EXPERT REVIEWS

Recent progress in the development of plant-derived vaccines

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Recombinant subunit vaccines have been with us for the last 30 years and they provide us with the unique opportunity to choose from the many available production systems that can be used for recombinant protein expression. Plants have become an attractive production platform for recombinant biopharmaceuticals and vaccines have been at the forefront of this new and expanding industry sector. The particular advantages of plant-based vaccines in terms of cost, safety and scalability are discussed in the light of recent successful clinical trials and the likely impact of plant systems on the vaccine industry is evaluated.

Keywords: oral delivery • plant virus • production platform • recombinant protein • subunit vaccine • transgenic plant • vaccine

Vaccination is the most effective and cost-efficient method for fighting human and animal diseases, preventing the spread of infectious diseases, completely eradicating some diseases and even helping to prevent cancer and autoimmune disorders [1]. There are now more than 25 vaccines licensed for use in humans with many more in the development pipeline. However, significant challenges still remain in developing vaccines for emerging and re-emerging diseases, producing vaccines in a cost-effective manner and providing a convenient way to deliver them. An effective vaccine should be inexpensive, confer protective immunity to the vaccinee with few side effects, and safe, stable and easy to administer.

Traditional vaccines are live, killed or otherwise attenuated/modified pathogens (e.g., influenza vaccines produced in specific pathogen-free eggs). However, they have been increasingly supplanted by recombinant subunit vaccines produced in genetically modified cells because these offer increased safety, less antigenic competition, the ability to target vaccines to specific sites and the ability to differentiate between infected and vaccinated animals [2]. In this approach, the gene encoding a protective antigen is expressed in a heterologous system and the resulting protein is purified and administered as a vaccine, hopefully provoking an immune response that will neutralize the intact pathogen on subsequent exposure. Recombinant subunit vaccines are simpler in composition than traditional vaccines and allow a wide range of different production hosts to be considered.

Production systems for recombinant subunit vaccines

Current commercial production systems

Recombinant subunit vaccines currently on the market are produced in bacteria, yeast or insect cells. The earliest work on heterologous subunit vaccine production was carried out in the bacterium *Escherichia coli*, which continues to dominate the field [3,4]; however, the *E. coli* system has been superseded in many cases by eukaryotic cells because the latter can produce more complex proteins that fold correctly and undergo forms of post-translational modification (e.g., N-glycosylation) that do not occur in bacteria. Furthermore, the presence of endotoxins and other pyrogens has limited the use of bacterial systems for vaccine production.

Yeast cells grow in a similar manner to bacterial cells and, like bacteria, require simple and relatively inexpensive media for growth. Since they are eukaryotes, they can fold and assemble complex recombinant proteins much more efficiently than bacteria. However, although they carry out N-glycosylation, the glycan structures often differ to those found in mammals. *Saccharomyces cerevisiae* was the first yeast used for recombinant protein production, a choice

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based on the widespread use of this organism as a laboratory model [5]. It is used commercially for the production of the HBV vaccine, a subunit vaccine based on the HBV surface antigen [6].

Mammalian cells are a logical choice for subunit vaccine production, particularly for viral antigens since these would be modified similarly to virus proteins expressed *in vivo* [7]. However, factors such as the cost of infrastructure and consumables, and the need for extensive product validation to prove that the final product is pathogen-free and does not contain oncogenic agents, make this platform commercially unfeasible for vaccines required on a large scale. Insect cell cultures share many features of mammalian cells but the culture medium is much less expensive and they tend not to harbor mammalian pathogens [8]. However, insect cells, as with mammalian cells, have limited scalability, and major differences in glycan structures could raise challenges for the production of some recombinant proteins.

