527–537
DOI 10.1007/s00299-011-1146-3

ORIGINAL PAPER

Expression of the cholera toxin B subunit (CT-B) in maize seeds and a combined mucosal treatment against cholera and traveler's diarrhea

S. Karaman · J. Cunnick · K. Wang

Received: 22 June 2011 / Revised: 29 August 2011 / Accepted: 31 August 2011 / Published online: 22 September 2011

Abstract The non-toxic B subunit (CT-B) of cholera toxin from *Vibrio cholerae* is a strong immunogen and amplifies the immune reaction to conjugated antigens. In this work, a synthetic gene encoding for CT-B was expressed under control of a γ-zein promoter in maize seeds. Levels of CT-B in maize plants were determined via ganglioside dependent ELISA. The highest expression level recorded in T_1 generation seeds was 0.0014% of total aqueous soluble protein (TASP). Expression level of the same event in the T_2 generation was significantly increased to 0.0197% of TASP. Immunogenicity of maize derived CT-B was evaluated in mice with an oral immunization trial. Anti-CTB IgG and anti-CTB IgA were detected in the sera and fecal samples of the orally immunized mice, respectively. The mice were protected against holotoxin challenge with CT. An additional group of mice was administrated with an equal amount (5 μg per dose each) of mixed maize-derived CT-B and LT-B (B subunit of *E. coli* heat labile toxin). In the sera and fecal samples obtained from this group, the specific antibody levels were enhanced compared to either the same or a higher amount of CT-B alone. These results suggest that a synergistic action may be achieved using a CT-B and LT-B mixture that can lead to a more efficacious combined vaccine to target diarrhea induced by both cholera and enterotoxigenic strains of *Escherichia coli*.

Keywords Cholera · ETEC · Maize · Plant-derived vaccine · Subunit vaccine

Communicated by P. Lakshmanan.

A contribution to the Special Issue: Plant Molecular Pharming in 2012 and Beyond.

S. Karaman
Interdepartmental Plant Biology Major, Iowa State University, Ames, IA 50011, USA

Present Address:
S. Karaman
Pioneer Hi-Bred International Inc., Ankeny, IA 50021, USA

J. Cunnick
Department of Animal Science, Iowa State University, Ames, IA 50011, USA

K. Wang (✉)
Department of Agronomy, Iowa State University, G405 Agronomy Hall, Ames, IA 50011-1010, USA
e-mail: kanwang@iastate.edu

Introduction

Cholera and traveler's diarrhea, caused by *Vibrio cholerae* and enterotoxigenic strains of *Eschericia coli* (ETEC), respectively, are two enteric diseases which are responsible for high mortality especially in young children in developing countries (Girard et al. 2006) and there is still urgent need for effective vaccines for these diseases. The toxins, cholera toxin (CT) and heat labile enterotoxin from ETEC (LT), which are responsible for the harmful effects of these infections, are composed of pentameric non-toxic B subunit and toxic A subunit. The B subunits bind to G_{M1} ganglioside (galactosyl-N-acetylgalactosamyl-syalil-galactosylglucosylceramide) receptors on intestinal epithelial cells. This binding aids a portion of the toxic A subunit to be internalized into the cytosol, where it starts a series of cellular events leading to diarrhea (Williams et al. 1999).

Although the B subunits are non-covalently linked as a pentamer, they are highly stable due to their structural properties and are resistant to trypsin and proteinase K

digestion and low pH (Ruddock et al. 1995). Therefore, oral immunization with CT-B and LT-B results in strong humoral and mucosal immune response (Rigano et al. 2003). Due to shared mechanism of toxin action, it is desirable and possible to develop combined vaccines, which would be effective against both ETEC and cholera. Accordingly, their potential as vaccine antigens against cholera and ETEC have been extensively exploited (Lebens et al. 1996; Mason et al. 1998; Chikwamba et al. 2002; Tacket et al. 1998, 2004). Many experimental ETEC vaccines included CT-B in combination with ETEC colonization factors to provide anti-toxin immunity along with antibacterial immunity (Qadri et al. 2003; Savarino et al. 2002). Although CT-B and LT-B share 85% homology (at the amino acid sequence level) in their mature proteins, some neutralizing epitopes in LT-B are missing in CT-B (Lebens et al. 1996). Therefore, utilization of both subunits in vaccine preparations might induce a mixture of polyclonal antibodies that could neutralize both toxins more effectively.

CT-B has been shown to be an effective carrier for the antigens that are chemically or genetically linked (Dertzbough and Elson 1993; George-Chandy et al. 2001). It was also reported that intravaginal (ivag) administration of bacterial CT-B chemically conjugated to ovalbumin (OVA) helps to induce OVA specific $CD4^+$ T cells. Neither OVA alone nor OVA co-administered with CT-B resulted in OVA specific $CD4^+$ cells (Luci et al. 2006). In fact, CT-B is an important immunogenic component in the only currently available cholera vaccine (Dukoral®, Crucell, The Netherlands), which is a killed whole-cell oral preparation (Schaetti 2009). Interestingly, some CT-B conjugates were reported to be highly tolerogenic (a substance which invokes immune non-responsiveness) when administered orally (Holmgren and Czerkinsky 2005). This function of CT-B has been successfully exploited to carry out proof of concept studies for treatment of human autoimmune diseases. For example, oral administration of the B chain of insulin conjugated to CT-B led to oral tolerance to the B chain of insulin. This in turn resulted in a lower dose and rate of insulin administration in murine models of diabetes to control the disease (Sadeghi et al. 2002). Similar success was attained against type II collagen-induced arthritis (Tarkowski et al. 1999).

CT-B has been expressed in a number of plant species including potato (Arakawa et al. 1997), carrot (Kim et al. 2009), tomato (Jani et al. 2002), tobacco (Jani et al. 2004) and rice (Nochi et al. 2007). All of these options present certain challenges for large-scale production of pharmaceuticals. Production in tobacco would require costly high-level purification due to the presence of toxic alkaloid by-products (Ma et al. 2003). On the other hand, seeds can be superior to vegetable crops due to their natural protein storage ability that allow a high level of recombinant protein production. In addition, seeds can provide a stable dry environment for long-term storage and long distance transportation. To date, rice seed has been reported to be an adequate system for the production of CT-B (Nochi et al. 2007). However, rice production requires large quantities of water continuously. Due to climate variability, increasing and competing demand for fresh water, and known effects of water shortages on agriculture (Brown and Halwell 1998), an edible crop with less water requirement and high yield potential, such as maize, can be an alternative option for pharmaceutical production.

In this work, CT-B was produced in endosperm of maize kernels via recombinant DNA technology. The transgene expression level was monitored for two generations. In vivo immunogenicity of maize derived CT-B was evaluated in mice with an oral feeding trial. Effectiveness of the CT-B and combined CT-B/LT-B treatment were assessed by including a group fed with a food pellet made of a mixture of ground CT-B and LT-B seeds.

Materials and methods

Plasmid construction

The CT-B coding sequence together with its signal peptide (PubMed Accession #: U25679) was optimized for maize expression by Genescript Corp. gene synthesis company (New Jersey, USA). The synthetic CT-B gene was cloned under the control of a maize endosperm specific γ-zein promoter (Marks et al. 1985) linked to the translational enhancer of the tobacco etch virus (Gallie et al. 1995). The 3′ terminator for the gene was from a soybean vegetative storage protein (Rhee and Staswick 1992). The plasmid was named as pCTB-BSG (Fig. 1a).

