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A functional antigen in a practical crop: LT-B producing maize protects mice against *Escherichia coli* heat labile enterotoxin (LT) and cholera toxin (CT)

Rachel Chikwamba[1,2], Joan Cunnick[3], Diane Hathaway[1,3], Jennifer McMurray[1], Hugh Mason[4] & Kan Wang[1,*]
[1]*Plant Transformation Facility, Department of Agronomy, Iowa State University, Ames, IA 50011-1010, USA*
[2]*Interdisciplinary Genetics Program, Iowa State University, Ames, IA 50011, USA*
[3]*Department of Microbiology and Immunology, Iowa State University, Ames, IA 50011, USA*
[4]*Boyce Thompson Institute for Plant Research, Tower Road, Ithaca, New York 14853-1801, USA*

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Abstract

We have produced a functional heat labile enterotoxin (LT-) B subunit of *Escherichia coli* in maize. LT-B is a multimeric protein that presents an ideal model for an edible vaccine, displaying stability in the gut and inducing mucosal and systemic immune responses. Transgenic maize was engineered to synthesize the LT-B polypeptides, which assembled into oligomeric structures with affinity for G_{M1} gangliosides. We orally immunized BALB/c mice by feeding transgenic maize meal expressing LT-B or non-transgenic maize meal spiked with bacterial LT-B. Both treatments stimulated elevated IgA and IgG antibodies against LT-B and the closely related cholera toxin B subunit (CT-B) in serum, and elevated IgA in fecal pellets. The transgenic maize induced a higher anti-LT-B and anti-CT-B mucosal and serum IgA response compared to the equivalent amount of bacterial LT-B spiked into maize. Following challenge by oral administration of the diarrhea inducing toxins LT and CT, transgenic maize-fed mice displayed reduced fluid accumulation in the gut compared to non-immunized mice. Moreover, the gut to carcass ratio of immunized mice was not significantly different from the PBS (non-toxin) challenged control group. We concluded that maize-synthesized LT-B had features of the native bacterial LT-B such as molecular weight, G_{M1} binding ability, and induction of serum and mucosal immunity. We have demonstrated that maize, a major food and feed ingredient, can be efficiently transformed to produce, accumulate, and store a fully assembled and functional candidate vaccine antigen.

Abbreviations: E. coli – Escherichia coli; LT-B – labile toxin B – subunit; PMSF – phenylmethanesulfonyl fluoride; PBS – phosphate buffered saline; SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis; BSA – Bovine serum albumin; ELISA – enzyme linked immunosorbent assay; EDTA – ethylene-diamine tetraacetic acid; Tris – Tris hydroxymethyl aminomethane; G_{M1} – galactosyl-N-acetylgalactosamyl-sialyl-galactosylglucosyl ceramide.

Introduction

The *E. coli* heat labile enterotoxin B subunit protein (LT-B) has qualities of an ideal oral vaccine, showing stability upon ingestion resulting in stimulation of secretory IgA antibodies against specific virulence determinants of invading pathogens in the gut. Functional LT-B is synthesized as monomers, which assemble into pentameric structures with high affinity for G_{M1} (galactosyl-N-acetylgalactosamyl-sialyl-gal-

*Author for correspondence: *E-mail:* kanwang@iastate.edu

actosylglucosyl ceramide) gangliosides. It is a potent oral immunogen and has been demonstrated to elicit specific serum and mucosal antibodies (Spangler, 1992; Dickinson & Clements, 1996). In addition, the protein is relatively small and has immunological and other physiochemical properties that could be readily tested to determine its functionality. These features made it an ideal protein for plant expression and production.

The potential of crop plants for the production of biopharmaceuticals and other novel proteins is beginning to be realized. To date, efforts to produce these proteins in plants have focused on dicotyledonous plants such as potato, tobacco, alfalfa, and *Arabidopsis* (Moffat et al., 1995; Mason et al., 1996; 1998; Kusnadi et al., 1997; Tackaberry et al., 1999). These plants have well established and reliable transformation procedures via *Agrobacterium tumefaciens*. Other attractive attributes of these plants include production of large volumes of green tissue in tobacco and alfalfa, which can produce several crops per year by cutting back foliage. Annual yields can be 25 and >100 metric tonnes (MT) per hectare for alfalfa and tobacco, respectively (Daniell et al., 2001). However, these plants have their drawbacks, especially for the purpose of convenient oral vaccine production and delivery. Green leaf tissues in which proteins are produced in these plants tend to have high amounts of phenolic and other potentially toxic compounds. Tobacco and *Arabidopsis* are not palatable, necessitating a purification or extraction step, which may add to the cost of production. An advantage of producing pharmaceuticals in potato is that the tuber is a good tissue for production and storage of proteins. Nevertheless, potato may require cooking to improve its palatability. Arakawa et al. (1998) have shown that boiling CT-B generating transgenic potato denatures up to 50% of the antigen. By contrast, although yields of cereal grains such as wheat, rice, and maize are less abundant than that of the green tissues of tobacco and alfalfa, high seed production in these plants makes scaling up quite easy. Unlike proteins synthesized in vegetative plant tissues, seed storage proteins are compartmentalized in protein bodies, specialized vacuoles in mature seed. These provide a stable environment devoid of significant amounts of enzyme activity prior to germination. Another advantage of producing functional proteins in cereal grains is the stability of protein during long-term storage. Levels of scFV antibody in rice seeds or tubers did not show a significant decline after storage at room temperature for 6 months (reviewed by Daniell et al., 2001). Maize is a convenient and practical crop for production of an edible vaccine. It has high yield, is a major ingredient in livestock feed and is a staple food in many countries. Additionally, maize does not require extensive heat during processing, which might lead to denaturation of antigens, among other advantages.

Work by Mason et al. (1998) and Tacket et al. (1998) showed that potato produced LT-B subunits were capable of inducing both systemic and mucosal antibodies in mice and humans, respectively. In this study we investigated the ability of maize-synthesized LT-B to retain properties of the native bacterial protein such as antigenicity, pentameric structure, G_{M1} ganglioside receptor binding capacity, and immunogenicity in mice. We used the maize gamma zein promoter (Marks et al., 1985), a seed endosperm specific promoter, to direct LT-B expression in maize kernels. We demonstrated that maize-synthesized LT-B folded properly into pentameric structures with affinity for G_{M1} gangliosides, like the native LT-B. When fed to mice, the maize-synthesized LT-B stimulated a protective immune response against diarrhea inducing toxins LT and its closely related cholera toxin (CT). In addition, feeding transgenic maize to mice did not result in the induction of tolerance to LT-B, as shown by the presence of significant systemic and mucosal anti-LT-B and CT-B antibodies. Our results indicated that maize could be efficiently transformed to produce, accumulate, and store fully assembled and functional antigen.

Results

Analysis of transgenic maize lines

A plant optimized coding sequence of LT-B (sLT-B) from *E. coli* strain H10407 (Yamamoto et al., 1982) was designed and synthesized by Mason et al. (1998). Construct pRC4 (Figure 1) was introduced into a maize hybrid line Hi II via particle bombardment as described (Frame et al., 2000). Transformed maize callus lines were analyzed by polymerase chain reaction (PCR) to confirm the presence of the sLT-B gene. Because an endosperm specific promoter was used, it was expected that the transgene would only express in endosperm of transgenic maize. Therefore, herbicide resistance and presence of the LT-B gene in PCR analysis were the criteria for selecting transgenic calli.

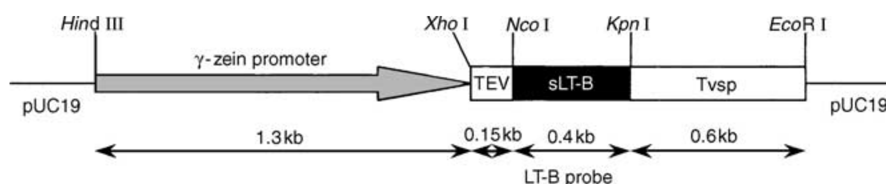

Figure 1. Schematic diagram of construct pRC4 with the synthetic gene encoding the B subunit of LT (sLT-B) under the control of the 27 kD gamma zein promoter, the translational enhancer leader sequence of tobacco etch virus (TEV) and the soybean vegetative storage protein terminator (vsp) in a pUC19 background. The 0.4 kb *Nco*I-*Kpn*I fragment was used as a probe in Southern analysis.

Sixty independent herbicide resistant events were analyzed, 47 were found to contain the LT-B gene. Three plants per event were regenerated from 20 of these events and grown in the greenhouse to maturity.

Nineteen of the 20 regenerated transgenic events (P77) were fertile. These plants were crossed with pollen from the non-transgenic inbred line B73. Mature transgenic kernels were analyzed for expression of the LT-B gene using ganglioside dependent ELISA as described by Haq et al. (1995) and in Materials and methods. The LT-B expression in R_1 seed from these events is summarized in Figure 2. LT-B expression ranged from less than 0.01% to greater than 0.05% LT-B of total soluble protein as shown. Nine events expressed less than 0.01% LT-B of total soluble protein, eight events had LT-B levels between 0.02 and 0.05% and two events showed expression above 0.05%.

Six events were chosen to increase seed for further analysis on the basis of seed availability and level of expression of LT-B. Seeds were planted from the expressing R_0 events to give R_1 generation plants, which

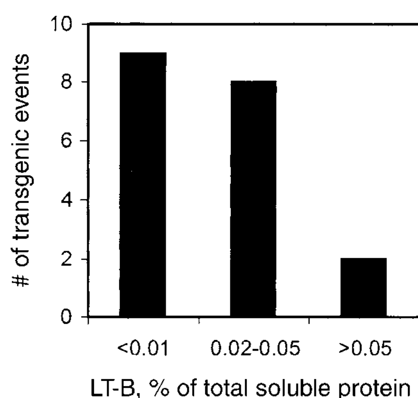

Figure 2. Accumulation of LT-B protein in endosperm of mature seed from 19 independent P77 R_0 transgenic events. Endosperm tissue was sampled as described in Materials and Methods and the LT-B expression assayed by ELISA. Twenty kernels from two ears per event were analyzed. The LT-B expression level (% of total soluble protein) of each event was determined as the mean of all ELISA positive kernels.

were grown to maturity in the greenhouse. These plants were either self-pollinated or pollinated with non-transgenic inbred line B73 or hybrid line Hi II (Table 1).

Southern analysis was carried out on selected events P77-2, -7 and -9 using leaf material of R_1 plants to determine transgene copy number (Figure 3). Since *Nco*I cuts only once in the transgene cassette, the hybridization banding pattern should give an estimation of the number of LT-B gene copies present in the maize genome. From the number and intensity of bands in the autoradiograph, it can be concluded that all three events had multiple gene insertions and possible tandem repeats in the same insertion site.

Immunoblot analysis was performed on five transgenic events using the R_2 kernels to detect the presence of the assembled LT-B subunits. The molecular weight of an LT-B monomer is 11.6 kD. However, only properly assembled LT-B subunits in a pentameric structure (55 kD) have G_{M1} binding capacity and hence native antigenic function (de Haan et al., 1998). As reflected in Figure 4, maize-synthesized LT-B from R_1 seeds of events P77-2, -7, -17, and -18 had similar molecular weight to the bacteria-derived LT-B in either pentameric (Figure 4(A)) or monomeric form (Figure 4(B)). No LT-B protein was detected from event P77-1 by western analysis. ELISA analysis also indicated that the LT-B expression of this event was low (Table 1).

For mice feeding experiments, dry kernels from each R_2 ear were harvested and ground to a fine meal. LT-B of the meal was assayed using ELISA (Table 1). Since the LT-B transgene in the R_2 seed population was segregating at a ratio of either 3:1 (for selfed plants) or 1:1 (for out-crossed plants, data not shown), the maize meal obtained from these events was a mixture of LT-B expressing and non-expressing seeds.

The expression observed in the meal of six events ranged from 0.004 to 0.19% LT-B of total soluble protein (Table 1). In event P77-9, the LT-B expression increased 10-fold compared to the level detected in

Table 1. Summary of transgene copy numbers, seed yield, and LT-B expression of six transgenic events

Event ID	Ear ID	Gene copy #	Pollen source	Seed yield	LT-B expression (% TSP)	
					R_1 whole kernels[a]	R_2 meal[b]
P77-1	3-1	nd	Hi II	254	0.01 ± 0.005	0.004
P77-2	2-1	4	Selfed	220	0.07 ± 0.007	0.049
	2-2		Hi II	66		0.033
	2-3		Selfed	220		0.034
	2-5		Selfed	128		0.020
	2-7		Hi II	152		0.047
	2-9		Selfed	51		0.036
	2-10		Selfed	188		0.022
P77-7	1-1	2	B73	23	0.05 ± 0.002	0.104
	1-2		Selfed	97		0.120
	1-3		Selfed	76		0.118
	1-4		Selfed	154		0.047
P77-9	2-1	5+	Selfed	196	0.02 ± 0.002	0.099
	2-2		Selfed	53		0.190
	2-6		Selfed	35		0.203
P77-17	1-1	nd	B73	23	0.02 ± 0.002	0.008
P77-18	2-1	nd	Selfed	9	0.02 ± 0.005	0.002

[a]Data from 10 expressing kernels from each event.
[b]ELISA was done on pooled kernels from one single ear.
nd: not determined

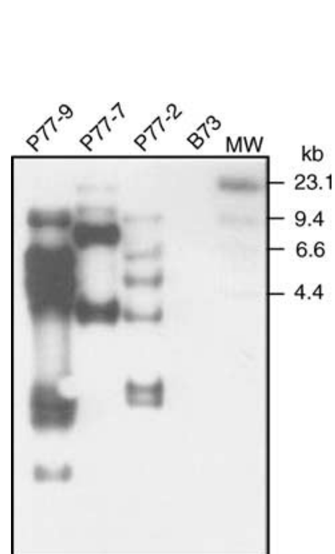

Figure 3. Southern blot analysis of selected transgenic events P77-2, -7, and -9. Ten micrograms of total leaf genomic DNA of R_1 plants was digested with *Nco*I, a restriction enzyme with a single cut within the sLT-B gene cassette (Figure 1). DNA was separated on a 0.8% agarose gel, blotted onto a Zeta-probe nitrocellulose membrane (BioRad) and hybridized with ^{32}P-labeled LT-B gene as probe. MW, molecular marker, lambda DNA digested with *Hin*d III. B73, non-transgenic maize DNA as negative control.

