Molecular Breeding **10:** 253–265, 2002.

Expression of a synthetic *E. coli* heat-labile enterotoxin B sub-unit (LT-B) in maize

Rachel Chikwamba[1,2], Jennifer McMurray[1], Huixia Shou[1], Bronwyn Frame[1], Sue Ellen Pegg[1,5], Paul Scott[4], Hugh Mason[3] and 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]Boyce Thompson Institute for Plant Research, Tower Road, Ithaca, NY 14853–1801, USA; [4]USDA-ARS, Department of Agronomy, Iowa State University, Ames, IA 50011–1010, USA; [5]Current address: Western Illinois University, Macomb, IL 61455, USA; *Author for correspondence (e-mail: kanwang@iastate.edu; fax: 515–294-2299)*

Received 7 March 2002; accepted in revised form 28 June 2002

Key words: Functional antigen, Gene expression, LT-B, *Zea mays*

Abstract

We have produced the B subunit of the enterotoxigenic *Escherichia coli* (ETEC) heat-labile enterotoxin (LT-B) in transgenic maize seed. LT-B is a model antigen that induces a strong immune response upon oral administration and enhances immune responses to conjugated and co-administered antigens. Using a synthetic LT-B gene with optimized codon sequence, we examined the role of promoters and the SEKDEL endoplasmic reticulum retention motif in LT-B accumulation in callus and in kernels. Two promoters, the constitutive CaMV 35S promoter and the maize 27 kDa gamma zein promoter, which directs endosperm-specific gene expression in maize kernels, regulated LT-B expression. Ganglioside-dependent ELISA analysis showed that using the constitutive promoter, maximum LT-B level detected in callus was 0.04% LT-B in total aqueous-extractable protein (TAEP) and 0.01% in R_1 kernels of transgenic plants. Using the gamma zein promoter, LT-B accumulation reached 0.07% in R_1 kernels. The SEKDEL resulted in increased LT-B levels when combined with the gamma zein promoter. We monitored LT-B levels under greenhouse and field conditions over three generations. Significant variability in gene expression was observed between transgenic events, and between plants within the same event. A maximum of 0.3% LT-B in TAEP was measured in R_3 seed of a transgenic line carrying CaMV 35S promoter/LT-B construct. In R_3 seed of a transgenic line carrying the gamma zein promoter/LT-B construct, up to 3.7% LT-B in TAEP could be detected. We concluded that maize seed can be used as a production system for functional antigens.

Abbreviations: BSA – bovine serum albumin, CaMV 35S – Cauliflower Mosaic Virus 35S RNA Promoter, CTAB – cetyltrimethylammonium bromide, EDTA – ethylene-diamine tetraacetic acid, ELISA – enzyme-linked immunosorbent assay, G_{M1} – galactosyl-N-acetylgalactosamyl-sialyl-galactosylglucosyl ceramide, LT-B – heat-labile toxin from *E. coli*, PBS – phosphate-buffered saline, PMSF – phenylmethanesulfonyl fluoride, SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis, Tris – Tris hydroxymethyl aminomethane

Introduction

Recombinant DNA technology and advances in plant transformation have expanded the utility of plants beyond the traditional uses of food, feed and fiber. Plants are now used as a source of proteins for industrial, pharmaceutical, and other purposes (Krebbers et al. 1993; Pen et al. 1993; Austin et al. 1994). Production of recombinant proteins in transgenic plants is emerging as a competitive and safe alternative to the traditional protein expression systems. This is particularly relevant for the production of monoclonal anti-

bodies and antigens for use as vaccines in humans and animals. A major advantage of producing antigens for this purpose in a crop like maize is that plant-derived antigens are free from human and animal pathogens normally associated with vaccines produced in conventional mammalian cell culture systems. To use plants as an efficient system for antigen production, a number of issues need to be considered. Transgenic lines should have high expression levels of the gene of interest in appropriate tissues. In addition, the recombinant proteins produced *in planta* should have structural integrity and be functionally active. Moreover, transgenic plants should be fertile and transmit and express the transgene predictably over generations. Novel protein production in plants has been reported in potato, tobacco, and Arabidopsis (reviewed by Daniell et al. (2001)). Advances in maize transformation (reviewed by Armstrong (1999) and Frame et al. (2000)) have allowed us to investigate the possibility of using this crop as a source of antigens to be used as oral vaccines.

In this study we report the production of a functional *Escherichia coli* (*E. coli*) heat labile enterotoxin B sub-unit (LT-B) in maize. LT-B is part of the heat-labile toxin (LT) produced by enterotoxigenic strains of *E. coli*, a leading cause of diarrhea in developing countries (Spangler 1992). The bacterium is ingested in contaminated food or water and colonizes the gut, where it secretes toxins, including LT. LT is an 84-kilodalton (kDa) oligomeric protein composed of two major noncovalently associated immunologically distinct peptides designated LT-A and LT-B. The A peptide (27 kDa) is responsible for the diverse biological effects of the toxin. LT-B is a 55 kDa homopentamer of 11.6 kDa peptides responsible for binding of the toxin to the host cell receptor G_{M1} (galactosyl-N-acetylgalactosaminyl-sialyl galactosyl glucosyl ceramide), which is found on the surface of eukaryotic cells.

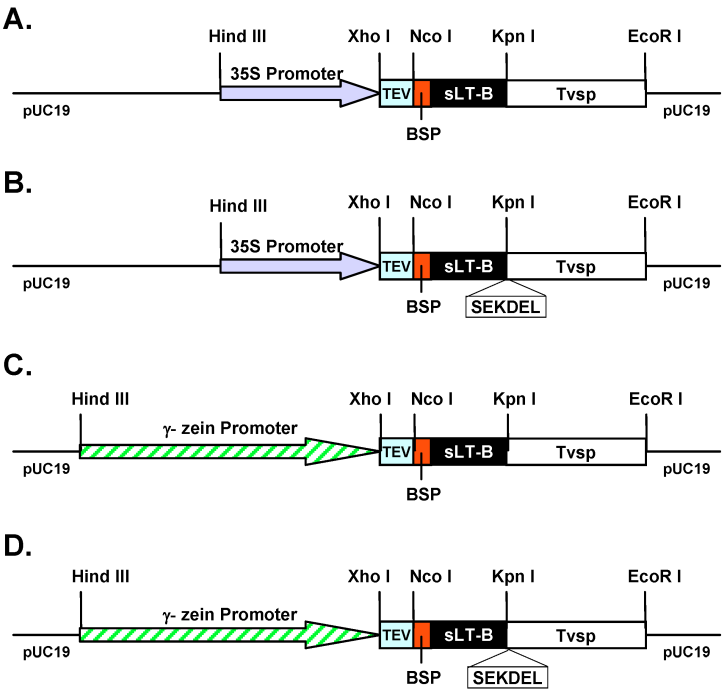
LT-B is strongly immunogenic, stimulating mucosal and systemic immune responses, and could be used as a vaccine against *E. coli* induced diarrhea (Dickinson and Clements 1996). In addition, LT-B could be used as an adjuvant when applied intranasally, stimulating immune responses against co-administered antigens (Millar et al. 2001). Adjuvants are substances that, either alone or linked to protein antigens, can stimulate mucosal responses and therefore significantly enhance the potency of vaccines in general. Moreover, LT-B can serve as an immunologic carrier in the traditional hapten-carrier configuration as well (Spangler 1992). Other antigens conjugated to LT-B can be immobilized for processing by the gut-associated lymphoid tissue (GALT) because of LT-B's affinity to G_{M1} gangliosides on the surface of the intestinal mucosa.

