Chapter 8
An Oral Vaccine for TGEV Immunization of Pigs

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8.1 Introduction

8.1.1 Structure of TGEV

Transmissible gastroenteritis virus (TGEV) is an economically important porcine pathogen that causes severe, contagious diarrhea and vomiting with high mortality in piglets under 2 weeks of age. TGEV can manifest endemically or epidemically in swine, and the virus may be vectored in by other animals such as dogs, cats, birds, and rodents. TGEV's prevalence and detrimental effects on commercial hog farms have spurred research into vaccines, particularly those suitable for convenient administration to large numbers of pigs. Large naïve droves are costly and inconvenient to immunize with current vaccines because each individual has to be isolated, vaccinated, and tagged. Inducing oral immunity through colostrum by vaccinating sows, or boosting immunity in piglets following single primary injection, will also be beneficial due to lower associated costs.

TGEV belongs to the subfamily *Coronavirinae* in the *Coronaviridae* family of enveloped viruses (Belouzard et al. 2012). Coronaviruses cause respiratory or enteric disease in avian, bovine, feline, canine, murine, and human hosts. The most widely known virus in this class is responsible for severe acute respiratory syndrome (SARS) (Nuttall and Dye 2013), and more recently, NCoV, a novel coronavirus isolated from the Arabian Peninsula (Buchholz et al. 2013; Hofer 2013). Structurally, coronaviruses are among the largest viruses, at about 100 nm in diameter, and have a large, positive-strand RNA genome. TGEV is related to other swine coronaviruses: the porcine respiratory coronavirus (PCRV), porcine

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J.A. Howard and E.E. Hood (eds.), *Commercial Plant-Produced Recombinant Protein Products*, Biotechnology in Agriculture and Forestry 68,
DOI 10.1007/978-3-662-43836-7_8,

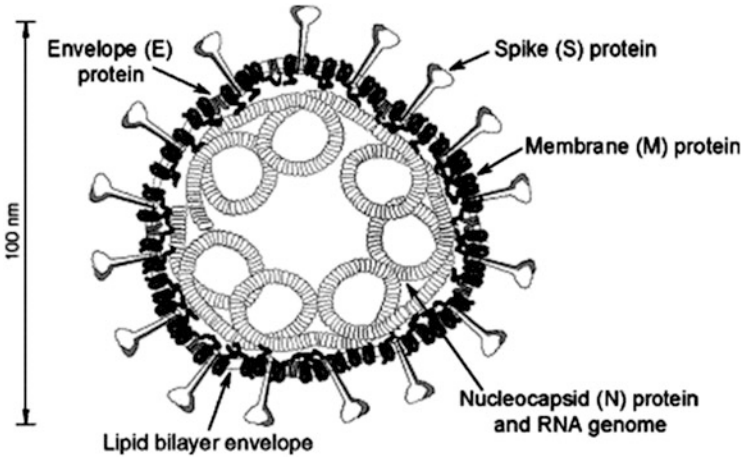

Fig. 8.1 Schematic of the coronavirus virion, with the minimal set of structural proteins. Reproduced with permission from Masters (2006)

epidemic diarrhea virus (PEDV), and porcine hemagglutinating encephalomyelitis virus (HEV) (Sestak and Saif 2008).

It is pertinent to examine the structure of TGEV and related viruses to explain the choices of epitopes available for the production of effective subunit vaccines. All coronaviruses have an envelope with radiating structures composed of trimers of the 220 kDa spike glycoprotein (S, also referred to as peplomer E2), as well as the smaller membrane glycoprotein (M, 29–36 kDa) and envelope protein (E1, 10 kDa) (Fig. 8.1). The M protein interacts with the nucleocapsid N protein and the viral RNA to form the icosahedral nucleoprotein core (Masters 2006). The S-protein, specifically the N-terminal domain between amino acids 522–744, binds aminopeptidase N receptor in the epithelium of the small intestine and mediates the fusion of the host and viral membranes and uptake (Belouzard et al. 2012).

8.1.2 Antigenicity of TGEV's Structural Components

The surface and subviral structural components of the virus have been assessed for antigenicity, and neutralizing antibodies were found to be associated with the surface components which function in recognition and binding with subviral and host proteins (Garwes et al. 1979). The M protein induces interferon production and also binds neutralizing antibodies (Laude et al. 1992). The S (or E2) protein has been found to be the most effective epitope at inducing neutralizing antibodies. The S-protein has four major antigenic sites at the N-terminal: A, B, C, and D; A and D are involved in antigen neutralization (Reguera et al. 2011; Correa et al. 1988;

Jiménez et al. 1986). Induction of cross-protection with the use of a related virus has been attempted. PCRV shows tropism towards respiratory tissues and differs from TGEV in having a deletion of the N-terminal 224–227 amino acids (containing antigenic sites A and D) of the S-protein, indicating that the A and D sites may be involved in tissue specificity. Immunization with PCRV was partially protective under the same conditions—33 % of piglets survived challenge (De Diego et al. 1994). The S-antigen was therefore selected as the most efficient immunogen for a subunit vaccine.

