Using transgenic plants as bioreactors to produce edible vaccines

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Expression of antigens as vaccines, and of immune modulating antibodies using transgenic plants as bioreactors is a convenient and inexpensive source for production of high-interest immunotherapeutic molecules. Toward development of edible vaccines, transgenes of various antigens and antibodies have been expressed successfully in plants, and have been shown to retain their native functionalities. Antigens from several human and veterinary pathogens have been expressed in transgenic plants, including Norwalk virus, rabies, HIV, measles, hepatitis B, anthrax, infectious bursal disease virus, avian reovirus and avian influenza virus. High consideration is being given to addressing technical challenges that can limit expression of immunotherapeutic proteins at sufficient levels in plants. Fully harnessing the efficiency of plant systems' production of recombinant proteins will further support their use as bioreactors and provide efficacious next-generation alternatives to traditional vaccine production and administration protocols. Production of edible subunit-based recombinant vaccine proteins in the form of leaves, seeds or fruit is expected to be cost effective, and products will be easily stored and transported under limited refrigeration without degradation. Administration of commercial edible vaccines will require significantly less labor and technical training of medical and veterinary personnel. Despite these promising attributes, there still remain concerns and challenges with edible vaccine development, such as achieving maximum expression levels, possible immune tolerance and allergy, as well as environmental contamination concerns. Notwithstanding these issues, expression of recombinant proteins in transgenic plant bioreactors is currently under development for a number of human and animal diseases. This article attempts to describe current approaches used in the preparation of prospective edible vaccine proteins, as well as a success story in production of vaccine-quality recombinant immunoprotective proteins against chicken infectious bursal disease virus (IBDV) in *Arabidopsis thaliana* as a solid step in proof-of-principle for the continued development of edible vaccines technologies in plants.

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Introduction

The use of plants as bioreactors for production of recombinant proteins has become well established due in part to specific developments within plant genetics, molecular biology and biotechnology. Introduced as a concept about a decade ago, plant bioreactors are genetically modified (GM) plants whose genomes have been manipulated to incorporate and express gene sequences of useful proteins derived from other biological sources. In this respect, plant-based bioreactor systems offer several advantages over other methods of biological protein production. They are economically grown on agricultural land or in glasshouses and use low-cost inputs such as light, water and minerals. Plant bioreactor systems can be easily adapted to large-scale operations by simply increasing the number of plants. For example, with the current state of technological development, enough hepatitis B antigens to vaccinate all of the approximately 133 million live births in the world each year could be grown on roughly 200 acres of land [1, 39]. Compared with using bacteria or animal

cells for production, there is minimal risk of contamination with potential toxins or human pathogens utilizing plant bioreactor systems. Oral ("edible") delivery of subunit vaccine proteins has been shown more efficient compared to subcutaneous or intramuscular injection vaccines due to the increased chance of provoking mucosal immune responses, which in turn stimulate cell mediated responses [2, 3]. Another crucial advantage of edible vaccine technology is the multi-component ability that is possible due to the crossing of two plant lines [4]. Resulting multi-component vaccine proteins are known as second-generation vaccines as they allow for several antigens to approach M (microfold) cells simultaneously [4]. A multi-component edible vaccine can also be multivalent in that it can be designed to confer simultaneous protection against multiple diseases, such as enterotoxigenic *E. coli* (ETEC), cholera and totavirus [4]. As general admixtures, injected vaccines lack this feature.

The challenge for making plant pharmaceutical protein production viable, as identified by the European Union (EU)'s 2007 – 2013 *7th Framework Programme*, is that recombinant protein yield must be improved, as well as the biomass production of transgenic plants. Current research efforts to substantially increase transgenic plant biomass production are underway primarily utilizing *Arabidopsis thaliana*, a plant that is gaining application as a favorable plant bioreactor systems candidate [39].

What are the high-value proteins that can be produced in plant bioreactor systems? Primarily, we envisage proteins that will be used for pharmacological purposes toward combating human and veterinary diseases. Such proteins could include insulin, human growth hormone (HGH), antihemopoietic proteins (e.g., factor VIII), antibodies, or, as the model proteins of focus in our studies, antigens for edible vaccine production.

