ANIMAL HEALTH

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INTRODUCTION

Recent technological advances in transgenic plants expressing recombinant proteins have resulted in the possibility of paradigm shifts in the area of animal healthcare (Hood *et al.*, 1997). By expressing proteins in edible tissue, the possibility of delivering a healthcare product directly by ingestion of the edible tissue is now becoming a reality. In addition to the potential ease of application, the low cost of production and the enormous amounts of product that can be produced in a plant-based system offer the possibility of disease control on a large scale. The types of products possible to be delivered in this manner could include therapeutic proteins, growth promotants, vaccines, monoclonal antibodies and proteins with antimicrobial activity. To date most activity in this area has focused upon the development of plant-based vaccines.

The utilization of transgenic plants for the delivery of animal healthcare has several potential benefits over traditional routes.

- Transgenic plants are usually constructed to express only a small antigenic portion of the pathogen or toxin, eliminating the possibility of infection or innate toxicity and reducing the potential for adverse reactions as in the case of whole virus vaccines.
- Since there are no known human or animal pathogens that are able to infect plants, concerns with viral or prion contamination are eliminated.
- Production in transgenic crops relies on the same established technologies to sow, harvest, store, transport, and process the plant as those commonly used for food crops, making transgenic plants a very economical means of large-scale vaccine production. Large quantities of protein-based therapeutics could be produced very economically.

E.E. Hood and J.A. Howard (eds.), Plants as Factories for Protein Production, 103–118.

- Expression of the recombinant protein comprising the therapeutic or vaccine in the natural protein-storage compartments of plants maximizes stability, minimizes the need for refrigeration and keeps transportation and storage costs low.
- Formulation of multicomponent therapeutics or vaccines is possible by blending the seed of multiple transgenic corn lines into a single vaccine.
- Direct oral administration is possible when immunogens are expressed in commonly consumed feed plants, such as grain, leading to the production of edible vaccines. In the case of vaccines, the immunogen could be produced in a safe, directly edible or easily purified form for edible, oral, or even parenteral vaccines. These vaccines could be used in a stand-alone vaccination strategy, as a booster, or in combination with other vaccines and vaccination routes.

Edible therapeutics and vaccines from plant material could be directly delivered in the feed and could be produced cheaply in large volumes thus avoiding many costs associated with the administration of conventional treatments. The use of transgenic plant material for animal health has, to date, been limited to the field of edible vaccines. Consequently, it is the focus of this review to highlight results obtained in the development of edible vaccines to animal diseases. Vaccines from plants are particularly suitable for stimulation of mucosal immunity, since edible plant products can be delivered orally to reach the gut mucosal tissue and elicit an immune response at mucosal surfaces. Recent advances in technology make it now possible to express vaccine antigens at high levels in plants (Streatfield *et al.*, 2001).

With the advent of improved transformation technology in the past decade, transgenic plants have now been successfully used to express a variety of genes. Numerous genes have been cloned into a variety of transgenic plants including many enzymes that demonstrate the same activity as their authentic counterparts (Hood *et al.*, 1997, Pen *et al.*, 1992, Trudel *et al.*, 1997). In 1999 over 60 million acres of transgenic plants were grown indicating that the system of production of transgenic plants is stable and robust. In particular, many additional genes have been expressed in plants solely for their immunogenic potential, including viral proteins (Lee *et al.*, 2001, Daniell *et al.*, 2001, Gomez *et al.*, 1999, Mason *et al.*, 1996, McGarvey *et al.*, 1995, Thanavala *et al.*, 1995, Modelska *et al.*, 1998, Wigdorovitz *et al.*, 1999) and subunits of bacterial toxins (Arakawa *et al.*, 1997, Arakawa *et al.* 1999, Haq *et al.*, 1995, Mason *et al.*, 1998). Animal and human immunization studies have demonstrated the effectiveness of many of these plant-derived recombinant antigens in stimulating the immune system. The production of antigen-specific antibodies and protection against subsequent toxin or pathogen challenge demonstrate the feasibility of plant derived-

antigens for immunological use. Many of these plant-derived antigens induced an immunological response comparable to that of the antigens in the original pathogen in mice (Gomez *et al*, 1998, Mason *et al*, 1998, Modelska *et al.*, 1998, Wigdorovitz *et al.*, 1999) humans (Kapusta *et al.*, 1999), poultry (ProdiGene, unpublished data) and swine (Streatfield *et al.*, 2001). Characterization studies of these engineered immunogens have shown that plants have the ability to express, fold and modify proteins in a manner that is consistent with the authentic source.