LT-B produced in potato and maize has been shown to be immunogenic in mice (Mason et al. 1998; Streatfield et al. 2001; Chikwamba et al. 2002 (in press)) and humans (Tacket et al. 1998). This protein is an ideal model for an oral vaccine, and its adjuvant properties make it attractive for use in co-expression or co-administration with other plant-synthesized or conventional vaccines. We have generated transgenic maize plants expressing LT-B in kernels using several constructs. We investigated the role of promoters and the SEKDEL amino acid motif, an endoplasmic reticulum-retention signal, in the accumulation of LT-B in transgenic maize. A high level of expression is important for meeting dosage requirements for plant-based biologics. We also examined the expression of LT-B over three generations in the greenhouse and the field. Our data indicate that maize seed, a key ingredient in livestock feed, can be used to produce functional antigens for use as feed-based vaccines.

Materials and methods

Constructs

A plant codon-optimized LT-B subunit gene (Mason et al. 1998) was cloned into four different constructs (Figure 1). Construct pTH210 (Mason et al. 1998), (Figure 1A), has the LT-B gene regulated by the CaMV 35S promoter, the tobacco etch virus (TEV) leader as a translational enhancer (Gallie et al. 1995), and the soybean vegetative storage protein (VSP) terminator (Rhee and Staswick 1992) in the 3′terminus. Transgenic maize calli and plants carrying this construct were designated P51. Construct pTHK210 (Mason, unpublished, Figure 1B) is identical to pTH210, except a SEKDEL (Ser-Glu-Lys-Asp-Glu-Leu) coding sequence is included in the C-terminus of the LT-B gene. Plants carrying this construct were designated P65. In construct pRC4 (Figure 1C), the CaMV 35S promoter was replaced by the maize 27 kDa gamma zein promoter (Marks et al. 1985). To construct pRC4, a 1.15 kb *Xho* I-*Eco*R I fragment including the TEV 5′, the synthetic LT-B coding sequence and the VSP terminator from pTH210 was cloned



Construct	Transgenic maize event ID	LT-B expression in
pTH210	P51	Callus and whole plant
pTHK210	P65	Callus and whole plant
pRC4	P77	Seed
pRC4-1	P112	Seed

Figure 1. Schematic diagram of constructs used for maize transformation to generate LT-B-expressing transgenic plants. All constructs contain the synthetic gene encoding the B subunit of LT in pUC19 vector background. 35S Promoter, CaMV 35S promoter; γ-zein promoter, 27 kDa gamma zein promoter from maize; TEV, tobacco etch virus translational enhancer leader sequence; sLT-B, the synthetic LT-B gene; Tvsp, soybean vegetative storage terminator; SEKDEL, endoplasmic reticulum-retention sequence motif; BSP, bacterial signal peptide from LT.

downstream of the gamma zein promoter in the vector pUC19. Plants carrying this construct were designated P77. Construct pRC4-1 (Figure 1D) is identical to pRC4 except it carried the LT-B gene with the SEKDEL motif in its C-terminus. Plants with this construct were designated P112. This construct was made by subcloning the 1.15 kb *Xho* I and *Eco*R I fragment from plasmid pTHK210 downstream of the maize 27 kDa gamma zein promoter in a pUC19 vector. In all cloning work, the resultant plasmids were 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 manufacturer's instructions.

Maize transformation

Maize transformation was conducted by the Iowa State University Plant Transformation Facility using the procedure described by Frame et al. (2000). Briefly, embryogenic calli generated from immature embryos of the Hi-II genotype were transformed using microprojectile bombardment. The constructs carrying the LT-B gene were co-bombarded with pBAR184 (Frame et al. 2000), a plasmid that carries the maize ubiquitin promoter-*bar* gene cassette as a selectable marker conferring resistance to the herbicide bialaphos. The bialaphos-resistant calli were analyzed by polymerase chain reaction (PCR), Southern blot, Northern blot and enzyme linked immunosorbent assay (ELISA) analyses as described below. Calli of transgenic events were regenerated and brought to maturity in the greenhouse. R_1 seed were obtained by out-crossing, with the transformants as female parents and untransformed inbred lines B73 or Hi-II as male parents.

PCR analysis

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 LT-B gene was amplified from 10 to 100 ng of total genomic DNA extracted from 0.1–0.25 g of callus as described by Chikwamba et al. (2002) (in press). Callus DNA was extracted in 400 μl extraction buffer [200 mM Tris-Cl (pH 7.5), 250 mM NaCl, 25 mM EDTA, and 0.5% SDS (w/v)], followed by extraction in an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, V:V:V). PCR primers and conditions, used as described (Chikwamba et al. 2002 (in press)), were the same in all cases: LTB-PCRF1 (5′-atc gat aca aaa caa acg aat ctc aag c-3′) and LTB-PCRR1 (5′-cca tgg cgt gga ttt tat gac att tta t-3′). These 28 bp primers amplify an 842 bp fragment from the TEV leader at the 5′ end to part of the VSP terminator at the 3′ end, including the LT-B-coding sequence.

Southern blot analysis

Southern blot analysis was undertaken on leaf tissue of young putative transformants or R_2 plants to confirm transformation and to estimate the copy number of the LT-B gene. Genomic DNA was extracted from leaves of maize plants using a cetyltrimethylammonium bromide (CTAB) protocol (Murray and Thompson 1980). Ten micrograms of leaf genomic DNA was digested with restriction enzyme *Hin*d III for P51 and P65, and *Nco* I for P77 and P112, at 37 °C overnight and separated on a 0.8% agarose gel. Linearized DNA gel blot analysis (Sambrook et al. 1989) was conducted on DNA samples with a ^{32}P-labeled LT-B gene fragment excised from pTH210 using the enzymes *Nco* I and *Kpn* I as probe (Figure 1).

Northern blot analysis

Northern blot analysis was conducted using the leaf tissue of selected transgenic plants. Total RNA was extracted from leaf tissue of R_0 and R_1 plants using Trizol reagent (Gibco Life Sciences, Rockville, MD, USA) according to the manufacturer's directions. Twenty micrograms of total RNA was separated on a 1% agarose gel in a denaturing gel buffer under RNAse-free conditions and then blotted onto a nitrocellulose membrane. The ^{32}P-labled LT-B gene fragment was also used as a probe in the hybridization procedure as described for Southern blot analysis.