Maize transformation

Immature embryos of maize Hi II genotype were used for transformation using microprojectile bombardment as described by Frame et al. (2000). The plasmid pCTB-BSG was co-bombarded with a selectable marker gene construct, pBAR184 (Frame et al. 2000). pBAR184 carries the phosphinothricin acetyl transferase (bar) gene (Thompson et al. 1987) driven by a maize ubiquitine promoter (Christensen and Quail 1996). This construct confers resistance to the herbicide bialaphos. Transgenic lines were regenerated and raised to maturity in the greenhouse. B73 inbred line was used as pollen donor for obtaining T_1 seeds. T_2 seeds were produced by self-pollination of T_1 plants.

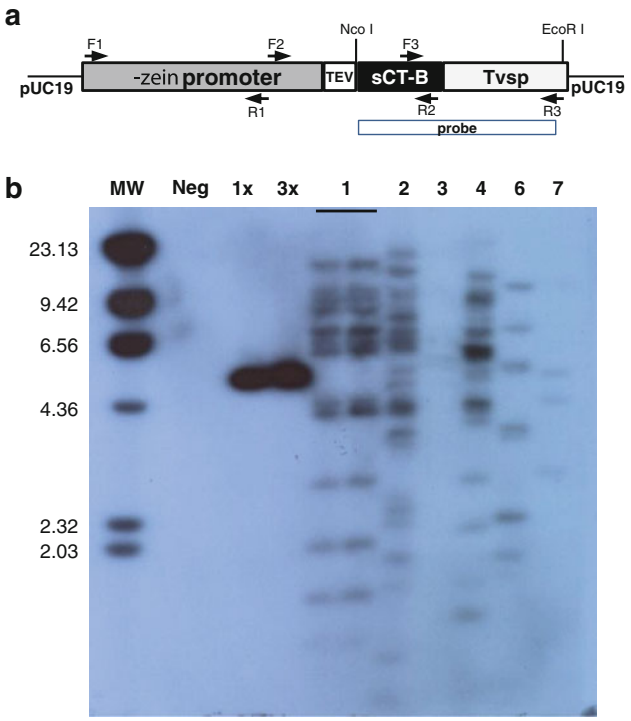

Fig. 1 Schematic vector map for pCTB-BSG (**a**) and Southern blot analysis of selected transgenic maize events (**b**). *MW* DNA marker ladder in kilo-bases (AM7720, Invitrogen Corp., Carlsbad, CA, USA), *Neg* negative control (untransformed maize DNA), *1x* and *3x* positive control (1 and 3 copies of the pCTB-BSG vector plasmid), *1–7* DNA from the transgenic maize

PCR analysis

The presence of the CT-B gene cassette in maize genome was verified by using Polymerase Chain Reaction (PCR) analysis. Genomic DNA isolation from maize calli was performed as follows: 0.1–0.25 g of bialaphos resistant callus was transferred to 1.5 ml sterile microcentrifuge tubes. The calli were homogenized by autoclaved pestles (Kontes Pellet Pestle, K749520-0000, Fisher Scientific, PA, USA) until juice exudes. Four hundred micro liters of extraction buffer (200 mM Tris-Cl [pH 7.5], 250 mM NaCl, 25 mM EDTA) was added to the tube. The tissue and buffer were mixed gently by inverting the tube a few times. An equal amount of saturated phenol was added to each sample and mixed gently in a similar fashion. The mixtures were centrifuged in a microcentrifuge for 10 min at 12,000 rpm at room temperature. The aqueous phase was transferred to new microcentrifuge tubes containing 350 µl of chloroform: iso-amyl alcohol mix (24:1 v:v) and mixed gently. This was followed by centrifugation for 10 min at 12,000 rpm at room temperature. The aqueous phase was transferred to a new tube and 300 µl of isopropanol was added. DNA was allowed to precipitate for 30 min at room temperature. Next, DNA was pelleted by centrifugation for 10 min at 12,000 rpm at room temperature. Isopropanol was discarded and pellets were washed with 500 µl of 70% ethanol by centrifuging at the same speed for 5 min. The pellets were air dried for 15–30 min and re-suspended in autoclaved distilled water.

Three sets of primers were used to verify the presence of promoter-leader-CT-B-terminator region in genomic DNA (Fig. 1a). The primer sets used are as follows: (1) F1: 5′-gct-atg-acc-atg-att-acg-cca-agc-3′ and R1: 5′-cat-tct-gta-ctt-ctg-cgt-ggc-tca-3′, (2) F2: 5′-tga-gcc-acg-cag-aag-tac-aga-atg-3′ and R2: 5′-ggg-tct-tat-tgt-tcc-aca-cgc-ag-3′, (3) F3: 5′-aag-acc-cca-cac-gct-att-gct-g-3′ and R3: 5′-gaa-ttc-gct-tca-aga-cgt-gct-ca-3′. These primers amplify 925, 738 and 629 bp fragments, respectively. The reaction mixtures and conditions were as follows: total volume of 25 µl containing 100 ng of maize callus genomic DNA, 1 µl of dNTP mix (containing 10 mM of each dNTP), 1 µl of each primer (2.5 µM stock), 1 µl of $MgCl_2$ (50 mM), 2.5 µl of 10× Taq polymerase PCR buffer and 0.2 µl of Taq polymerase (Biolase USA Inc., City, NJ, USA). PCR was initiated at 95°C for 4 min. This was followed by 30 cycles of amplification steps of which are; denaturation at 95°C for 1 min; annealing at 62°C for 40 s, and extension at 72°C for 1 min.

Southern blot analysis

Southern blot analysis to estimate copy numbers of the CT-B gene in transgenic maize lines was carried out on DNA extracted from leaf tissue of T_1 young plants using the CTAB protocol (Saghai-Maroof et al. 1984).

Ten micrograms of maize genomic DNA was digested with the restriction enzyme *Nco*I at 37°C overnight and resolved on 0.8% agarose gel. The 950 bp fragment containing synthetic CT-B gene and VSP terminator was excised from pCTB-BSG plasmid with *Nco*I and *Eco*RI restriction enzymes and used as probe following labeling with ^{32}P (Fig. 1a). DNA blot was carried out following standard procedures (Sambrook et al. 1989).

Protein extraction from corn seeds

For CT-B expression analysis of T_1 seeds, endosperm tissue from 20 seeds of each event was collected individually using a non-destructive method described by Sangtong et al. (2001). About 15–20 mg of endosperm tissue was removed from seeds using a mini mechanical drill (Multi-pro-225 T2, DREMEL, Racine, WI, USA) and the powder was transferred to a 1.5 ml microcentrifuge tube. For ELISA, extraction buffer [25 mM sodium phosphate (pH: 6.6), 100 mM sodium chloride, 1 mM EDTA, 0.1% Triton X-100, 10 µg/ml Leupeptin, 0.25 mM Pefabloc SC] was added at a ratio of 10 µl/mg of powder. The samples were

incubated at 37°C on a shaker incubator at room temperature for 2 h. Highest expressers among T_1 seeds were identified and planted to obtain T_2 seeds. For Western analysis, ground whole seeds from T_2 plants were used. Ground seed powder were suspended in extraction buffer [200 mM Tris-HCl, (pH 8.0), 100 mM NaCl, 400 mM sucrose, 10 mM EDTA, 14 mM 2-Mercaptoethanol, 0.05% Tween-20] at the rate of 10 µl buffer per milligram of powder. The powder was incubated for 1 h on a vortex shaker at room temperature and further incubated in refrigerator overnight. Before loading to protein gels, samples were centrifuged on a bench-top micro-centrifuge at maximum speed for 15 minutes to remove cellular debris. The amount of total soluble proteins (TASP) were determined by the Bradford Assay (Bradford 1976) with the BioRad (Hercules, CA, USA) protein dye concentrate using a standard curve derived from bovine serum albumin (BSA).