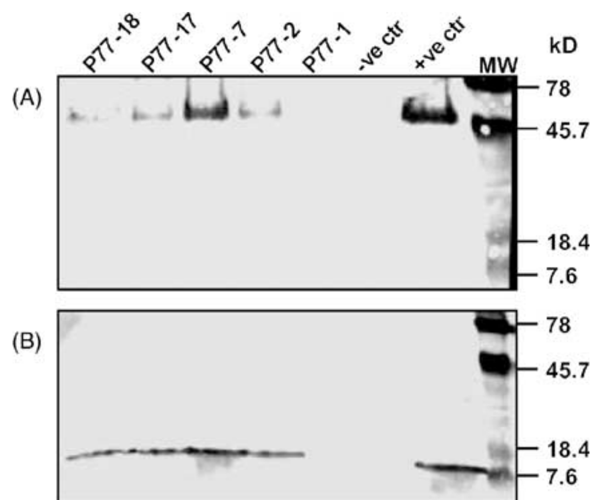

Figure 4. Western analysis of selected transgenic events P77-1, -2, -7, -17, and -18. One hundred micrograms of total soluble protein from R_2 meal was separated on an analytical discontinuous SDS (12%) PAGE, transferred to a 0.45 μm nitrocellulose membrane and probed with goat anti-LT-B and rabbit anti-goat alkaline phosphatase conjugate as primary and secondary antibodies, respectively. Panel (A), protein samples not boiled prior to loading. Panel (B), protein samples boiled (95°C, 5 min) prior to loading. MW, Molecular weight standards; +ve ctr (100 ng), bacterial LT-B as positive control; −ve ctr, non-transgenic maize extracts as negative control.

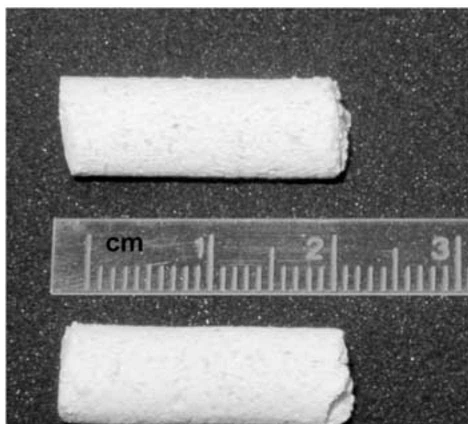

Figure 5. Maize meal pellets used to feed BALB/c mice for oral administration of LT-B. Pellet was made from either non-transgenic maize kernel meal spiked with 10 μg of bacterial LT-B, or transgenic maize kernel expressing 10 μg of LT-B. Each pellet weighed 1 g (length scale in centimeters).

their R_1 kernels. The LT-B expression level varied not only between independent events, but also between ears within one event. Event P77-2 was used for the mice feeding experiment because of its adequate LT-B expression and sufficient seed yield at the time of experiments. A total of five P77-2 ears from selfed plants were pooled for the feeding experiment.

Anti-LT-B antibody response in mice

Mice were fed with maize pellets (Figure 5) containing 10 μg of maize-synthesized or bacterial LT-B on days 0, 3, 7, and 21. Figure 6 depicts the anti-LT-B antibody responses detected in the blood and fecal pellets of orally immunized mice. Results are presented as mean ± SD. Serum anti-LT-B IgG levels in blood were first detected on day 13 following initial immunization. For mice fed with both transgenic maize pellets and maize pellets spiked with the bacterial LT-B, this was reflected as a late stage primary response as antibody class switching to IgG occurred (Figure 6(A)). A secondary immune response observed as a peak mean antibody response (5.7 ± 1.1 mg/ml) at day 27 (1 week after the fourth feeding). The IgG concentration about double the amount observed in the primary response. The response of the transgenic maize fed group was similar to that observed in mice immunized with bacterial LT-B, which also peaked (5.8 ± 0.7 mg/ml) at day 27. There were no changes in specific antibody production in mice fed with maize pellet made from non-transgenic maize (negative group) throughout the experiment.

Fecal secretory IgA plays an important role in attenuation of toxin effects, possibly more so than the serum IgG and IgA because it is secreted in the gut at the point of contact with the toxin. Figure 6(B) shows the anti-LT-B IgA concentrations detected from fecal pellets of immunized mice. The secretory IgA of transgenic maize-fed mice increased gradually over the course of the experiment and peaked (278 ± 50μg/g of dry fecal matter) at day 27. Mice fed with maize spiked with the bacterial LT-B also developed anti-LT-B IgA in their fecal pellets. However, the secondary response of these mice was significantly lower (149 ± 21μg/g dry fecal matter, $p < 0.001$) than that of transgenic maize-fed mice. No substantial production of fecal anti-LT-B IgA was detected in mice fed with non-transgenic maize pellet.

The serum anti-LT-B IgA was determined during the course of the experiment and the results are shown in Figure 6(C). Both the maize-synthesized and bacterial LT-B protein induced serum anti-LT-B IgA in mice, and both show clearly defined primary and secondary antibody responses. However, transgenic maize induced a significantly higher serum IgA (428 ± 68 μg/ml, $p < 0.001$) on day 27, compared to the bacterial LT-B (250 ± 36 μg/ml), which also induced a significantly higher serum IgA response than the negative control ($p < 0.001$).

Anti-CT-B antibody response in immunized mice

The cholera toxin (CT) is physically, chemically, and antigenically similar to LT (Dickinson & Clements, 1996). To investigate whether mice immunized with maize synthesized or bacterial derived LT-B protein would also generate cross-reacting antibodies that would bind CT-B, we performed anti-CT-B antibody analysis on mice sera and fecal samples (Figure 7). Figure 7(A) shows the concentration of serum anti-CT-B IgG over the course of the experiment. In contrast to anti-LT-B IgG, no marked responses to primary immunization were detected in mice fed with either transgenic maize or bacterial LT-B spiked maize pellets. Elevated serum anti-CT-B IgG antibodies were detected at day 27. The IgG level in transgenic maize-fed mice was significantly higher (2.3 ± 0.8 mg/ml) than that of LT-B spiked maize-fed mice (1.0 ± 0.5 mg/ml). No anti-CT-B IgG antibodies were detected in the negative control mice fed with non-transgenic maize pellets.

Figure 7(B) shows the cross-reacting anti-CT-B fecal IgA antibody production. Although some anti-

