

Transgenic plants as vaccine production systems

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Transgenic plants that express foreign proteins with industrial or pharmaceutical value represent an economical alternative to fermentation-based production systems. Specific vaccines have been produced in plants as a result of the transient or stable expression of foreign genes. It has recently been shown that genes encoding antigens of bacterial and viral pathogens can be expressed in plants in a form in which they retain native immunogenic properties. Transgenic potato tubers expressing a bacterial antigen stimulated humoral and mucosal immune responses when they were provided as food. These results provide 'proof of concept' for the use of plants as a vehicle to produce vaccines.

The development of genetic transformation technology for plants has facilitated the study of plant gene expression, and has resulted in great progress toward the genetic design of plants with enhanced production traits (such as herbicide, insect and disease resistance). Recently, several academic and industrial laboratories have begun experimenting with transgenic plants as novel manufacturing systems. We introduced the concept of vaccine production in transgenic plants in 1992 (Ref. 1). This effort was stimulated by interest in evaluating the capacity of plants to produce different classes of proteins of pharmaceutical value, and because of the practical need for new technology for the production and delivery of inexpensive vaccines, especially in the developing world. Candidate vaccines should be a useful test system for evaluating the capacity of transgenic plants to produce pharmaceutically active proteins, because the immune system would amplify the biological response to even relatively low levels of foreign protein.

On a more applied level, the announcement of the Children's Vaccine Initiative² documented the need for new vaccine technology to combat infectious diseases. We hypothesized that plants could be a useful system for producing vaccines, because large amounts of antigen could be produced at a relatively low cost, using agriculture instead of sophisticated and expensive cell culture-based expression systems. In this review, we will discuss the progress that has been made by several groups in what is now an expanding area of vaccine research that utilizes transgenic plants.

Oral vaccines and mucosal immune responses

Many infectious agents colonize or invade epithelial membranes; these include bacteria and viruses that are

transmitted in contaminated food or water or by sexual contact. Vaccines that are effective against these infections must stimulate the mucosal immune system to produce secretory IgA (S-IgA) at mucosal surfaces such as the gut and respiratory epithelia^{3,4}. In general, a mucosal immune response is more effectively achieved by oral, rather than parenteral, antigen delivery. Several particulate antigens have proven to be effective oral immunogens, including live and killed microorganisms. By comparison with parenteral immunizations, oral immunization using subunit or soluble antigens is often inefficient at stimulating an immune response, and requires larger amounts (mg versus μ g) of antigen⁵.

Subunit vaccines based upon recombinant cell-culture expression systems are feasible but, for commercial-scale production, these systems require fermentation technology and stringent purification protocols so that sufficient amounts of recombinant protein can be obtained for oral delivery. Even with technological improvements, fermentation-based subunit vaccine production may be a prohibitively expensive technology for developing countries where novel oral vaccines are urgently needed. Transgenic plants that express antigens in their edible tissue might be used as an inexpensive oral-vaccine production and delivery system⁶; therefore, immunization might be possible simply through consumption of an 'edible vaccine'.

The choice of which antigens to use in the initial studies has been strongly influenced by the desire to determine if transgenic plant materials containing foreign antigens will result in oral immunization and stimulate a mucosal immune response. Because of the need in the developing world for new oral-vaccine technology against diarrheal diseases², antigens from enteric pathogens have been the early targets for plant-based expression. In addition, effort has focused on the

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production of antigens that assemble into ordered structures, such as virus-like particles (VLP), with the hope that they will be more resistant to digestion, more likely to reach the gut-associated lymphoid tissue (GALT) and, thus, more likely to be perceived as a foreign antigen once it reaches the GALT (Refs 3,4).