8.2 Description of the System Used to Produce the Antigen

8.2.1 Theoretical Advantages of the Maize Process Over Other Technologies

High-level expression of heterologous eukaryotic proteins has been successfully attempted in both prokaryotes and eukaryotes. However, many eukaryotic proteins require posttranslational modifications such as glycosylation, which are not carried out by prokaryotes, and so eukaryotic systems are sometimes necessary to obtain functional end products.

The benefits of plants as production systems are primarily the low cost of production and rapid scale-up that make it more responsive than other methods to high production targets. Traits introduced by transgenic techniques into separate lines can be combined by traditional breeding methods to produce lines with a combination of these traits. The production of bulk plant material requires only space, fertile soil, and sunlight, which are less onerous to provide than sterile fermentation chambers and animal care. Plants do not harbor animal pathogens, and the concern for contamination of products with animal pathogens, such as prions or viruses, is alleviated. Plant material has been shown to serve to bioencapsulate proteins, permitting slow release in the gut (Kong et al. 2001; Verma et al. 2010; Bailey 2000). Thus, there may be a degree of dosage flexibility with oral antigens administered in plant tissue, and the feed can be administered over a larger window to accommodate time frames for induction of secretory and humoral immunoglobulins (Bailey 2000; Daniell et al. 2001).

Important considerations before attempting expression of heterologous eukaryotic proteins in plants are the requirements for posttranslational protein modifications, such as glycosylation, and the presence of allergens and toxins. Glycosylation moieties and patterns differ between animals and plants which has raised concerns over the potential for nonfunctional proteins or an allergic reaction to the plant glycosylated protein. These differences, however, do not appear to be significant functionally (Ma et al. 2003; Suzuki et al. 2003), and plant carbohydrate-specific IgEs in allergic patients were shown to be clinically irrelevant when the protein is orally administered (Mari et al. 2008; Bosch and Schots 2010). This lack of allergenicity

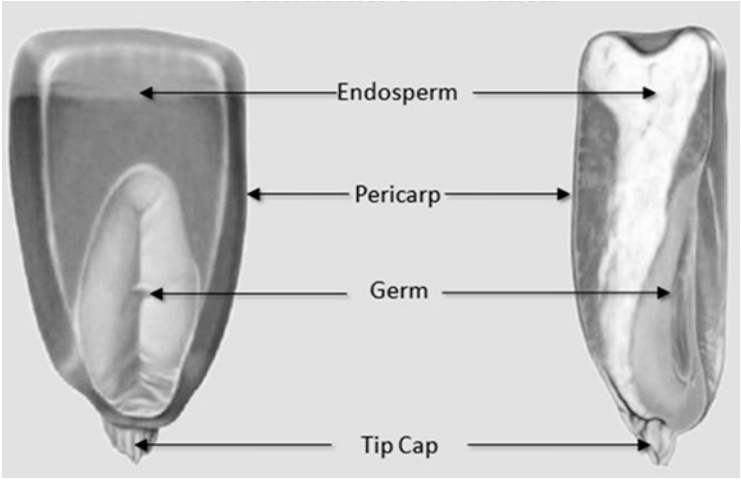

Fig. 8.2 Cross section of maize seed. Reproduced with permission from Cereal Process Technologies, LLC

may be partly explained by tolerance to plant glycosylation induced by oral exposure (see Ghaderi et al. 2012 for an overview of glycosylation of biotherapeutic proteins). Potential concerns about glycosylation-induced allergenicity may also be overcome by the removal of plant glycosylation sites, inactivation of plant glycosylases, and the expression of plant deglycosylases and mammalian-type glycosylases (Sethuraman and Stadheim 2006; Desai et al. 2010; Mamedov et al. 2012). Host plants should also be screened for toxic or allergenic metabolites that may co-purify or be co-administered with the target protein.

Edible tissues such as grain from plants with generally recognized as safe (GRAS) status may be directly used for feed-based vaccines (Naqvi et al. 2011). Seeds are a natural, desiccated, storage system lacking proteases, and recombinant proteins produced in seed have been stable at room temperature for extended periods of time (Naqvi et al. 2011). Selection and backcrossing into parent seed and hybrid lines can establish uniform concentrations of antigen expression (Hood et al. 2012) and standard processing and formulation methods can reliably produce consistent concentrations of antigens for administration, which is not straightforward with perishable edible tissues such as lettuce leaves and bananas. Bioencapsulation of the antigen in plant tissue prevents premature degradation resulting in the antigen persisting in the intestine, a key factor for the induction of a protective sIgA response, important for protection against ingested pathogens. A system that exemplifies the GRAS system is maize (or corn) grain which was used in the work described here. Its structure and specific advantages are described below.