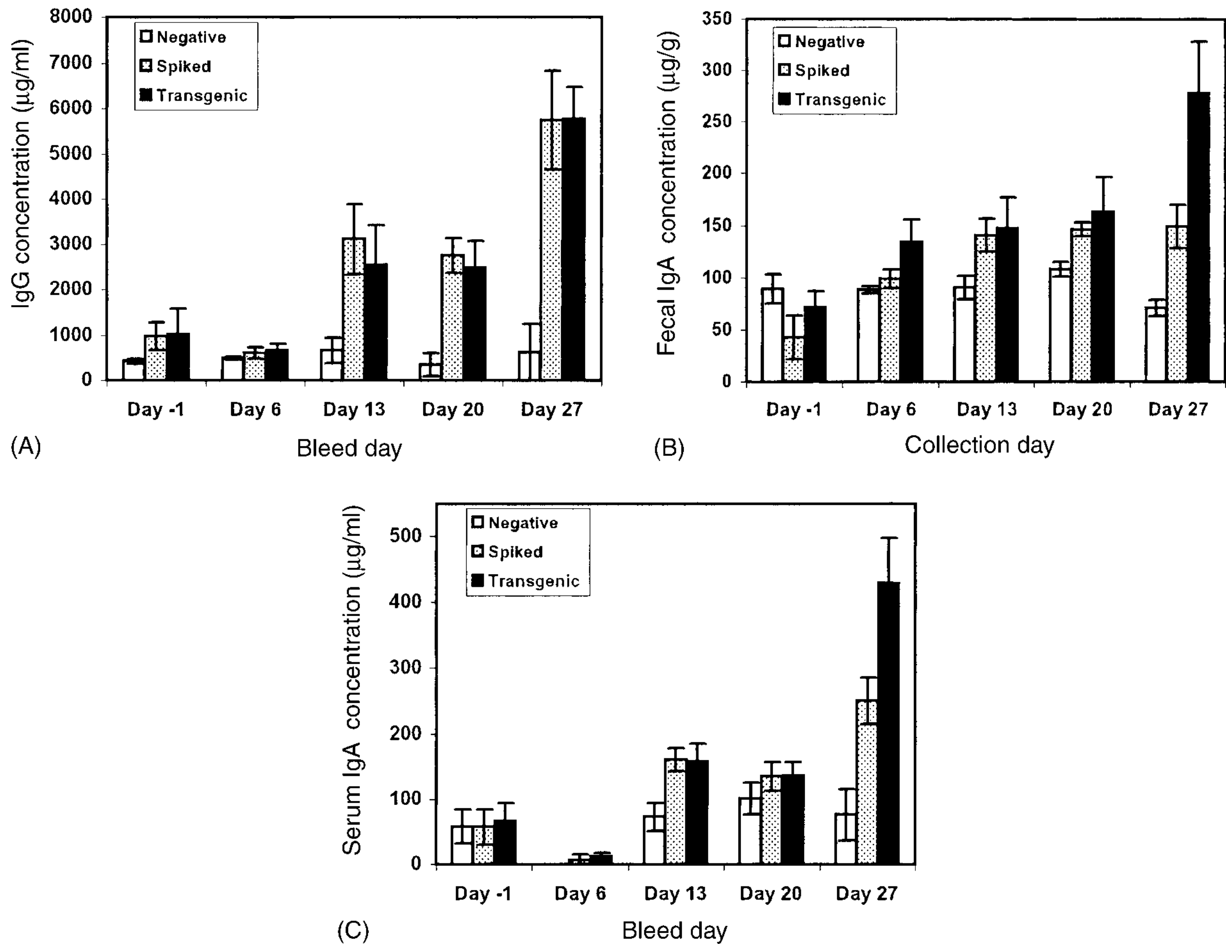

Figure 6. Anti-LT-B antibody analysis in orally immunized mice. Twelve mice in each feed category were fed with maize meal pellets from non-transgenic maize (negative), non-transgenic maize spiked with bacterial LT-B (spiked) and transgenic maize expressing LT-B (transgenic) at days 0, 3, 7, and 21. (A) The levels of serum anti-LT-B IgG. (B) The levels of fecal anti-LT-B IgA. (C) The levels of serum anti-LT-B IgA.

CT-B IgA antibody was detected in mice fed with either maize-synthesized or bacterial LT-B over the course of the experiment, they were not very high above the background. Mice fed with the control maize pellets (negative group) also displayed measurable production of anti-CT-B antibody. The fecal anti-CT-B cross-reacting antibodies were only marginally higher ($p < 0.02$) in mice immunized with transgenic maize at the end of the experiment (day 27).

Figure 7(C) shows the cross-reacting serum anti-CT-B IgA antibodies at the end of the experiment (day 27). The antibody production over the course of immunizations could not be tracked over the course of the experiment because of limitations in serum availability from earlier bleeds. Transgenic maize pellets induced a higher response (196 ± 69 µg/ml, $p < 0.01$) compared to the bacterial LT-B spiked maize pellet in mice. Interestingly, LT-B spiked maize pellets did not induce significantly higher (69 ± 40µg/ml) serum anti-CT-B IgA in mice compared to non-transgenic negative control pellets.

Response of immunized mice to challenge with LT and CT toxins

To investigate whether maize-synthesized LT-B provides protection against toxin challenge, we performed the patent mouse assay (Guidry et al., 1997) in the orally immunized mice (Figures 6 and 7), using LT or CT to challenge the mice. These toxins activate adenylate cyclase and result in fluid secretion into the lumen of the bowel. The fluid secretion exceeds

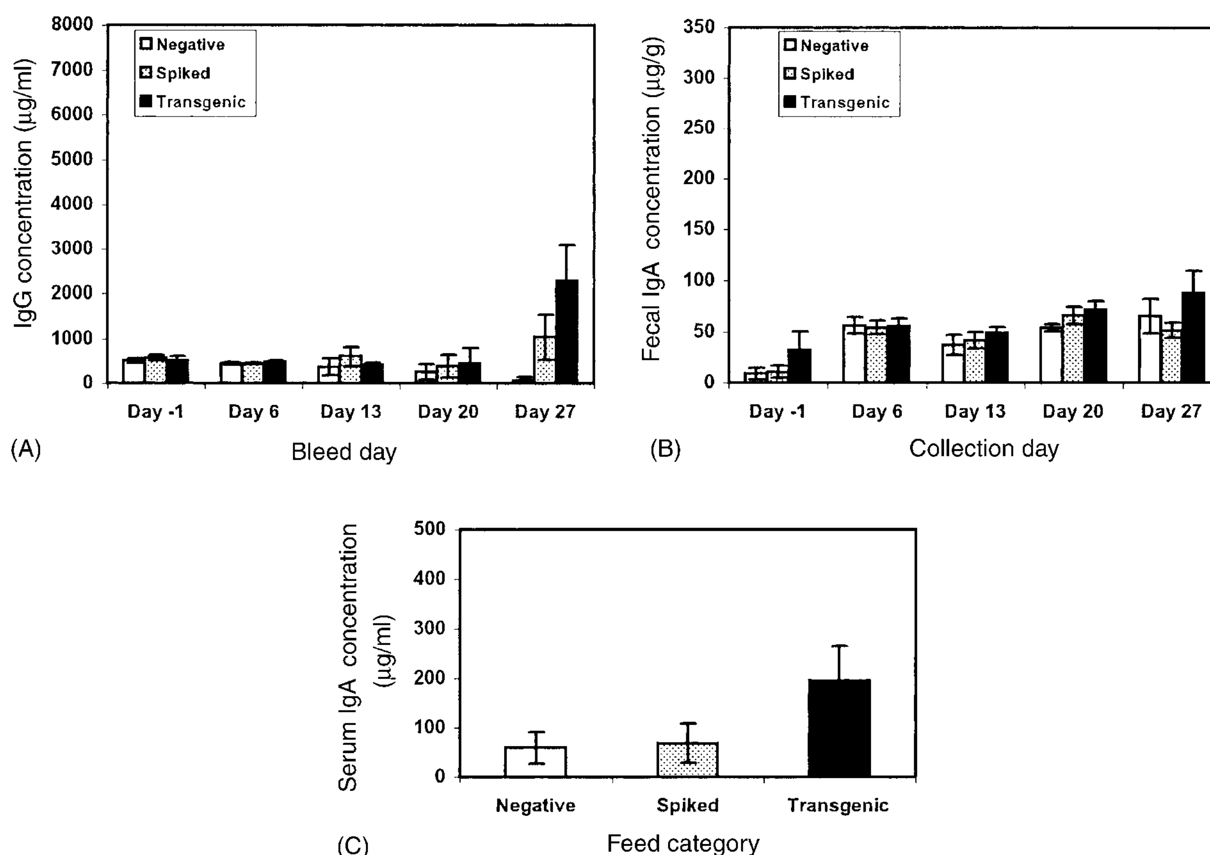

Figure 7. Cross-reacting anti-CT-B antibody analysis in orally immunized mice. Mice were fed with maize meal pellets from non-transgenic maize (negative), non-transgenic maize spiked with bacterial LT-B (spiked) and transgenic maize expressing LT-B (transgenic) at days 0, 3, 7, and 21. (A) Levels of serum anti-CT-B IgG. (B) Levels of fecal anti-CT-B IgA. (C) Levels of serum anti-CT-B IgA at day 27.

the bowel's capacity to reabsorb it and, therefore, it accumulates (Mason et al., 1998). The movement of fluid from the body into the lumen of the bowel decreases the weight of the carcass and increases the relative weight of the gut. The gut/carcass ratio, therefore, changes in a dose dependent fashion. In a toxin dose-response experiment (Figure 8(A)), we measured the gut/carcass ratio of non-immunized mice gavaged with two levels of LT and CT (25 and 50 µg). Significant fluid influx was detected in mice gavaged with both levels of LT and CT toxins when compared to mice gavaged with phosphate buffered saline (PBS) as a negative control ($p < 0.02$ for 25 µg and $p < 0.001$ for 50 µg). A significant difference in gut/carcass ratio between LT and CT ($p < 0.02$) was observed when 50 µg of toxin were administered. The CT was significantly more potent than the LT as expected (Bowman & Clements, 2001). There was no significant difference in fluid influx observed between LT and CT at 25 µg/dose level.