Using 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 of the biotechnology industry 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 (Arakawa *et al.*, 1997, Gomez *et al.*, 1998, Mason, *et al.*, 1992). When administered orally by feeding, such antigens can induce an immune response (Streatfield *et al.*, 2001, Haq *et al.*, 1995, Mason *et al.*, 1996) and, in some cases, result in protection against a subsequent challenge with the pathogen (ProdiGene, unpublished data, Arakawa *et al.*, 1997, Mason 1998). Certain antigens expressed in plants have shown sufficient promise to warrant human clinical trials (Tacket *et al.*, 1998, 2000). This has led to optimism that the inherent advantages of plants can be used to dramatically change the way in which vaccines can be delivered, 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 competitively with the cost of other approaches.

A number of different plant systems have recently been under investigation for use as edible oral delivery systems. Of these, a system based on the use of transgenic maize seed appears to be the most realistic for a number of reasons. Among these reasons are the ability to introduce a grain-

based product directly into a feed or food system, the ability to utilize the already existing infrastructure for the production, harvesting, transportation, storage, and processing of the grain, the ability to deliver a product (both monovalent and multivalent) at a cost competitive with contemporary vaccines due to a low cost of goods, and a plant system amenable to transformation with highly developed and characterized genetics. The use of corn grain is being explored as a particularly convenient delivery system for edible vaccines using a commercial animal example, a vaccine against swine transmissible gastroenteritis virus (TGEV). Additionally, a vaccine directed against enterotoxigenic strains of *Escherichia coli* (ETEC) is being developed as a model system to further develop vaccines for animal health. 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 (Sixma *et al.*, 1991). Approximately 66% of ETEC strains harbor LT, and in about half of these strains LT is the only toxin present (Svennerholm and Holmgren, 1995). ProdiGene has expressed LT-B in corn and demonstrated its immunogenicity and efficacy when fed to mice as a model system for the development of edible vaccines for animal health (Streatfield *et al*, 2001).

Swine transmissible gastroenteritis (TGE) (Saif and Wesley, 1992) is recognized as one of the major causes of sickness and death in piglets particularly in areas with high concentrations of pigs or regions with poor sanitation. TGE is a highly contagious enteric disease that is characterized by vomiting, severe diarrhea and high mortality in piglets less than two weeks of age. The causal agent of TGE is a pleomorphic, enveloped single-stranded RNA virus belonging to the genus *Coronavirus* of the family Coronaviridae. The virion 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 (Laude *et al.*, 1990) Replication of virus in the villous epithelial cells of the small intestine results in the destruction or alteration of function of these cells. These changes lead to a reduction in the activity of the small intestine that disrupts digestion and cellular transport of nutrients and electrolytes. In small piglets this can lead to a severe and fatal deprivation of nutrients and dehydration. Following infection, pigs that have survived the infection are immune to subsequent infections presumably due to local immunity in the intestinal mucosa. Thus, since active immunity towards TGEV involves local immunity, presumably through the activation and secretion of intestinal SIgA, edible vaccines that target activation of the intestinal mucosa immune system are particularly attractive in the control of this disease. ProdiGene has generated transgenic maize plants that express the spike protein at high levels. Corn expressing the S protein of TGEV was fed to 13-day-old piglets for ten

days and subsequently challenged with a virulent Purdue strain of TGEV. This group of piglets was significantly protected from the disease in contrast to the control group that was fed non-transgenic corn. Results from a second trial duplicated these results (Streatfield *et al.*, 2001) demonstrating that the delivery of antigens in an edible oral form is efficacious.