The maize kernel contains three main regions: the embryo, the endosperm, and the pericarp (Fig. 8.2). The pericarp includes the seed coat and fruit layers. The pericarp originates from the ovary wall and functions to protect the seed. The

aleurone layer is the outermost layer of the endosperm and lies directly below the pericarp. Aleurone grains contain enzymes involved in the breakdown of starch and proteins in the germinating seed. The embryo and endosperm are well suited to heterologous protein accumulation as both are rich in protein and have associated tissue-specific promoters. The volume of the endosperm is greater than that of the embryo allowing, in theory, more protein accumulation but, in practice, embryo-preferred promoters have been used to produce some of the highest recorded concentrations of heterologous proteins (Hood et al. 2012; Egelkrout et al. 2013). Desiccation of the seed provides an environment that protects proteins from enzymatic degradation (Ma et al. 2003), and maize seed has cystatins (Yamada et al. 2000; Massonneau et al. 2005) and other protease inhibitors (Jongsma and Bolter 1997) as a defense against proteolysis by insect pathogens.

8.2.2 Theoretical Considerations for an Optimal Vaccine for Piglets

The ideal vaccine for TGEV will provide mucosal, lactogenic, and systemic immunity, while being simple to administer to droves which may number in the many thousands. Potentially, the methods to protect naïve piglets at highest risk from TGEV infection are to provide immune colostrum by vaccinating sows and an oral/nasal vaccine to boost secretory neutralizing antibody levels. Since TGEV is an enteric disease, the induction of sIgA is inferred to be more protective. It has been observed that the mucosal immunoglobulin, IgA, is more efficiently induced through gut-associated lymphoid tissue (GALT) if the antigen persists in the intestine (Foss and Murtaugh 2000). Induction of passive immunity transmitted through colostrum can be mediated by either of the soluble immunoglobulins IgG or IgA. Initially, IgG is more abundant and protects against systemic infection, whereas IgAs in colostrum provide protection to the gut lumen where the enterocytes in which TGEV replicates reside. IgA production and mucosal immunity is thus the response desired. There have been three main routes taken to effect protection in young piglets: (1) parenteral immunization with inactivated virus, (2) oral/nasal administration of attenuated virus, and (3) oral administration of viral subunit antigens in feed.

Parenteral immunization of young piglets with inactivated virus is ineffective as it does not induce the local immune response in the gut, nor are piglets at this age capable of mounting an immune response. However, parenteral immunization of pregnant sows can induce production of protective immunoglobulins in colostrum and milk. The disadvantage of this approach is the necessity of individually vaccinating sows, which can be time-consuming and expensive. Immunization must also be done on a schedule prior to birth that maximizes immunoglobulin presence in the colostrum, and that can be difficult, especially with large numbers. Nasal administration of attenuated viruses does overcome this difficulty, but each

pregnant animal still needs to be isolated and the vaccine delivered separately. As with parenteral injection, the vaccination schedule is important because, while the attenuated virus does replicate in the gut, thus stimulating antibody production over an extended period, viral replication is suboptimal. While the oral vaccine can be more easily administered, the frequency of inoculation has to be modulated to maximize colostrum immunoglobulin timed with birth.

The option of using the oral route to deliver viral subunit antigens in feed is fairly straightforward in terms of administration: the food is put out in feeding troughs and animals are monitored by observation to ensure all animals have had access to the food. However, dosage for orally administered vaccines in feed is particularly significant, because antigens could be ingested at greater or lesser doses depending on the amount of feed consumed, and some of the antigens would be expected to be destroyed by the digestive process. The typical vaccine dosage in feed may be up to 100 times greater than that used for parenteral administration, but this may be reduced by the use of suitable targeting or carrier molecules (Carter and Langridge 2002). Still, common consensus is that a precise dose, taking into account degradation in the gut, must be given to all animals. Interestingly, Verma et al. (2010) have shown that the response from a 20-fold range of antigen levels given encapsulated in plant materials is comparable.

Finally, with any vaccine, oral or parenteral, there is a concern over the potential induction of tolerance, which has been shown to occur for orally administered antigens with repeated doses of antigens over a long term and is mediated by regulatory T-cells. A single large dose has also been shown to induce anergy—a mechanism of adaptive tolerance that cause T-cells, in an environment presumably low in co-stimulators, to become unresponsive to antigens (Weiner 1997; Weiner et al. 2011). Therefore, prior to widespread vaccination, tests must be conducted to ensure this is not an issue. With these caveats addressed, plants have proven to be efficient hosts for heterologous protein production.