The choice of plant system for testing recombinant antigen production was initially driven by convenience and the need to evaluate genetic constructs quickly. For this reason, tobacco plants were generally utilized^{1,7} but, because of the high levels of toxic alkaloids in the leaves, studies on animals feeding on tobacco are not practical without substantial purification of tobacco-derived antigens. Therefore, potato has been used as the plant of choice in several of the studies reported below^{6,7}. This decision was based upon the observation that mice would accept raw potato tubers in lieu of laboratory feed, and because tubers can be generated for feeding studies within a few months of the transformation process⁸. With the purpose of acquiring a delivery system for humans, we have recently developed a genetic transformation system for banana⁹, and are investigating the requirements for expressing abundant proteins in banana fruit. Crops used for animal feed, such as alfalfa, grains and beans, are obvious choices for animal vaccines, although the difficulty of generating uniform transgenic samples of these species has prohibited their use in most early studies.

Systems for expressing foreign proteins in plants

Two different strategies for transgene expression (for candidate vaccine production) in plants have been evaluated (Fig. 1). These involve either: (1) stable genomic integration, with foreign DNA introduced either by *Agrobacterium* T-DNA vectors or by direct means (including microprojectile bombardment); or (2) transient expression using viral vectors. Stable expression affords the advantage of the subsequent generation of large numbers of transgenic plants, either by vegetative or sexual means, and the opportunity to introduce more than one gene for possible multi-component vaccine production. In addition, judicious choice of genetic regulatory elements allows organ and tissue-specific expression of foreign antigens. Transient expression is less easy to initiate, because the viral vector must be inoculated into individual host plants; however, greater yields of foreign protein can usually be recovered. Published reports on both types of expression system are summarized below.

Stable genomic transformation using genes encoding foreign antigens

Streptococcus mutans spaA protein

The first report of the concept of using a plant expression system for the production of an edible vaccine appeared in a patent application published under the International Patent Cooperation Treaty¹⁰. It described a means to express a surface protein (spaA) from *S. mutans* in tobacco plants to a level of approximately 0.02% of the total leaf protein; the gene had been stably inserted by *Agrobacterium*-mediated trans-

formation. Data were presented on the oral immunogenicity of spaA produced in *Escherichia coli*, which stimulated the production of S-IgA in saliva. No further reports of these studies have been published.

Hepatitis B surface antigen (HBsAg)

The expression of HBsAg at levels equal to 0.01% of total soluble protein in tobacco has been demonstrated¹. The tobacco-derived recombinant HBsAg (rHBsAg) was recovered from leaf extracts as a VLP with an average size of 22 nm, which is important because the particle form of HBsAg is required for immunogenicity¹¹. The plant-derived VLPs mimic the appearance of recombinant yeast-derived HBsAg particles¹², which is the material that is used in the currently available recombinant vaccine for hepatitis B (Recombivax®; distributed by Merck, Sharpe, and Dohme). In addition, the plant-derived material had similar buoyant density and antigenicity to human- and yeast-derived HBsAg, indicating faithful preservation of protein folding characteristics in the plant system¹.

A crude extract of rHBsAg from plants was used in parenteral immunization studies with mice¹³. The extract caused an immune response that was similar to the one achieved with Recombivax®, and included all the IgG subclasses, as well as IgM. Because T-cell-mediated immunity is essential for the prevention of hepatitis B, the fidelity of the T-cell epitope expressed by the tobacco-derived rHBsAg was investigated¹³. T cells isolated from the lymph nodes of mice, which were primed by parenteral immunization with the tobacco-derived rHBsAg, could be stimulated to proliferate *in vitro* by the tobacco-derived and yeast-derived rHBsAg. In total, the studies of rHBsAg from plants conclusively demonstrate that B- and T-cell epitopes of HBsAg are preserved when the antigen is expressed in transgenic plants, and that the recombinant antigen is produced as a VLP that mimics the currently available commercial vaccine.

E. coli heat-labile enterotoxin B subunit and cholera-toxin B subunit

In developing countries, diarrheal disease is an important cause of mortality, especially among children. Bacteria which cause diarrhea include *Vibrio cholerae* and the related enterotoxigenic *E. coli*. An oral vaccine composed of the cholera-toxin B subunit (CT-B) with killed *V. cholerae* cells gives protection against cholera and enterotoxigenic *E. coli* (Ref. 14). However, international health organizations have not distributed this vaccine because the cost of production of CT-B is too high for developing countries to afford.