Thus the development of edible vaccines offers the potential to aid in the control of enteric diseases such as ETEC and TGE. Edible vaccines from plant material could be directly delivered in the feed and could be produced cheaply in large volumes thus avoiding many costs associated with the administration of conventional vaccines. Vaccines from plants are particularly suitable for stimulation of mucosal immunity, since edible plant products can be delivered orally to reach the gut mucosal tissue and elicit an immune response at mucosal surfaces. Recent advances in technology make it now possible to express vaccine antigens at high levels in plants.

SELECTED EXAMPLES

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

It was first investigated whether LT-B produced in corn would induce an immune response when fed to mice. Mice were fed ground transgenic corn seed, then 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 mg 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 measured 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 mg 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.

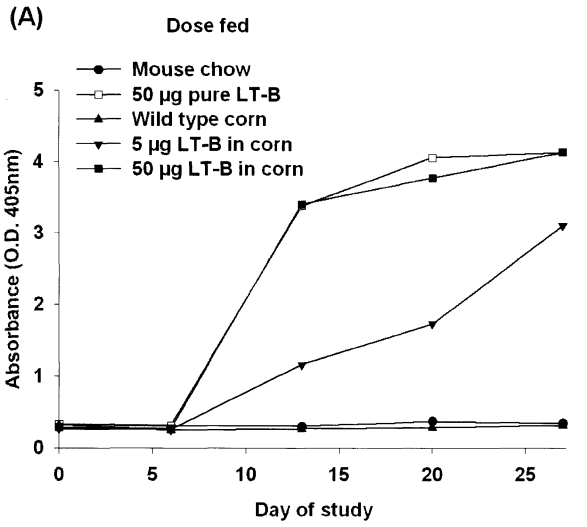

Figure 1A. *Protective immune responses of mice fed transgenic LT-B corn. Anti-LT-B specific Ig in serum. The mean response for the seven mice in each group is shown.*

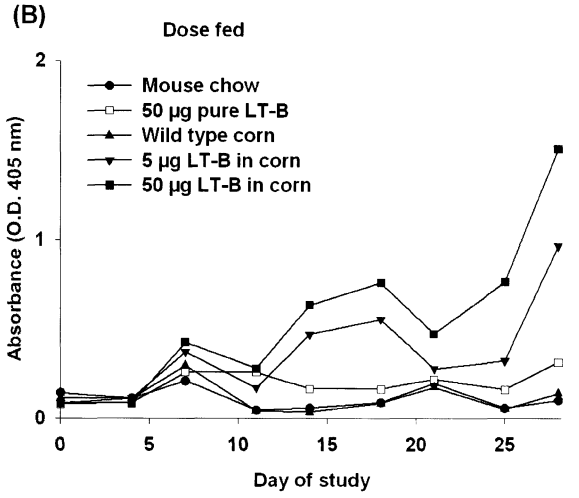

Figure 1B. *Anti-LT-B specific IgA in fecal material. The mean response for the seven mice in each group is shown; (C) The degree of gut swelling following challenge with LT holotoxin. Mean values for the weight ratios are shown with 95% confidence levels, and the sample size is given (n).*

In order to assess the efficacy of LT-B expressed in corn, LT-B corn was examined to determine if oral ingestion of LT-B corn could prevent gut swelling in mice exposed to the LT holotoxin. The upper intestines of a control group of mice swell when gavaged with LT, whereas those of mice fed LT-B expressed in corn do not swell when challenged with LT (Fig. 1C). Thus, LT-B expressed in corn appears to be protective against LT.

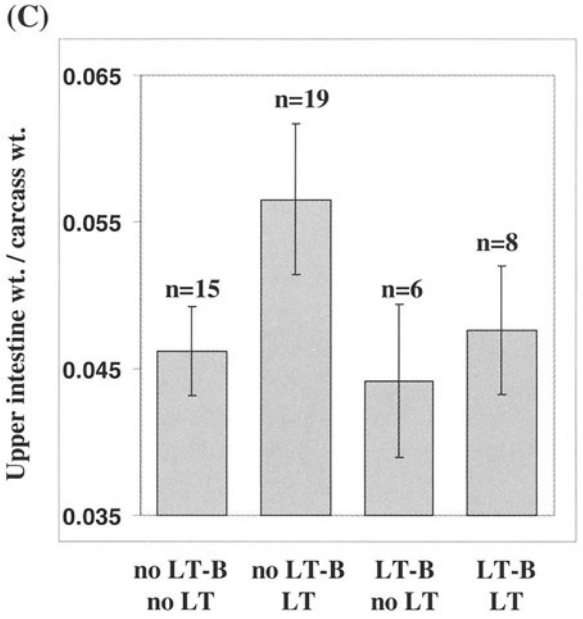

Figure 1C. *The degree of gut swelling following challenge with LT holotoxin. Mean values for the weight ratios are shown with 95% confidence levels, and the sample size is given (n).*

The S protein of TGEV expressed in corn is protective against viral infection.

Following the encouraging results with Lt-B, ProdiGene progressed to developing an edible vaccine against an economically important animal disease, TGE in swine. ProdiGene 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). Percent Morbidity Incidence was calculated as the number of animals with clinical signs > 2 divided by the total number of animals. Animals were monitored for clinical symptom development and scored on the appearance of various TGEV symptoms.

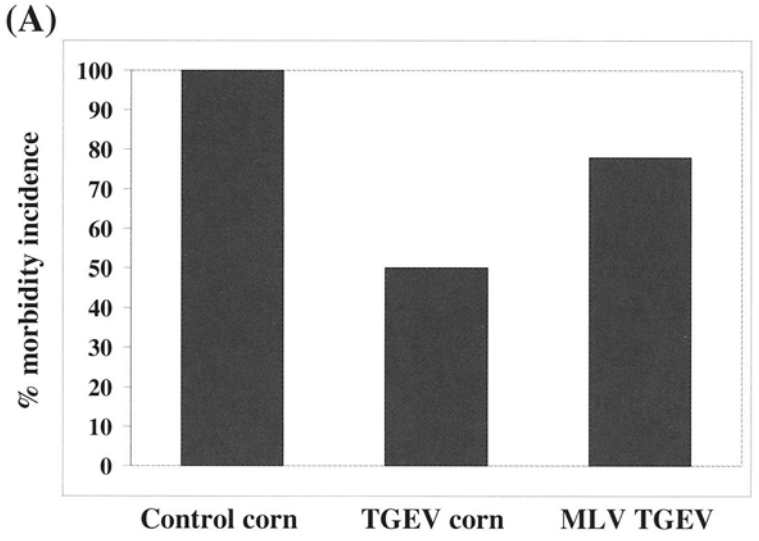

Figure 2A. *Percent morbidity incidence. Protection against TGEV of piglets fed transgenic corn expressing the S protein or modified live vaccine (MLV) TGEV.*

By comparison, only 50% of those animals 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. However, when duration of symptoms is considered, (Fig. 2B) along with the clinical severity index, (Fig. 2C) it appeared that piglets that received the modified live vaccine recover as quickly as those that were fed transgenic corn expressing the S protein. Further studies with higher levels of orally delivered antigens should refine these results.