8.2.3 Review of Past Efforts to Produce TGEV Vaccines

De Diego et al. (1994) showed that oral immunization with TGEV was more effective at inducing the presence of neutralizing antibodies in colostrum and milk compared to intranasal immunization with PCRV. They attributed this difference to GALT being more effective than bronchus-associated lymphoid tissue (BALT), going even to the extent of questioning if BALT was indeed an integral mammalian structure (Pabst 1992). Pigs have been shown to not have BALT constitutively (Delventhal et al. 1992; Pabst and Gehrke 1990). Thus, the use of intranasal stimulation by PCRV may have been less effective for this reason. Peyer's patches in the gut have long been known to be associated with GALT, and oral immunization has been shown to be effective in producing IgA and IgG globulins in milk and was 100 % effective in protecting 3–5 day piglets obtained from seronegative sows against challenge. This experiment (De Diego et al. 1994)

established that persistence in the gut and therefore a longer-term mucosal response in older animals can be achieved by using an attenuated live vaccine which replicates in the gut.

A variety of viruses have been used as vectors for expression of full length and truncated versions of S-antigen. Baculovirus-infected cells containing the N-terminal fragment of the S-antigen induced neutralizing serum antibodies in piglets (Tuboly et al. 1994). Furthermore, oral immunization of piglets using S-antigen-recombinant porcine adenovirus induced both neutralizing antibodies in sera and mucosal antibodies in the intestine (Tuboly et al. 2001). Fusions of the S-protein expressed in *E. coli* were immunogenic and produced cognate antibodies but did not neutralize the virus. S-protein expressed in vaccinia virus produced neutralizing antibodies when injected into animals (Hu et al. 1985). The expression of S-antigen in nuclear polyhedrosis virus and its expression in insect s9 cells allowed the production of a secreted version which was immunogenic in rats despite lack of proper glycosylation (Godet et al. 1991). Mucosal and serum antibodies were also elicited in rabbits orally inoculated with an attenuated strain of *Salmonella* Typhimurium expressing a fusion of the D-epitope of the S-antigen to *E. coli* heat-labile toxin B-subunit (Lt-B) (Smerdou et al. 1996).

Various TGEV antigens have been produced in plants. Gomez et al. (1998) expressed either the N-terminal 750 residues, or the entire S-protein, under the control of the CaMV 35S promoter in *Arabidopsis*. The antigen was purified from leaves and administered to mice. The mice produced antibodies that reacted specifically with TGEV and neutralized infective particles. Subsequently, the same group expressed the N-terminal domain in potatoes and obtained a serum response to both intraperitoneally delivered tuber extracts and orally administered tubers. Immunoprecipitation and ELISA results showed that antibodies were detecting the native protein; however, the sera did not neutralize the virus in vitro (Gomez et al. 2000). Tuboly et al. successfully produced neutralizing serum antibodies in piglets using tobacco plants expressing various permutations of the S gene under the control of a synthetic super promoter (Tuboly et al. 2000).

8.3 Technical Progress

8.3.1 Achievement of High Levels of Expression of TGEV-S Antigen

In an early experiment using S-antigen, Gomez et al. (1998) used a CaMV 35S promoter to drive expression of an un-optimized sequence in *Arabidopsis*. While the protein produced was immunogenic, its levels were too low to be visualized by SDS-PAGE and could only be detected by Western blotting. Subsequently, Tuboly et al. (2000) used plant-optimized S-antigen sequences driven by a super promoter and increased levels to 0.1–0.2 % total soluble protein (TSP) S-antigen expression in tobacco.

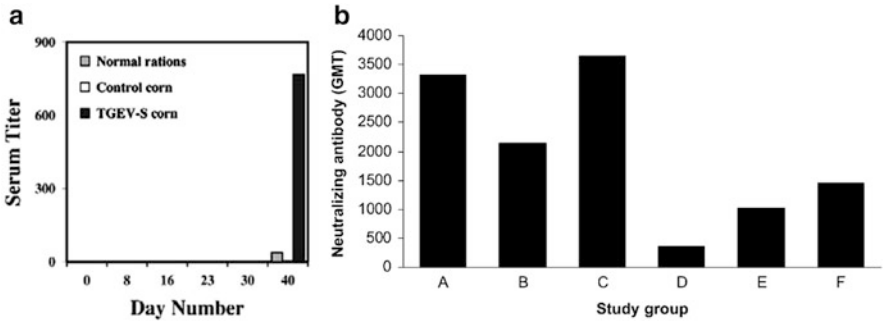

Fig. 8.3 (**a**) Induction of TGEV-neutralizing antibodies in serum from 10–12-day-old piglets fed transgenic corn seed expressing the S-protein of TGEV. Reproduced with permission from Lamphear et al. (2002). (**b**) Colostrum TGEV neutralization titers on the day of farrowing. Pregnant gilts received the following treatments: group A (oral corn vaccine on days −35 to −29 and −14 to −8), group B (oral corn vaccine on days −35 to −33 and −14 to −12), group C (oral corn vaccine on days −35 and −14), group D (oral corn placebo on days −14 to −8), group E (intramuscular live vaccine on days −35 and −14), and group F (oral corn vaccine on days −14 to −8), where the "−" sign represents days before farrowing. Reproduced with permission from Lamphear et al. (2004). See text (Sect. 8.3.2) for discussion