Quantification of CT-B expression in seeds

CT-B expression in endosperm and ground whole kernels was determined by ganglioside dependent ELISA (Mason et al. 1998; Chikwamba et al. 2002) modified for quantification of CT-B. Anti-sera and CT-B standard were obtained from Sigma chemical company. Anti-CT antibodies raised in rabbit (C-3062, Sigma, St. Louis, MO, USA) and biotinylated anti-rabbit goat IgG was used as primary and detection antibodies, respectively. Both antibodies were diluted 1/20,000 in phosphate buffered saline. After detection antibody, wells were loaded with streptavidin horse radish peroxidase diluted 1/1,000 (BD Biosciences, 554066, MD, USA). The color development was obtained by addition of ABTS substrate (3-ethylbenzthiazoline-6-sulfonic acid, Sigma A-1888, MO, USA) prepared according to manufacturer's instructions. End point readings were measured spectrophotometrically at 405 nm in a PowerWave XS plate reader (BT-MQX200R, Bio-Tek Instruments Inc., Winooski, VT, USA). All samples were measured in duplicates and non-transgenic maize protein extracts were used as negative controls. Calculations of CT-B concentrations were carried out using a standard curve of purified bacterial CT-B (C-9903, Sigma, St. Louis, MO, USA).

Western Blot

Approximately 70 mg of total protein extracted from ground maize seeds were separated by SDS-PAGE (Laemmli 1970). The resolved proteins were transferred to 0.45 µm nitrocellulose blotting membrane (BioRad Laboratories, Hercules, CA, USA) using a BioRadTransblot apparatus following manufacturer's instructions. After blocking with 5% milk, membranes were incubated at room temperature on a shaker for 1 h in primary antibody raised against CT (rabbit-anti-CT, Sigma C-3062) diluted 1/3,000 in 1% milk. This was followed by incubation in alkaline phosphatase conjugated secondary antibody diluted at the ratio of 1/5,000 in 1% milk (Sigma A-9919) in a similar manner for 1 h. Detection was performed following procedures of Blake et al. (1984).

Preparation of feeding pellets and immunizations

Seeds were ground in a coffee grinder (Braun, Type 4041: Model KSM2) to a size able to pass through a 425-micron size sieve, and a homogenous mix was prepared as described by Chikwamba et al. (2002). Food pellets of ground transgenic maize and non-transformed corn were prepared to the same size and contained one of the following five feed treatments. Five micrograms of CT-B (CTB-5 group), 10 µg of CT-B (CTB-10 group), 5 µg of maize derived LT-B (Chikwamba et al. 2002; LTB-5 group), a combination of 5 µg of CT-B with 5 µg LT-B (CTB5-LTB5 group), or non-transformed corn (WT group; which served as the negative control). Four-week-old female BALB/c mice were obtained from Harlan (Indianapolis, IN, USA). Prior to start of the experiment, mice were allowed a 2-week adjustment period with a reverse light–dark cycle (lights-off at 9 A.M.) in the Iowa State University (ISU) animal facility where they were housed throughout the experiment. All animal procedures were approved through the ISU Committee on Animal Care. Eight mice were randomly assigned to each treatment group. Mice were fed a basic diet of mouse chow with water ad libitum. Mice were fasted overnight (during the light phase) prior to feeding corn pellets on days 0, 7, 21 and 49 of the study. Throughout the study, two mice were housed in each cage. However, during feeding of the corn pellet they were caged individually and one maize pellet was placed on the lid of each cage (without bedding) 30 min before lights off. Mice were allowed to eat the maize pellet in the dark cycle for 5 h until the pellet was completely consumed (approximately 3 h) and then returned to their home cage with their normal mouse chow.

Preparation of serum and fecal samples

Approximately 100 µl of blood and fecal samples (as available) were collected weekly over 8 weeks, starting the day before the first oral immunization feeding treatment. Blood samples were collected from the sephanous vein except for the day of necropsy. On day 55, mice were euthanized and blood was collected from hearts via cardiac puncture. Sera were isolated from blood samples immediately after collection by centrifugation at 14,000 rpm for 15 min and kept at −20°C until analyzed. For ELISA,

dilutions for sera ranged from 1/60 to 1/400. Fecal samples were lyophilized and kept at −20°C until analyzed. On the day of analysis, fecal samples were dissolved in PBS (10:1 w/v) and centrifuged at 15,000 rpm. The fecal extracts were used in ELISA without dilution.

Quantification of anti-CT-B and anti-LT-B antibodies

Quantification of anti-CT-B and anti-LT-B antibodies via ELISA was conducted as described earlier (Karaman et al. 2006) with a minor modification. Twenty-five nanograms of pure LT-B (provided by John Clements) or CT-B (Sigma) was loaded in each well instead of 1 μg as described before (Karaman et al. 2006). This modification was determined based on comparison of different amounts of LT-B and CT-B with sera of known antibody levels (data not shown).

Patent mouse assay

To assess protection from toxin challenge a patent mouse assay was undertaken (Guidry et al. 1997). Due to logistic constraints, only mice from the CTB-5 group and the WT group were tested. Three mice from each of these groups were challenged with either 30 μg of LT (kindly provided by John Clements) or with 30 μg of CT (Sigma, C3012) toxins. Two mice from each of these groups were given an equal volume of (200 μl) water as negative control. Toxins in 200 μl of PBS and water were administered via gavage. The mice were fasted overnight ad libitum prior to the gavage administration. Three and half-hours after the challenge, mice were euthanized and their carcasses were weighed. Following dissection, guts were removed from duodenum to anus and weighed separately. The gut:carcass ratios (Richardson et al. 1984) were calculated to determine the amount of water influx into the gut.

Statistical analysis

Data were analyzed by Analytical Software Statistix® 8 (Tallahassee, FL, USA). ANOVA and repeated measures analysis of variance tests were used for analysis of gut:carcass ratios, and IgG or IgA levels, respectively. Comparisons were considered significant at a P value ≤ 0.05 and marginally significant at P value between 0.09 and 0.05.

Results and discussion

Analysis of transgenic corn lines carrying CT-B gene under a seed specific promoter

Transgenic events carrying the pCTB-BSG and pBAR184 constructs were referred to as P248. The putative transformant (herbicide resistant) calli were screened with PCR for the presence of CT-B gene. Seven out of nine bialaphos resistant events were shown to carry the intact CT-B gene (data not shown). T_0 plants were regenerated from these events and Southern analysis was carried out on DNA obtained from young leaves of six lines. The Southern analysis result is shown in Fig. 1b. Event specific integration of the CT-B gene was observed. Two out of six (33%) of events carried low copy number transgene insertions (≤3 copies) and three out of six (50%) of the events carried high copy number insertion (>10 copies). The percentages of low and high copy number transgene insertion events are similar to what was previously reported in transgenic maize lines generated by the biolistic method (Shou et al. 2004).