The heat-labile enterotoxin (LT) from *E. coli* is a multimeric protein that is structurally, functionally and antigenically very similar to cholera toxin (CT). X-ray crystallography¹⁵ has been used to determine that LT has one A subunit (LT-A), with an M_r of 27 kDa, and a pentamer of B subunits (LT-B), each of which has an M_r of 11.6 kDa. Specific binding of the nontoxic LT-B pentamer to the G_{M1} gangliosides present on

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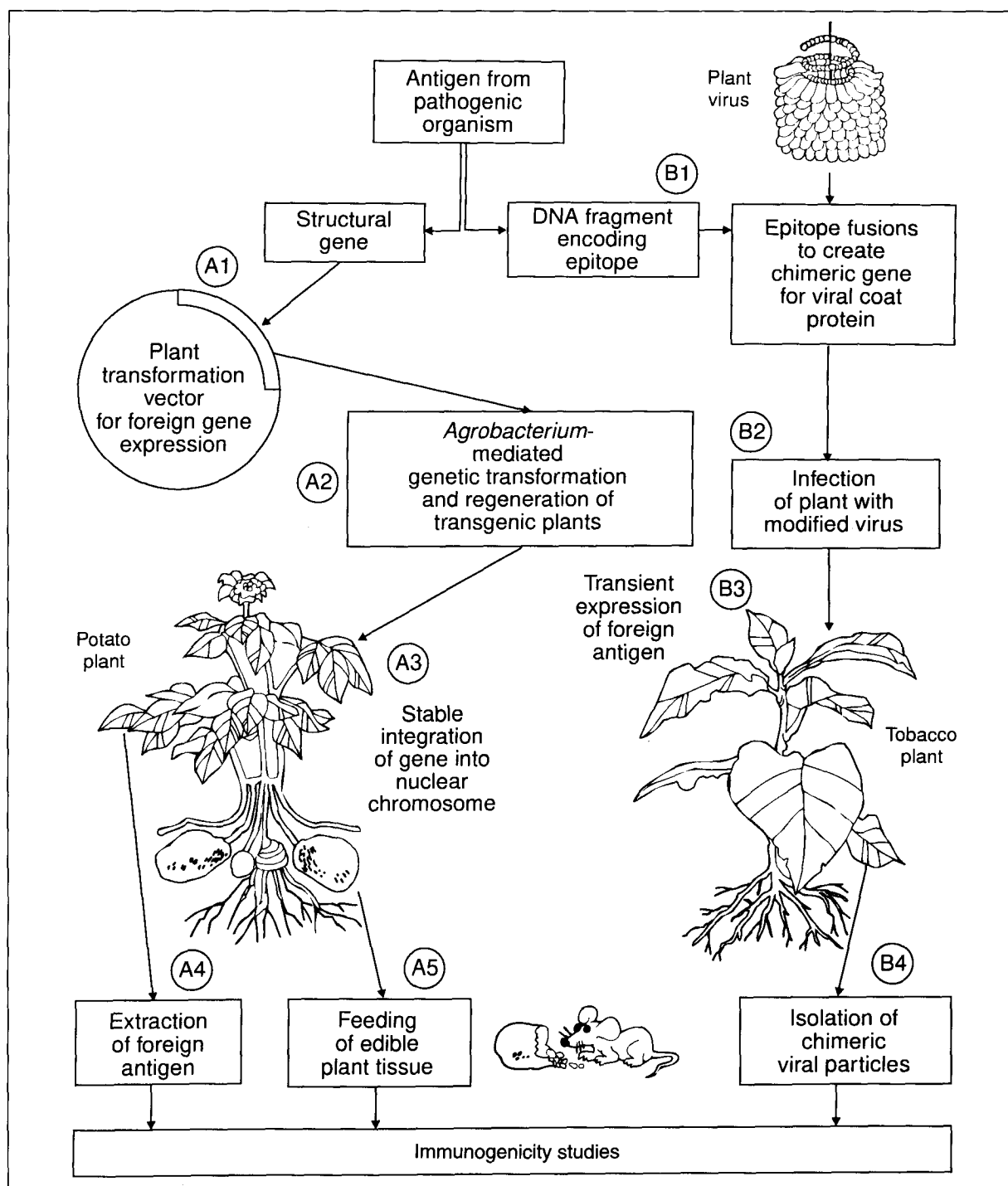