Using codon-optimized sequences for maize fused to the barley alpha-amylase signal sequence (BAASS), Lamphear et al. (2004) achieved levels of S-antigen expression in maize seed of 13 mg/kg. BAASS targets protein to the cell wall and is later cleaved—a process that permits higher levels of accumulation in the apoplast than is possible in the cytoplasm. Using standard plant techniques, F1 lines of maize that had high levels of expression were selected and backcrossed to commercial maize lines to obtain stable, increased levels of protein expression. Since the protein was produced in maize, a GRAS plant, further purification before oral administration was not necessary.

8.3.2 Induction of Neutralizing Antibodies

Lamphear et al. (2002) studied the levels of serum neutralizing antibodies induced by administration of TGEV-S corn alone in piglets. 10–12-day-old TGEV seronegative piglets were divided into three groups (normal rations, control corn, TGEV-S corn) of four piglets each, with 2 mg of the antigen administered in ground corn mixed with medicated milk replacer. 8 days after the last antigen administration, the piglets were challenged with 1 mL of orally administered virulent TGEV. Serum was collected and neutralizing antibody levels measured (Fig. 8.3a). Piglets fed TGEV-S corn showed more than tenfold greater neutralizing serum response as well as milder symptoms (a geometric mean titer of 768.5 in TGEV-S-corn fed piglets compared to titers of 64 in the control groups). While the presence of serum antibodies is significant, greater protection will potentially be afforded by mucosal antibodies.

The induction of neutralizing antibodies in both serum and colostrum was examined in gilts following the administration of oral TGEV vaccine in maize comprising a subunit vaccine of the S-protein expressed in corn (Lamphear et al. 2004). Two kg doses of corn containing 26 mg of antigen were administered to gilts previously sensitized with three doses of modified live vaccine (MLV-TGEV, Intervet) with two oral administrations 115 and 102 days before farrowing and one intramuscular injection 88 days before farrowing. Subsequently, they were divided into six groups for testing (A–F; see Fig. 8.3b) and administered oral TGEV-S corn or control corn at various schedules. TGEV-S corn was given to group A (oral corn vaccine on days −35 to −29 and −14 to −8), group B (oral corn vaccine on days −35 to −33 and −14 to −12), group C (oral corn vaccine on days −35 and −14), group D (oral corn placebo on days −14 to −8), group E (intramuscular live vaccine on days −35 and −14), and group F (oral corn vaccine on days −14 to −8), where the "−" sign represents days before farrowing. Serum antibody levels dropped by up to half at the time of farrowing but were still relatively high compared to control (data not shown). Colostrum obtained on the day of farrowing showed neutralizing antibodies were present. Antibody levels in animals in group C administered test corn for 2 days were almost 3.5 times higher than animals administered intramuscular injection with modified live virus, indicating that corn was more effective at inducing secreted antibody than parenteral vaccine and that the window of administration may be important. That secretory antibodies were produced in colostrum is a clear indication of the stimulation of a mucosal response.

8.3.3 Protection of Piglets Against Challenge with Live Virus

Because it was not clear if the antibodies induced by oral administration were protective against infection, a trial to test protection against challenge was carried out with 10–12-day-old, specific pathogen-free piglets (Streatfield et al. 2001; Lamphear et al. 2002). Piglets were fed TGEV-S corn, control placebo corn, and orally administered MLV-TGEV controls. All piglets were challenged with virulent TGEV on day 18 and monitored twice daily for symptoms for 9 days until the end of the study. Following challenge, piglets were scored twice daily for signs of diarrhea (normal = 0, creamy = 1, watery = 2) and other symptoms (dehydration and depression, anorexia = 1, vomitus = 3, moribund or death = 10) to give a total clinical score. Clinical symptoms for each study group were scored as follows: percent morbidity incidence [(number of animals with clinical signs scoring ≥ 2 divided by total number of animals) × 100], percent morbidity incidence and duration [(total number of clinical observations $\geq$ divided by the product of the total number of pigs and days scored) × 100], or clinical severity index (total clinical score divided by the product of the total number of pigs and days scored).

Half of the control group and about 10 % of the MLV-TGEV group showed morbidity, compared to 0 % of the TGEV-S corn (4-day administration) group.

Morbidity duration and clinical severity were highest in control corn, as expected, but TGEV-S corn showed better protection even than MLV-TGEV. Increased duration of administration showed slightly higher morbidity levels (Fig. 8.4), but piglets administered TGEV-S corn for 4 days showed no symptoms.