T_0 plants were grown to maturity to obtain T_1 seeds. A total of 25 ears representing 6 independent events were obtained. The expression of CT-B gene in T_1 seeds from each event were analyzed using a non-destructive method described by Sangtong et al. (2001). This method allows for removal of small amount (15–20 mg) of endosperm tissue for molecular analysis with a mini mechanical drill without damaging the germ. After removal of parts of the endosperm, the drilled seed can still be germinated to plant.

Expression levels of CT-B in seed were determined by ganglioside dependent ELISA. Twenty randomly selected seeds representing each event were pooled. Four out of six lines expressed CT-B at varying levels and two remaining lines failed to express CT-B at detectable level even though the CT-B gene was present in the genome. The CT-B contents in the four expressing lines (P248-1, -2, -4 and -7) ranged from 0.0002% CT-B of total aqueous soluble protein (TASP) or 0.009 μg CT-B per gram of dry seed weight (DSW) to 0.0014% CT-B of TASP or 0.05 μg CT-B per gram of DSW. Two CT-B high expressing lines, P248-1 and P248-4, were further grown to maturity for T_2 seeds by either self- or sib-pollination. The average CT-B content from eleven T_2 ears of P248-1 was measured as 0.04 μg CT-B per gram of DSW, which was two times higher than its T_1 parents. The average CT-B content from nine T_2 ears of P248-4 was measured as 1.56 μg per gram of DSW, which was more than 30 times higher than the T_1 parents. The enhanced transgene expression in some events in advanced generations was observed previously with the expression levels of LT-B (Chikwamba et al. 2002) and avidin (Hood et al. 1997) in corn seeds. Due to small sample size, we did not observe an obvious correlation between transgene expression and insertion copy numbers.

Western analysis was performed in protein extracts obtained from T_2 seeds of two events (P248-1 and P248-4). Figure 2 shows the results with non-boiled protein samples

(Fig. 2a) and the protein samples (Fig. 2b) boiled prior to gel electrophoresis. The bacterial pentameric CT-B has an approximate size of 55 kDa while monomers have approximate size of 11.6 kDa (Zhang et al. 1995). Non-boiled bacterial CT-B used as control (Lane b-CTB, Fig. 2a) showed multiple bands, suggesting that the commercial bacterial CT-B may contain a mixture of CT-B multimer conformations. This pattern was observed by other groups (Arakawa et al. 1997; Jani et al. 2002, 2004). The second strongest band (indicated by black star in Fig. 2a) was considered as the pentamer. As shown in Fig. 2a, three ears of P248-4 and T_2-Mix (seeds used for feeding experiments) had a band detected by the anti-CT-B antibody. Interestingly, this band (indicated by black triangles) has a lower electrophoretic mobility than that of the bacterial CT-B standard (black star). This result was also reported in recombinant CT-B produced in potatoes (Arakawa et al. 1997), tobacco (Jani et al. 2004) and tomato (Jani et al. 2002). It was suggested that this was due to the inability of plant cells in cleaving the bacterial signal peptide of CT-B. This result is different from what we have observed previously with maize-derived LT-B in which bacterial signal was found to be cleaved from the mature LT-B protein in corn seeds (Chikwamba et al. 2002). The differential mechanisms by which the signal peptides of these two highly homologous proteins are processed in maize kernels remain to be elucidated.

Figure 2b shows the outcome of a parallel experiment using the boiled protein samples (Fig. 2b). Maize-derived monomeric CT-B (white triangle in Fig. 2b) also has a lower electrophoretic mobility when compared with its bacterial CT-B control (white star in Fig. 2b), confirming the observation with the non-processed bacterial CT-B as can be seen in Fig. 2a. Sample #3 of P248-4 in Fig. 2b was not observed and was likely degraded after heat treatment. On the other hand, the lack of CT-B bands in protein extracts from P248-1 ears was likely due to low expression level of CT-B in this line as it was not seen in either Fig. 2a or b.

Evaluation of immune response of mice fed with corn-derived CT-B and LT-B

BALB/c mice were divided into five feeding treatment groups with eight mice per group to receive maize-derived CT-B and/or LT-B four times in the study. Mice were orally fed (on days 0, 7, 21, and 48 relative to the first feeding) with maize pellets containing: (1) 5 μg of CT-B (CTB-5), (2) 10 μg of CT-B (CTB-10), (3) 5 μg of LT-B (LTB-5), (4) 5 μg of CT-B plus 5 μg of LT-B (CTB5-LTB5), or (5) non-transgenic maize (WT). Blood and fecal samples were collected weekly and analyzed for the presence of anti CT-B and anti LT-B antibodies.

Serum IgG

Anti-CT-B and anti-LT-B IgG antibodies in sera of all groups were measured (Fig. 3). For both analyses, there was a significant treatment effect over time (P values < 0.05). Anti-CT-B IgG antibody levels observed in sera of all treatment groups were lower than anti-LT-B IgG levels observed in sera of the LTB-5 and the CTB5-LTB5 groups (Fig. 3). While serum anti-LT-B IgG cross-reacts with CT-B (LTB-5 group; Fig. 3), serum anti-CT-B IgG did not strongly cross-react with LT-B (CTB-5 and CTB-10 groups; Fig. 3b). Starting from day 13, anti-CT-B levels in sera of the LTB-5 group (111.58 ± 6.6 μg/ml; Fig. 3a) were significantly different than that of the WT group (37.29 ± 3.13 μg/ml) throughout the study (P values < 0.05). On the other hand, anti-LT-B antibody levels measured in sera of the CTB-5 and CTB-10 groups (Fig. 3b) were not different than that of the WT group at any bleed day.

Anti-CT-B antibodies in sera of the CTB-5 and CTB-10 groups (Fig. 3a) were significantly different than that of the WT group starting from day 27 until day 55 (P values < 0.05). Anti-CT-B antibody levels in the CTB-5 and CTB-10 groups were not different from each other until day 55. On day 55 anti-CT-B antibodies detected in sera of

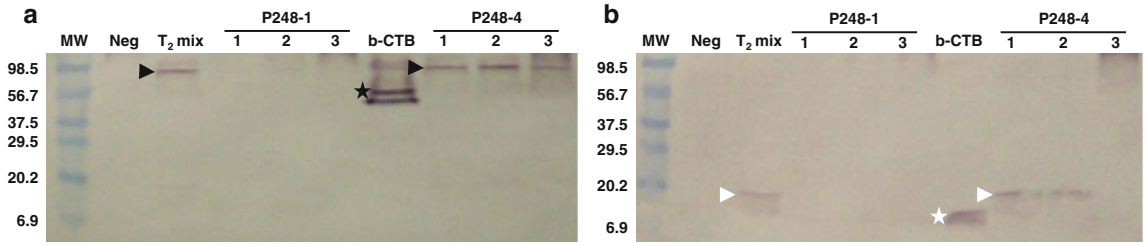

Fig. 2 Western blot analysis of P248-1 and P248-4 T_2 generation maize seeds Unboiled (**a**) and boiled (**b**). *MW* molecular weight marker in kilodaltons, *Neg* negative control (crude protein extract of the untransformed maize seeds), *T_2 mix* crude protein extract of the mill used in mice feeding experiment, *1–3* crude protein extracts of different lines of T_2 generation maize seeds, *b-CTB* positive control (bacterial CTB). *Black arrowhead* plant-derived CTB pentamer, *black star* bacterial CTB pentamer, *white arrowhead* plant derived CTB monomer, *white star* bacterial CTB monomer