Plant-based production of high-value proteins as raw materials for pharmaceutical biologics is an emerging approach that is currently gaining substantial attention worldwide. The term "molecular farming" has been coined to describe this type of transgenic production of recombinant proteins in plants. (www.cost.esf.org/domains_actions/fa/Actions/Molecular_Farming). A number of studies on production of proteins in plants have been published in recent years showing the viability of the technique from a scientific point of view. Proteins such as somatropin and tetanus toxin have been readily manufactured [5, 6]. A large number of vaccine antigens have been successfully produced in plants and manufacturing of plant-produced antigens can very well be the best application for this technique in the future. Such antigens include the OspA protein of *Borrelia burgdorferi,* hepatitis B virus surface antigen, the B subunit of the *E. coli* heat labile enterotoxin, Norwalk virus antigen and measles antigens [7-11]. Oral immunization (i.e. feeding) of small animals with some of these edible antigens has induced significant vaccine-specific immune responses [12-15].

In humans, pilot experiments have shown measurable serum antibody responses after oral ingestion of transgenic plants expressing specific antigens [12, 16-19]. A promising HIV-1 subunit vaccine antigen candidate is the p24 protein. This structural capsid protein is relatively conserved between subtypes and is capable of eliciting a strong immune response since it contains several B- and T-cell epitopes [20-25]. Furthermore, an antibody response to the p24 protein can be detected early in infected individuals. Maintenance of a high anti-p24 response has been shown to correlate to non- or slow progression to AIDS, thus indicating a possible role of the p24 protein for induction of protective immune responses [25-27]. These characteristics make the p24 antigen an interesting subunit vaccine antigen candidate. In plants, the intact HIV-1 p24 protein has been expressed in transgenic

tobacco [28]. A genetically engineered construct consisting of sequences encoding antigenic determinants of HIV-1 *env* (gp41) and *gag* (p24) proteins together with HBsAg expressed in tomato has also been described [29].

Of great importance is the fact that a first commercial plant-produced pharmaceutical protein is currently going through clinical trials in North America and Europe. The Canadian company Sembiosys has obtained regulatory permission to perform clinical trials with human insulin produced in the plant safflower [30]. In this chapter we will highlight the facts and detailed procedures utilized in plant bioreactor studies, and potential applications for these technologies in therapeutic and prophylactic pharmaceuticals production.

Preparation of edible vaccines

1. Selection of the desired gene and plant

As the first important step, developing edible vaccines involves introduction of selected desired genes into plants and then inducing these altered plants to manufacture the encoded proteins. This process is known as transformation, and the altered plants are called transgenic plants. Toward development of edible vaccine subunit proteins, selection of important epitope region(s) from the pathogen of interest is the one of the key factors that determines the success of potential edible vaccines. A successful edible vaccine should ultimately be safe, non-pathogenic, and able to induce both mucosal and systemic immunity upon entry into the digestive tract. Efficacious edible vaccines should be able to resist the rigid acidic environment of the stomach, and reach the target cells in bioactive form. Selected antigen genes and their required expression machinery should be compatible with the selected plant type. Antigens in transgenic plants are delivered through bioencapsulation within the tough outer wall of plant cells. Bioencapsulation of recombinant antigens with transgenic plant cell vesicles protects the integrity of the antigens from gastric secretions until the plant cell walls degrade in the intestines. Upon degradation, antigens are released, taken up by M cells in the intestinal lining that overlay Peyer's patches and gut-associated lymphoid tissue (GALT). Subsequent antigen processing includes passage to macrophages, other antigen-presenting cells, and local lymphocyte populations. Following vaccination and subsequent exposure to the native pathogen, serum IgG, IgE and local IgA responses, and memory cells are triggered, which would promptly neutralize the attack by the real infectious agent. Like conventional subunit vaccines, edible vaccines are composed of antigenic proteins and are devoid of pathogenic genes. As such, edible vaccines cannot establish infection, which better assures safety, a particularly important consideration for vaccine regimens involving susceptible populations such as immunocompromised patients, children and the elderly. Conventional subunit vaccines can be expensive and technology-intensive, require complex purification, refrigeration, and produce poor mucosal responses. Oral administration protocols greatly reduce the need for trained medical personnel. Production of potential edible vaccine-quality proteins in transgenic plants is highly efficient and can be readily scaled up for commercial production. Transgenic plants can be engineered to produce immunoprotective proteins against infectious diseases, as well as some autoimmune diseases and human tumors. Transgenic potatoes, tomatoes, maize, rice, and soybeans have been developed and used in various plant bioreactor studies. The results of human trials that have tested several transgenic plant-produced recombinant therapeutic proteins have shown positive responses and no major safety concerns [39]. Transgenic plant-made vaccines are also being used in veterinary medicine. Livestock animals have been fed transgenic plants, including *Arabidopsis thaliana*, alfalfa and potato, expressing antigens to protect them from various pathogens, including foot-and-mouth disease virus (FMDV),