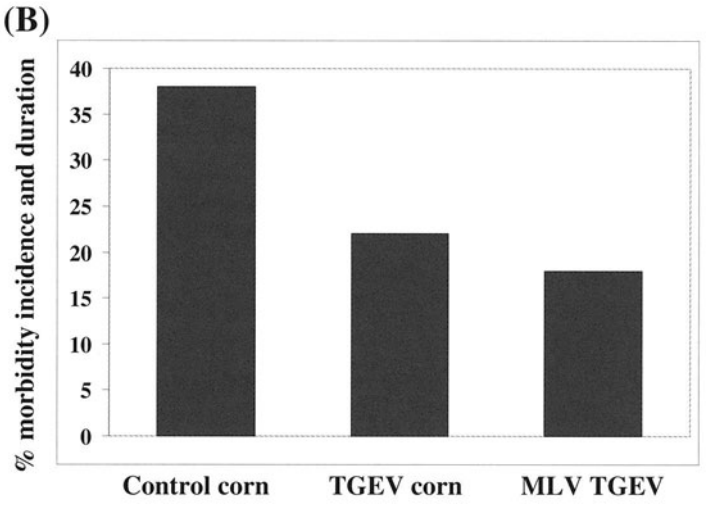

Figure 2B. *Percent morbidity incidence and duration*

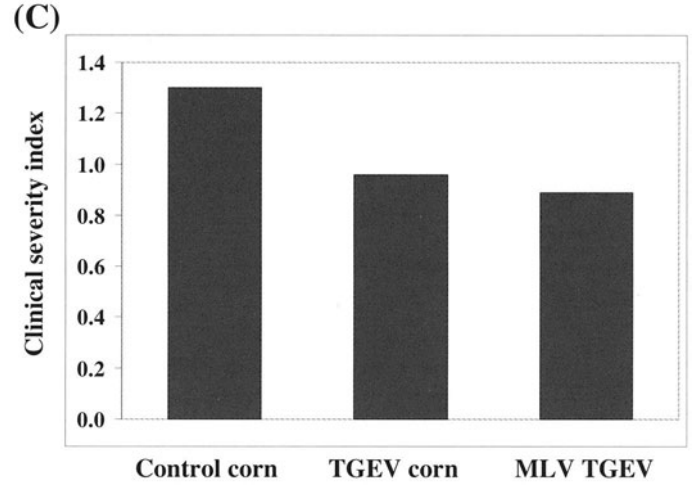

Figure 2C. *Clinical severity index. See text for definitions of clinical indices.*

Previously the full spike (S) protein was expressed in *Arabidopsis* (Gomez *et al.*, 1998). In this case, expression of the S protein was not detectable, yet plant extract injected intramuscularly into mice resulted in

the production of detectable anti- S serum. More recently, the S protein has been expressed in tobacco (Tuboly *et al.*, 2000). The S protein was expressed at levels that could be detected by ELISA and a protein of the expected size was seen when analyzed by Western blotting. Leaf extracts from these plants were injected into 28-day old pigs. In comparison to those pigs that were injected with nontransgenic plant extract, measurable TGEV-specific antibodies were detected in the pigs. ProdiGene has extended these results by generating transgenic corn expressing the S protein that was fed to pigs in a virulent TGEV challenge study. This is the first demonstration of protection of an economically important animal from a naturally occurring disease by an oral vaccination using an edible system. Moreover this system uses conventional feed materials, e.g. corn, to deliver the antigen. One report (Modelska *et al.,* 1998) has shown in the laboratory the amelioration of rabies symptoms in mice fed multiple doses of a chimeric plant virus expressing the rabies glycoprotein following inoculation with an attenuated rabies strain. To our knowledge, until our report no animals in conventional food animal husbandry have been vaccinated with edible vaccines and shown to be protected from the disease. The level of protection seen in this study includes general health and vigor, a decrease in clinical symptoms, lack of virus shedding and other observations known to be criteria in 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 a viral infection, or interference with parts of the viral replicative process.

THE FUTURE

Over the past decade, transgenic plants have been successfully used to express a variety of genes from bacterial and viral pathogens. Many of the resulting peptides induced an immunologic response in mice (Gomez *et al.*, 1998, Mason *et al.*, 1998, Wigdorovitz, *et al.*, 1999) and humans (Kapusta *et al.,* 1999) comparable to that of the original pathogen. Characterization studies of these engineered immunogens have proven the ability of plants to express, fold and modify proteins in a manner that is consistent with the authentic source.

Numerous genes have been cloned into a variety of transgenic plants including many enzymes that have demonstrated the same enzymatic activity as their authentic counterparts (Hood *et al.*, 1997, Hood and Woodard, this volume, Moldoveanu *et al.*, 1999, Trudel *et al.,* 1992). Animal and human immunization studies have demonstrated the effectiveness of many plant-

derived recombinant antigens in stimulating the immune system. (See Table 1).

Table 1: *Examples of edible vaccines under development.*

Antigen Source	Plant System	Reference:
Bovine Pneumonic Pasteurellosis	White clover	**Lee RW *et al.*** 2001.
Cholera toxin	Tobacco	**Daniell H *et al.*** 2001.
B subunit of the Escherichia coli enterotoxin (recLT-B)	Potato	**Lauterslager TG *et al.*** 2001.
Hepatitis B surface antigen (HBsAg)	Potato	**Richter LJ *et al.*** 2000.
Respiratory syncytial virus (RSV)	Tomato	**Sandhu JS *et al.***2000.
Spike (S) protein of transmissible gastroenteritis virus (TGEV)	Tobacco	**Streatfield *et al.*** 2001 **Tuboly T *et al.*** 2000.
Hepatitis B virus surface antigen	Lupin (*Lupinus luteus* L.) and lettuce (*Lactuca sativa* L.) cv. Burpee Bibb	**Kapusta J *et al.*** 1999.
Foot and mouth disease virus (FMDV).	Alfalfa	**Wigdorovitz A *et al.*** 1999.
Rabies	Lettuce	**Modelska A *et al.***1998.
Hepatitis B surface antigen	Potato	**Tacket CO *et al.*** 1998.
B subunit of the Escherichia coli enterotoxin (recLT-B)	Potato	**Mason HS *et al.*** 1998.
Norwalk virus	Tobacco, potato	**Mason HS *et al.*** 1996.

Some of the first attempts to make edible vaccines included transgenic potatoes expressing the *E. coli* heat-labile enterotoxin (LT-B) (Haq *et al.*, 1995) and a Norwalk virus surface protein (Mason *et al.*, 1996). In both cases, mice fed the antigenic tubers produced serum and secretory antibodies specific to the authentic antigen. Subsequently, many plant-expressed antigens, including those referenced above, have been shown to elicit an

immune response when administered through an oral route. Several of these antigens have shown sufficient promise to warrant human clinical trials (Mason *et al.*, 1998).

One of the most promising aspects of edible vaccines is the ability of orally administered immunogens to stimulate a mucosal immune response (Arakawa *et al.,* 1997) Mucosal surfaces, the linings of the respiratory, gastrointestinal, and urogenital tracts, play an important physical and chemical role in protecting the body from invading pathogens and harmful molecules. The mucosal immune system is distinct and independent of the systemic, or humoral, immune system, and is not effectively stimulated by parenteral administration of immunogens (Czerkinsky *et al* 1993). Rather, the mucosal immune system requires antigen presentation directly upon the mucosal surfaces. Since most invading pathogens first encounter one or more of the mucosal surfaces, stimulation of the mucosal immune system is often the best first defense against many transmissible diseases entering the body through oral, respiratory and urogenital routes (Holmgren *et al.*, 1994). Transgenic plants could produce large quantities of immunologically active recombinant antigen, very economically, for vaccine production. Multicomponent vaccines could easily be formulated from the seed of multiple transgenic plant lines to generate an increased chance for successful virus neutralization, in a stand-alone vaccination strategy, as a booster, or in combination with other vaccines and vaccination routes.

The use of transgenic grain for the delivery of animal healthcare based on preliminary results appears to have a legitimate place in the treatment and prevention of animal diseases. Such an approach will blur the line between traditional animal healthcare products and animal nutrition. Many parameters remain to be explored in the development of this technology. Some of these parameters include marketing and delivery of the product to the customer, the ability to fit the product into conventional husbandry techniques and the value and pricing of such products.

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