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Plant-based vaccines: unique advantages

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Abstract

Numerous studies have shown that viral epitopes and subunits of bacterial toxins can be expressed and correctly processed in transgenic plants. The recombinant proteins induce immune responses and have several benefits over current vaccine technologies, including increased safety, economy, stability, versatility and efficacy. Antigens expressed in corn are particularly advantageous since the seed can be produced in vast quantities and shipped over long distances at ambient temperature, potentially allowing global vaccination. We have expressed the B-subunit of *Escherichia coli* heat-labile enterotoxin and the spike protein of swine transmissible gastroenteritis virus at high levels in corn, and demonstrate that these antigens delivered in the seed elicit protective immune responses.

Keywords: Plant vaccines; Transmissible gastroenteritis virus (TGEV); *Escherichia coli* heat-labile enterotoxin (LT)

1. Introduction

Following recent advances in molecular biology, there is a growing potential for new classes of vaccines. The dissection of pathogens into their various components allows the development of specific subunit vaccines that are just as efficacious but are safer than whole pathogen vaccines. However, despite recent advances in vaccine research, the most common route of vaccination remains that of parenteral injection. The development of a broadly applicable oral delivery system remains a goal for the efficient widespread administration of vaccines, but unfortunately this has proven impractical in most cases to date. The use of subunit vaccines for oral delivery has been generally resisted because of the obvious likelihood of protein degradation in the gut. Furthermore, even if the protein were to survive within an oral delivery system, there is no certainty that trafficking the protein to the gut would be sufficient to mount an immune response.

Recently, transgenic plants have been investigated as an alternative means to produce and deliver vaccines. There are several reports demonstrating that antigens derived from various pathogens can be synthesized at high levels and in their authentic forms in plants [1–3]. When administered orally, by feeding, such antigens can induce an immune response [4,5] and, in some cases at least, result in protection against a subsequent challenge with the pathogen [6,7]. Certain antigens expressed in plants have shown sufficient promise to warrant human clinical trials [8,9]. This has led to optimism that the inherent advantages of plants can be used to dramatically change the way in which we deliver vaccines, and indeed that plants can become the delivery vehicle of choice for future vaccines. Combining the normal use of plants as human foods and as animal feed, with the production of vaccine subunit components in plant tissues, should allow vaccines to be produced at a fraction of the cost of other approaches. For the same cost, the recipient may take 100 or even 1000 times the dose of antigen that would be delivered by the parenteral injection route. We are exploring the use of corn grain as a particularly convenient delivery system for edible vaccines using both human and commercial animal examples, including

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vaccines against enterotoxigenic strains of *Escherichia coli* (ETEC) and swine transmissible gastroenteritis virus (TGEV).

Among children under five in developing countries, ETEC are responsible for over 650 million cases of diarrhea resulting in about 800 000 deaths each year [10]. About 20% of visitors to developing countries also get travelers' diarrhea from ETEC [11]. A major disease agent of ETEC is the heat-labile toxin (LT). This toxin has a multi-subunit structure very similar to cholera toxin and consists of a pentamer of receptor binding (B) subunits and a single enzymatic (A) subunit [12]. Approximately 66% of ETEC strains harbor LT, and in about half of these strains LT is the only toxin present [13]. An oral vaccine against ETEC is under development and consists of an inactivated whole cell *E. coli* component, together with an LT-B subunit component [14]. We have expressed LT-B in corn and demonstrate its immunogenicity and efficacy when fed to mice.

Swine transmissible gastroenteritis (TGE) is recognized as a major sickness and cause of death in piglets, particularly under conditions of intensive farming [15]. It is a highly contagious enteric disease that is characterized by vomiting, severe diarrhea and high mortality in piglets less than 2 weeks of age. The causal agent of TGE is a multi-subunit, enveloped, single-stranded RNA virus, TGEV, belonging to the genus *Coronavirus* of the family Coronaviridae [15]. It contains three structural proteins designated M, N and S. Protein M is an integral membrane protein, N is a phosphoprotein that encapsulates the viral RNA genome, and S (spike) is a large surface glycoprotein [15]. Pigs that survive a first infection are immune to subsequent infections of the virus [16], probably due to local mucosal immunity in the intestine through the activation of S-IgA. Thus, vaccines that target the activation of the mucosal surface of the intestine are particularly attractive in the control of TGE and similar diseases. We have expressed the S protein of TGEV in corn and show that it is protective in a piglet feeding trial.