Evaluation of protein production

Evaluation of protein expression was carried out in callus and maize kernels. About 0.25 g of fresh callus was homogenized in 500 μl of protein extraction buffer [25 mM Sodium phosphate (pH 6.6), 100 mM NaCl, 0.5% Triton X-100 (v/v), 10 μg ml^{-1} leupeptin (w/v)]. Two methods were used for processing dry maize kernels, one manual and the other partially mechanized. In the manual procedure, individual kernels were partially crushed with a mortar and pestle, frozen in liquid nitrogen, and then ground to a fine powder. In the mechanized method, individual kernels and a single ball bearing (3/8 inch, Dynasteel) were placed in a polycarbonate tube (1/2 × 2 inches, Spex CertiPrep, Metuchen, NJ, USA). The tubes were shaken in a Spex CertiPrep GenoGrinder for 5–10 minutes at 1400 rpm, until the kernels were reduced to a fine powder.

Protein extraction buffer was added at a ratio of 10 μl per mg of dry, finely ground kernel tissue in an Eppendorf tube; 30 to 50 μg of tissue was used per sample. The samples were placed on a vortex shaker for 30 to 60 minutes for complete resuspension and allowed to sit on the bench for another hour, after which they were centrifuged at 14,000 rpm in a microcentrifuge for 15 minutes at room temperature. One hundred microliters of the supernatant (50 μl per well, 2 replications/sample) was used for ganglioside-dependent ELISA analysis as previously described (Mason et al. 1998). Total soluble protein concentrations 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).

To identify kernels expressing LT-B for planting for the next generation, individual kernels from each ear (two ears per event) were analyzed by a partial destruction method (Sangtong et al. 2001). With the use of a fine drill and without damaging the embryo, part of the endosperm material from each kernel was removed for ELISA analysis. Protein was extracted from the endosperm material as described for whole kernels. Kernels expressing LT-B were planted to give rise to the subsequent generation.

Quantitation of LT-B expression in maize

LT-B expression in maize was determined using ganglioside-dependent ELISA (Mason et al. 1998; Chikwamba et al. 2002 (in press)). 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; 0.01 M Na_2HPO_4, 0.003 M KH_2PO_4, (pH 7.2), 0.1 M NaCl, 0.05% Tween-20 (v/v)]. Each step of the ELISA was carried out at 37 °C throughout the process unless otherwise specified. Briefly, LT-B from plant extracts was captured in microtiter plates (Costar 3590, Fisher Scientific, PA, USA) coated with Type III G_{M1} gangliosides (1.5 μg/well) from bovine brain (G2375, Sigma, St Louis, MO, USA), dissolved in sodium carbonate coating buffer [15 mM Na_2CO_3, 35 mM $NaHCO_3$ (pH 9.6), 3 mM NaN_3] for 1 hour at room temperature. The plates were blocked to prevent nonspecific antibody binding with 5% dry milk (w/v, DIFCO, Becton Dickinson, MD, USA) in PBS for one hour at room temperature. The LT-B in crude extracts was captured for one hour at 37 °C or at 4 °C overnight, followed by incubation with goat anti-LT-B antiserum (1:1500 dilution, Biogenesis, Kingston, NH, USA) at 37 °C for one hour. Rabbit anti-goat alkaline phosphatase conjugate KPL [1:2500 dilution in 1% dry milk (w/v) in PBS] was added, and the plate incubated for one hour. This was followed by addition of phosphatase substrate, which consisted of 1 mg ml^{-1} ρ-nitrophenyl phosphate (ρNPP, Sigma) in alkaline phosphatase buffer [0.1 M Tris (pH 9.5), 0.05 M $MgCl_2$, 0.1 M NaCl]. Absorbance at 405 nm was immediately measured spectrophotometrically over a two-hour period in a Dynatech MRX Plate Reader (Dynex Technologies, VA, USA). Sample wells were blanked against nontransgenic maize protein extracts, and all measurements were performed in duplicate. Raw ELISA data were converted to percentage LT-B of total aqueous extractable protein by reference to an ELISA standard curve constructed using purified bacterial LT-B (kindly provided by John Clements, Tulane University, New Orleans, LA, USA).

Screening of transgenic plants in field

To identify transgene-expressing plants in the field, the segregating R_2 plants were sprayed with 200 mg l^{-1} Liberty® (w/v) with 0.05% Tween-20 (v/v) two weeks post-emergence. The gene expressing plants were either self-pollinated or crossed with their siblings. The segregation ratio of herbicide resistance was approximately 3:1 (resistant: susceptible), as expected, and the herbicide-resistant plants were tagged for identification. Both tassels and ears were bagged, and because plants were both male- and female-fertile, they were, in most cases, self-pollinated to produce R_3 seed.

Data analysis

To determine the expression level for each ear, twenty kernels per ear were analyzed for LT-B expression, and an average expression level was obtained from only the LT-B positive kernels. The data is presented as the mean ± standard deviation. For P51 and P77 R_3 kernels, ELISA analyses were carried out on eight ears, twenty whole kernels per ear as described. For each ear, values of 10 randomly selected kernels were selected from LT-B positive kernels and used to determine the mean expression per ear. Analysis of variance was carried out using the statistical analysis package SAS (The SAS Institute Inc., Cary, NC, USA).

Results

Generation of LT-B-producing transgenic maize plants

The constructs used for maize transformation are shown in Figure 1. The LT-B gene in all constructs was synthesized with a codon bias for expression in potato and maize (Mason et al. 1998). In constructs pTH210 and pTHK210, the CaMV 35S promoter, the TEV enhancer, and the VSP terminator regulated the LT-B gene. In construct pTHK210, a DNA sequence encoding the SEKDEL amino acid motif, which binds to the SEKDEL receptor in the endoplasmic reticulum (Munro and Pelham 1987), was included in the C-terminus of the LT-B coding sequence. This motif has been shown to enhance the expression of a non-optimized LT-B gene in transgenic potato (Haq et al. 1995). Transgenic maize events carrying these two constructs were designated P51 and P65, respectively. In constructs pRC4 and pRC4-1, the maize 27 kDa gamma zein promoter replaced the CaMV 35S promoter in pTH210 and pTHK210, respectively. Construct pRC4-1 has the SEKDEL motif in the C-terminus of LT-B. Transgenic events carrying these two constructs were designated P77 and P112, respectively (Figure 1).