the CTB-10 group (179.73 ± 25.75 μg/ml) was significantly higher than that of the CTB-5 group (110.08 ± 22.26 μg/ml, $P = 0.04$). Interestingly, on days 13 and 20, serum anti-CT-B IgG levels in LTB-5 group (111.58 ± 18.63 and 144.72 ± 35.51 μg/ml) were found to be significantly higher than that of the CTB-10 group (52.75 ± 15.46 and 69.35 ± 20.99 μg/ml) (P's = 0.0001). However, on days 27 and 34 the differences were not significant. On days 41 and 55, serum anti-CT-B IgG of the CTB-10 group exceeded and was higher than that of the LTB-5 group ($P = 0.07$, $P = 0.04$, respectively).

On days 34, 41 and 55, serum anti-CT-B antibody levels in CTB5-LTB5 group were significantly higher than that of the LTB-5 group (P values ≤ 0.0061). On days 34 and 41, serum anti-CT-B antibody levels in CTB5-LTB5 group were higher than that of the CTB-10 group (day 34; $P = 0.02$ significant and day 41; $P = 0.076$ marginal). These observations suggest that a combined treatment could perform better than higher doses of CT-B alone for vaccine formulations which aim at the prevention of cholera. The presence of LT-B in the mix may contribute a 2-fold benefit because the induction of antibodies is faster and anti-LT-B antibodies cross-react with CT-B. Using a related and stronger antigen (LT-B) may help to reduce the need for high doses of CT-B while, the presence of CT-B in the vaccine, provides the antigenic epitopes necessary for protection from toxigenic cholera. As LT-B is a related antigen and responsible for 2/3 of the cases of traveler's diarrhea, the incorporation of LT-B with CT-B should act to focus the immune response to cross-reactive epitopes using a lower amount of each antigen.

Serum antibodies that develop in response to CT-B alone do not cross-react with LT-B. Analysis of anti-LT-B IgG levels indicated significant treatment effect throughout the study ($P = 0.0$). However, anti-LT-B antibody levels in sera of the CTB-5 and the CTB-10 groups were not significantly different than that of the WT group on any bleed day. Anti-LT-B levels in sera of the LTB-5 and the CTB5-LTB5 groups were significantly higher than that of the other three groups (P values < 0.05) starting from day 13 until the day of necropsy (day 55). Due, presumably, to lack of cross-reactivity of anti-CT-B IgGwith LT-B, anti-LT-B antibody levels in sera of the LTB-5 and the CTB5-LTB5 groups were not significantly different from each other on any bleed days.

Comparison of anti-CT-B IgG antibody and anti-LT-B IgG antibody levels induced by the CTB-5 and LTB-5 groups indicated that antibody induction by CT-B occurs after more immunization exposures and the antibody levels are lower. The highest anti-CT-B IgG in the CTB-5 group was recorded on day 41 with 129.58 ± 22.7 μg/ml whereas the peak anti-LT-B IgG in the LTB-5 group was observed earlier, on day 27 with 640.91 ± 75.1 μg/ml.

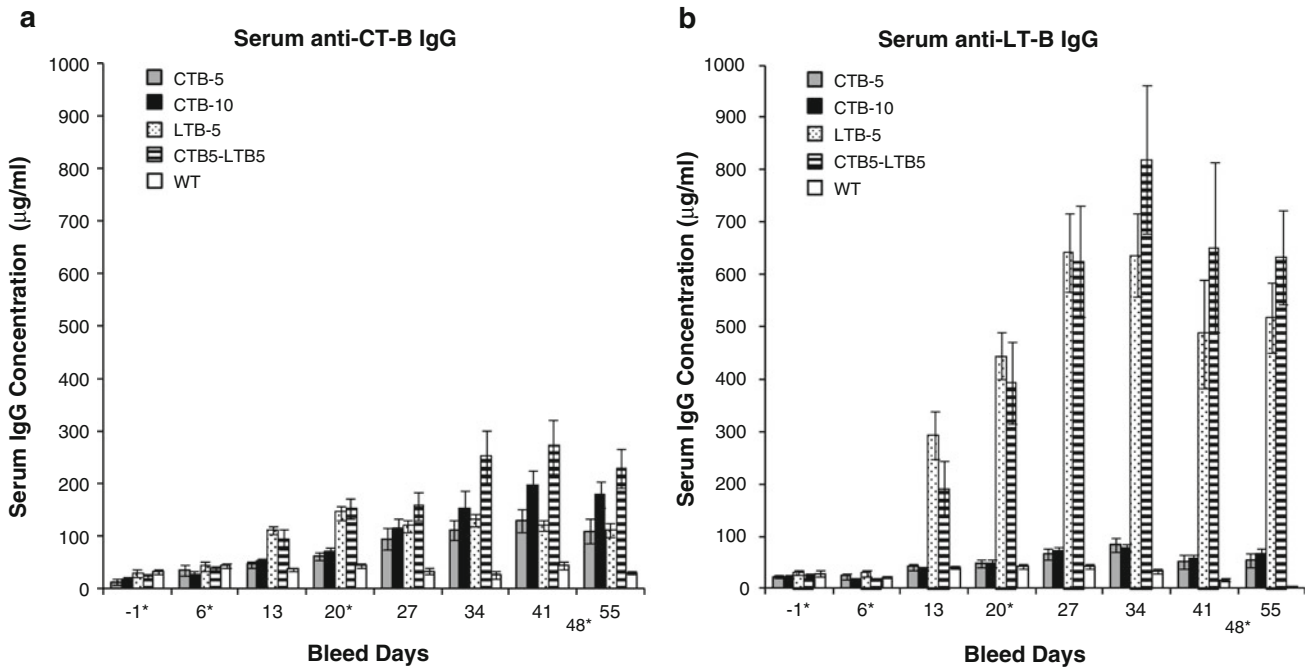

Fig. 3 Serum antibody responses Anti-CT-B-IgG concentration (**a**) and anti-LT-B-IgG concentration (**b**). Mice were orally administered feed made of untransformed maize (WT) or transgenic maize (CT-B maize, LT-B maize or a combined feed) containing 5 μg of CT-B (CTB-5), 10 μg of CT-B (CTB-10), 5 μg of LT-B (LTB-5) or 5 μg of CT-B and 5 μg of LT-B (CTB5-LTB5) four times. Data are provided as mean ± SE ($n = 8$). *Asterisk* indicates the day before the dose administration

The lower antibody induction to CT-B and IgG cross reactivity with LT-B might be explained by the signal peptide which remained attached to CT-B. The signal peptide may mask an epitope essential for production of the type of anti-CT-B IgG that can bind to LT-B. Arakawa et al. (1997) indicated that potato derived CT-B might possess a signal peptide. When mice were orally immunized with potato derived CT-B, anti-CT-B antibody levels were shown to be significantly lower than those immunized with an equal amount of bacterial CT-B (Arakawa et al. 1998). They also reported that 30 and 90 μg of potato derived CT-B induced similar levels of anti-CT-B antibodies (Arakawa et al. 1998). Interestingly, recent reports of immunization with a rice-based CT-B vaccine resulted in a strong cross-reactivity of serum IgG against LT-B (Tokuhara et al. 2010). In their study, Tokuhara et al. observed mixed length of CT-B monomers and wide bandwidth range of pentamers. The presence of a fraction of CT-B in its native structure, without the signal peptide (Nochi et al.2007), could have resulted in this outcome (Tokuhara et al. 2010).