Figure 8(B) shows the results of the patent mouse assay performed on day 28 of the experiment. Compared to mice that were fed with non-transgenic maize, mice immunized with LT-B expressing transgenic maize had reduced gut/carcass ratio when challenged with 25 µg LT toxin. The gut/carcass ratios for transgenic maize-fed mice challenged by LT toxin was not significantly different from non-transgenic maize-fed mice gavaged with PBS only (0.109 ± 0.001 v.s. 0.095 ± 0.0002), indicating that mice orally immunized with transgenic maize were protected from challenge with 25 µg LT toxin. Mice fed with LT-B-spiked maize also showed a similar reduction in the gut/carcass ratio. There was significant fluid influx in mice that were given the non-transgenic maize meal compared to the PBS control group (0.130 v.s. 0.095,

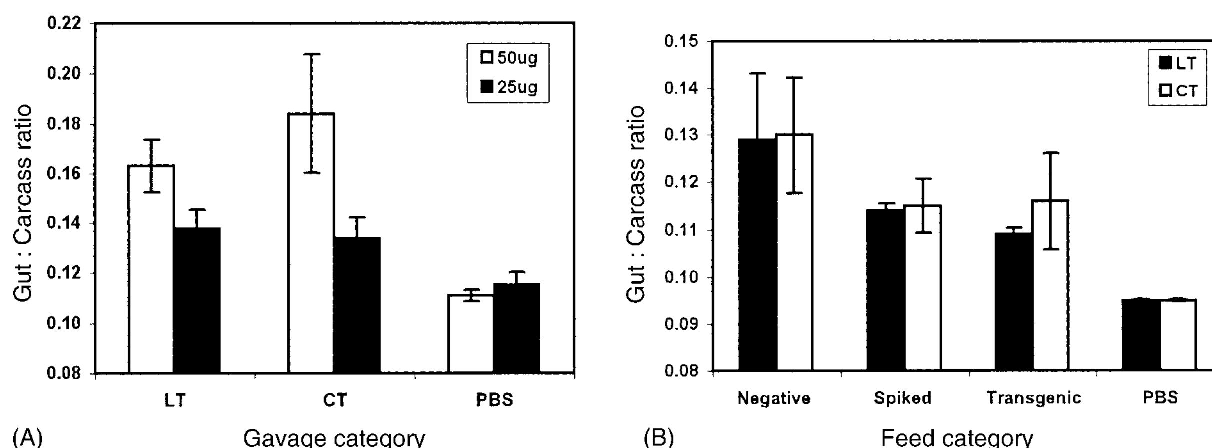

Figure 8. Patent mouse assay for toxin challenge of mice. (A) Dose responses of LT, CT, and PBS buffer alone. Mice were gavaged with two levels (25 and 50 μg) of LT and CT toxins and dissected 3 h later to determine the gut/carcass ratio. For each group $n = 2$. (B) Response of mice fed with maize meal pellets from non-transgenic maize (negative), non-transgenic maize spiked with bacterial LT-B (spiked) and transgenic maize expressing LT-B (transgenic) to toxin challenge. Each mouse was gavaged with 25 μg of LT or CT or PBS buffer alone at day 28 and gut/carcass ratio was determined 3 h later as described in Materials and Methods. For each group $n = 2$.

$p < 0.01$). The gut of mice from all feed categories gavaged with PBS (shown as PBS group in Figure 8(B)) did not accumulate fluid indicating that the gavage procedure itself did not cause an inflammatory response resulting in fluid accumulation.

Similar results were observed when orally immunized mice were challenged with 25 μg CT-B (Figure 8(B)). Mice fed with either transgenic maize meal and LT-B-spiked maize meal also had reduced fluid influx compared to mice fed with non-transgenic maize and gavaged with CT toxin. The gut to carcass ratio for mice in these two categories (transgenic maize and spiked) was not significantly different from mice fed with negative maize and gavaged with PBS (0.095 ± 0.0002).

Discussion

We have demonstrated that a synthetic gene encoding *E. coli* LT-B can be successfully expressed in transgenic maize seed and accumulate in pentameric G_{M1} binding form. Oral immunization of BALB/c mice with maize-synthesized LT-B induced elevated IgA and IgG serum antibodies and elevated fecal IgA against diarrhea-inducing toxins LT and CT. In addition, transgenic maize-fed mice challenged with LT or CT toxins displayed reduced gut/carcass ratios that were not significantly different from the PBS challenged negative control group, indicating that the maize-synthesized LT-B has biological and immunological functions comparable to the native LT-B protein that can protect immunized mice from LT or CT toxins challenge.

In this study, we inserted into the maize genome a synthetic LT-B gene that was optimized for expression in both potato and maize (Mason et al., 1998). To achieve a high level of gene expression, we used the 27 kD maize gamma zein promoter to drive transcription of the sLT-B gene. The 27 kD gamma zein promoter is one of the strongest seed specific promoters in maize, and zeins comprise 50–60% of the total seed storage proteins (Marks et al., 1985). In contrast to other plant tissues, seeds provide a relatively dry and stable environment for protein storage. Seeds also present potentially practical and protective delivery vehicles for targeting edible vaccines into the gut because of their palatability and high protein content. The antigenic protein may be delivered within protein bodies, and, therefore, not in readily soluble form. Presentation of proteins in soluble form has been shown to be conducive to oral tolerance (Simmons et al., 2001). The initial level of LT-B expression in R_1 seed ranged from less than 0.01 to 0.07% LT-B of total soluble proteins (TSP). Considerable variation in LT-B expression was observed between and within each independent event. Southern analysis was conducted on the best performing events P77-2, -7, and 9. The events have multiple transgene copy numbers and variable insertion patterns. Gene copy numbers and the relative positions of transgene insertion in the plant

genome (position effects) may be factors that affect level of transgene expression (Christou, 1996).

Compared to the LT-B expression in R_1 seed, we observed an increased LT-B expression in the R_2 seed for the same event. LT-B expression ranged from 0.004 to 0.2% of TSP. The reason for this increase is not clear. However, this phenomenon has also been reported by Hood et al. (1997) who observed a similar increase in avidin gene expression in transgenic maize in subsequent generations. We observed but did not follow up events with decreased LT-B expression over the generations. It is worth noting that the LT-B levels obtained from R_2 seed was from a mixture of segregating seed population from each event, with expression ratio of 3:1 for selfed and 1:1 for out-crossed plants. It is conceivable that the LT-B yield would be higher if only expressing kernels had been included in the meal.