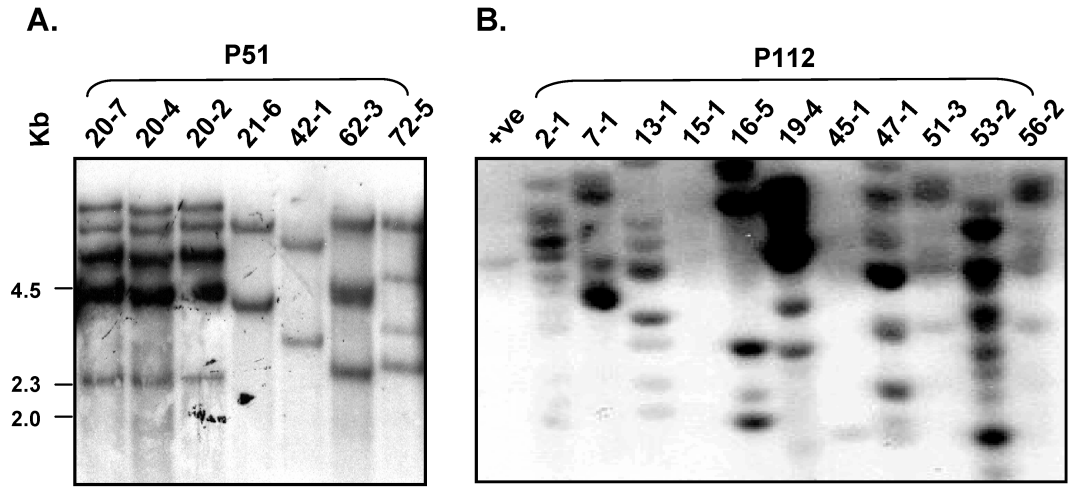


Figure 2. Southern blot analysis of total leaf genomic DNA of P51 (A) and P112 (B) events digested with *Hin*d III and *Nco* I respectively and probed with the LT-B gene.

PCR analyses using primers for the LT-B gene were carried out on herbicide-resistant calli. The co-transformation rate for the selectable marker gene *bar* and the gene of interest LT-B in all four constructs ranged from 57.5 % to 88.9 %, with an average of 77%. Southern blot analysis was carried out on genomic DNA isolated from leaf tissue of putative transformants. Results of representative events from construct pTH210 (P51) and pRC4-1 (P112) are shown in Figure 2A and 2B, respectively. Genomic DNA was digested with restriction enzymes that cut once within the construct; *Hin*d III was used for P51 (Figure 2A) and P65 (data not shown). *Eco*R I and *Nco* I were used for P77 (Chikwamba et al. 2002 (in press)) and P112 (Figure 2B), respectively. Results showed integration of the LT-B gene cassette into the maize genomic DNA. Each event had a unique insertion pattern, and plants within the same event had similar transgene insertion patterns as expected (P51-20, Figure 2A). Most of the events were shown to contain multiple copies of the transgene. Estimated copy number ranged from two to more than ten, as is typical of plants transformed via microprojectile bombardment (Christou 1996).

Figure 3 shows the Northern blot analysis results on representative events of P51. A transcript with ~700 nucleotides hybridized with the LT-B probe and could be detected in leaf RNA of transgenic plants that had been regenerated on the basis of herbicide resistance, PCR analysis, and callus expression of LT-B (Table 1). Different levels of RNA expression were observed, and P51-23 had no detectable LT-B

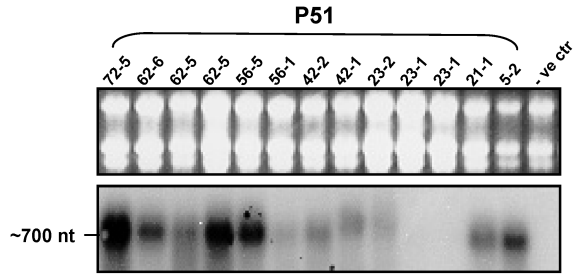


Figure 3. Northern blot analysis of total leaf RNA of P51 probed with a ^{32}P-labeled LT-B gene fragment RNA from non-transgenic B73 plant used as negative control.

RNA, despite having been shown to contain multiple copies of the transgene (data not shown). These results showed the LT-B gene was being transcribed in maize leaf tissue in transgenic plants carrying pTH210.

Constitutive expression of LT-B

Table 1 summarizes the level of LT-B gene expression in callus under the constitutive promoter CaMV 35S in maize. LT-B expression was quantified using the ganglioside-dependent ELISA procedure (Haq et al. 1995; Chikwamba et al. 2002 (in press)). Because ganglioside binds only multimeric LT-B protein, these positive ELISA results indicate that the protein was being properly assembled in maize tissue. The pentameric nature of maize generated LT-B was also confirmed by western analysis (Chikwamba et al. 2002 (in press)).

Table 1. Level of assembled LT-B protein (% LT-B in total aqueous-extractable protein) in transgenic maize callus.

Transgenic plant ID	Total # events tested	# Events expressing LT-B at various levels*		
		< 0.005	0.005–0.01	0.01–0.04
P51	54	42	9	3
P65	51	33	13	5

*Level of assembled LT-B protein in total aqueous extractable protein was determined by ganglioside-dependent ELISA.

Forty-two (77.7%) of the 54 independent P51 transgenic events were shown to express LT-B at levels lower than 0.005% of total aqueous-extractable protein (TAEP). Nine events (16.7%) had LT-B protein levels between 0.005 and 0.01% in TAEP. Only three (5.6%) of the callus lines had LT-B protein level above 0.01% in TAEP. A similar range of LT-B expression was observed for P65 transgenic events. Of 51 events tested, five callus events (9.8%) had LT-B levels higher than 0.01% of TAEP. The majority of events (90.2%) had LT-B levels less than 0.01% of TAEP. Maximum expression for both constructs (Events P51-62 and P65-69) was 0.04% LT-B of TAEP. While the number of events with LT-B expression above 0.01% of TAEP increased, the SEKDEL microsomal retention signal included in the construct pTHK210 (P65) did not substantially improve the level of the LT-B protein accumulation in maize callus compared with P51 events (Table 1).

The level of LT-B protein in young leaf tissue of P51 and P65 plants was also analyzed. In general, LT-B levels in R_0 transgenic leaf tissue were consistent with levels in corresponding callus tissue, with a few exceptions in which the LT-B level declined in leaf tissue (data not shown). We also observed that plant regeneration, vigor, and seed set were generally poor in both P51 and P65 events that showed high expression of LT-B protein in callus (data not shown).

For determination of the level of LT-B protein in seed, R_1 kernels from each event were homogenized manually or mechanically and assayed individually. Based on the levels of total protein extracted from kernels using these two methods, there were no differences in the protein yield between these two methods. A total of three P51 events and nine P65 events were tested. Poor seed set was a limiting factor for P51 events. Most events tested showed less than 0.005% LT-B in TAEP in whole seed. The maximum level of LT-B (0.01% LT-B in TAEP) was detected in one P51 event (P51-62) and one P65 event (P65-69) (data not shown). When embryo and endosperm tissue from a single kernel were analyzed separately, the LT-B protein was detected primarily but not exclusively in the embryo as expected (data not shown).

We observed that LT-B expression was not always consistent between callus and R_1 kernels. For example, event P51-62 had an LT-B callus expression level of 0.02% in TAEP, but a kernel expression of 0.01%. Event P51-23 had LT-B callus expression of 0.01% in TAEP, but no expression in R_1 kernels. However, event P65-69 had a consistent expression in callus and R_1 kernels of 0.01% LT-B in TAEP (data not shown).

Seed-specific expression of LT-B

Transgenic maize plants carrying the constructs pRC4 and pRC4-1 (Figure 1C and 1D) were also produced. Since the LT-B gene in these constructs was regulated by the 27 kDa gamma zein promoter, a seed-specific promoter, no LT-B expression was detected in callus tissues as expected (data not shown). A total of 19 P77 events and 20 P112 events were generated. For analysis of the LT-B protein levels in R_1 seed, proteins from 20 kernels per ear representing each event were extracted separately and assayed for LT-B expression by ELISA. Data from all LT-B positive kernels in each ear were pooled to determine the mean and standard deviation (SD) for each ear.

Levels of LT-B accumulation in P77 (one ear per event) and P112 (two ears per event) are presented in Figures 4 and 5, respectively, and summarized in Table 2. Of 19 LT-B-expressing P77 events, 11 (58%) had LT-B protein levels higher than 0.01% of TAEP (Figure 4). Two events (P77-2 and P77-3) had LT-B levels of up to 0.07%.

P112 transgenic events showed the highest level of LT-B accumulation in R_1 seeds (Figure 5). P112 events carry the construct pRC4-1, in which the endoplasmic reticulum-retention signal sequence SEKDEL was included in the C-terminus of LT-B gene. All 15 P112 events tested had LT-B levels of 0.01% or higher (Table 2). In addition, six events had LT-B levels higher than 0.1%. One event (Event

Table 2. Level of assembled LT-B protein (% LT-B in total aqueous-extractable protein) in R_1 transgenic seeds.

Transgenic plant ID	Total # events tested	# Events expressing LT-B at various levels*			
		< 0.005	0.005–0.01	0.01–0.1	> 0.1
P77	19	2	6	11	0
P112	15	0	0	9	6

*Level of assembled LT-B protein in total aqueous extractable protein was determined by ganglioside-dependent ELISA.

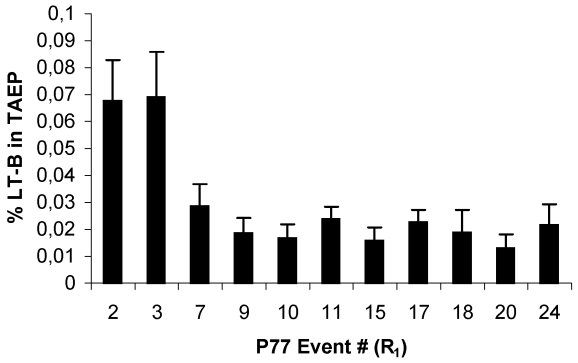


Figure 4. LT-B level [% in total aqueous extractable protein (TAEP)] in R_1 seeds of P77 events. Error bars indicate ± standard deviation.

P112-44) accumulated up to 0.9% of LT-B in TAEP, about 13-fold higher than the highest-expressing P77 R_1 event (P77-3). This is in contrast to the observations for transgenic events P51 and P65, in which the SEKDEL motif did not improve the LT-B level in callus tissue. Inclusion of the SEKDEL motif in the LT-B gene under the regulation of the seed-specific promoter greatly enhanced the LT-B level in P112 events.

Plant performance comparison

Compared to transgenic maize plants carrying CaMV 35S promoter/LT-B constructs (P51 and P65), plants carrying zein promoter/LT-B constructs (P77 and P112) were more vigorous in overall performance. Table 3 summarizes the seed set from transgenic lines P51, P65, P77, and P112. The seed set of P51 was the poorest; only 23% of events set more than 50 kernels per event. For P65, although the level of assembled LT-B protein did not increase, the fertility of transgenic plants was markedly improved over P51 plants. Sixty-four percent of events set more than 50 seeds. Both P77 and P112 had good fertility. Seventy-four percent of P77 events and 55% of P112 events produced more than 50 kernels per event, respectively. The transgene segregated 1:1 or 3:1 as expected in most cases.

Evaluation of LT-B protein levels in R_2 and R_3 seeds

To evaluate stability of LT-B gene expression under control either of a constitutive promoter or a seed-specific promoter over generations, we carried out further analysis using one event of P51 (Event P51-62) and eight events of P77 (Events P77-2, 3, 7, 9, 10, 11, 17, and 18) (Tables 4 and 5).

P51-62 had the highest LT-B accumulation (0.01% TAEP) in R_1 seed. Five plants of this event were grown in the greenhouse, and two were successfully self-pollinated to produce R_2 seeds. The LT-B levels measured in R_2 seeds had a four-fold increase compared with R_1 kernels, with 0.04% LT-B of TAEP (data not shown). P51-62 R_2 seeds were grown in the field in the summer of 2001. Transgene-expressing plants were first identified by resistance to the herbicide bialaphos sprayed 2 weeks post-emergence and subsequent self-pollination to produce R_3 seeds. LT-B expression was tested in eight ears (20 kernels per ear) of event P51-62 (Table 5). Mean expression was obtained from 10 randomly selected LT-B expressing kernels for each ear. The levels of LT-B varied greatly between ears derived from the same event. These values ranged from 0.03% (ear #8) to 0.28% (ear #1), with an average of 0.17% in TAEP. Within the same ears, large standard deviation values indicate differences in expression from kernels on the same ear. The LT-B level in R_3 ear #1 (0.28%) represented a further seven-fold increase when compared with the level in the R_2 seed (0.04%).