2. Materials and methods

2.1. Construction of plasmids

A synthetic version of a gene encoding a variant of LT-B (GenBank accession M17874) was synthesized in which codon usage was optimized for highly expressed maize genes. A series of overlapping complementary oligonucleotides was annealed and the resulting sequence was amplified by the polymerase chain reaction. A maize codon optimized version of the barley α-amylase signal sequence was also incorporated to provide a cell secretion signal at the N-terminus of LT-B. This signal sequence is cleaved upon protein export and results in high levels of protein accumulation in the cell wall [17]. A maize codon optimized version of the S protein of TGEV was constructed in a similar manner and also fused to the barley α-amylase signal sequence. The synthetic genes were sub-cloned into a maize expression cassette within a transformation vector that included right and left border sequences of an *Agrobacterium tumefaciens* Ti plasmid and the *pat* gene of *Streptomyces viridichromogenes* conferring resistance to the herbicide Basta.

2.2. Agrobacterium-mediated maize transformation

The plant expression constructs were introduced into maize using an *A. tumefaciens* mediated approach [18]. Ears of the maize line Hi-II were harvested at 9–12 days after pollination, surface sterilized in 50% bleach with Tween-20 for 30 min and rinsed twice with sterile water. Immature zygotic embryos were isolated from the ears under sterile conditions and washed twice with co-cultivation medium. A suspension of *A. tumefaciens* was added directly to the embryos and the mixture was vortexed vigorously for 30 s and incubated at room temperature for 5 min. Embryos were placed scutellum side up onto co-cultivation medium and were incubated at 19°C in the dark for 3–5 days. They were then transferred to antibiotic-containing medium without herbicide selection and were incubated for a further 3 days in the dark at 27–28°C. Subsequently, embryos and calli were transferred to fresh herbicide containing selection medium every 2 weeks, and the callus was finally transferred onto regeneration medium. Mature somatic embryos were placed in the light and allowed to germinate. Ten plants from each transformation event were transplanted to soil and were grown in a greenhouse to generate T_1 seed.

2.3. Preparation of soluble protein extracts from corn seed

Dry seeds were pulverized using a mortar and pestle and were then shaken vigorously in a tube with a steel ball bearing in the presence of approximately 500 μl of PBST per seed. Extracts containing soluble protein were recovered by centrifugation of the homogenized tissue and collection of the supernatant. Protein concentrations were determined using an assay for protein-dye binding [19].

2.4. Quantification of recombinant LT-B in transgenic corn

A sandwich-ELISA was deployed. Between each step the 96-well assay plates were repeatedly washed with PBST. Plates were coated with 133 ng ml^{-1} of LT-B antibody in 0.05 M carbonate/bicarbonate buffer at pH

9.6 by incubating for several hours at 4°C. The wells were then blocked by incubating with 3% BSA in PBST at 37°C for 1 h. Soluble protein extracts were added to the wells and incubated at 4°C for several hours. Next, 50 ng ml^{-1} of biotinylated LT-B antibody in 1% BSA/PBST was added to the wells and incubated at 37°C for 1 h. Streptavidin-alkaline phosphatase in PBST/1% BSA was then added to the wells and incubated at 37°C for 30 min. Finally, *para*-nitrophenyl-phosphate was added to each well and incubated at 37°C for 30 min, and the absorbance at 405 nm was recorded. A dilution series of a recombinant LT-B standard and non-transgenic corn were included in the assay.