The ganglioside dependent ELISA used to determine the level of LT-B expression demonstrated the ability of maize-synthesized LT-B to bind G_{M1} gangliosides, a property of native pentameric LT-B (Dickinson & Clements, 1996). Our ELISA results indicated that the maize-produced LT-B was similar to the bacterial LT-B that properly folded and assembled into pentamers. Immunoblot analysis indicated that the maize-synthesized LT-B was similar in molecular weight to the bacterial LT-B, the non-boiled form was oligomeric and dissociated into monomers upon boiling.

Mice fed with LT-B expressing transgenic maize developed serum and mucosal antibodies that are specific to LT-B and cross-react with the closely related CT-B. It is difficult to accurately quantify the secretory IgA in the gut as the amounts measured in fecal pellets may be a reflection of the remaining antibody after degradation by microbes that live in the gut, among other processes. Serum IgA, while not involved in protection of host from invading pathogens and toxins, was used to corroborate the differences in the effect of the different feed types (compare Figures 6(B) and (C)).

Interestingly, compared to spiked LT-B-fed mice, we observed a significantly higher level of anti-LT-B IgA in fecal and serum samples of transgenic maize-fed mice (Figure 6(B) and (C)) especially at day 27 when the maximum amount of antibody was expected. One possible explanation could be that the maize-synthesized LT-B is more stable within the maize tissues and was released slowly as the meal was digested. The spiked bacterial LT-B, on the other hand, could be more vulnerable to proteolytic degradation during the process of pellet preparation and feeding as it is merely mixed in with regular maize meal. An alternative explanation could be that there was more LT-B in transgenic maize than the ELISA assay indicated depending on the fineness of the meal. We observed that the finer the maize meal was ground, the higher the LT-B yield we measured, suggesting that a finer maize powder allows better protein extraction. However, very fine maize meal did not mould well to make pellets for feed, so we used an average textured meal to make the pellets for feeding.

Cross-reacting anti-CT-B antibodies could also be detected in mice fed with LT-B expressing transgenic maize or LT-B-spiked maize. There were overall more LT-B binding antibodies compared to CT-B binding IgG and IgA (compare Figures 6(A) with 7(A) and Figures 6(B) with 7(B)). This is not unexpected since the B subunits from the two toxins share some common epitopes but are not identical, (there is a 20% difference in nucleotide and amino acid sequence levels between them (Dallas & Falkow, 1980)). Therefore, among antibodies produced in response to LT-B immunization, only those produced to the common epitopes will cross-react with CT-B.

It is important that an oral vaccine should elicit an immune response in the gut. It has been shown that in many cases, it is possible to prevent the initial infection by stimulating production of mucosal secretory IgA against specific virulence determinants on the pathogen. Specific secretory IgA prevents interaction of the pathogen with the mucosal surface by blocking attachment and/or colonization to prevent invasion of host cells or by neutralizing surface acting toxins (Dickinson & Clements, 1995). We show here that the maize-synthesized LT-B stimulates secretory IgA in the gut and is, therefore, functional in that regard.

We have demonstrated that mice orally immunized with the transgenic maize or LT-B spiked maize have reduced fluid influx in their guts (gut/carcass ratio) compared to the non-transgenic maize-fed mice, indicating that the LT-B-immunized mice were protected from the toxin challenge. In this study, 25 μg of LT used for challenge was determined previously (Mason et al., 1998). It was found that in a dose response curve, the gut/carcass ratio reached a plateau at 0.16 at a dose of 25 μg of LT (Mason et al., 1998). This level of LT and CT used for the challenge was quite substantial for mice considering their low body mass. For comparison, in one human trial, 25 μg of native CT administered orally was shown to elicit a 20-liter cholera purge in human volunteers (Levine et al.,

1993), while 25 μg of LT administered with a whole cell B subunit cholera vaccine was shown to elicit up to 6 liters of fluid (Bowman & Clements, 2001).

The gut/carcass ratio used for the efficacy assessment is toxin dose dependent. In our patent mouse assay, challenge with 25 μg of toxin showed attenuation of both LT and CT toxin effects in orally immunized mice (Figure 8(B)). When 50 μg of toxin were used, the gut/carcass ratio of the transgenic maize fed group was no longer different from that of toxin-challenged non-transgenic maize group (data not shown), suggesting that at this level of toxin, orally-immunized mice were not protected from these diarrhea-inducing toxins.

While we detected more LT-B binding IgG and IgA compared to CT-B binding antibodies, there was no significant difference in protection between LT and CT challenged mice. The apparent low anti-CT-B antibody concentrations observed could merely be a reflection of the limit of detection of the ELISA. Data for anti-CT-B binding antibodies showed more variability compared to anti-LT-B serum data, especially at lower titers. This may be an artifact in measuring these antibodies. Alternatively, this could be due to the simple reason that there are not as many cross-reacting anti-CT-B antibodies, and the few that are present are adequate to attenuate the effect of CT.

Despite the attractiveness of mucosal vaccination, mucosally-administered proteins are frequently not immunogenic (Bowman & Clements, 2001) and require more antigen relative to parenteral vaccination (Simmons et al., 2001). A number of strategies have been developed to facilitate and enhance the immune response obtained after mucosal vaccination and to prevent induction of oral tolerance. The use of adjuvants is one such strategy and LT-B has been shown to be a potent one. Millar and co-workers (2001) showed that contrary to previous opinion (reviewed by Dickinson & Clements, 1996), the non-toxic subunits LT-B and CT-B are potent oral immunogens. These subunits avoid tolerance induction when administered mucosally and generate strong serum and mucosal responses (reviewed by Snider, 1995). They are arranged in a pentameric ring and contain five receptor-binding pockets for high avidity association with cellular G_{M1} gangliosides. We have demonstrated here that maize-synthesized LT-B has a mitigating effect against both LT and CT toxins. Protein less immunogenic than LT-B may not stimulate an immune response when administered orally in an edible transgenic plant or may even induce tolerance against itself. LT-B can, therefore, be co-expressed in the same plants with the less immunogenic vaccines to achieve a more vigorous immune response. As an adjuvant, LT-B could play a key role in providing safe and cost effective mucosal immunization against enteric pathogens, which can be effectively controlled by recombinant subunit vaccines.

LT-B could also be used as a cost effective carrier for induction of an increased mucosal response to antigens to which it is chemically or genetically conjugated. Because of its ganglioside binding properties, LT-B has the capacity to bring conjugated antigens in contact with the mucosal system, making oral vaccination more efficient. Increased immunogen concentration in the mucosal lymphoid tissues may reduce the requirement for high levels of antigen biosynthesis in transgenic plants.

Maize-synthesized LT-B did not induce tolerance as indicated by the production of antibodies over the vaccination period. We have shown that maize-synthesized LT-B conferred protection against both LT and its closely related CT. Maize is highly palatable and as such, a reasonable crop for vaccine production. Fasted mice easily consumed 1 g of pellet containing LT-B protein within 3 h. Production of LT-B in kernels and palatability of maize pellets make it easier and convenient to administer an oral vaccine in maize relative to other plants. Feeding periods for administration of adequate doses with potato reported by Mason et al. (1998), were quite prolonged, up to 24 h. Because of the high level of expression of the LT-B that could be achieved in maize, only 1 g was required to administer an adequate dose (10 μg) of LT-B.

Recent research has demonstrated the potential of plant-derived antigens for immunization against infectious agents that invade through mucosal surfaces (Tacket et al., 1998). Maize is a major feed ingredient and could potentially play a key role in the production of edible vaccines for livestock. Oral vaccine delivery from maize seed would be convenient, practical and would allow long-term storability and stability of the proteins. Storage of antigen in protein bodies is expected to protect it from immediate enzymatic degradation and from the acidic environment in the stomach (Simmons et al., 2001). In addition, it will allow the protein to be released slowly. This is advantageous because delivering oral vaccines in soluble form is conducive to oral tolerance. Questions under further investigation in our laboratory include increased expression to address dosage requirement and dosage stability in grain.