Eight P77 R_1 events with LT-B levels ranging from 0.017% (Event P77-10) to 0.069% (Event P77-3) were followed to the R_2 generation (Table 4). Two to three R_1 plants representing each event were self-pollinated to give rise to R_2 seeds. Table 4 summarizes the LT-B levels in R_1 seeds (one ear per event) and corresponding R_2 seeds (one to two ears per

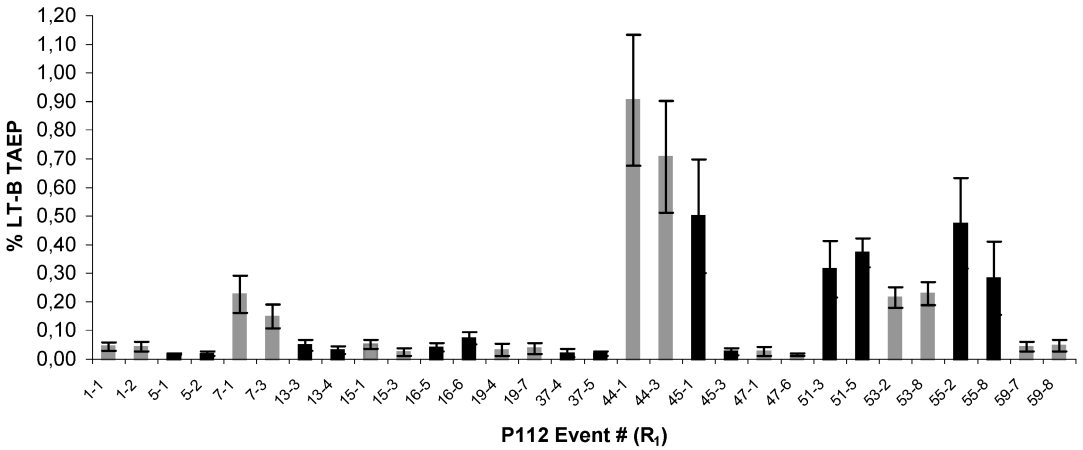


Figure 5. LT-B level [% in total aqueous extractable protein (TAEP)] in R_1 seeds of P112 events. Data from ears in the same event are shaded similarly. Error bars indicate ± standard deviation.

Table 3. Summary of seed set from transgenic maize carrying four different constructs.

Transgenic plant ID	Total # events tested	# events with seed set*				% events set seed > 50 kernels
		0	< 50	50–100	101–200	
P51	13	5	5	1	2	23
P65	14	0	5	7	2	64
P77	19	0	5	5	9	74
P112	20	1	8	8	3	55

*Seed set: number of events with range of seed harvested per ear.

event). The LT-B levels in the R_2 seeds were greatly increased for all events (up to 100-fold in Event P77-9), compared with the LT-B levels in R_1 seeds. While Event P77-7 had a moderate LT-B level in R_1 seed (0.029%, Table 4), it reached over 2% of TAEP in two ears tested in R_2 generation. The R_2 plants of this event were grown in the field in the summer of 2001 for R_3 seeds. Results of LT-B levels from eight R_3 ears representing event P77-7 are presented in Table 5. Significant differences ($p > 0.0001$), were observed between ears descending from the same R_2 plant, and like P51 R_3 kernels, large standard deviation values indicate differences in expression from kernels on the same ear. The highest expressing ear (#1) reached 3.7%. Based on mass of the powder used and the volume of buffer added, the LT-B level in the ear could be expressed as 350 μg g^{-1} or 35 kg ton^{-1} LT-B of dry ground kernel.

Discussion

We describe the generation and characterization of LT-B expressing maize plants transformed with four different constructs. Several factors can be manipulated to control the level of expression of foreign genes in transgenic plants. These include foreign gene sequence optimization, promoters, terminators, and targeting and retention sequences. We examined the role of two of these factors, promoters and the microsomal retention signal SEKDEL, in expression of the synthetic LT-B gene and accumulation of the functional product in callus and seed.

The codon bias of a transgene is important in enhanced expression of a foreign protein in plants (Gribskov et al. 1984). Mason et al. (1998) synthesized the LT-B gene used in this study with a codon bias optimized for expression in potato and maize and demonstrated that the synthetic gene could be expressed in potato. The construct pTH210 (P51), with the constitutive CaMV 35S promoter, was used to determine if the codon usage in the synthetic LT-B gene had been adequately optimized for expression in

Table 4. Levels of assembled LT-B protein (% LT-B in total aqueous-extractable protein) in R_1 and R_2 seeds of P77.

R_1 seed			R_2 seed			Fold increase (R_2/R_1)
Event ID	Mean	SD	Ear ID	Mean	SD	
2	0.068	0.015	2–1	1.155	0.342	18.5
			2–2	1.356	0.373	
3	0.069	0.017	3–1	1.319	0.627	9.1
7	0.029	0.008	7–1	2.071	0.816	84.5
			7–2	2.761	0.817	
9	0.019	0.006	9–1	1.634	0.358	104.7
			9–2	2.245	1.077	
10	0.017	0.005	10–1	0.324	0.141	19.3
			10–2	0.327	0.140	
11	0.024	0.005	11–1	0.463	0.246	14.3
			11–2	0.211	0.100	
17	0.023	0.005	17–1	0.127	0.057	5.6
18	0.019	0.008	18–1	0.343	0.222	16.9
			18–2	0.292	0.117	

maize. Expression of LT-B in callus of P51 and P65 events indicated that the synthetic LT-B gene was adequately optimized for expression in maize. LT-B levels in both P51 and P65 plants varied considerably between events, and did not exceed 0.04% of total aqueous extractable protein.

Results of Southern blot analysis show that most transgenic events had multiple copies of the transgene cassette, ranging from two to more than 10 copies in most cases. While we could not conclusively associate the level of LT-B expression with transgene copy number within individual constructs, high LT-B expression was associated with relatively low gene copy number. Event P51-62 was estimated to have about three copies of the gene, event P77-7 had about five copies and event P112-51 had about three copies. All these events had substantially higher LT-B expression relative to other events transformed with the same construct. Northern analysis was undertaken for the P51 events to determine the size of the transcription unit. An $\sim$700-nucleotide transcript was observed, a size consistent with a transcript containing part of the TEV leader, the LT-B gene, and part of the terminator sequence. In most cases, plants that were confirmed to be transgenic by Southern blotting were also shown to express the 700-nucleotide gene transcript in Northern blot analysis and to express the protein in ELISA analysis.

We observed that transgenic callus events with high levels of LT-B protein in P51 but not P65 were associated with poor plant growth and fertility. Reduced performance in plant growth, vigor, and seed set in P51 plants relative to plants carrying other constructs could be attributed to several possible causes. Poor plant performance in some events could have been induced by *in vitro* tissue culture stress. Alternatively, it could be attributed to possible toxic effects of the LT-B protein in the tissues of the growing plant and tissues involved in seed development. This phenomenon was also observed by Mason et al. (1998) in transgenic potato plants producing high levels of LT-B protein. One of their higher-expressing transgenic potato events had poor growth and stunting, leading to the speculation that LT-B could in fact be toxic if expressed in the growing plant cells. P65 events did not have enhanced expression but had improved plant vigor compared with P51 plants. Retention of the LT-B protein in the ER could have limited its secretion into the cell wall and thus have reduced the effect of the protein on cell growth. Kernel expression using the CaMV 35S promoter was low, the highest expression observed was 0.01% LT-B of TAEP in event P51–62. LT-B expression was also not consistent between callus and R_1 transgenic kernels in the same transgenic events, possibly due to differences in tissue types between callus and seed. Other mechanisms such as transgene silencing could also contribute to such phenomenon (Matzke and Matzke 1995). Therefore, the level of LT-B expression in callus is not necessary a good indicator for kernel expression. It was concluded that low LT-B expression levels with the CaMV 35S promoter warranted inves-

Table 5. Comparison of LT-B levels (% LT-B in total aqueous-extractable protein) in R_3, ($p > 0.0001$) seed of P51 and P77.