2.5. Quantification of recombinant TGEV S protein in transgenic corn

A sandwich-ELISA was deployed. Between each step the 96-well assay plates were repeatedly washed with PBST. Plates were coated with antibody raised against feline infectious peritonitis virus (FIPV) which recognizes TGEV (VMRD, Inc., Pullman, WA) in 0.05 M carbonate/bicarbonate buffer at pH 9.6 by incubating for several hours at 4°C. The wells were then blocked by incubating with 5% milk/PBST at 37°C for 1 h. Soluble protein extracts were added to the wells and incubated at 4°C for several hours. Next, 387.5 ng ml^{-1} of biotinylated FIPV antibody in 5% milk/TPBST was added to the wells and incubated at 37°C for 1.5 h. Streptavidin-alkaline phosphatase in PBST/1% BSA was then added to the wells and incubated at 37°C for 1 h. Finally, *para*-nitrophenyl-phosphate was added to each well and incubated at 37°C for 30 min, and the absorbance at 405 nm was recorded. A dilution series of a TGEV standard and non-transgenic corn were included in the assay.

2.6. Production of transgenic grain

Transgenic lines expressing high levels of recombinant protein in T_1 seed were backcrossed to commercial maize lines. Pollen from the transgenic lines was used to pollinate the commercial lines in order to bulk up transgenic seed rapidly.

2.7. Immunization of mice

BALB/c mice were housed individually and fed a basic diet of mouse chow with water allowed ad libitum. The mouse chow was removed overnight prior to administering test samples on days 0, 7 and 21 of the study. The mice were divided into five groups, with seven individuals in each group. Test samples consisted of either mouse chow with or without 50 μg pure recombinant LT-B, wild type corn, or transgenic corn expressing 5 or 50 μg LT-B. All the mice consumed each dose in full. Blood samples were collected by conducting tail bleeds prior to the first feeding of test samples and on days 6, 13, 20 and 27 of the study. Fecal samples corresponding to material excreted over the previous 24 h were collected prior to the first feeding of test samples and on days 4, 7, 11, 14, 18, 21, 25 and 28 of the study. For the protection assay, two groups of mice were fed either wild type corn or transgenic corn expressing 50 μg LtB on the same feeding program as above. The patent mouse assay was conducted 34 days after initial feeding.

2.8. Detection of anti-LT-B Ig in mouse serum

96-well plates were coated with LT-B pentamer, repeatedly washed with PBS and blocked with 3% BSA for 1 h at 37°C. The blocking solution was replaced with serum recovered from mouse blood and diluted 11-fold in the blocking solution. Samples were incubated for 2 h at 37°C and then repeatedly washed with PBST. Anti-mouse Ig polyvalent conjugated to alkaline phosphatase and diluted 1000-fold in blocking solution was added to each well and incubated for 2 h at 37°C. Plates were repeatedly washed with PBST and 1 mg ml^{-1} *para*-nitrophenyl-phosphate was added to each well. Following 30 min of incubation at 37°C the absorbance at 405 nm was determined.

2.9. Detection of anti-LT-B IgA in mouse fecal samples

Between each step the 96-well assay plates were repeatedly washed with PBS. Plates were coated with the ganglioside G_{M1} at 4°C for several hours and blocked with 3% BSA for 30 min at 37°C. A 1 μg ml^{-1} solution of LT-B in PBS was added to each well and the plates were incubated at 37°C for 1 h. Lyophilized fecal pellets were resuspended in 1% BSA/PBST, incubated for 30 min and spun to remove solid matter. Samples of fecal supernatants were added to each well and incubated at 37°C for 1 h. A 1000-fold dilution of goat *anti*-mouse IgA was added to each well and incubated at 37°C for 1 h. Next, a 5000-fold dilution of *anti*-goat alkaline phosphatase conjugate was added to each well and incubated at 37°C for 1 h. Finally, *para*-nitrophenyl-phosphate was added to each well, the plates were incubated at 37°C for 30 min and the absorbance at 405 nm was determined.

2.10. LT protection assay

The patent mouse assay used here was a modification of the sealed adult mouse assay [20]. Following completion of the feeding regime, mice were fasted but allowed

water ad libitum for 24 h. For this period they were housed in cages with raised wire meshes to prevent coprophagy. Each study group was split and the mice were administered by gavage either 20 μg LT in PBS buffer or just PBS buffer. They were left for 4 h, sacrificed and dissected to assess gut swelling. The upper intestine and carcass weights were recorded, and the ratio calculated.