In summary, for serum IgG production, presence of LT-B in the combined treatment may strengthen the immune response to cholera, while presence of CT-B may not intensify the immune response against LT-ETEC. Addition of LT-B to a CT-B vaccine could broaden the usefulness of the vaccine.

Fecal IgA

Unlike serum IgG analysis, fecal IgA data were only available for days 13 through 41 for all groups (Fig. 4). For both analyses (anti-CT-B IgA and anti-LT-B IgA), there was a significant treatment effect (P values < 0.05) and anti-CT-B IgA antibodies were found to cross react with LT-B, as well as, anti-LT-B IgA antibodies cross-reacting with CT-B (Fig. 4a, b).

Fecal anti-CT-B IgA levels of the CTB-5 and the CTB-10 groups, although both higher than that of the WT group, were not different from each other throughout the study (Fig. 4a). Anti-CT-B IgA levels in the CTB-5 and CTB-10 groups were significantly different than that of the WT group throughout the study (days 13–41; Fig. 4; P values ≤ 0.05).

Anti-CT-B IgA level in LTB-5 group was significantly higher than ($P = 0.0423$) that of WT group on day 13 indicating cross-reactivity of anti-LT-B IgA with CT-B. Anti-CT-B IgA levels in the CTB5-LTB5 group were not different than those of the CTB-5 and the CTB-10 groups on days 27 and 34 (Fig. 4a). Interestingly, the anti-CT-B IgA level in the CTB5-LTB5 group was less than that of in the CTB-5 and the CTB-10 groups on days 6, 13, 20 and 41 (P values < 0.04; Fig. 4a). On day 34, anti-CT-B IgA levels in the CTB5-LTB-5 group were significantly higher than those of the LTB-5 group ($P = 0.03$).

Analysis of anti-LT-B fecal IgA levels showed significant treatment effect from day 13 through day 41 ($P = 0.04$; Fig. 4b). Fecal anti-LT-B IgA levels in the LTB-5 group and the CTB5-LTB5 group were significantly different than that of the WT group on days 13, 20, 27, 34 and 41 (P values ≤ 0.05; Fig. 4b). Anti-LT-B IgA levels in the LTB-5 group and the CTB5-LTB5 group were significantly different than the combined CTB-5 and the CTB-10 groups on days 20, 27, and 34 (P values ≤ 0.05). Anti-LT-B IgA levels in the CTB-5 group were significantly different than that of the WT group only on days 13 and 27 (P values < 0.05).

Corn derived CT-B may induce a longer lasting immune response at the mucosal surface than LT-B. The levels of anti-LT-B IgA in the LTB-5 group decreased on days 27 and 41 while the anti-CT-B IgA levels in the CTB-5 and CTB-10 groups were higher on day 41 in comparison to day 13.

Responses of immunized mice to CT and LT toxin challenges

Due to logistic constraints, only the mice in the CTB-5 and the WT groups were challenged with CT or LT. These toxins cause fluid secretion to the lumen of the bowel (Williams et al. 1999). The secreted fluid cannot be re-absorbed (Mason et al. 1998) and thus accumulates in the gut. This results in the weight of the gut to increase relative to the weight of the body. Therefore the gut:carcass ratios serves as a measure of response toward toxin challenge. If immunizations provide protection we would expect similar gut:carcass ratios with unchallenged control mice that were given water in place of toxin. Larger gut:carcass ratios indicate diarrhea therefore no protection of the treatments.

As can be seen on Fig. 5, significant protection against CT was observed in the CTB-5 group ($p = 0.004$). However, although the mean gut:carcass ratio in CTB-5 group was lower than that of the WT group, significant protection against LT challenge was not achieved in this CT-B corn immunized mice, suggesting that cross protection of LT by CT-B immunization at this dose is not effective. There could be two reasons for this outcome. First, the amount of antibodies produced by 5 mg of CT-B may not be sufficient to provide protection against the dose of LT used for the challenge. Second, the affinity of anti-CT-B IgA to LT-B may not be strong enough. In humans, it is known that serum IgG can be transported to the gut lumen via FcRN. Although FcRN in mice do not appear in the adult gut epithelium, there exists a role of IgG in protection against pathogens and toxins at the intestinal mucosal surface (Roopenian and Akilesh 2007; Kuo et al. 2010).

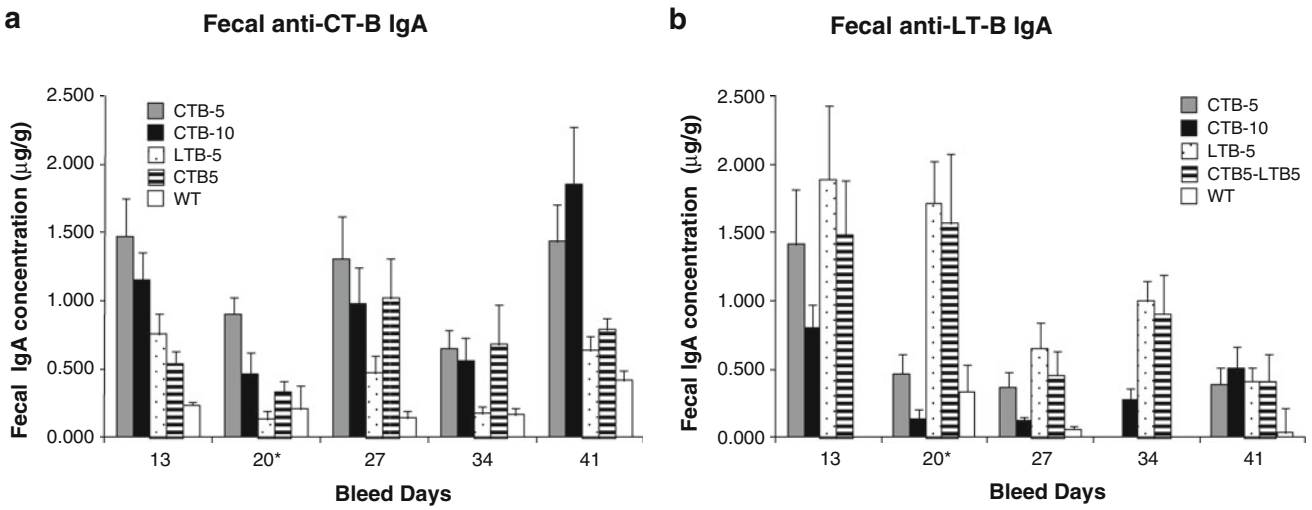

Fig. 4 Gut antibody responses Anti-CT-B-IgA concentration (**a**) and anti-LT-B-IgA concentration (**b**). Mice were orally administered feed made of untransformed maize (WT) or transgenic maize (CT-B maize, LT-B maize or a combined feed) containing 5 µg of CT-B (CTB-5), 10 µg of CT-B (CTB-10), 5 µg of LT-B (LTB-5) or 5 µg of CT-B and 5 µg of LT-B (CTB5-LTB5) four times (day 0, 7, 21 and 48). Sufficient amounts of fecal extracts to complete both types of IgA analysis were not available on days which are not included in the graphs. Data are provided as mean ± SE ($n = 8$). *Asterisk* indicates the day before the dose administration

Cross-reactivity between anti-CT-B IgG (produced by maize-derived CT-B) and LT-B may contribute to increased protection. This also supports the need for a combined CT-B/LT-B vaccine to protect against both toxigenic *V. cholera* and *E. coli*.