Table 1. Advantages and disadvantages of different plants as transgenic bioreactors. (Adapted from Lal *et al* [4].)

Plant/fruit	Advantages	Disadvantages
Tobacco	Good model for evaluating recombinant proteins Low-cost preserving system (numerous seeds, stored for long time) Easy purification of antibodies stored in the seeds, at any location Large harvests, number of times/year	Produces toxic compounds*
Potato	Dominated clinical trials Easily manipulated/transformed Easily propagated from its "eyes" Stored for long periods without refrigeration	Needs cooking, which can denature the antigens and decrease immunogenicity**
Banana	Do not need cooking Proteins not destroyed even if cooked Inexpensive Grown widely in developing countries	Trees take 2-3 years to mature Transformed trees take about 12 months to bear fruit Spoils rapidly after ripening Contains very little protein, so unlikely to produce large amounts of recombinant proteins
Tomato	Grow quickly Cultivated broadly High content of vitamin A may boost immune response Overcome the spoilage problem by freeze-drying technology Heat-stable, antigen-containing powders***, made into capsules Different batches blended to give uniform doses of antigen	Spoils readily
Rice	Commonly used in baby food because of low allergenic potential High expression of proteins/ antigens Easy storage/transportation Expressed protein is heat-stable	Grows slowly Requires specialized glasshouse conditions
Lettuce	Fast-growing Direct consumption	Spoils readily
Soybean and Alfalfa	Large harvests, number of times/year	
Musk melon (cantaloupe)	Fast growing Easily propagated by seed Easily transformed	
Others	Carrots, peanuts, wheat, corn	

*Currently, therapeutic proteins in tobacco are being produced. **Some kinds of South American potatoes can be eaten raw. Although some studies show that cooking does not destroy full complement of antigen in potatoes.[3] ***Freeze-dried tomato powder containing NV capsid and LT-B was found immunogenic. Same technology is also used for potatoes and carrots.

bovine rotavirus (BRV) and bovine viral diarrhea virus (BVDV).

Ideal plants for edible vaccine production should meet certain requirements, including accumulation of the antigen of interest in sufficient quantities, retention of the recombinant antigen immunomodulatory properties, and produce no antigen processing interfering effects. A summary of advantageous and undesirable characteristics among certain plants that have been studied for transgenic expression is presented in Table 1.

2. Vectors with plant-specific super promoters

Edible vaccine development has been challenged by low expression levels of foreign proteins in transgenic plants. Reported expression rates range from 0.01-2% total soluble protein (TSP), which can render edible vaccine proteins less immunogenic. Selection of strong plant-specific super promoters to improve expression levels is another key factor that can determine the success of edible vaccines. Our lab obtained a plant-specific super promoter (gift of Dr. S. Gelvin, Purdue University), and we have subsequently tested its expression of several immunologically

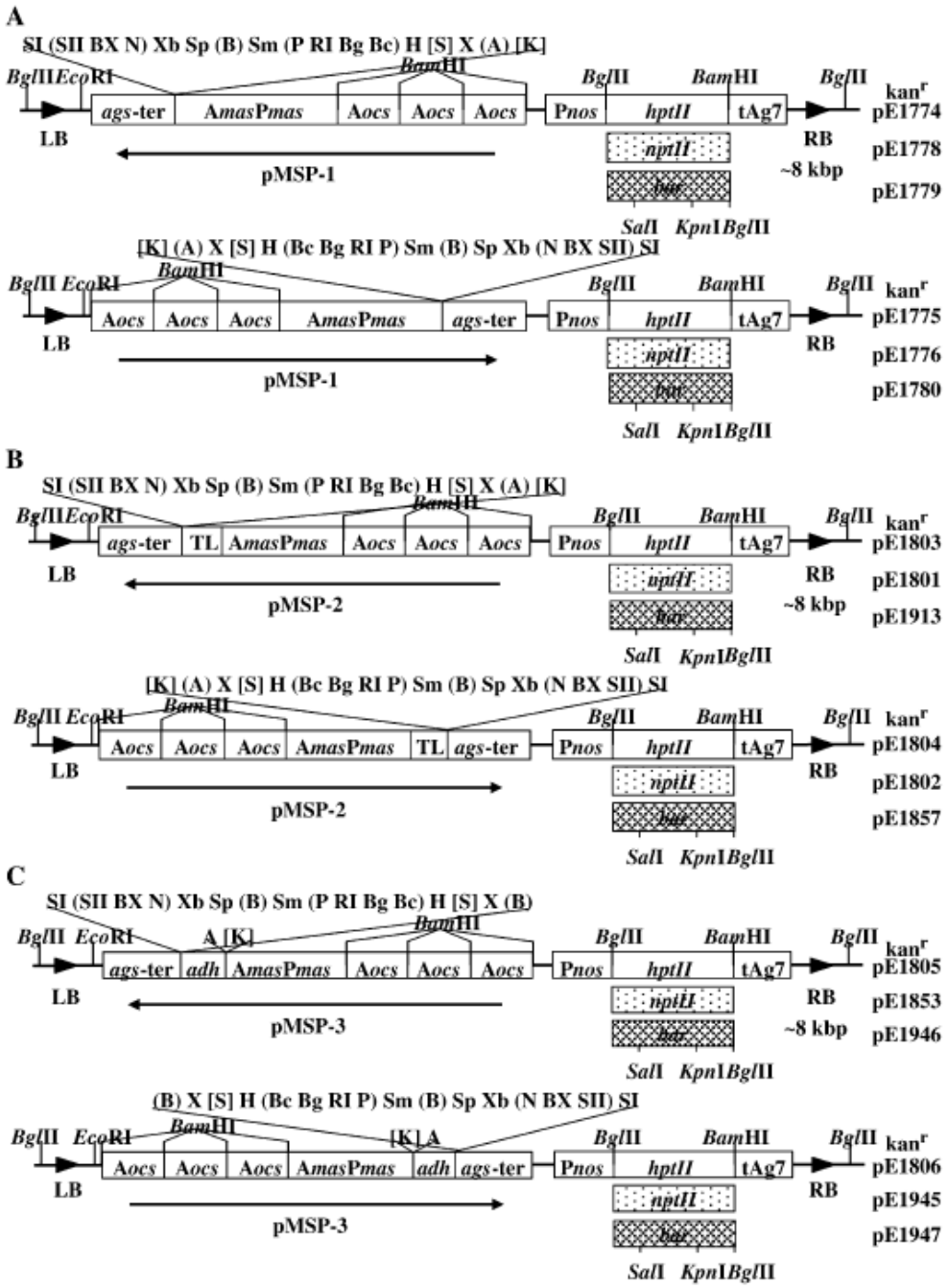

Figure 1. Physical map of super-promoter vector. (Adapted from Lee *et al* [31].)

important avian virus proteins over the past ten years, including infectious bursal disease virus (IBDV) VP2 protein, avian reovirus (ARV) sigma C, and avian influenza virus (AIV) HA antigen. Our studies confirmed the high efficiency of this vector, as our expression levels for IBDV VP2 protein reached 4.8%[37, 38]. The detailed vector construct physical map used in our expression studies is shown in Figure 1.

This super-promoter consists of a trimer of the octopine synthase transcriptional activating element affixed to the mannopine synthase2' (mas2') transcriptional activating element plus

minimal promoter sequence. Lee's group tested a super-promoter-β-glucuronidase A fusion gene in stably transformed tobacco *(Nicotiana tabacum*) and maize (*Zea mays*) plants and in transiently transformed maize Black Mexican Sweet protoplasts. In both tobacco and maize, superpromoter activity was much greater in roots than in leaves. In tobacco, superpromoter activity was greater in mature leaves than in young leaves, whereas in maize superpromoter activity differed little among the tested aerial portions of the plant. When compared with other commonly used promoters (cauliflower mosaic virus 35S, mas2', and maize ubiquitin), superpromoter activity was approximately equivalent to those of the other promoters in both maize Black Mexican Sweet suspension cells and in stably transformed maize plants [39].