2.11. Swine feeding trial

The trial subjects were 10 day-old specific pathogen free piglets that were TGEV sero-negative and were from a herd with a low incidence of disease. On days 0–10 of the study all piglets in each of three groups of 10 animals were withheld from feed overnight, prior to administering corn to two of the study groups. The corn ration for a piglet consisted of either 100 g of wild type corn or 50 g of transgenic corn (corresponding to approximately 2 mg of the S protein of TGEV) mixed with 50 g of wild type corn. The corn was mixed with medicated milk replacer to give a thick oatmeal-like consistency. For the two groups of piglets receiving corn, a line of prepared meal sufficient for the whole group was placed on a clean dry floor and attempts were made to ensure that each piglet received an adequate portion. The piglets were then returned to regular water and medicated weaning rations. Piglets in the third group were orally vaccinated with the current commercially available modified live vaccine MLV TGEV on days 0 and 7 of the study according to label directions. On day 12 of the study all animals were orally challenged with a 2 ml dose of virulent TGEV (Purdue strain, titer $10^{7.6}$ $FAID_{50}$'s per dose). Prior work had shown that this dose should produce a clinically typical TGE watery diarrhea in 21–28 day old piglets that would persist for 7–10 days, but would not be lethal. Following challenge, piglets were scored twice daily for signs of diarrhea (normal = 0, creamy = 1, watery = 2) and other symptoms (dehydration and depression, or anorexia = 1, vomitus = 3, moribund or death = 10) to give a total clinical score. The clinical symptom data for each study group are presented as a percent morbidity incidence (number of animals with clinical signs > 2 divided by total number of animals), a percent morbidity incidence and duration (total number of clinical observations > 2 divided by total number of pig days), and a clinical severity index (total clinical score divided by total number of pig days). To confirm viral challenge, fecal samples were collected from randomly selected animals within any group that produced watery diarrhea. These samples were checked for TGEV activity by inoculating confluent ST cells and staining by specific immunofluorescence.

3. Results

3.1. LT-B corn fed to mice induces an immune response that combats LT holotoxin

We first investigated whether LT-B produced in corn would induce an immune response when fed to mice. Mice were fed ground transgenic corn seed and serum and fecal samples were analyzed for immune responses. Notably, equivalent amounts of pure LT-B or transgenic corn expressed LT-B induce similar *anti*-LT-B specific Ig responses in serum Fig. 1A. The response is clearly evident at 13 days after the first feeding and remains elevated for the course of the study. Doses of 5 μg of LT-B expressed in corn are sufficient to give a strong Ig response in serum, demonstrating that corn is an effective oral delivery vehicle for LT-B.

As a guide to mucosal immunogenicity, *anti*-LT-B specific IgA levels were recorded in fecal material of mice that had been fed LT-B expressed in corn. Responses are evident after 7 days and clearly cycle with peak responses about 1 week after each dose Fig. 1B. As with the serum Ig response, doses of 5 μg of LT-B expressed in corn are sufficient to induce a strong mucosal IgA response. Strikingly, LT-B expressed in corn induces a much greater *anti*-LT-B specific mucosal IgA response than pure LT-B.

In order to assess the efficacy of LT-B expressed in corn, we examined whether it could prevent gut swelling in mice exposed to the LT holotoxin. The upper intestines of a control group of mice swell up when gavaged with LT, whereas those of mice fed LT-B expressed in corn do not swell Fig. 1C. Thus, LT-B expressed in corn appears to be protective against LT.

3.2. The S protein of TGEV expressed in corn is protective against the virus

We then progressed to developing an edible vaccine against an economically important animal disease, TGE in swine. We conducted a study to compare transgenic corn expressing the S protein of TGEV with a commercial modified live TGEV vaccine. A negative control group fed wild type corn was also included. The percent morbidity incidence shows that all the piglets fed only wild type corn developed TGE clinical symptoms Fig. 2A. By comparison, only 50% of those that received the transgenic corn expressing the S protein exhibited symptoms. Interestingly, 78% of the piglets receiving the commercial modified live vaccine developed symptoms, indicating that the edible transgenic corn vaccine is more effective. By contrast, the percent morbidity incidence and duration, and the clinical severity index indicate that piglets that received the modified live vaccine appear to recover quicker than those that were fed transgenic corn Fig. 2B and C.