Materials and methods

Cloning and bacterial transformation

The enzymes *Xho*I and *Eco*RI were used to cut out the 1.15 kb fragment including the tobacco etch virus (TEV) 5′ untranslated region that mediates enhancement of translation initiation (Gallie et al., 1995), the synthetic LT-B gene (Mason et al., 1998) and the soybean vegetative storage protein (VSP) terminator (Mason et al., 1993) from the plasmid pTH210 (H. Mason, unpublished). This fragment was cloned in front of the maize 27 kD gamma zein promoter (Marks et al., 1985) in a pUC19 vector to give the plasmid pRC4 (Figure 1). The resultant plasmid pRC4 was sequenced to ensure correct orientation and fidelity of ligation junctions. DNA for maize transformation was obtained using the Qiagen (Qiagen GmbH, Germany) Maxiprep kit according to the manufacturers instructions.

Maize transformation

Embryogenic maize Hi II callus was transformed using microprojectile bombardment as described by Frame et al. (2000). Briefly, the plasmid pRC4 (Figure 1) encoding the synthetic LT-B gene was co-bombarded with a selectable marker gene construct, pBAR184. This construct contains the ubiquitin promoter/*bar* gene cassette that confers resistance to the herbicide bialaphos (Frame et al., 2000). Herbicide-resistant calli were analyzed using the polymerase chain reaction (PCR) for presence of the LT-B gene cassette. Calli of transgenic events designated P77 were regenerated and plants brought to maturity in the greenhouse.

DNA extraction from maize callus and leaf tissue

Plant DNA was extracted from callus as follows: 0.1–0.25 g of callus were collected in a 1.5 ml ependorf tube and ground with a plastic Kimble pestle (Fisher Scientific, Pittsburgh, PA, USA) in 400 μl extraction buffer [200 mM Tris–HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA, and 0.5% SDS]. An equal volume of phenol : chloroform : isoamyl alcohol (25:24:1, v/v/v) was added and mixed well by inverting the tube several times. The tubes were centrifuged at maximum speed on a bench top centrifuge for 10 min. The aqueous phase was transferred to a clean eppendorf tube and the DNA precipitated with 2-propanol and washed with 70% ethanol. Leaf genomic DNA was extracted from R_1 maize plants using a cetyltrimethylammonium bromide (CTAB) protocol, as described by Murray and Thompson (1980). Three grams of fresh leaf tissue were ground to a fine powder in liquid nitrogen and resuspended in 25 ml of CTAB buffer (1% CTAB, 50 mM Tris, 0.7 M NaCl, 1 mM phenathroline, pH 8.0) supplemented with 0.5% beta-mercaptoethanol just before use. Samples were incubated for 30 min at 65°C, then allowed to cool to room temperature. Twenty milliliters of chloroform : isoamyl alcohol (24:1, v/v) were added and the samples mixed by inverting on an orbital shaker for 10 min. The tubes were centrifuged at 2500 g and the aqueous phase was collected. DNA was precipitated from the aqueous phase in 2/3 volume of 2-propanol, with gentle inversion, and removed with a glass hook. The DNA was washed for 30 min at room temperature in 80% ethanol plus 15 mM ammonium acetate. The liquid was removed and the DNA was air-dried and dissolved in TE (10 mM Tris, 1 mM EDTA, pH 8.0).

Polymerase chain reaction

Transgenic calli were initially selected on the basis of resistance to bialaphos and the presence of the LT-B gene was confirmed by PCR analysis. The forward and reverse primers, respectively, used were, LT-B-PCRF1 (5′-atc gat aca aaa caa acg aat ctc aag c-3′) and LT-B-PCRR1 (5′-cca tgg cgt gga ttt tat gac att tta t-3′). These primers amplify an 842 bp fragment from the TEV leader to part of the VSP terminator, including the LT-B gene. PCR reactions were carried out in a total volume of 50 μl containing 50–250 ng of maize callus genomic DNA, 5 μl of 10 × PCR buffer, 2 μl of dNTP mix (containing 10 mM of each dNTP), 12 μl of each primer (2.5 μM stock), and 1 μl of Biolase Taq Polymerase (Biolase USA Inc., NJ, USA). Reaction conditions were as follows: Initial PCR activation (95°C, 3 min) was followed by 30 amplification cycles (denaturing, 94°C, 30 s; annealing, 60°C, 30 s, and extension, 72°C, 45 s) and a final extension step at 72°C for 5 min.

Southern blot analysis

Ten micrograms of leaf genomic DNA were digested with restriction enzyme *Nco*I at 37°C overnight and separated on a 0.8% agarose gel. Linearized DNA gel blot analyses (Sambrook et al., 1989) were conducted on DNA samples using the ^{32}P-labeled LT-B gene fragment as probe (Figure 1).

Protein extraction from maize kernel

Individual mature dried kernels frozen in liquid nitrogen were ground to a fine powder in a mortar and pestle. The fine powder was transferred into a 1.5 ml eppendorf tube and weighed. For ELISA analysis, 25 mM sodium phosphate buffer (pH 6.6) containing 100 mM NaCl, 10 mM EDTA, and 0.5% Triton X-100 (v/v) was added at the rate of 10 μl buffer per milligram of maize powder. For western analysis, 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] was added at the rate of 10 μl buffer per milligram of maize powder. The powder was soaked for 1 h on a vortex shaker at room temperature and centrifuged at maximum speed on a bench top centrifuge for 15 min to remove insoluble debris. Protein concentrations were determined as total soluble protein (TSP) 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).

To identify transgenic R_0 events expressing LT-B, individual kernels from each ear (two ears per event) were analyzed by a partial destruction method, where kernels were partially drilled to remove part of the endosperm without damaging the embryo as described by Sangtong et al. (2001). Protein was extracted from endosperm as described for whole kernels. Kernels expressing LT-B were planted to give the R_1 generation.

Quantitation of LT-B expression in maize

LT-B expression in maize was determined using ganglioside dependent enzyme linked immunosorbent assay (ELISA). Reagents and antisera for the ELISA were obtained from Biogenesis Inc. (Kingston, NH, USA) and KPL (Gaithersburg, MD, USA). Volumes of 50 μl were used throughout all ELISA assays unless otherwise specified. Wells were washed three times between each step using 300 μl of phosphate buffered saline Tween-20 [PBST; 0.01M Na_2HP0_4, 0.003M KH_2PO_4, 0.1 M NaCl, (pH 7.2), 0.05% Tween-20 (v/v)]. ELISA was carried out at 37°C throughout the process unless indicated otherwise.

Microtiter plates (Costar 3590, Fisher Scientific, PA, USA) were coated with Type III G_{M1} gangliosides (1.5 μg/well) from bovine brain (G2375, Sigma, St Louis, MO, USA) in sodium carbonate coating buffer [15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3 (pH 9.6)] for 1 h. The plates were blocked to prevent non-specific antibody binding with 5% dry milk (w/v, DM, DIFCO, Becton Dickinson, MD, USA) for 1 h at room temperature. The protein extracts were added to the ganglioside coated microtiter dish, incubated for 1 h, followed by incubation with goat anti-LT-B antiserum (1:1500 dilution, Biogenesis) for 1 h. Rabbit anti-goat alkaline phosphatase conjugate [1:2500 dilution in 1% DM (w/v) in PBS] was added, and the dish incubated for 1 h. This was followed by addition of phosphatase substrate, 1 mg/ml ρ-nitrophenyl phosphate (Sigma) in alkaline phosphatase buffer (pH 9.5). Absorbance at 405 nm was measured spectrophotometrically over a 2 h period in a Dynatech MRX Plate Reader (Dynex Technologies, VA, USA). Sample wells were blanked against non-transgenic maize protein extracts and all measurements were performed in duplicate. Raw ELISA data were converted to nanogram/milliliter of LT-B of total soluble protein by reference to an ELISA standard curve constructed using purified bacterial LT-B (kindly provided by John Clements, Tulane University, LA, USA).