Event ID	Ear ID	Mean	SD	t Grouping*
P51–62	1	0.282	0.168	a
	2	0.252	0.199	a
	3	0.183	0.111	ab
	4	0.167	0.039	b
	5	0.166	0.091	abc
	6	0.148	0.052	c
	7	0.121	0.086	cd
	8	0.026	0.019	d
P77–7	1	3.660	2.740	a
	2	2.096	1.334	b
	3	1.743	1.094	bc
	4	1.350	0.552	bc
	5	1.318	0.780	bc
	6	1.299	0.752	bcd
	7	0.949	0.659	cd
	8	0.190	0.123	d

* values with same letter(s) were not significantly different from each other

tigation of LT-B expression using a seed-specific promoter.

Zeins constitute 50–60% of the seed protein in maize (Marks et al. 1985), and the 27 kDa gamma zein promoter is one of the strongest seed-specific promoters characterized to date. Substantially higher levels of LT-B expression were observed in the R_1 kernels of P77, up to 0.07% LT-B in TAEP, compared with 0.01% in P51 R_1 kernels. In the R_3 generation, the gamma zein promoter resulted in 13-fold higher levels of LT-B in the seed than the constitutive CaMV 35S promoter (0.28% in P51-62 compared with 3.7% in P77-7, Table 5). In P77 plants, LT-B protein accumulation was observed predominantly in the endosperm. This was determined through embryo vs. endosperm extraction and quantitation of LT-B (data not shown).

Events from P112 had the highest level of expression in the R_1 seed (Figure 5). Since this construct was identical to P77 except for the SEKDEL motif included in the LT-B gene in construct pRC4-1, this increase in LT-B level could be attributed to the presence of this endoplasmic reticulum-retention sequence in the transgenic plants carrying pRC4-1. The SEKDEL motif is expected to sequester the protein in the ER (Munro and Pelham 1987). Haq et al. (1995) showed that the SEKDEL motif resulted in significantly higher LT-B accumulation when compared with the LT-B gene without SEKDEL. These authors proposed that the cellular compartmentation of the SEKDEL protein could have facilitated oligomerization of LT-B monomers into pentamers detectable by ganglioside-dependent ELISA. We observed no marked improvement in the level of LT-B protein in maize callus of P65 events in which the LT-B gene had the SEKDEL motif compared with P51 callus events, which did not. Why the enhancement effect of the SEKDEL motif was not observed in callus is not clear. It is possible that callus is a tissue that inherently does not contain as much protein as seed, and so does not accumulate protein beyond a certain level. No substantial SEKDEL enhanced expression was observed in kernels using the constitutive promoter either; the highest level of expression observed in P65 R_1 kernels was 0.01% in event P65-69.

There appears to be interaction between promoter and/or target tissue and the SEKDEL motif. More likely there is an interaction between the tissue and the SEKDEL motif; SEKDEL works in some tissues better than others due to differences in endomembrane system functions. To propose promoter-SEKDEL interaction would imply interaction at the DNA level, which could be possible but not likely. These observations suggest the possible benefits of using the SEKDEL motif in improving the expression of a novel protein in maize kernels could be dependent on target tissue and promoter used, among other factors.

In all constructs, variability in LT-B levels was observed in plants from different transgenic events in all generations studied, and also between plants within the same event. Variability between events within the same generation could be explained as differences in transgene copy number and position effects in independently transformed events. From results of Southern analysis, plants from the same transgenic event appeared to have the same transgene insertion pattern (Figure 2). However, despite having similar transgene insertion patterns, significant variation in LT-B expression was observed between plants from the same event, especially in the R_3 generation (Table 5). This variation could be attributed to the microenvironment in which the plants developed, and some could be attributed to the genotypes of the plants. While the plants should be generally similar in genetic makeup, the Hi-II germplasm used for transformation in this work was generated from crossing Hi-II parents A and B, which were derived from the inbred lines A188 and B73. The germplasm was

therefore heterozygous to some degree, and segregated to give some of the observed variation (Armstrong et al. (1991); M. Lee, personal communication). Some of the variation could be attributed to epigenetic effects. Other researchers have reported similar variation and proceeded to select the highest-expressing plant for subsequent generations (Hood et al. 1997; Zhong et al. 1999). These observations have important implications for commercial production of a novel protein. It cannot be assumed that plants from the same event have similar levels of expression, and it may take several generations of selection of individual lines to establish a stable transgenic line with a desired and consistent level of transgene expression.

Substantial increases in LT-B levels were observed between subsequent generations for both P51 and P77 events. In one case an increase of more than a 100-fold was achieved (Event P77-9, Table 4). It is possible that a foreign protein is poorly expressed in the R_1 generation because of the stress of the *in vitro* culture, transformation, and regeneration processes. Self-pollination in subsequent generations may increase the dose of the transgene in the endosperm, which might result in increased LT-B levels. It was also observed that while large differences were observed between different generations of the same transgenic event, the events did not always rank in the same order. An event with a high level of LT-B expression in the R_1 generation would not necessarily exhibit high expression in the next generation (see Table 4). Other workers have observed that subsequent generations have been shown to have increasing novel protein levels relative to the R_1 generation (Zhong et al. 1999).

Streatfield et al. (2002) recently reported the production of a synthetic LT-B in transgenic maize. The LT-B gene was optimized for expression in maize and was driven by an unspecified maize constitutive promoter. They reported that LT-B was highly expressed in maize seed at up to 1.8% of the total soluble protein in the R_1 generation, and further increased about 5-fold in subsequent generations. We observed up to 3.7% LT-B in TAEP in an R_3 generation ear from a transgenic line carrying the LT-B gene driven by the gamma zein promoter. The highest level of expression in an individual kernel from this ear was 6.4%. It is noteworthy that while both expression levels are comparable, the LT-B quantification methods used by our group and Streatfield's group were different. LT-B is expressed as a monomer, and assembles into a pentameric ganglioside-binding form that is functional. Whereas the polyclonal antibody capture sandwich ELISA used by Streatfield and co-workers measured total LT-B protein in both monomeric and multimeric forms; we used G_{M1} gangliosides-dependent ELISA to capture the functional pentameric form of LT-B. Streatfield's group also reported an up to 40% difference when a polyclonal antibody or G_{M1} gangliosides were used as capture in sandwich ELISA to quantify LT-B expression, with the former resulting in higher values when used in conjunction with a biotinylated antibody to detect the captured LT-B.