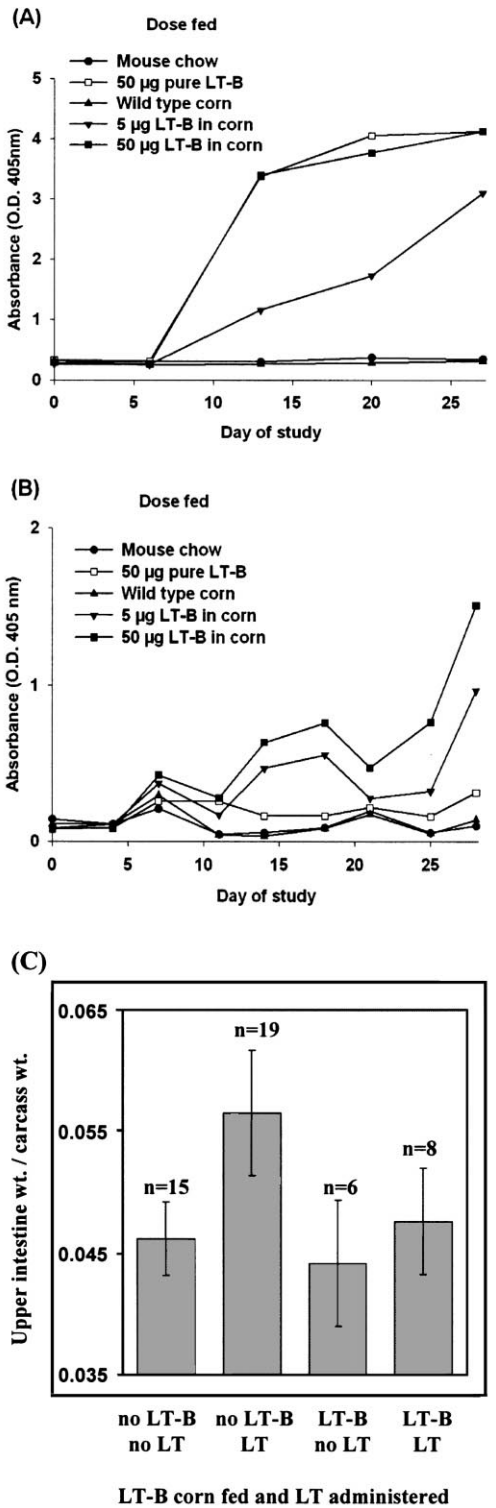

Fig. 1. Protective immune responses of mice fed transgenic LT-B corn. (A) *Anti*-LT-B specific Ig in serum. The mean response for the seven mice in each group is shown; (B) *Anti*-LT-B specific IgA in fecal material. The mean response for the seven mice in each group is shown; (C) The degree of gut swelling following challenge with LT holotoxin. Mean values for the weight ratios are shown with 95% confidence levels, and the sample size is given (*n*).