Plants as an alternative production system

Plants have been used to produce over 200 medically relevant proteins and their many benefits now make them a serious competitor to mammalian cells for biopharmaceutical production [9]. In terms of vaccine production, plants share the advantages of other eukaryotic systems (ability to produce complex, correctly folded and post-translationally modified proteins) but lack their principal disadvantages in terms of cost, safety and scalability. With plants, there is no need to build and run expensive fermenters, hire skilled workers and pay for expensive culture media. Plants can be grown and harvested using traditional agricultural practices [10]. Plants tend not to harbor human pathogens and any colonizing bacteria or animal-derived material can be removed using appropriate sanitary measures before processing. The cost of scaling fermenter-based production up or down in response to demand can be high, whereas with plants the same can be achieved simply by using more or less glasshouse space or land. Another issue addressed by the use of plant production systems is vaccine delivery and storage, especially in countries where there is a poor health infrastructure. The vaccination of humans and animals with partially processed plant material containing a vaccine subunit, either by oral or topical delivery, could circumvent many of the current challenges with vaccine distribution, including the absence of a cold chain [11]. Proteins expressed in certain plant tissues (e.g., cereal seeds) remain stable for years at ambient temperatures without loss of activity [12]. Orally delivered plant-derived subunit vaccines are capable of inducing a mucosal response in animal models and such animals can also withstand pathogen challenge. Plant-derived vaccines survive in the stomach through bioencapsulation, which allows gradual release [11] and, in some cases, this makes the vaccine more efficacious than the same subunit delivered through the parenteral route [13]. A selection of vaccine candidates produced in plants that have provided protective efficacy in animals or immunogenicity in humans is provided in Table 1.

Diversity of plant-based systems

Many plant species have been used to produce vaccines, including tobacco, corn, potato, tomato, carrot and lettuce, but species range is not the only form of diversity inherent in the use of plants to produce recombinant vaccines. As well as different species, a number of different gene transfer and expression strategies have been considered, the most prevalent of which are now discussed.

Transgenic plants

The most widely used strategy for vaccine production in plants is the nuclear transgenic system, in which the antigen transgenes are transferred to the plant nuclear genome. The advantages of this approach when used in a major terrestrial crop species include:

- Transformation is a fairly routine procedure in many species and can be achieved by a range of methods, the two most common of which are *Agrobacterium*-mediated transformation and the delivery of DNA-coated metal particles by microprojectile bombardment;
- A stable transgenic line can be used as a permanent genetic resource;
- Among the various plant systems it is the simplest to maintain (once the producer line of transgenics is available) and is, ultimately, the most scalable;
- It is possible to establish seed master banks, which will probably become adopted from a regulatory perspective as the equivalent of master cell banks for good manufacturing practice (GMP)-compliant production.

Disadvantages, compared with other plant systems, include the relatively long development time (required for transformation, regeneration, analysis of transgenics, selection and bulking up of the producer line), the unpredictable impact of epigenetic events on transgene expression (e.g., post-transcriptional gene silencing, position effects) and the potential for transgene spread from some crops through outcrossing. The latter has been a significant hurdle to regulatory and public acceptance, although this has been addressed to a certain extent by growing transgenic plants in containment. It has also been necessary to carry out a great deal of R&D to that ensure the yields of recombinant proteins are adequate for commercial exploitation [14].

Transplastomic plants

Instead of introducing transgenes into the nuclear genome, they can be targeted to the chloroplast genome using particle bombardment or other physical DNA delivery techniques, ensuring that the transgene is embedded in a chloroplast DNA homology region [15]. The main benefits of the chloroplast system are that there are thousands of chloroplasts in a typical leaf cell, yet only one nucleus – therefore, the number of transgene copies in the cell following plastid transformation and the establishment of homoplasmy is much higher, promising greater product yields. This is enhanced by the absence of epigenetic phenomena, such as transgene silencing in the chloroplast genome. Chloroplasts,

Table 1. Selection of plant-derived vaccine antigens conferring protection against disease challenge in animal studies or conferring protective immunity in humans.

Antigen	Production system	Efficacy data	Ref.
Veterinary vaccines			
Actinobacillus pleuropneumoniae ApxIIA	Transgenic tobacco	Mice fed transgenic leaf powder were protected from an injection of a lethal dose of *A. pleuropneumoniae*	[51]
Anthrax protective antigen	Tobacco chloroplasts	Mice administered antigen survive lethal challenge with anthrax toxin	[20]
Bovine herpes virus (type 1) glycoprotein D	Tobacco leaves	Humoral and cellular immune response in mice and cows following parenteral administration. Alleviation of symptoms in cows challenged with the virus	[52]
Canine parvovirus VP2 epitope	Cowpea mosaic virus epitope display in cowpea	Immunogenic in mice following parenteral or nasal administration. Protective against lethal challenge in parenterally immunized dogs	[53]
Enterotoxigenic *Escherichia coli* fimbrial subunit FaeG	Tobacco leaves	Prevented bacterial colonization of piglet villi in an *in vitro* assay	[54]
Foot-and-mouth disease virus VP1 epitope	Alfalfa leaves	Specific antibody response in mice. Immunogenic in mice following parenteral or oral administration, protected against viral challenge	[55]
	Arabadopsis thaliana leaves	Immunogenic in mice following parenteral administration, protected against viral challenge	[56]
	Potato tuber	Immunogenic in mice, protected against viral challenge	[57]
	Tobacco infected with bamboo mosaic virus	Immunogenic in swine, protected against viral challenge	[36]
Foot-and-mouth disease virus polyprotein P1–2A/protease 3C	Tomato	Immunogenic in guinea pigs, protected against viral challenge	[58]
Infectious bronchitis virus S1 glycoprotein	Potato tuber	Neutralizing antibodies produced. Immunogenic in chickens following parenteral, oral or nasal administration. Mice and chickens protected against viral challenge	[59]
IBDV VP2 protein	Rice grains	Specific pathogen-free chickens orally vaccinated with transgenic rice seeds expressing VP2 protein produced neutralizing antibodies against IBDV and were protected when challenged with a highly virulent IBDV strain, BC6/85	[60]
Mink enteritis virus VP2 epitope	Cowpea mosaic virus epitope display in cowpea	Immunogenic in mink following parenteral administration. Protective against viral challenge	[61]
Murine hepatitis virus glycoprotein S 5B19 epitope	Tobacco mosaic virus epitope display in tobacco	Immunogenic in mice following parenteral or nasal administration. Protected mice against virulent strain of the virus	[62]
Rabbit hemorrhagic disease virus VP60 epitope	Plum pox virus epitope display in tobacco	Neutralizing antibodies produced. Immunogenic in mice and rabbits following parenteral administration. Rabbits survived lethal challenge following parenteral administration	[63]
	Potato leaves	Neutralizing antibodies produced. Immunogenic in rabbits following parenteral administration. Rabbits survived lethal challenge following parenteral administration	[64]
Transmissible gastroenwteritis coronavirus N-terminal domain of the spike glycoprotein	Maize seeds	Neutralizing antibodies produced in piglets. Reduced symptoms in piglets when challenged with virus following oral delivery	[65]
Human vaccines			
Synthetic peptides against the cysticercosis pathogen *Taenia solium*	Transgenic papaya	Complete protection against cysticercosis was induced in 90% of immunized mice	[66]

IBDV: Infectious bursal disease virus; VP: Virus protein.

Table 1. Selection of plant-derived vaccine antigens conferring protection against disease challenge in animal studies or conferring protective immunity in humans (cont.).

Antigen	Production system	Efficacy data	Ref.
Human vaccines (cont.).			
Enterotoxigenic *Escherichia coli* heat-labile toxin, B subunit	Maize seeds	Immunogenic and protective in mice after oral administration. Serum and secretory antibodies produced in humans	[67]
	Potato tubers	Immunogenic and protective in mice after oral administration. Immunogenic in human after oral administration	[68]
		Expressed as fusion with cholera toxin B subunit and rotavirus VP6 protein. Mice developed neutralizing antibodies. Immunogenic in mice against enterotoxigenic *E. coli*, rotavirus, and *Vibrio cholerae* following oral administration	[69]
	Carrots	Immunogenic and protective against cholera toxin challenge	[70]
E. coli O157:H7 intimin	Tobacco leaves	Immunogenic and protective in primed mice after oral administration	[71]
Hepatitis B virus surface antigen	Lettuce leaves	Immunogenic in humans following oral administration	[72]
Norwalk virus capsid protein	Tobacco leaves, potato tubers	Immunogenic in mice and humans following oral administration	[73]
Pseudomonas aeruginosa membrane protein F epitope	Cowpea mosaic virus epitope display in cowpea	Specific antibodies produced. Immunogenic in mice following parenteral administration. Protected mice against challenge with *P. aeruginosa*	[74]
	Tobacco mosaic virus epitope display in tobacco	Specific antibodies produced. Immunogenic in mice following parenteral administration. Protected mice against challenge with *P. aeruginosa*	[75]
Rabies virus glycoprotein and nucleoprotein	Viral vectors in tobacco and spinach leaves	Immunogenic and protective in mice when delivered orally and parenterally. Immunogenic in humans following oral administration	[76]
Respiratory syncytial virus G and F proteins	Alfalfa mosaic virus epitope display in tobacco	Neutralizing antibodies produced. Immunogenic in mice following parenteral administration. Mice protected from viral challenge	[77]
Rotavirus VP6 protein	Potato tubers	Expressed as fusion with cholera toxin B subunit and enterotoxigenic *E. coli* heat-labile toxin, B subunit. Specific serum and intestinal antibodies produced. Immunogenic in mice against Enterotoxigenic *Escherichia coli*, rotavirus and *V. cholerae* following oral delivery. Symptoms reduced following rotavirus challenge in pups	[69]
Recombinant vaccinia virus B5 antigenic domain (pB5) (protective against smallpox)	*Arabidopsis thaliana*	Mice immunized intramuscularly with pB5 generated an antibody response that reduced virus spread *in vitro* and conferred protection from challenge with a lethal dose of vaccinia virus	[78]
V. cholerae cholera toxin, B subunit (as single antigen)	Potato tubers	Immunogenic and protective in mice when delivered orally	[79]
Yersinia pestis F1 and LcrV antigens	Transient expression in *Nicotiana benthamiana*	When administered to cynomolgus macaques the purified plant-produced antigens induced serum IgG and IgA responses specific to F1 and LcrV, and conferred complete protection against lethal challenge with *Y. pestis*	[33]

IBDV: Infectious bursal disease virus; VP: Virus protein.

derived from ancient bacteria, also support operon-based transgenes allowing the expression of multiple proteins from a single transcript. Finally, and perhaps most importantly from the regulatory perspective, chloroplasts are absent from the pollen of most of our food crops, which limits the potential for outcrossing.

There are two disadvantages to the chloroplast system – first, chloroplast transformation is not a standard procedure and is thus far limited to a relatively small (although growing) number of crops [16]. Second, since chloroplasts are derived from ancient bacteria they lack some of the eukaryotic machinery for post-translational modification, for example, they are unable to synthesize

glycan chains. Even so, functional vaccine antigens have been produced in chloroplasts against a number of viral, bacterial and parasitic diseases, including cholera [17], tetanus [18], anthrax [19,20], plague [21] and amebiasis [22].

Plant viruses as expression vectors

Engineered plant viruses have been shown to be efficient in the production of soluble antigens or virus-like particles (VLPs) that present and deliver candidate peptide epitopes [23]. In the first strategy, the gene of interest is introduced into the plant virus genome so that its expression is controlled by a viral promoter, most often, the coat protein subgenomic mRNA promoter [24]. The coat protein is the most highly expressed protein in an infected host and, therefore, this is the promoter of choice for the expression of a foreign gene. There are several examples of target antigens produced in plants using this strategy, including a vaccine against non-Hodgkin's lymphoma based on single-chain Fv antibodies that has entered clinical trials (Table 2). There are, however, some concerns regarding the environmental safety of using replicating plant viruses as vectors and another drawback is the tendency for recombinant viruses to shed the transgene during replication, resulting in the systemic spread of transgene-negative variants throughout the plant.

Virus-like particles are being pursued actively for vaccine development [25]. This is because viruses have defined surface properties, regular geometries because of the extensively repetitive structure and are excellent models in nanoassembly [26]. Furthermore, their nanoscale dimensions make them attractive in vaccine development and drug delivery. The first recombinant vaccines were VLPs that were based on HBV surface antigens that were produced in yeast [5,6] but there is now great interest in achieving the analogous process in plants, using plant viruses to present multiple copies of a target epitope. The peptide sequence can be fused either to the N-terminus, within the coding sequence or to the C-terminus of the coat protein, depending on the plant virus. A simple and relatively inexpensive polyethylene-glycol procedure generates very pure virus particles. The plant viruses that have been the most amenable for peptide display are tobacco mosaic virus (TMV), alfalfa mosaic virus, cowpea mosaic virus and bamboo mosaic virus [23].

Plant VLPs have been effective for the presentation and delivery of peptide epitopes, but less so for the presentation of larger polypeptides, as these larger molecules hinder assembly of the virus. However, because of the advantages of using VLPs to enhance immunogenicity there have been extensive studies to modify the surface of the virus particles so that the polypeptides of interest can be conjugated to the VLP surface. Smith and colleagues modified the surface of TMV so that candidate antigens could be fused to a surface-exposed lysine via streptavidin [27]. The authors fused the canine oral papillomavirus L2 protein to streptavidin and displayed it on TMV particles. The L2 protein fused to the TMV particles was more immunogenic in mice than nonconjugated L2 protein. This approach, although in its infancy, is very promising for vaccine development. There is also increasing interest in the use of plant virus particles as nanoparticles for drug delivery. For example, Rae and colleagues showed that orally delivered cowpea mosaic virus nanoparticles were stable in the GI tract, thus showing that these plant virus particles have great potential for oral delivery of target molecules [28].

Virus-based & Agrobacterium*-based transient expression strategies*

Stable transgenic plants are produced by permanently integrating DNA into the plant genome, which is a very rare occurrence and so selection must be used to propagate the few transformed cells and regenerate them into whole plants. However, many cells are initially transformed with DNA, which is degraded before it can integrate. Before this happens, the DNA can be expressed and proteins can be produced. Such transient expression has been developed as a shortcut for the production of vaccines and other proteins in plants, allowing relatively large amounts of protein to be produced quickly. The advantages of

Table 2. Plant-derived antigens (and antibodies for passive immunization) in humans that have reached clinical trials.

Product	Class	Indication	Company/organization	Crop	Status
Various single-chain antibodies	Antibody (passive)	Non-Hodgkin's lymphoma	Large Scale Biology Corporation [now insolvent]	Viral vectors in tobacco	Phase I
CaroRX	Antibody (passive)	Dental caries	Planet Biotechnology	Transgenic tobacco	Phase II
Escherichia coli heat-labile toxin	Antigen (active)	Traveler's disease	Prodigene Inc. [now insolvent]	Transgenic maize	Phase I
			University of Arizona	Transgenic potato	Phase I
HBV surface antigen	Antigen (active)	Hepatitis B virus	University of Arizona	Transgenic potato	Phase I
			Thomas Jefferson University/ Polish Academy of Sciences	Transgenic lettuce	Phase I
Norwalk virus capsid protein	Antigen (active)	Norwalk virus	University of Arizona	Transgenic potato	Phase I
Rabies glycoprotein	Antigen (active)	Rabies virus	Thomas Jefferson University	Viral vectors in spinach	Phase I

this approach include the minimal set-up costs and the rapid onset of protein expression. An example of a transient expression system is the agroinfiltration method, where recombinant *Agrobacterium tumefaciens* is infiltrated into leaf tissue under vacuum and milligram amounts of protein can be produced within a few weeks. This system was pioneered in tobacco but can be applied to any leafy species and has been developed commercially in alfalfa by the Canadian biotechnology company Medicago Inc., Québec, Canada.

Other transient expression systems have been developed to remove the safety concerns surrounding the use of intact virus expression vectors and to avoid transgene loss during systemic spreading (see earlier). We and others have addressed this issue by developing the 'launch vector' system (Figure 1), which combines the use of plant viruses and *Agrobacterium* binary plasmids [29]. Millions of copies of the recombinant viral vector are introduced ('launched') into the plant cells by vacuum infiltration using an *A. tumefaciens* culture harboring the launch vector. Primary transcripts (which contain the recombinant viral genome) are then produced in the nucleus and transported into the cytoplasm, where on replication, very high copy numbers of the viral vector accumulate in the plant cell. As a result, in a matter of days, high levels of target protein (mg to g quantities) accumulate in the plants. Since the launch vector is introduced into nongenetically modified seedlings, large quantities of source material can readily be made available for production, thus creating an immediate capability for scale-up. Accumulation of target protein can be achieved in less than a week after infiltration. A similar system known as magnifection [30] has been used to express plague antigens in plants [31]. The magnifection strategy, developed by Icon Genetics (now part of Bayer CropSciences, Monheim am Rhein, Germany), also renders the systemic spread of the virus unnecessary through the use of *A. tumefaciens* as a delivery vehicle. The bacterium delivers the viral genome to so many cells that local spreading is sufficient for the entire plant to be infected. Taking the systemic spreading function away from the virus and relying instead on the bacterium to deliver the viral genome to a large number of cells allows the same viral vector to be used in a wider range of plants than the virus natural host range. In the launch vector system, we use a modified TMV lacking the coat protein, thus removing instability issues related to the presence of coat protein and phloem-mediated movement, and environmental issues related to the presence of infectious particles. Using this system we have produced a wide variety of target antigens from several pathogens, including *Bacillus anthracis*, *Yersinia pestis* and influenza virus [32–34].

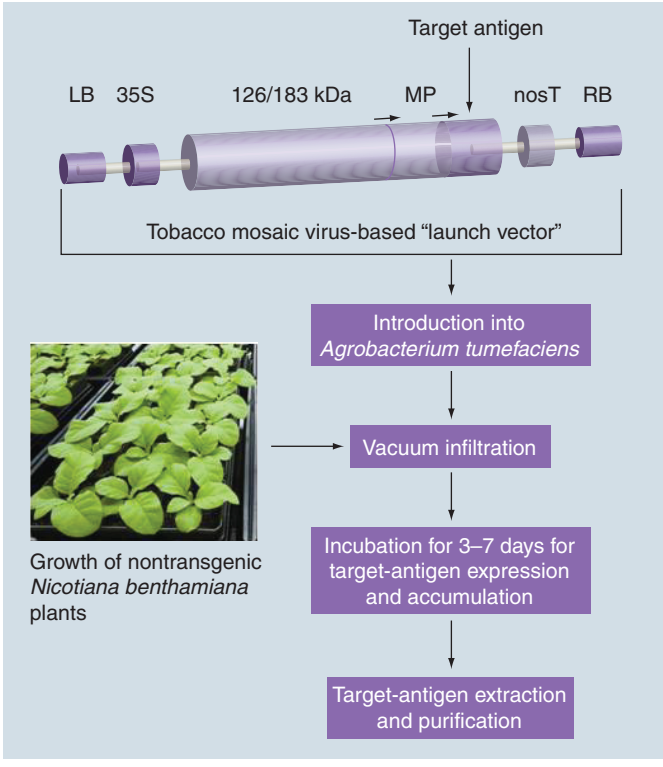

Figure 1. Schematic diagram of a tobacco mosaic virus-based launch vector system for production of vaccine antigens in nontransgenic plants. Arrows indicate positions of subgenomic mRNA promoters. The target antigen replaces the coat protei-coding sequence.
LB and RB refer to left border and right border, respectively, of the T-DNA in the *Agrobacterium tumefaciens* binary vector; this T-DNA is transferred into the nuclei of the plant cells following agroinfiltration; 35S: 35S promoter from cauliflower mosaic virus (a plant DNA virus) that drives transcription of the transgene; MP: Movement protein required for cell-to-cell movement; nosT: nos transcriptional terminator from *A. tumefaciens* nopaline synthase gene; 126/183kDa: replicase proteins of tobacco mosaic virus required for replication of the virus.

Plant suspension cells

Plant suspension cells are individual plant cells and small aggregates growing in liquid medium in a fermenter [35]. Suspension cell cultures are usually derived from callus tissue by the disaggregation of friable callus pieces in shake bottles, and are later scaled up for fermenter-based production. Recombinant antigen production is achieved by using transgenic explants to derive the cultures, or transforming the cells after disaggregation, usually by cocultivation with *A. tumefaciens*. Suspension cultures share advantages with whole plants grown in contained bioreactors (e.g., the 'Lemna' system developed by Biolex Therapeutics Inc. [Pittsboro, NC, USA]), that is, controlled growth conditions, batch-to-batch reproducibility, containment and production under current GMP. Although commercial systems based on rice and carrot cells are in development (e.g., a carrot cell system developed by the Israeli biotechnology company Protalix [Karmiel, Israel]), tobacco cells are the most widely used and are thus far the only system to be used for a veterinary vaccine approved by the US FDA, a vaccine against Newcastle disease in poultry successfully licensed by Dow AgroSciences (Indianapolis, IN, USA) in 2006.

Immunogenicity & efficacy of plant-derived vaccines

Several reviews have been published to date that summarize the wealth of data showing the feasibility of using plants to produce biologically active recombinant proteins, including vaccines [9,10].

These plant-produced vaccines have been shown to generate target-specific systemic and mucosal immune responses, and are also effective in stimulating T-cell-mediated immunity. Importantly, several recent reports show that the target-specific immune responses confer protection to model animals when challenged by the relevant pathogen. Furthermore, these vaccines show efficacy not only in the favored mouse model, but also in larger animals, such as ferrets (for influenza) or nonhuman primates (Table 1). Several successful Phase I clinical trials have also been performed with plant-based vaccines (Table 2). Some recent developments are now discussed.

Using a bamboo mosaic virus-based expression vector, Yang and colleagues produced chimeric VLPs presenting a 37-amino acid epitope of foot and mouth disease virus coat protein VP1 [36]. The purified virus particles were used to immunize swine. Following a second boost, all swine challenged subcutaneously with foot-and-mouth disease virus were protected from infection. A chloroplast-derived protective antigen from *B. anthracis*, which was partially purified from tobacco plants and delivered subcutaneously to mice, conferred protective immunity; 100% of the mice survived challenge with lethal doses of toxin [37]. Recombinant subunit vaccines against *Y. pestis* have been produced in plants using the three strategies described above. Using a TMV-based expression vector, Santi and colleagues produced F1, V and an F1–V fusion protein in *Nicotiana benthamiana* [31]. Following subcutaneous vaccination of guinea pigs and subsequent challenge with aerosolized *Y. pestis*, up to 75% of the vaccinated animals that received the F1–V fusion or only the V candidate antigen were protected. Using a similar approach to produce F1 and V in plants, Mett and colleagues showed that three subcutaneous doses of a mixture of plant-produced and purified F1 and V antigens, administered to the nonhuman primate model of pneumonic plague, cynomolgus macaques, conferred protection to all animals when challenged with aerosolized *Y. pestis* [33]. More recently, hemagglutinin domains produced using the plant virus-based approach when delivered with or without neuraminidase (produced in plants), conferred a high degree of protective immunity to ferrets when challenged with the homologous egg-grown influenza/Wyoming/3/03 virus [38].

In addition to the aforementioned studies, there have also been several successful Phase I clinical trials with plant-based vaccines (Table 2). Recently, Tacket reported the results of three independent clinical studies with transgenic corn and potato that expressed the B subunit of the heat-labile enterotoxin from enterotoxigenic *E. coli* and transgenic potato expressing the norovirus capsid protein [39]. These candidate vaccines are targeted against diarrheal diseases. Human volunteers ingested three doses of the transgenic potato or corn; both mucosal and systemic immune responses were induced.

Expert commentary

The advantages and diversity of plant-based production systems for vaccines, coupled with the successes achieved in veterinary and clinical trials, promises a bright future for this emerging field, especially considering the recent approval of Dow's plant cell culture-based vaccine against Newcastle disease in poultry. The plant cell system and the use of deconstructed plant viruses for transient expression are particularly attractive because of the speed with which new vaccines could be produced, which would be advantageous for protection against bioterrorism and in the rapid development of vaccines against pandemic influenza virus strains. However, in order for plant-based vaccines to become more widely available, several challenges still need to be overcome, even though increasingly these are political rather than technological in nature. Initially, the main problems facing the use of plants in general were the poor and inconsistent expression levels, the low yields after downstream processing, the potential impact of plant-specific glycans, and lack of public acceptance. The technological hurdles have been largely overcome and the major plant systems being developed for commercial use regularly achieve high and consistent yields [9]. The issue of plant glycans is being addressed through a number of strategies that include the *in vivo* modification of proteins in the secretory pathway, or the targeting of proteins to intracellular sites to avoid the addition of complex glycans [40]. Even public acceptance has increased with the trend towards glasshouse-based production as opposed to cultivation in open fields [41].

Plant-specific glycans

Although the protein synthesis and folding pathways are highly conserved between plants and animals, there are some differences in the capacity for post-translational modification. Plants do not, for example, hydroxylate proline residues in recombinant collagen. There are also various differences in glycan structure: plant-derived recombinant human glycoproteins tend to contain the carbohydrate groups $\beta(1\rightarrow2)$-xylose and $\alpha(1\rightarrow3)$-fucose, which are absent in mammals, but generally lack the terminal galactose and sialic acid residues that are found on many native human glycoproteins. Since glycan structures can impact on the solubility, stability, immunogenicity and biological activity of recombinant proteins, the 'humanization' of glycan structures produced in plants has been an important topic of research and debate in the scientific community. There has been considerable interest in modifying the plant glycosylation pathway to humanize the glycan profile of recombinant proteins. Several changes in the pathway are required to produce proteins with typical human glycan structures [40]. Strategies used include the *in vitro* modification of plant-derived recombinant proteins by purified human $\beta(1,4)$-galactosyltransferase and sialyltransferase enzymes and the expression of human $\beta(1,4)$-galactosyltransferase in transgenic plants to produce recombinant proteins with galactose-extended glycans [42,43]. To remove the non-mammalian $\beta(1\rightarrow2)$ -xylose and $\alpha(1\rightarrow3)$-fucose residues, some researchers have explored the possibility of inhibiting the enzymes responsible for synthesizing these groups, while in one case this goal has been achieved by gene-knockout-interference techniques [44,45]. Another approach is to prevent the glycoproteins passing through the Golgi, so only high-mannose glycans are added [46]. Although plant glycans