Immunoblot analysis

A 10–20 μl aliquot containing 50–100 μg of total protein from maize kernels was analyzed by SDS-PAGE (Laemmli, 1970). Protein samples were either boiled for 5 min at 95°C or loaded directly on the gel without heat treatment. The separated proteins were transferred to a 0.45 μm nitrocellulose membrane using the BioRad Transblot apparatus according to the manufacturer's instructions. Proteins were evaluated for presence of monomeric or oligomeric LT-B in boiled and non-boiled samples, respectively, by western blot analysis. The analysis used goat anti-LT-B antiserum and an alkaline phosphatase conjugated anti-goat IgG (KPL) at 1:3000 and 1:10,000 dilutions, respectively, using the procedures of Blake et al. (1984).

Preparation of feeding pellets

Kernels harvested from individual ears of transgenic R_1 plants were milled separately in a coffee grinder (Braun, Type 4041: Model KSM2). The LT-B content of each batch of maize powder was determined by ELISA and results are shown in Table 1. Maize powder from self-pollinated transgenic event P77-2 was selected for feeding mice. The LT-B concentrations in milled maize powder ranged between 10 M to 13 μg/g. Non-transgenic maize kernels were added to adjust the concentration of LT-B to 10 μg/g. To prepare the pellets for feeding mice, a fixed amount of

maize meal was weighed out and sterile distilled water added to make firm dough. The dough was then molded into a cylinder (Figure 5) using a 3 ml disposable syringe barrel with the Leur tip cut off. The cylindrical dough was cut into blocks (2 cm in length) that weighed 0.9–1.2 g each when dried. The pellets were allowed to air-dry overnight and then weighed again prior to feeding to ensure that they weighed as close to 1 g as possible. One extra pellet was prepared for each feeding, to be assayed for LT-B content at the end of the experiment. Non-transgenic maize meal was treated in a similar manner to make negative control pellets. To make the positive control, the non-transgenic maize powder was weighed out and mixed with bacterial LT-B in sterile water. Each dried pellet weighed 1 g and contained 10 µg of the bacterial LT-B protein.

Mice feeding

Five-week-old female inbred BALB/c mice were obtained from Harlan (Indianapolis, IN, USA) and were allowed a 2-week adjustment period with reverse light dark cycle prior to onset of experiment. The mice were kept in the Iowa State University animal facility. All animal procedures were approved by the Iowa State University Laboratory Animal Resources Committee on Animal Care prior to experimentation.

Mice were fasted 12 h prior to feeding with water *ad libitum*. Three feed categories were used for this experiment, non-transgenic B73 maize pellets, non-transgenic maize spiked with 10 µg/g bacterial LT-B and LT-B expressing transgenic maize pellets. These feed types are referred to in the figures as Negative, Spiked, and Transgenic, respectively. Twelve mice were used per feed category. Two mice were placed in each cage and two pieces of the appropriate maize pellets were placed in a 15 × 60 mm dish on either end of the cage just before lights out. Bedding in the feeding cages was removed to ensure that all pellets were completely consumed during feeding. The dishes were then removed 3 h later and the mice returned to their main cages with their normal rodent food. At the end of the 3 h period the mice had consumed all the maize pellets supplied. Mice were fed on days 0, 3, 7, and 21.

Serum and fecal sample preparation

For anti-LT-B and anti-CT-B antibody quantity determination, fecal pellets were collected and blood (approximately 100 µl) obtained once a week prior to fasting and feeding and once before euthanasia (days -1, 6, 13, 20, and 27). Mice were not bled prior to feeding at day 3. Mice were bled sephanously. Immediately after collection, the blood sample from each animal was centrifuged at 17,000 × *g* in a microcentrifuge for 10 min to separate serum from clotted blood, and the sera collected and stored at −20°C until assayed. For ELISA evaluation of anti-LT or CT antibodies, sera collected on day 27 were diluted in PBS at 1:50 ratio for IgG assays and 1:20 ratio for IgA assays. Lower dilutions (1:10 to 1:25 for IgG and 1;10 to 1:20 for IgA) were used for sera from earlier bleeds with lower antibody titers. Fecal pellets were stored at −80°C until assayed. Fecal pellets were removed from −80°C and lyophilized for 36–48 h in open eppendorf tubes set upright in a Virtix Freezemobile 12LX lyophilizer. Dry fecal pellets were weighed, and PBS buffer supplemented with 0.2 mg/ml (w/v) trypsin inhibitor, 12.5% sodium azide (w/v), and 1 mM PMSF was added at 10 µl for every milligram of dry fecal matter. Samples were soaked in buffer overnight at 4°C, and then centrifuged at 17,000 × g on the micro centrifuge for 10 min and the supernatant removed for ELISA analysis as described below.

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

Reagents and antisera for the ELISA were obtained from Sigma. Antisera for ELISA were diluted in PBS buffer as described above. For anti-LT-B or anti-CT-B determinations, microtiter plates were pre-coated with 1.5 µg of mixed gangliosides (Type III form Bovine, Sigma G2375) per well diluted in sodium carbonate coating buffer (see above) for 1 h. This was followed by incubating with 1 µg/well purified LT-B (John Clements) or CT-B (Sigma C9903) at 37°C for 1 h. Plates were blocked with 5% DM (w/v) in PBS for 1 h at room temperature. Diluted serum samples were added and the dishes incubated at 37°C for 1 h. Anti-LT-B or anti-CT-B IgG levels in serum were determined by incubating with rabbit anti-serum against mouse IgG conjugated to alkaline phosphatase (Sigma A2418, diluted 1:7000 in 1% dry milk) at 37°C for 1 h. IgA antibodies were similarly determined in parallel using rabbit anti- serum against mouse IgA conjugated to alkaline phosphatase (Sigma A4937, also diluted 1:7000 in 1% dry milk). Plates were read over a 2-h period as described above. Extracted fecal samples were measured without further dilution. Values for IgG and IgA were determined from a standard curve with purified mouse myeloma proteins [MOPC 21γG1, MOPC 315 γA(IgAλ2)] obtained from Sigma.

Patent mouse assay

The patent mouse assay (Guidry et al., 1997) was used to determine protection from toxin challenge. It is a modification of the sealed adult mouse assay (Richardson et al., 1984). The mice were challenged with LT (John Clements) and CT (Sigma C3012) toxins. The mice were fasted for 12 h prior to the challenge with water available *ad libitum* and orally gavaged with 200 μl of PBS (pH 7.2) containing (25 or 50 μg) LT or CT toxins. After toxin administration (3 h), the mice were euthanized by carbon dioxide inhalation, dissected, and the gut removed from the duodenum to the anus. The gut, with the fat pad and the mesentery left intact, and the remaining body were weighed separately. The gut/carcass ratio (Richardson et al., 1984) was then calculated to determine the extent of toxin induced water influx into the gut.

Statistical analysis

Data was analyzed using the package Statistix for Windows, Version 4.0 (Analytical Software, Tallahassee, FL) and the Students *t*-test. The results are reported as mean values ± standard deviation (SD).

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