Ther Adv Vaccines
2015, Vol. 3(5-6) 139–154
DOI: 10.1177/2051013615613272

Plant-based vaccines for animals and humans: recent advances in technology and clinical trials

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***Abstract*:** It has been about 30 years since the first plant engineering technology was established. Although the concept of plant-based pharmaceuticals or vaccines motivates us to develop practicable commercial products using plant engineering, there are some difficulties in reaching the final goal: to manufacture an approved product. At present, the only plant-made vaccine approved by the United States Department of Agriculture is a Newcastle disease vaccine for poultry that is produced in suspension-cultured tobacco cells. The progress toward commercialization of plant-based vaccines takes much effort and time, but several candidate vaccines for use in humans and animals are in clinical trials. This review discusses plant engineering technologies and regulations relevant to the development of plant-based vaccines and provides an overview of human and animal vaccines currently under clinical trials.

Keywords: GMP-compliant, human vaccine, plant-based vaccine, plant transformation, veterinary vaccine

Introduction

In the past quarter century, plant genetic engineering technologies have progressed dramatically. Barta and colleagues were the first to transcribe a chimeric gene of nopaline synthase and human growth hormone in sunflower and tobacco plants using the Ti plasmid [Barta *et al.*1986]. Shortly thereafter, mouse monoclonal antibody was produced and functionally assembled in tobacco leaf segments [Hiatt *et al.* 1989]. As bioreactors, plants may yield high amounts of recombinant proteins; these proteins are not contaminated with pathogens of animals or humans and can be stored without refrigeration at low cost. A number of recombinant proteins have been produced in plants, and the production of protein-based pharmaceuticals has partially shifted from bacterial, fungal, and mammalian cell cultures to plants and plant cell cultures [Lico *et al.* 2012; Merlin *et al.* 2014; Twyman *et al.* 2005]. Commercialized enzymes and reagents produced in plants are available. For instance, human type I collagen, which can self-assemble into fine homogenous fibrils, is manufactured in tobacco plants [Shoseyov *et al.* 2014]. Bovine trypsin produced in maize, TrypZean (Sigma-Aldrich), has been on the market since 2002. TrypZean is particularly useful in animal cell cultures because it has no contaminants of animal origin. Rice has been used to manufacture human lysozyme and lactoferrin [Hennegan *et al.* 2005; Yang *et al.* 2002]. Protalix, an Israeli company, has developed a method to produce plant-based biopharmaceuticals in cultured transgenic carrot or tobacco cells [van Dussen *et al.* 2013; Zimran *et al.* 2011]. In 2012, Protalix and its partner Pfizer received approval from the United States Food and Drug Administration (FDA) of the United States for taliglucerase alfa for Gaucher's Disease.

On the other hand, plant-based human vaccines are not yet commercialized, although production of dozens of viral and bacterial subunit vaccines is attempted in transgenic plants. Recombinant subunit vaccines are safer than traditional vaccines, because they contain no live pathogens. Various plants such as tobacco, rice, maize, potato, alfalfa, lettuce, tomato, carrot, peanut, and soybean are used as hosts for gene introduction, which is achieved *in vitro* by using protoplast or cell culture, or hairy root culture. