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Vaccine antigen production in transgenic plants: strategies, gene constructs and perspectives

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Abstract

Stable integration of a gene into the plant nuclear or chloroplast genome can transform higher plants (e.g. tobacco, potato, tomato, banana) into bioreactors for the production of subunit vaccines for oral or parental administration. This can also be achieved by using recombinant plant viruses as transient expression vectors in infected plants. The use of plant-derived vaccines may overcome some of the major problems encountered with traditional vaccination against infectious diseases, autoimmune diseases and tumours. They also offer a convenient tool against the threat of bio-terrorism. State of the art, experimental strategies, safety and perspectives are discussed in this article.

Keywords: Edible vaccines; Genetically-modified plants; Plant viruses; Bio-terrorism

1. Introduction

Subunit vaccines are commercially produced in genetically engineered bacteria, yeast or mammalian cells. With the advent of genetic engineering of higher plants, attempts have been made to add transgenic plants to the list. The goal is to produce plant organs (leaves, fruit), crude extracts (dry protein powder) or purified proteins that upon oral or parenteral administration deliver one (or more) immunogenic protein(s) in a manner that triggers an immune response.

The applications of plants as protein production systems are wide and varied. The first demonstration of expression of a vaccine antigen within plants occurred in 1990 when Curtiss and Cardineau expressed the *Streptococcus mutans* surface protein antigen A (SpaA) in tobacco [22]. This demonstration was closely followed by plant expression of the hepatitis B surface antigen (HbsAg) [1,2], the *E. coli* heat–labile enterotoxin responsible for diarrhoea [1], the Norwalk virus capsid protein [1] and the rabies virus glycoprotein [3]. Proteins produced in these plants induced synthesis of antigen specific mucosal IgA and serum IgG when delivered orally to mice and humans. References [4,5] list proteins that have been expressed in genetically-modified plants (GM-plants) and are now being tested for their potential use as human or animal vaccines. The production of autoantigens in plants for oral tolerance therapy of autoimmune diseases has also been shown to be feasible [5,6]. In addition, attention is being directed to the production of epitopes in plants that target cytotoxic activity against tumours. Plants can also serve as bioreactors for the production and scale-up of functional antibodies used in immunotherapy [7], however the focus within this paper will be restricted to plant-derived therapeutics for active immunisation.

Approaches to meet the present public concern on the use of GM-plants and the spread of GM-pollen have been proposed [4,5,8,9].

2. Is there a need for plant-derived vaccines?

Definitely, the answer is yes. As outlined in Table 1, the production of recombinant vaccines in plants may overcome some of the major difficulties encountered when using traditional or subunit vaccines in developing and developed countries. In developing countries difficulties include vaccine affordability, the need for “cold chains” from the producer to the site of use of the vaccine and the dependence on injection. Plant-derived vaccines do not face these issues. In developed countries plant-derived vaccines offer increased safety, envisaged low cost of program for mass vaccination, and the wider use of vaccination for veterinary use [10].

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Table 1
Technical and social benefits envisaged in plant-derived edible vaccines

No.	Benefit	Characteristics
1	Oral delivery	The plant cell wall, consisting essentially of cellulose and sugars, provides protection in the stomach and gradual release of the antigen in the gut
2	Use as raw food or dry powder	The vaccinogenic plant tissue may be used as raw food, dried or, alternatively, proteins may be partially or fully purified and administered in capsules as dry powder
3	No need for "cold chain"	The vaccinogenic plant parts or plant extracts can be stored and shipped at room temperature
4	Mucosal and serum immune response	Plant-derived vaccines are primarily designed to trigger the mucosal immune system (IgA), thus preventing pathogen entry at mucosal surfaces; they also elicit serum and, possibly, cytotoxic responses
5	Cost efficiency	Production cost will be reduced 100–1000 times as compared with that of traditional vaccines
6	Optimised expression system	Plants may be engineered to accumulate the antigen in convenient intracellular compartments (endoplasmic reticulum, chloroplast)
7	Ease of genetic manipulation	Procedures essentially rely on established molecular and genetic manipulation protocols; these are already available in developing countries
8	Ease of production and scale-up	GM-plants can be stored as seeds. Unlimited vaccine quantity can be produced from these in limited time; production and management is suitable for developing countries
9	Safer than conventional vaccines	Lack of contamination with mammalian pathogens
10	Ideal to face bio-weapons	Safety and cost efficiency propose plants plant-derived vaccines as an ideal tool to face bio-terrorism
11	Ideal for veterinary use	Cost affordable Ready for use as food additive

3. Plant species

To date many plant species have been used for vaccine-production. Early studies used tobacco and potato but now tomato, banana, corn, lupine, lettuce and others are being used for this purpose [1,4,5]. The choice of the plant species (and tissue in which the protein accumulates) is important and is usually determined through how the vaccine is to be applied in the future. For example an edible, palatable plant is necessary if the vaccine is planned for raw consumption. This limitation is overcome in non-edible plants by vaccine antigen extraction and purification. Antigen extraction is often performed when using tobacco, a plant that offers considerable experimental advantages such as ease of transformation and extensive genomic sequence knowledge. Heat treatment is feasible only if there is no deleterious effect on antigen stability. Recently, a "cooked" GM corn snack that accumulates the *E. coli* heat–labile enterotoxin has been proposed. In the case of vaccines for animal use, the plant should preferentially be selected among those consumed as normal component of the animals' diet.

4. What are the targets for plant-derived vaccines?

4.1. Vaccines against infectious diseases

There is a large and fast growing list of protective antigens from microbial and viral pathogens that have been expressed by plants. The initial focus was upon human pathogens. However, today attention has also spread to animal pathogens (e.g. Newcastle and foot and mouth disease). There is no limit to the number and range of antigens that can be produced in plants if the DNA sequences coding for the appropriate genes are available.

4.2. Vaccines against autoimmune diseases

Transgenic plants expressing autoantigens are being produced in attempt to cure diseases in which the immune system recognises the body's own proteins as foreign. The diseases include arthritis, multiple sclerosis, myasthenia gravis, and type I diabetes. The rational is that an appropriate oral dose of a plant-derived autoantigen will inhibit the development of the autoimmune disease. Pioneering and recent work is described in [5,6].

4.3. Vaccines against human tumours

Particular proteins have been shown to over-express on the cell surface of many tumours, including melanoma and breast cancer. Naturally acquired, actively induced or passively administered antibodies against these antigens have been able, in some cases, to eliminate circulating tumour cells and micrometastasis. However, cancer vaccine development is complicated due to the tumour antigens also being auto-antigens [11,12].

In the last decade, immunologists have identified and characterised epitopes specific for different human tumours. For instance, an epitope specific, cytotoxic T lymphocyte response in mice was stimulated after injecting naked recombinant plasmid DNA carrying a poly-epitope isolated from a human melanoma tumour [13]. This DNA is now being integrated into the nuclear and chloroplast DNA of tobacco in attempt to develop a plant-derived melanoma vaccine (collaboration: Pasteur Institute, Paris, University of Milano, Italy, and University of Central Florida, USA).

5. The biotechnological approach: construction of appropriate gene expression cassettes, plant transformation, and efficiency of antigen expression

The production of a vaccine in plants depends upon the availability of a DNA sequence coding for a protective antigen and on the construction of an expression "cassette" suitable for plant transformation. Stable plant transformation currently offers two options: insertion of the foreign gene into the nuclear genome or into the chloroplast genome. Transient plant transformation has also been used for plant expression of vaccine antigens through integration of the gene of interest into a plant virus and subsequent infection of susceptible plants. Plants producing two or more antigens may also be obtained through transformation with multiple gene constructs or through sexual crossing. The strategies for plant expression cassette construction and plant transformation depend on the desired goal. Points worth noting are summarised in Table 2.

5.1. Stable integration of genes into the plant genome

The quantity of plant tissue that may constitute a vaccine dose must be of practical size both for field production and for consumption. Since the demonstration that low levels of a recombinant hepatitis B surface antigen (HbsAg) could be produced in GM potato and that the antigen assembled into spherical particles similar to those seen in infected human serum, efforts have been directed to increasing antigen expression and accumulation to a reasonable level [1,7,18].

A number of factors may modulate gene expression in plants. They include: codon usage; promoter, leader and polyadenylation signals; DNA sequences that target antigen accumulation to a specific tissue or cell compartment and others found listed in Table 2. The use of carrier proteins may also be required, especially for small, non-particulate subunit vaccine antigens. The observation that the LT-B, CT-B and HBsAg antigens are highly immunogenic when assembled into multi-subunit structures led to the finding that these structures may act as carriers for different candidate epitopes [1,2].

The site of gene integration into the genome also influences epitope and transgene accumulation in plants. *Agrobacterium tumefaciens* infection is most frequently used to achieve permanent integration into the nuclear DNA, where integration occurs at random chromosomal sites. A second promising approach is based on the integration of the gene or epitope into the circular chloroplast DNA (cpDNA) that is present in multiple copies within defined plant cells. In this case transformation is usually achieved through the use of the "particle gun" and results in site-specific integration [4,15]. Both nuclear and chloroplast genomes accept large and multiple gene inserts [1,17].

Advantages envisaged for cpDNA transformation are manifold: the cpDNA molecule (a circular DNA molecule of about 150 Kb) is fully sequenced in a number of important plants and is present to up to 10.000 copies per cell. Furthermore, it has been shown that chloroplasts can properly process eukaryotic proteins, including correct folding and disulfide bridges [17]. Integration into cpDNA has two

Table 2
Gene constructs, expression signals and peptide design for optimal vaccine-production in GM-plants

No.	Purpose	Approach and notes	References[a]
1	Optimise codon usage	Adapt codon usage to that preferred by plant genes	[1]
2	Optimise epitope sequence	Adapt A + T composition to that found in plant genes Eliminate sequences that destabilise or splice mRNA Minimise secondary structure hairpins	[1]
3	Select promoter	This may be: plant constitutive, tissue specific, inducible by environmental factors	[1]
4	Use leader and 3′-polyadenilation signals	Alternative signals affect protein accumulation Use TEV (the 5′untranslated region of the tobacco etch virus)	[7,14]
5	Target protein to the chloroplast	Integrate the DNA sequence in the nuclear DNA and use an N-terminal chloroplast transit peptide: the protein is accumulated in the chloroplast	
6	Target protein to the endoplasmic reticulum	Use an endoplasmic reticulum retention signal, such as SEKDEL	[1]
7	Integrate the epitope DNA in the chloroplast DNA	Integrate the DNA sequence in the chloroplast DNA under appropriate expression signals: the protein will be synthesised and accumulated in the chloroplast	[14,15]
8	Integrate the epitope DNA into a plant virus vector	Use a viral promoter when the epitope is integrated into a plant virus Use a defective virus for improving yield and for environmental safety	[9,16]
9	Express polycystronic mRNA	Integrate into the plant DNA a poly-epitope under a single expression signals	[1,17]
10	Choose selectable marker genes	Use an appropriately selected gene Remove the gene after selection	[4,18]

[a] The cited references give further recommended readings on construction of plant expression cassettes, expression signals and peptide design listed in the table.

important advantages, the first being the foreign sequence is targeted, by homologous recombination through the use of appropriate flanking sequences, to a precise cpDNA site. This eliminates variability in gene expression and gene silencing, which may occur in the case of gene integration in the nuclear DNA. The second advantage lies in the increased accumulation of the recombinant protein (up to 46% of total soluble protein, as compared with 0.01–0.4% with nuclear inserted genes). Apparently, accumulation of the foreign protein in the chloroplast does not significantly impair photosynthetic efficiency.

The current limitation to frequent use of cpDNA transformation is that although cpDNA transformation is routine in tobacco, it is more difficult and still requiring optimisation in other edible plant species [14,15,17].

5.2. The use of plant viruses as transient expression vectors

Plus-sense, single-stranded plant RNA viruses have been proposed as an effective alternative to produce vaccine antigens in plants. In this technique the epitope of interest is engineered into a plant virus, usually within the coat protein gene. Infection of a susceptible non-GM-plant results in intracellular production and accumulation of the epitope. The epitope sequence, as well as the viral genome, never become integrated into the plant genome and hence are only expressed by the generation of infected cells [1,9,16].

A recombinant cowpea mosaic virus was shown to elicit protective immunity in mink when engineered to express the antigenic epitope against mink enteritis virus [9]. Other successes are listed in [1,9]. A limitation of the recombinant cowpea mosaic virus approach is the failure of the virus to assemble when peptides of more than 25 amino acids are incorporated into their coat protein. More flexibility was obtained when epitope sequences were inserted at the N-terminal end of the coat protein of the alfalfa mosaic virus (A1MV). Recombinant AlMV has enabled expression of significant quantities of rabies virus and HIV epitopes upon integration of their respective coding sequence into the A1MV coat protein, and infection of tobacco plants. The extra sequences were found to protrude from virion surface without interfering with virus assembly [9]. Results of these studies demonstrated that in order to retain antigenic capacity, the virus particle must retain its potential to self assemble while displaying the antigenic epitope on its surface. Recombinant A1MV coat protein molecules have also demonstrated the ability to assemble into particles containing three different epitopes from HIV and rabies [9,16]. This demonstrates the ability of plant viruses to produce multicomponent vaccines. Claimed advantages of transient viral expression of transgenes over transgenic plants are: shorter time for cloning of the foreign gene in the viral genome as compared with time required to transform plants, the ease at which antigen production can be scaled up and the wide host range of plant viruses that allow the use of multiple plant species as biofactories [9].

6. Oral delivery, mucosal and systemic antibody responses

Most infectious agents enter the body through mucosal membranes. Induction of mucosal immunity is best achieved by direct vaccine delivery to mucosal surfaces. This stimulates production of sIgA, the predominant antibody isotope in mucosal secretion. Whilst effective inducers of systemic immunity, vaccines delivered by injection are not efficient at inducing mucosal responses [1,5,7,9,17].

Plant-derived vaccines have demonstrated the ability to induce both systemic and mucosal immune responses [1,19]. The major obstacle to oral vaccination is the digestion of the antigenic protein in the stomach. Vaccines derived and delivered by plant cells have been shown to overcome this problem through the protective effect of the plant cell wall. Like liposomes and microcapsules, the plant cell wall allows gradual release of the antigen onto the vast surface area of the lower digestive tract. Further problems may be associated with poor immunogenicity or the induction of tolerance. Binding to a targeting molecule or carrier peptide, such as HbsAg, has been shown to overcome poor immunogenicity of orally delivered subunit vaccines [1,5]. In specific circumstances, for example cancer therapy, injection of the drugs, after purification from the producing plant, may be preferred.

7. Safety and public acceptance

Plant-derived vaccines are certified free from animal pathogen contaminants. Furthermore plant DNA is not known to interact with the animal DNA and plant viral recombinants do not invade mammalian cells. Further safety of plant-derived vaccines is obtained through following the same regulations established for traditional vaccines. Nevertheless, the present concern over the use of GM-plants is now affecting research in this important field, especially in Europe.

One of the fears is that GM-pollen may outcross with sexually compatible plants (related crops or weeds) and affect biodiversity. In order to address this alarm, several pollen containment approaches have been developed. These are essentially based on the exploitation of different forms of male sterility (suicide genes, infertility barriers, apomixis). An alternative way of solving the problem is engineering vaccines into the cpDNA, which is not transmitted to the sexual progeny through the pollen grains [14,15]. An additional safety feature would be the recognition of GM-plants that produce vaccines by the addition of genes encoding coloured plant pigments [5].

It is important to recognise that plants that produce vaccines are medicinal plants and should be grown, processed

and regulated as pharmaceutical products. It is thought that pharmaceutical crops will be able to be grown on relatively small extensions of land, preferably contained within greenhouses using controlled environmental conditions. In the majority of earlier papers, level of antigen accumulation in the plant organ was in the order of 0.1–0.4% of total soluble protein [1], while the more recent developments on cpDNA integration promises to increase this value to 30% or more [20]. At the latter value, land requirements for industrial plant-derived vaccine-production will be in the order of a few thousand square meters. This will definitely enable vaccine-producing plants to be set apart from field grown crop plants and offer added safety when engineered plant viruses are used for transient antigen expression. A further point of public concern in GM-plants is the presence of antibiotic resistance genes (used as selective marker in most transgenic plants). Approaches have now been developed to generate GM-plants (with both nuclear or cpDNA integration) that do not carry these genes [4,18,20].

8. Future perspectives

Although still at an early stage of development, the experimental know-how and results strongly suggest that plant-derived edible vaccines are likely to become a reality in the next few years. Future research will demonstrate if these vaccines meet the standards of quality (purity, potency, safety and efficacy) defined for vaccines by the World Health Organization [21].

When is this expected to happen? A realistic appraisal of the state of the art should consider that after the ongoing event of *discovery* (i.e. the demonstration that plants can be engineered as to produce edible vaccines that trigger an immune response in mice and humans), we are now confronted with the successive problems of *clinical trials*, *process development*, *registration* and *marketing*. *Clinical trials* with populations at risk are already under way in some laboratories. The definition of the overall immune response to plant-derived edible vaccines is of the utmost importance. With the growing availability of plant-derived vaccines, this will soon be verified. *Process development* primarily concerns achieving sufficiently high levels of expression of the recombinant antigen, and defining the optimal way of antigen administration. Solutions to the first point are well under way, as described above, while approaches to the second will be manifold. While the initial concept was to induce an immune response by directly feeding a crude edible plant portion (fruit, leaf, tuber), it is now felt that this may not be the ideal solution as it would be difficult to standardise antigen concentration in different harvests of the same crop. Furthermore, fresh products may have short shelf life. Dried products, for instance banana slices, may offer a partial solution, but the best solution (as for shelf-life, stability and title standardisation) would be delivery in the form of a dry powder. This can be achieved by using low cost food processing technology. A dried tomato powder has been stored for one year in C. Arntzen's laboratory without loss of antigen activity. In cases in which effectiveness is much more relevant than cost, for example with cancer antigens, administration may be through injection of appropriately purified antigens.

Field and clinical trials are required to define the risk/benefit ratio of a GM-plant before *registration* is granted. In most countries of the world, plants engineered to produce vaccines fall under the very restrictive rules set up to control GM-crop plants. The present concern, especially in Europe, over the use of biotechnology for the genetic improvement of crop plants also negatively affects the acceptance of GM-plants for medicinal use. As a consequence, while the demonstration that plant-derived vaccines are effective on populations at risk is expected to arrive within 1–2 years, a further quarantine of 2–3 years will be required in order to fulfil requirements for *registration* and *marketing*. It is hoped that simpler rules will be set up for GM-plants producing vaccines and that they are seen as clearly and legally distinct from GM-plants grown for nutrition purposes.

Important social questions still exist. Who will benefit from this development? Who will be able to perform research, produce and control plant-derived edible vaccines? Will the resultant vaccines be affordable to developing countries? Definitely, the answer is that there is no danger of monopoly in the hands of powerful economic groups. Many countries in the world are already greatly involved in research on plant vaccines; these include the USA, the European Community, China, Japan, India, Korea and others. The reason for this is that the applications are based on established gene cloning and plant transformation technology and that development requires relatively limited investment.

9. A unique opportunity against the threat of bio-weapons

A number of infectious diseases, including smallpox, anthrax and plague have recently raised concern for their possible use in actions of bio-terrorism. Nations at risk are now faced with the need to be ready to vaccinate part or all of their population within limited periods of time. This means that millions of vaccine doses have to be prepared, stored and renewed at intervals of time. The economic and technical benefits offered by plant-derived vaccines (Table 1) propose these vaccines as ideal substitutes for traditional vaccines. Research on plants that produce antigens against major pathogens feared in case of bio-terrorism is already under way.

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