This unexpected finding suggests the possible induction of oral tolerance to extended exposure of antigen for longer periods than 4 days. The levels of humoral and secreted (serum and stool) antibodies in these piglets were not monitored, but it seems likely that the levels were diminished by the extended exposure. This is an important result, as it indicates that a shorter duration of administration of oral vaccine is more effective and more economical as well.

8.3.4 Tissue Targeting and Stability of Antigen in Maize Seed

S-antigen expression in maize seed was directed by a constitutive polyubiquitin promoter and the protein was present in endosperm, aleurone, and embryonic tissues. Whole seed expressed antigen at a level of 25 μg/g. Fractionation to determine the sites of protein accumulation within the seed showed that the embryo (germ tissue) accumulated the highest concentration of recombinant protein of 50 μg/g—double the concentration of whole grain. The levels in bran (aleurone and pericarp layers) were very low, and endosperm contained levels in between those in grain and pericarp (Lamphear et al. 2002).

Stability of antigen was examined in whole grain stored for 10 months at ambient temperature without humidity monitoring, at 10 °C in a seed storage facility at 50 % humidity, and in corn meal stored at 4 °C (Fig. 8.5). Levels of antigen in stored grain were compared to freshly harvested grain, and no difference was found. This important result indicates that antigen is stable even under uncontrolled conditions at ambient temperature, making it less onerous for storage and transport over long periods and permitting elimination of the cold chain for transport and delivery.

8.3.5 What Expected or Unexpected Hurdles Were Overcome to Reach Target

TGEV-S antigen was not a novel target for expression, but several hurdles that limited its efficacy were overcome. Maize, as the bioencapsulating agent, presumably prolonged exposure to GALT tissues. High levels of expression and stability were achieved using a constitutive promoter and an apoplast targeted BAASS sequence. The antigen was stable in stored grain, overcoming a major economic hurdle.

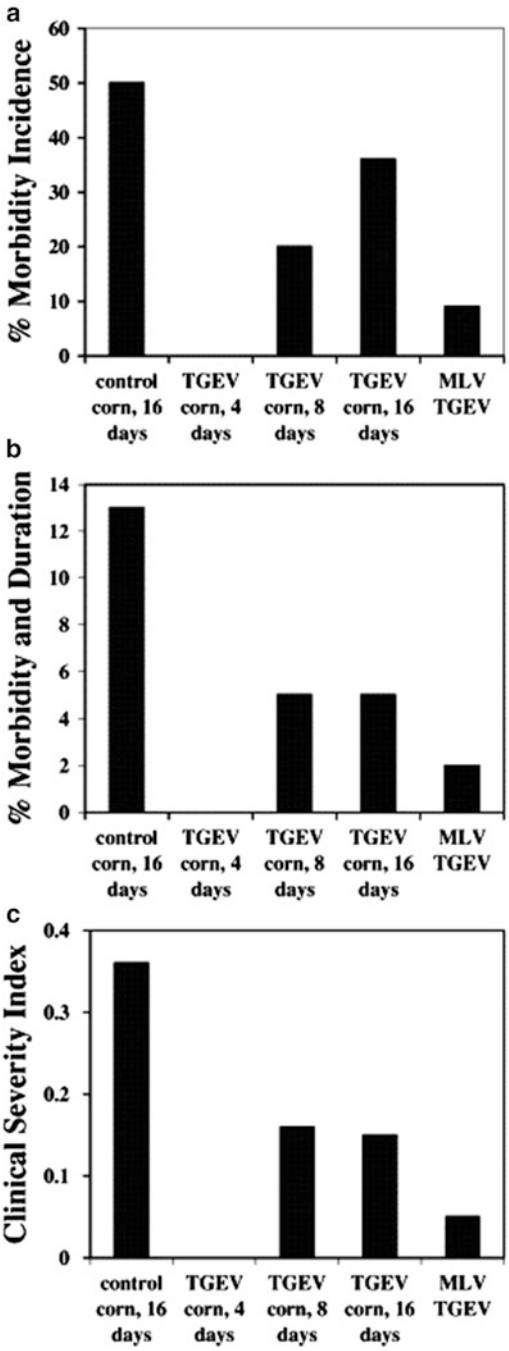

Fig. 8.4 Protection against TGEV of 10–12-day-old specific pathogen-free piglets fed with either control corn or transgenic corn expressing the S-protein (TGEV corn) for 4, 8, or 16 days as indicated. Positive controls were administered modified live vaccine (MLV-TGEV) orally. The panels show: (**a**) percent morbidity incidence, (**b**) percent morbidity incidence and duration, and (**c**) clinical severity index. See text for description of methods. Reproduced with permission from Lamphear et al. (2002)