Figure 1

Strategies for the production of candidate vaccine antigens in plant tissues. Genes encoding antigens from pathogenic organisms (viruses, bacteria or parasites) that have been characterized and for which antibodies are available, can be handled in two ways. In one case, the entire structural gene is inserted into a plant transformation vector between 5' and 3' regulatory elements (**A1**); this will allow transcription and accumulation of the coding sequence in all, or selected, plant tissues. This vector is then used for the *Agrobacterium*-mediated transformation of plant cells (**A2**), or for stable integration of the expression cassette by other means, and regeneration of transgenic plants. The resulting plants contain the expression cassette stably integrated into the nuclear chromosomal DNA (**A3**), and can be used either for extraction and partial purification of the foreign antigen (**A4**), or for direct feeding of plant tissues (**A5**; in this case, a potato tuber) for assessment of immunogenicity. Alternatively, if epitopes within the antigen are identified, DNA fragments encoding these can be used (**B1**) to construct chimeric genes by fusion with a coat protein gene from a plant virus, e.g. tobacco mosaic virus (TMV) or cowpea mosaic virus (CPMV). The recombinant virus, or in the case of TMV and CPMV, even the viral RNA made *in vitro* from the plasmid clone, is then used to infect established plants (**B2**). Virus replication and systemic spread allow high-level transient expression of the chimeric coat protein in most plant tissues (**B3**). The viral particles, expressing the foreign epitope on their surfaces, are then purified (**B4**) and used for immunogenicity studies.

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epithelial cell surfaces allows entry of the toxic LT-A subunit into cells. LT-B and CT-B are both potent oral immunogens¹⁶.

LT-B has been expressed in transgenic plants, although the levels of expression were low⁷. The recombinant LT-B (rLT-B) produced in tobacco and potato showed enhanced accumulation when a C-terminal microsomal-retention sequence (Ser-Glu-Lys-Asp-Glu-Leu) was added. Sequestration of the rLT-B within microsomal vesicles may favor the association of individual subunits into the more stable pentameric form of LT-B. The tobacco-derived rLT-B appeared to be at least partially pentamerized, as judged by gel-permeation chromatography and its ability to bind gangliosides⁷.

The oral immunogenicity of rLT-B was tested in mice and compared with bacterial LT-B (Ref. 7). When given orally to mice by gastric intubation, the plant-derived antigen stimulated humoral and mucosal immune responses, with titers comparable to the bacteria-derived LT-B. In addition, the antibodies produced against the tobacco-derived LT-B were able to neutralize LT activity, indicating the potential protective value of the immune response. The oral immunogenicity of unpurified rLT-B was also assessed by feeding raw transgenic potato tubers to mice. After only four feedings of 5 g tuber samples to mice, mucosal and serum antibodies were recovered. No immune response was observed in animals that were fed non-transformed tubers. This demonstrates that a food source containing a foreign antigen can induce oral immunization.

It should also be noted that CT and LT are excellent oral adjuvants, which stimulate immune responses against co-fed antigens^{16,17}. Co-ordinate expression of A and B subunits to form the holotoxin in plants could enhance the vaccine value of other less immunogenic plant-expressed vaccine antigens expressed in the same tissues. This strategy is feasible because CT and LT function as adjuvants at concentrations well below those that cause diarrhea^{16,17}.

Transient expression of candidate vaccines using viral vectors

Using viral vectors for transient expression in plants represents a potentially useful means of producing high levels of recombinant antigens (Fig. 1). With tobacco mosaic virus (TMV), there are two ways to attain foreign-protein expression: (1) foreign-gene transcription, driven from a subgenomic promoter; and (2) fusion of foreign proteins or peptides with the capsid protein that normally coats the virus. The first strategy has been used to produce high-level expression of α -trichosanthin, an antiviral protein, in transfected plants¹⁸, but the length of the foreign DNA insert that can be tolerated is undetermined. Capsid-protein fusions may be a better strategy, especially because the foreign protein is in particulate form (TMV virions), which is highly immunogenic¹⁹. Although the recombinant virus would need to be highly purified for parenteral administration, or partially purified for oral adminis-

tration, this strategy may prove to be a cost-effective alternative to cell culture-based recombinant expression.