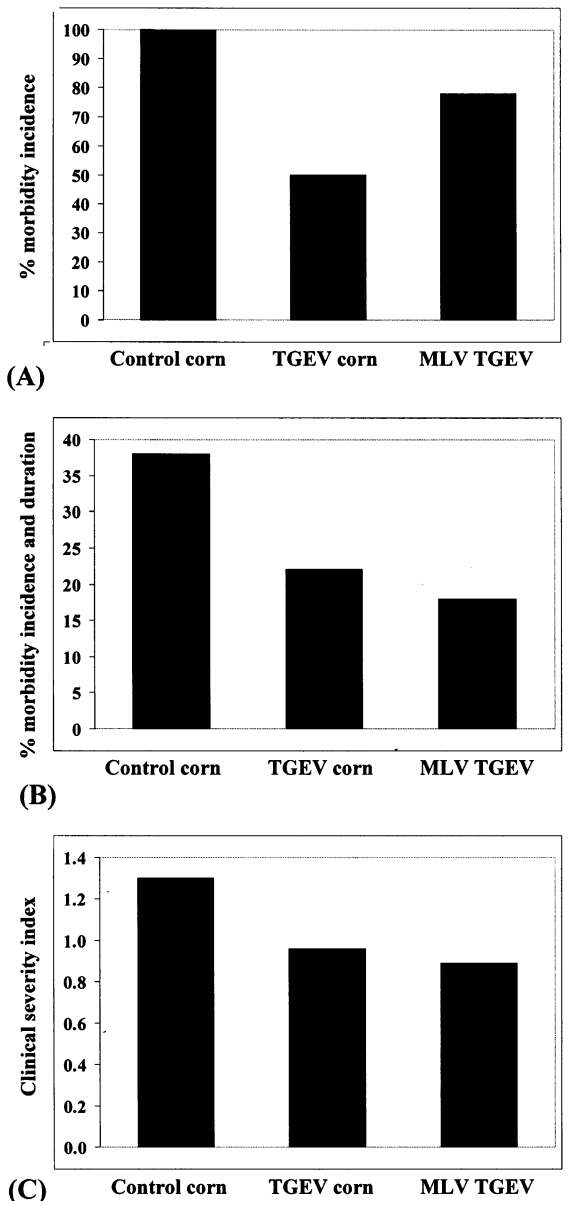

Fig. 2. Protection against TGEV of piglets fed transgenic corn expressing the S protein or modified live vaccine (MLV) TGEV. (A) Percent morbidity incidence; (B) Percent morbidity incidence and duration; (C) Clinical severity index. See text for definitions of clinical indices.

However, differences between these two groups in the percent morbidity incidence and duration, and in the clinical severity index are slight, whereas both groups are clearly protected against TGEV compared to piglets fed wild type corn. Fecal samples from piglets showing watery diarrhea were shown to harbor TGEV (data not shown).

4. Discussion

The use of transgenic plants, and in particular cereals, for vaccine production has several potential benefits

over traditional methods. Firstly, transgenic plants are usually engineered to express only a small antigenic portion of a pathogen or toxin, eliminating the possibility of infection or innate toxicity and reducing the potential for adverse reaction. Secondly, since there are no known human or animal pathogens that are able to infect plants, concerns with viral or prion contamination are eliminated. Thirdly, the successful synthesis of foreign proteins in transgenic crops rely on the same established technologies to sow, harvest, store, transport and process plant material as those currently used for food crops, making transgenic plants a very economical means of large-scale vaccine production. Fourthly, the expression of foreign proteins in the natural storage tissues of plants maximizes protein stability, so reducing the need for refrigeration and keeping transportation and storage costs low [17]. Fifthly, the formulation of multi-component vaccines is possible by blending the seed of transgenic lines expressing different proteins. Sixthly, direct oral administration is possible when the chosen proteins are expressed in commonly consumed food plants to give edible vaccines.

Orally administered vaccines are particularly efficient at stimulating local mucosal immune responses at the intestinal surface, and the integrated nature of the mucosal immune system allows other mucosal sites to also be primed [21]. The mucosal immune system is distinct and independent of the humoral immune system and is not effectively stimulated by parenteral administration of candidate antigens. Rather, the mucosal immune system requires antigen presentation directly to a mucosal surface [22]. Since mucosal surfaces form the first defense against transmissible diseases entering the body through oral, respiratory, urinary and genital routes, edible plant vaccines are an attractive means of protection against a wide range of pathogens. They could potentially be used alone or in combination with other vaccination routes.

To our knowledge the study reported here is the first example of an animal used in conventional food husbandry acquiring protection from a major disease through ingesting an edible vaccine. Moreover, by using corn as the delivery vehicle for the antigen, we utilized a conventional feed material of the animal. The protection observed in this study includes aspects of general health and vigor, reduced clinical symptoms and other factors known as criteria for disease protection. The mechanism of protection is unknown but may be an active immune response by the animal, competitive inhibition of viral receptor sites leading to non-establishment of viral infection, or interference with the viral replication process.

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