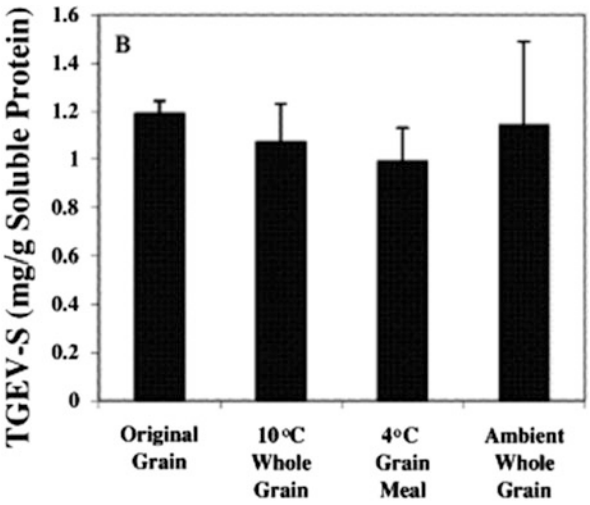

Fig. 8.5 Antigen stability in tissues from transgenic corn seed: measurement of extracted TGEV-S antigen as mg antigen per g extracted soluble protein from grain or grain meal stored for 10 months at either 4 °C, 10 °C, or at ambient temperature in a grain storage facility in Iowa. Values represent mean ± one standard deviation. Figure reproduced with permission from Lamphear et al. (2002)

In this work, it was also unexpectedly discovered that the smallest dose regimens, administered purely by the oral route, were also the most effective. Significantly, when young pigs were fed a constant diet of the antigen for 2 weeks, protection was reduced, an indication that tolerance may develop with long-term exposure (Lamphear et al. 2002). The duration of protection for piglets following antigen administration in feed was not tested. Administration of antigen over a 4-day period gave the highest levels of protection against live challenge—even higher than oral vaccination with a modified live virus (Fig. 8.4; see Sect. 8.3.3).

In gilts, as well, the shortest oral regimen of two boosters showed the highest colostrum levels of serum neutralizing antibodies. Thus, if passive immunity transferred by colostrum is protective in suckling pigs, then this regimen would be more economical as well as less onerous. In gilts, colostrum levels of antibodies persisted for about 3–5 days following piglet birth (Lamphear et al. 2004), which provides passive immunity while the piglets developed their gut flora and become suitable subjects for the administration of vaccines in feed.

8.4 Nontechnical Hurdles

8.4.1 Regulatory Compliance

Regulatory approval must be obtained for each step of the process, including compliance for growing transgenic plants to licensure of the vaccine product. Plant-based vaccines have already been approved for use with livestock and with humans. In 2006, the USDA approved a plant-produced vaccine against Newcastle disease virus for administration to fowl by Dow AgroSciences. In 2012, FDA approved the first pharmaceutical product produced in plants, Elelyso™ (taliglucerase alfa), to Protalix Biotherapeutics and Pfizer. Thus, precedents now exist for both human and animal pharmaceuticals produced in plants. The existence of these plant-based vaccines smooths the path for the development and regulatory approval of future plant-based vaccines. Some essential regulatory procedure for growth of transgenic plants and approval of veterinary vaccines in the USA are described below.

8.4.1.1 APHIS Permits

Similar to the requirement for other vaccines produced in yeast or eggs, the plant crop must not inadvertently be intermixed with commodity food. One important feature of the plant production system is that field-grown plants have the potential to pollinate other food or feed crops. The APHIS arm of the USDA issues permits for the growth of transgenic corn. These include a number of growing restrictions to prevent intermixing of the crop inadvertently with other food and feed crops. To this point, the USDA has developed a highly restrictive set of isolation conditions for growing the crop, over and above the standard conditions for producing vaccines, including a one-mile isolation corridor from other corn. As maize pollen is relatively heavy, it normally only pollinates other corn plants within a few meters (Luna et al. 2001) making this an extreme precaution. Nevertheless, as the growing acreage needed for such a product is low (~70,000 acres, or $\leq$0.1 % of the total acreage of corn in the USA to produce two doses of 26 mg of vaccine at 13 mg antigen/kg corn for each of the estimated 65 million swine and assuming a yield of 150 bushels/acre), it is quite reasonable to find isolated areas to grow the crop. After harvesting, the corn can be processed into corn meal, blended to obtain precise dosing, and formulated to the final product.

8.4.1.2 Obtaining USDA Safety Clearance for Marketing Livestock Vaccine

Production of a vaccine is only the first step in the process towards licensing. The vaccine must be safe and effective in order to obtain USDA approval for use through the Veterinary Biologics program. Guidelines are set out by the USDA under the Virus-Serum-Toxin Act (21 U.S.C. 151–159).