This observation extends our previous study in which mice fed with maize-derived LT-B were protected from LT and partially protected from CT challenges (Chikwamba et al. 2002). Others have demonstrated that potato-derived (Mason et al. 2002) and carrot-derived (Rosales-Mendoza et al. 2008) LT-B were shown to protect mice from both LT and CT challenges. Recently, Tokuhara et al. (2010) reported that mice immunized with rice-derived CT-B were cross-protected against LT toxin challenge. As mentioned above, the lack of cross-protection effect from the maize-derived CT-B in the study could be due to the unprocessed signal peptide in mature CT-B or differences in toxin challenge.

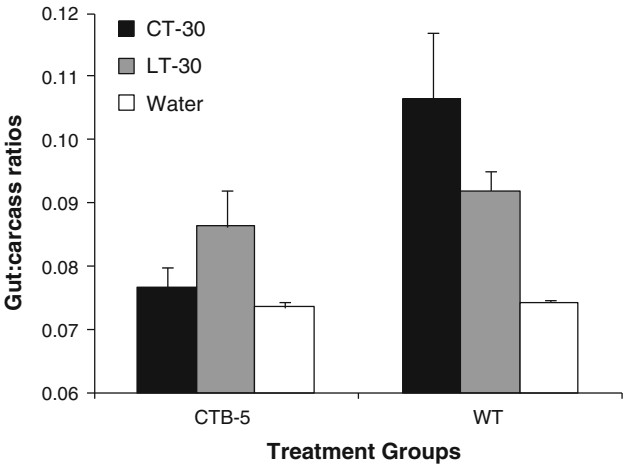

Fig. 5 Immune protection against toxin challenge Mice from CTB-5 and WT groups were challenged orally via gavaging with 30 µg of cholera toxin (*CT-30*; $n = 3$) or 30 µg of *E.coli* heat labile enterotoxin (*LT-30*; $n = 3$). Two mice from each group were administered water as negative control (*Water*). Gut:carcass ratio indicates level of water retention in response to challenge. Data are provided as mean ± SE

Conclusion

A transgenic corn expressing B subunit of cholera toxin (CT-B) from *Vibrio cholerae* in its seeds was developed to explore its utility as a mucosal vaccine or vaccine component against cholera and traveler's diarrhea. The seed expression of the CT-B could be enhanced through further breeding to advanced generations. While the mature CT-B in maize tissue was not processed for the removal of its signal peptide, the pentameric assembly of CT-B was demonstrated via ganglioside dependent ELISA.

Our study shows that maize derived CT-B can be immunogenic in mice. Ten micrograms of maize derived CT-B may be better at priming anti-CT-B IgG antibody production in comparison to 5 µg of maize derived CT-B. Because the maize does not process the bacterial native signal peptide effectively, anti-CT-B antibodies induced by maize derived CT-B may be different than that induced by bacterial CT-B.

Our initial intention of this study was to establish a combined vaccination system that can be effective for both CT-caused cholera and LT-caused traveler's diarrhea diseases. On certain days of sample collection, the antibody

levels observed were higher in the mice from the CTB5-LTB5 combined treatment group than that of the CTB or LTB only groups. However, due to incomplete CT-B signal peptide processing in maize and other limitations, our data will need further verification and improvement. For instance, a mature CT-B should be produced in maize by using a signal peptide from maize seed zein protein (Moeller et al. 2009) instead of its bacterial native signal peptide. Repeating combined treatments with a higher dose of maize-derived CT-B would help to deduce more sound results for adjuvant/synergistic actions of CT-B and LT-B.

Acknowledgments The authors wish to thank Iowa State University Plant Transformation Facility for providing maize transformation service. We are thankful to John Clements of Tulane University for providing bacterial LT-B and to Chad Niederhuth for his technical assistance. This work was partially supported by the United States Department of Agriculture (USDA-NRI 99–35504-7799) and Iowa State University Research Foundation to KW, the Biopharmaceutical/Bioindustrial Initiative of the Plant Sciences Institute at Iowa State University to KW and JC, and The Scientific and Technical Research Council of Turkey to SK.

References

Arakawa T, Chong DKX, Merrit JL, Langridge WHR (1997) Expression of cholera toxin B subunit oligomers in transgenic potato plants. Transgenic Res 6:403–413

Arakawa T, Chong DKX, Langridge WH (1998) Efficacy of a food plant-based oral cholera toxin B subunit vaccine. Nat Biotech 16:292–297

Blake MS, Johnston KH, Russell-Jones GJ, Gotschlich EC (1984) A rapid sensitive method for detection of alkaline phosphatase conjugated antibody on Western blots. Anal Biochem 136:175–179

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Brown LR, Halwell B (1998) China's water shortage could shake world food security. World Watch 11:10–16

Chikwamba RK, Cunnick J, Hathaway D, McMurray J, Mason H, 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

Christensen AH, Quail PH (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res 5:213–218

Dertzbough MT, Elson CO (1993) Comparative effectiveness of the cholera toxin B subunit and alkaline phosphatase as carriers for oral vaccines. Infect Immun 61:48–55

Frame BR, Zhang H, Cocciolone SM, Sidorenko LV, Dietrich CR, Pegg SE, Zhen S, Schnable PS, Wang K (2000) Production of transgenic maize from bombarded type II callus: effect of gold particle size and callus morphology on transformation efficiency. In Vitro Cell Dev Biol-Plant 36:21–29

Gallie DR, Tanguay RL, Leathers V (1995) The tobacco etch viral 5′ leader and poly(A) tail are functionally synergistic regulators of translation. Gene 165:233–238

George-Chandy A, Eriksson K, Lebens M, Nordström I, Schön E, Holmgren J (2001) Cholera toxin B subunit as a carrier molecule promotes antigen presentation and increases CD40 and CD86 expression on antigen-presenting cells. Infect Immun 69:5716–5725

Girard MP, Steele D, Chaignat CL, Kieny MP (2006) A review of vaccine research and development: human enteric infections. Vaccine 24:2732–2750

Guidry JJ, Cardenas L, Cheng E, Clements JD (1997) Role of receptor binding in toxicity, immunogenicity and adjuvanticity of *Escherichia coli* heat labile enterotoxin. Infect Immun 65:4943–4950

Holmgren J, Czerkinsky C (2005) Mucosal immunity and vaccines. Nat Med 11:S45–S53

Hood EE, Witcher DR, Maddock S, Meyer T, Baszczynski C, Bailey M, Flynn P, Register J, Marshall L, Bond D, Kulisek E, Kusnadi A, Evangelista R, Nikolov Z, Wooge C, Mehigh RJ, Hernan R, Kappel WK, Ritland D, Li CP, Howard JA (1997) Commercial production of avidin from transgenic maize: characterization of transformant, production, processing, extraction and purification. Mol Breed 3:291–306