are immunogenic in some animals, the potential adverse effects of plant-derived recombinant glycoproteins in humans have yet to be demonstrated in clinical trials [47,48].

Downstream processing

One of the often-cited advantages of plants for vaccine production is that vaccines can be administered orally in unprocessed or part-processed plant material (e.g., fruit juice or tomato paste), which will eliminate the costs of downstream processing (DSP). The DSP stage of production is generally the most expensive, representing up to 80% of overall production costs (the majority for chromatography and associated materials, labor and capital equipment). Although it is possible that some vaccines could be administered orally, it is unrealistic to assume this will be the major route for all vaccines derived from plants. Therefore, in some cases the vaccine antigens will need to be purified as is the case with any other production system. In many cases, it will be necessary to develop specific processing steps for each product, although certain classes of product can be isolated using a standardized approach (e.g., protein A-based affinity chromatography to isolate vaccines based on full-length recombinant antibodies). Several aspects of downstream processing have to be customized specifically for plant systems, including the removal of fibers, oils and other byproducts from certain crops, and process optimization for the treatment of different plant species and tissues [49]. Therefore, a significant challenge for the future is how to ensure that the economic benefits of plant systems are not absorbed by processing costs further downstream.

Regulatory landscape

Part of the industry inertia discussed earlier reflects the regulatory environment, which makes companies unwilling to abandon tried and tested production systems in favor of those with an uncertain regulatory pedigree. Plant-based production is evolving and the regulatory environment is changing around it. In a few short years, we have moved from a situation where the regulatory agencies were stepping on each other, uncertain where plants fitted into their precisely structured framework for biotechnology-derived drugs, to a more welcoming climate in which the regulators are beginning to work with the scientists to develop guidelines specifically for recombinant pharmaceuticals derived from plants [50].

The launch vector technology described in this review provides many advantages in a regulatory context because it generates high levels of target protein expression, and is easily scalable for manufacturing purposes, but does not involve the production of transgenic plants, therefore, circumventing legislation focusing on stable genetic resources. Furthermore, with the high levels of expression attainable with this technology, the processes can be performed in contained facilities, without the need for field-grown plants, thus avoiding environmental concerns related to the use of recombinant viruses. Although systems based on plant cells share the advantage of containment, the need for fermenters means they are not as scalable. The launch vector technology, therefore, shows great promise for the commercial production of recombinant proteins, including vaccines and therapeutics.

Five-year view

With success in the expression and production of a wide range of functionally active recombinant proteins and immunologically active vaccines in plants, there has been a push towards the building of commercial scale plant and plant cell culture-based production facilities. This thrust is forcing the industry to address several issues, including consistency of expression levels and product quality, detailed biochemical characterization and toxicity testing of the final product, detailed cost estimates, containment and, finally, public acceptance. As these issues are addressed and more parties become interested in this technology, financial support to carry out clinical trials beyond Phase I will become more readily available. The trend towards close collaborations between academia and industry will facilitate progress in the field. With the commercialization of vaccines produced in yeast and insect cell cultures, and more recently the approval of a poultry vaccine produced in plant cell cultures, the vision of many to have a whole plant-produced vaccine will soon become a reality.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Key issues

- An effective vaccine should be inexpensive, confer protective immunity to the vaccine with few side effects and be safe, stable and easy to administer.
- Plants potentially provide an inexpensive platform for the production of safe and stable vaccines, once processing and regulatory requirements have been addressed.
- There are diverse plant-based systems to suit a variety of manufacturing scenarios.
- Plant-based production is easily scalable, allowing it to cope with massive and fluctuating demand and to respond to sudden disease outbreaks.
- Many technological issues, which occurred early in the development of plant-derived pharmaceuticals and vaccines, are now being overcome.

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