3. **Plant transformation**

The production of transgenic plants is the same as farming regular crops; the differences lie in the transformation process of instilling proteins [32]. There are currently three methods used to produce transgenic plants: 1) gene-gun biolistic particle delivery, 2) *Agrobacterium tumefaciens*-facilitated transformation, and 3) electroporation, with the two most common methods being gene-gun and *A. tumefaciens* transformation [2]. Gene-gun transformation inserts the desired DNA into a target plant genome by bombarding embryonic suspension cell cultures [4]. Multi-copy and multi-site transgene insertions resulting in gene silencing is common issue using the gene gun method [2]. *A. tumefaciens*-mediated transformation is the most commonly used in producing transgenic plants. *A. tumefaciens* is a naturally occurring bacterium found in soil that is able to insert segments of foreign DNA into the plant by entering through wounds such as scratches [4]. It has a circular Ti plasmid (tumor inducing), which enables it to infect plant cells, integrate into their genome and produce a hollow tumor (crown gall tumor), where it establishes infection. These attributes can be exploited for insertion of foreign DNA into the plant genome. The Ti plasmid can be disarmed by deleting the genes for auxin and cytokinin synthesis such that tumor formation is eliminated.

4. Transgenic plant screening

Genes for antibiotic and herbicide resistance are used as markers to select for transformed cells and whole plants, which contain the foreign gene(s), and for expressing the desired product, at which time selected (transformed) cells and/or plants can be regenerated [33]. The gene(s) of interest integrate randomly into plant genomes, resulting in a different antigen expression level for each independent line [34]. As a result, 50-100 plants can be transformed simultaneously, and plants expressing the highest levels of antigen and least number of adverse effects can be selected for further analysis. Production of transgenic plants is species-dependent and takes 3-9 months. Reducing this time to 6-8 weeks is becoming possible by using real-time quantitative PCR(qPCR), a genetic approach that can help accelerate the selection process. Some antigens, like viral capsid proteins, require posttranslational modification and self-assembly into VLPs (virus like particles). These VLPs mimic the virus without carrying DNAor RNA and therefore are not infectious.

5. Evaluation of the protein in animal model

Each single antigen expressed in plants must be tested for its proper assembly, which can be verified by animal studies and Western blots, and quantified by enzyme-linked immunosorbent assay (ELISA)[35]. Specific protocols for orally administering high-value proteins (e.g. pharmaceutically interesting substances produced in plants) to humans and farm animals requires more scientific study in order to advise the future use of these compounds in industry and for pharmaceutical purposes. Formulations that are optimized for tablet production and retain the biological activity of the high-value therapeutic or prophylactic protein have the potential to introduce new applications based on edible vaccine research.

Concerns

There are many concerns which need to be answered in the future before edible vaccines can begin to gain a niche in the clinical and veterinary pharmaceutical markets, such as antigen selection, efficacy in systems, choice of plants, delivery, dosage, safety, the public's perception, quality control and licensing. Government policy on genetically modified (GM) foods may also influence the future of investment in edible vaccine research and development. We remain confident in the potential of this technology to improve the tools available to efficiently treat and prevent important human and veterinary diseases.

Conclusions

Edible plant-derived vaccines may lead to a future of safer and more effective immunization. Resulting therapeutic products would overcome some of the difficulties associated with traditional vaccines, like costly production, distribution and delivery. Edible vaccine studies demonstrate encouraging progress toward resolving major hurdles in these emerging commercial vaccine technologies. Before plant-based edible vaccines become product-ready, many technical and policy issues must be addressed. However, with limited access to essential health care in much of the world, and with the scientific community still struggling with complex diseases like HIV, malaria, etc, a cost-effective, safe and efficacious delivery system in the form of edible vaccines has the potential to become an essential approach to bringing new weapons forward in modern disease treatment and prevention.

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