Jani D, Meena LS, Rizwan-ul-Haq QM, Singh Y, Sharma AK, Tyagi AK (2002) Expression of cholera toxin B subunit in transgenic tomato plants. Transgenic Res 11:447–454

Jani D, Singh NK, Chattacharya S, Meena LS, Singh Y, Upadhyay SN, Sharma AK, Tyagi AK (2004) Studies on the immunogenic potential of plant-expressed cholera toxin B subunit. Plant Cell Rep 22:471–477

Karaman S, Cunnick J, Wang K (2006) Analysis of immune response in young and aged mice vaccinated with corn-derived antigen against *Eschericia coli* heat-labile enterotoxin. Mol Biotech 32:31–42

Kim YS, Kim MY, Kim TG, Yang MS (2009) Expression and assembly of cholera toxin B subunit (CTB) in transgenic carrot (*Daucuscarota L.*). Mol Biotechnol 41:8–14

Kuo TT, Baker K, Yoshida M, Qiao S, Aveson VG, Lencer WI, Blumberg RS (2010) Neonatal Fc receptor: from immunity to therapeutics. J Clin Immunol 30:777–789

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lebens M, Shahabi V, Backstrom M, Houze T, Lindblad N, Holmgren J (1996) Synthesis of hybrid molecules between heat-labile enterotoxin and cholera toxin B subunits: potential for use in a broad-spectrum vaccine. Infect Immun 64:2144–2150

Luci C, Hervouet C, Rousseau D, Holmgren J, Czerkinsky C, Anjuère F (2006) Dendritic cell-mediated induction of mucosal cytotoxic responses following intravaginal immunization with the nontoxic B subunit of cholera toxin. J Immunol 176:2749–2757

Ma JK, Drake PM, Christou P (2003) The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 4:794–805

Marks MD, Lindell JS, Larkins BA (1985) Quantitative analysis of the accumulation of zein mRNA during endosperm development. J Biol Chem 260:16445–16450

Mason HS, Haq TA, Clements JD, 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 TS, Arntzen CJ (2002) Edible plant vaccines: applications for prophylactic and therapeutic molecular medicine. Trends Mol Med 8:324–329

Moeller L, Gan Q, Wang K (2009) A bacterial signal peptide is functional in plants and directs proteins to the secretory pathway. J Exp Bot 60:3337–3352

Nochi T, Takagi H, Yuki Y, Yang L, Masumura T, Mejima M, Nakanishi U, Matsumura A, Uozumi A, Hiroi T, Morita S, Tanaka K, Takaiwa F, Kiyono H (2007) Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination. Proc Natl Acad Sci USA 104:10986–10991

Qadri F, Ahmed T, Ahmed F, Bradley Sack R, Sack DA, Svennerholm AM (2003) Safety and immunogenicity of an oral, inactivated enterotoxigenic *Escherichia coli* plus cholera toxin B subunit vaccine in Bangladeshi children 18–36 months of age. Vaccine 2:2394–2403

Rhee Y, Staswick PE (1992) Nucleotide sequence for the soybean vegetative storage protein vspB gene. Plant Physiol 98:794–795

Richardson SH, Giles JC, Kriger KS (1984) Sealed adult mice: a new model for enterotoxin evaluation. Infect Immun 43:482–486

Rigano MM, Sala F, Arntzen CJ, Walmsley AM (2003) Targeting of plant-derived vaccine antigens to immunoresponsive mucosal sites. Vaccine 21:809–811

Roopenian DC, Akilesh S (2007) FcRn: the neonatal Fc receptor comes of age. Nat Immunol 7:715–725

Rosales-Mendoza S, Sora-Guerra RE, López-Revilla R, Moreno-Fierros L, Alpuche-Solís ÁG (2008) Ingestion of transgenic carrots expressing the *Eschericia coli* heat-labile enterotoxin B subunit protects mice against cholera toxin challenge. Plant Cell Rep 27:79–84

Ruddock LW, Ruston SP, Kelly SM, Price NC, Freedman RB, Hirst TR (1995) Kinetics of acid-mediated disassembly of the B subunit pentamer of *Escherichia coli* heat-labile enterotoxin. J Biol Chem 270:29953–29958

Sadeghi H, Bregenholt S, Wegmann D, Peterson JS, Holmgren J, Lebens M (2002) Genetic fusion of human insulin B-chain to the B subunit of cholera toxin enhances in vitro antigen presentation and induction of bystander suppression in vivo. Immunology 106:237–245

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci USA 81:8014–8018

Sambrook J, Frittsch EF, Maniatis TH (1989) Molecular cloning, a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, NY

Sangtong V, Mottl EC, Long MJ, Lee M, Scott MP (2001) Serial extraction of endosperm drillings (SEED)—a method for detecting transgenes and proteins in single viable maize kernels (*Protocols*). Plant Mol Biol Rep 19:151–158

Savarino SJ, Hall ER, Bassily S, Wierzba TF, Youssef FG, Peruski LF Jr, Abu-Elyazeed R, Rao M, Francis WM, El Mohamady H, Safwat M, Naficy AB, Svennerholm AM, Jertborn M, Lee YJ, Clemens JD (2002) Pride Study Group. Introductory evaluation of an oral, killed whole cell enterotoxigenic *Escherichia coli* plus cholera toxin B subunit vaccine in Egyptian infants. Pediatr Infect Dis J 21:322–330

Schaetti C (2009) Vaccines for enteric diseases: update on recent developments. Vaccine 8:1653–1655

Shou S, Frame BR, Whitham S, Wang K (2004) Assessment of transgenic maize events produced by particle bombardment or Agrobacterium-mediated transformation. Mol Breed 13:201–208

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

Tacket CO, Pasetti MF, Edelman R, Howard JA, Streatfield S (2004) Immunogenicity of recombinant LT-B delivered orally to humans in transgenic corn. Vaccine 22:4385–4389

Tarkowski A, Sun JB, Holmdahl R, Holmgren J, Czerkinsky C (1999) Treatment of experimental autoimmune arthritis by nasal administration of a type II collagen-cholera toxoid conjugate vaccine. Arthritis Rheum 42:1628–1634

Thompson CK, Movva NR, Tizard R, Crameri R, Davies JE, Lauwereys M, Botterman J (1987) Characterization of the herbicide-resistance gene bar from *Streptomyces hygroscopicus*. EMBO J 6:2519–2523

Tokuhara D, Yuki Y, Nochi T, Kodama T, Mejima M, Kurokawa S, Takahashi Y, Nanno M, Nakanishi U, Takaiwa F, Honda T, Kiyono H (2010) Secretory IgA-mediated protection against *V. cholerae* and heat-labile enterotoxin-producing enterotoxigenic *Escherichia coli* by rice-based vaccine. Proc Natl Acad Sci USA 107:8794–8799

Williams NA, Hirst TR, Nashar TO (1999) Immune modulation by the cholera-like enterotoxins: from adjuvant to therapeutic. Immunol Today 20:95–101

Zhang RG, Scott DL, Westbrook ML, Nance S, Spangler BD, Shipley GG, Westbrook EM (1995) The 2.4 A crystal structure of cholera toxin B subunit pentamer: choleragenoid. J Mol Biol 25:550–562