Malarial epitope fusions with TMV capsid protein

Turpen *et al.* have described a method for engineering the capsid protein of TMV as either internal or C-terminal fusions with peptides carrying epitopes derived from malarial sporozoites¹⁹. Both internal and C-terminal fusion constructs yielded high titers of genetically stable recombinant virus when used to infect tobacco plants. Antigenicity, measured by enzyme-linked immunosorbent assay (ELISA) and western blot, showed that the recombinant capsid proteins were recognized by the appropriate monoclonal anti-malarial antibodies.

Zona pellucida protein fusion with TMV capsid protein

The zona pellucida ZP3 protein of mammalian oocytes has been a target for immune contraception, and an epitope of 13 amino acids from murine ZP3 has now been expressed in plants as a fusion with TMV capsid protein²⁰. The recombinant virus accumulated to high levels in infected plants, although systemic movement was somewhat slower than for the wild-type virus, and the viral particles were smaller. Mice immunized with the recombinant virus developed antibodies against ZP3 that were recruited to the zona pellucida; ovarian pathology was seen, but there was no observable effect on the fertility of the treated mice. Further work with extended epitopes or epitope mixtures may yield better results.

Cowpea mosaic virus capsid protein fusion

Cowpea mosaic virus (CPMV) has recently been developed as an expression system for the presentation of foreign peptides^{21,22}. The advantages of CPMV are: high yield (1–2 g of virus per kg of host tissue), thermostability and ease of virus purification. CPMV is an icosahedral particle containing 60 copies each of large (L) and small (S) coat proteins. Earlier work showed that a foot and mouth disease virus epitope could be expressed as an S-protein fusion²¹, but problems arose with the loss of the inserted RNA during serial passage. Modification of the chimeras resulted in genetically stable fusions, and the system has now been used to produce virus-expressing epitopes derived from human rhinovirus 14 and human immunodeficiency virus (HIV-1) that are immunogenic in test animals²². Furthermore, the antibodies raised against the CPMV-HIV chimera were able to neutralize three different strains of HIV-1 (Ref. 23). Inserts as long as 30 amino acids have been used in CPMV chimeras²¹, although a particular sequence may have unpredictable effects on the systemic spread of the virus, thus compromising virus yield.

Future prospects

The demonstration that vaccine antigens can be produced in plants in their native, immunogenic forms opens exciting possibilities for the 'bio-pharming' of vaccines. If the antigens are orally active, food-based

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'edible vaccines' could allow economical production and delivery in developing countries. Some engineering challenges remain, including maximizing the expression of the antigenic proteins, stabilizing the foreign protein during post-harvest storage in plant tissues, and enhancing the oral immunogenicity of some antigens.

Concerns about allergy and immune tolerance of orally applied antigens must be addressed as this technology develops, and will be resolved only by collaborative research efforts with medical specialists in these fields. It must be determined whether the levels of antigen required to induce the desired oral immune responses are less than the levels that could induce tolerance, as happens with proteins that occur as normal components of our food. If so, a delivery scheme must be developed to provide only the 'edible vaccine' as a medicinal product at the required dosage level and not as a routine food source. The specific oral response (immunogenicity versus tolerance) may be antigen specific and may, therefore, need to be considered on an individual vaccine basis. Research will be needed to determine which types of plants are most suitable for vaccine delivery; this must be coupled with the discovery of means to express the protein at the desired levels in the appropriate plant cells and tissues. Lastly, a thorough study of the relationship between immunogenic dose responses and antigen levels in various foods needs to be undertaken. In the short term, vaccines for animals are a more likely target for edible-vaccine technology than vaccines for humans, and studies in this area are likely to increase our understanding of the basic mechanisms, which can then be applied to the development of all vaccines.

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