Maize generated LT-B was shown to be similar in biochemical and physical properties to the bacterial LT-B (Streatfield et al. 2001; Chikwamba et al. 2002 (in press)). Mice feeding experiments (Streatfield et al. 2001; Chikwamba et al. 2002 (in press)) showed that when orally administered to mice, transgenic corn meal expressing LT-B was capable of inducing strong serum and mucosal antibodies, and to protect mice from challenge with the *E. coli* labile toxin. In a previous study (Chikwamba et al. 2002 (in press)), we showed that four doses of 10 μg LT-B per gram of corn meal pellet were adequate to induce an immune response in mice. Using the gamma zein promoter the LT-B levels of up to 350 $\mu g\ g^{-1}$ of dry kernel tissue were attained. This expression level is therefore more than was required to induce a protective immune response in experimental mice. Mason et al. (1998) suggested that up to 1.1 mg would be required to induce a protective immune response in humans, and this dosage requirement could be met in 3 grams of dry maize meal from P77-7 R_3 kernels. We harvested three generations of functional LT-B-expressing plants; the transgene in the selected events was not silenced over generations and transmission was in a normal Mendelian fashion. Assays of LT-B in greenhouse and field showed an increase in accumulation of LT-B over the generations. Stability of expression is critical for high-expressing events that would be used for protein production in a practical situation.

We showed in this work that the gamma zein promoter resulted in higher kernel expression of LT-B than the CaMV 35S promoter. Other modifications such as the SEKDEL motif show a marked improvement in expression of this gene when combined with the gamma zein promoter. Future work will focus on further enhancement of LT-B expression levels by out-crossing the transgenic plants to an elite line with good agronomic qualities, among other breeding techniques.

Acknowledgements

We thank John Clements for supplying bacterial LT-B and Brian Larkins for providing the gamma zein promoter. Rachel Chikwamba is supported by the Rockefeller Foundation as a graduate student. This work was supported by USDA grant number 99–35504-7799. Journal Paper No. J-19767 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. 3703, and supported by Hatch Act and the State of Iowa.

References

Armstrong C.L., Green C.E. and Phillips R.L. 1991. Development and availability of germplasm with high type II culture formation response. Maize Genet. Coop. Newsl. 65: 92–93.

Armstrong C.L. 1999. The first decade of maize transformation: a review and future perspective. Maydica 44: 101–109.

Austin S., Bingham E.T., Koegel R.G., Matthews D.E., Shahan M.N., Straub R.J. et al. 1994. An overview of a feasibility study for the production of industrial enzymes in transgenic alfalfa. Ann. NY Acad. Sci. 721: 234–244.

Bradford M.M. 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.

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

Christou P. 1996. Transformation technology. Trends Plant Sci. 1: 423–431.

Daniell H., Streatfield S.J. and Wycoff K. 2001. Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci. 6: 219–226.

Dickinson B.L. and Clements J.D. 1996. Use of *Escherichia coli* heat-labile enterotoxin as an oral adjuvant. In: Kiyono H., Ogra P.L. and McGhee (eds), Mucosal Vaccines. Academic Press, pp. 73–87.

Frame B.R., Zhang H., Cocciolone S.M., Sidorenko L.V., Dietrich C.R., Pegg S.E. et al. 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. B. 36: 21–29.

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

Gribskov M., Devereux J. and Burgess R.R. 1984. The codon preference plot: Graphic analysis of protein coding sequences and prediction of gene expression. Nucl. Acids Res. 12: 539–549.

Haq T.A., Mason H.S., Clements J.D. and Arntzen C.J. 1995. Oral immunization with a recombinant bacterial antigen produced in transgenic plants. Science 268: 714–716.

Hood E.E., Witcher D.R., Maddock S., Meyer T., Baszczynski C., Bailey M. et al. 1997. Commercial production of avidin from transgenic maize: Characterization of transformant, production, processing, extraction and purification. Mol. Breed. 3: 291–300.

Krebbers E., Bosch D. and Vandekekerekhove J. 1993. Prospects and progress in the production of foreign proteins and peptides in plants. In: Shewry P.R. and Gutteridge S. (eds), Plant Protein Engineering. Cambridge University Press, Cambridge, UK, pp. 315–325.

Marks M.D., Lindell J.S. and Larkins B.A. 1985. Quantitative analysis of the accumulation of zein mRNA during endosperm development. J. Biol. Chem. 260: 16445–16450.

Matzke A.M. and Matzke A.J.M. 1995. How and why do plants inactivate homologous transgenes? Plant Physiol. 107: 679–685.

Mason H.S., Haq T.A., Clements J.D. and Arntzen C.J. 1998. Edible vaccine protects mice against *Escherichia coli* heat labile enterotoxin (LT): Potatoes expressing a synthetic LT-B gene. Vaccine 16: 1336–1343.

Millar D.G., Hirst T. and Snider D.P. 2001. *Escherichia coli* heat labile enterotoxin B sub-unit is a more potent mucosal adjuvant than its closely related homologue, the B sub-unit of Cholera toxin. Infect. Immun. 69: 3476–3482.

Munro S. and Pelham H.R.B. 1987. A C-terminal signal prevents secretion of luminal ER proteins. Cell 48: 899–907.

Murray M.G. and Thompson W.F. 1980. Rapid isolation of high-molecular-weight plant DNA. Nucl. Acids Res. 8: 4321–4325.

Pen J., Sijmons P.C., Van Ooijen A.J.J. and Hockema A. 1993. Protein production in transgenic crops: Analysis of plant molecular farming. In: Industrial Crops Production. Elsevier, Amsterdam, pp. 241–250.

Rhee Y. and Staswick P.E. 1992. Nucleotide sequence for the soybean vegetative storage protein vspB gene. Plant Physiol. 98: 794–795.

Sambrook J., Frittsch E.F. and Maniatis T.H. (eds) 1989. Molecular Cloning, A Laboratory Manual. Chapters 9–31 to 9–62. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Sangtong V., Mottl E.C., Long M.J., Lee M. and Scott M.P. 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.

Spangler B.D. 1992. Structure of Cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. Microbiol. Rev. 56: 622–647.

Streatfield S.J., Jilka J.M., Hood E.E., Turner D.D., Bailey M.R., Mayor J.M. et al. 2001. Plant based vaccines: Unique advantages. Vaccine 19: 2742–2748.

Streatfield S.J., Mayor J.M., Barker D.K., Brooks C., Lampear B.J., Woodard S.L. et al. 2002. Development of an edible subunit vaccine in corn against enterotoxigenic strains of Eschericia coli. In Vitro Cell Dev. B. 38: 11–17.

Tacket C.O., Mason H.S., Lasonsky G., Clements J.D., Levine M.M. and Arntzen C.J. 1998. Immunogenicity in humans of a bacterial antigen delivered in a transgenic potato. Nature Med. 4: 607–609.

Zhong G.Y., Peterson D., Bailey M., Witcher D.R., Register J., Bond D. et al. 1999. Commercial production of aprotinin in transgenic maize seed. Mol. Breed. 5: 345–356.