8.4.2 Economic Considerations

8.4.2.1 Cost of Production

The cost of production is always a potential limitation for any product to be commercialized. In this case, since the product is produced in corn which is a feed source for pigs, it does not need to be purified thereby reducing a major expense. Furthermore, the corn itself has value as a feed product, further lowering the effective cost of the vaccine. The actual cost of the vaccine can therefore be calculated by the cost to keep the grain segregated from food and feed crops and loss in yield over commodity crops. Both of these costs are further reduced by having high levels of antigen in maize, thereby limiting the amount of corn needed to be grown. In theory and in practice, the cost should be lower than the traditional injected vaccines. The indirect benefit of not having to physically inject the animals provides another value proposition making this even more desirable.

8.4.2.2 Public Acceptance of Transgenics

The first group that needs to accept this product is the swine producers. This is largely dependent on their perceived need for added protection from the disease. Once this hurdle is overcome, there is always the concern over trying any new product. Acceptance by swine farmers, however, should be relatively straightforward since this product should cost less to administer than traditional vaccines.

Even though farmers may accept this relatively readily, the general public is leery of transgenic crops. On a rational basis, this product would not pose a threat to the general public for several reasons: (1) the product will be quickly digested and will not persist in the animal at the time of slaughter; (2) the protein itself is present in the food chain when animals are infected by the virus, and the use of vaccines limits its presence; (3) the protein itself poses no threat as it has no toxicity or enzymatic activity; (4) as a protein, the degradation products are amino acids that are common to all living organisms; (5) as pork is cooked, any protein would be quickly denatured prior to human consumption; and (6) growing maize expressing S-antigen will involve very small acreage, thus a nationwide concern would not be triggered in the event of an inadvertent exposure.

Unfortunately, logical arguments are not always sufficient; undoubtedly a subset of the general public will fear the product for reasons other than safety arguments. This can be due to a general fear of transgenic crops, vaccines, fear of new products, aversion to technology, or other fears. While all of these are considerable hurdles, they can be overcome if the benefits of this product outweigh the risks. In this case, the benefits can be determined by how much of a threat is perceived by the disease and the cost and efficiency of this approach can eliminate the threat.

8.4.3 Barriers to Commercialization

While the goal of most vaccine production in plants is commercialization, the dearth of products on the market speaks to the many barriers that must be crossed before the goal is achieved: (1) the product must be made at a level in plants that makes it commercially viable; (2) for scale-up with plants such as corn, adequate barriers to dissemination must be incorporated and APHIS approval obtained; (3) the product must be tested in the target animal to rule out negative side effects, and approval for marketing must be obtained; and (4) finally, a large investment to scale-up and market the product is needed. For large companies such as Dow AgroSciences, which take a product from the lab to the market, this is less formidable than for small biotechnology companies which need to move to market at speed lest funding wane while the process is still underway.

8.5 Conclusions

8.5.1 Overall Significance of This Work

Protection with a subunit antigen expressed in corn, exclusively by the oral route, is shown for the first time to be effective in piglets, the target species for immunization. This demonstration, using corn-encapsulated S-antigen administered orally as both primer and booster, could circumvent the need for parenteral vaccinations or oral immunizations with modified live virus, making the process of vaccinating large herds much more economical and less time-consuming than using injected vaccines.

This work has three significant outcomes: (1) the demonstrated use of an oral subunit vaccine in production of neutralizing antibodies in both adults and young pigs, (2) neutralizing antibodies from both active and passive immunity being protective to a direct challenge with live virus, and (3) short duration of oral exposure of antigen (4 days) being sufficient to develop complete protection from challenge.

The use of maize seed that can be administered directly through feed clearly shows that this approach provides protection that can be as good if not better than injectable products. Using antigen-expressing corn as a top dressing on feed has the additional advantage of also bioencapsulating the antigen, which extends its contact with GALT in the gut and provides a greater immune response. Storage stability has also been demonstrated, with both the whole seed and meal, at room temperature and with refrigeration. In short, this approach demonstrates that a practical, low-cost, heat-stable, orally delivered vaccine is achievable.

8.5.2 Additional Improvements That May Make This Product More Valuable

Production of subunit antigens in well-tolerated, edible plant tissue opens the door to a lot of possibilities. Several antigens to different diseases can be combined into a single vaccine by standard breeding techniques. Since antigens expressed in maize seed are tolerant to storage over long periods at ambient temperature, they can be stockpiled against zoonotic outbreaks. The elimination of the cold chain can be highly significant in rural areas. The use of the edible vaccine as both primary and booster increases convenience and lowers duration of administration to animals. Adjuvants can be co-expressed as needed to improve immunogenicity. Clearly, there is ample room for improvement of this technology.

8.6 Future Directions

The use of TGEV-S protein clearly shows commercial potential as described above. Using more recent technology, improvements could be made to produce a higher concentration of the antigen or having it targeted specifically to the embryo. This study also represents one of the first clear demonstrations of providing protection against a pathogen in animals, paving the way for other vaccine antigens to be tested.

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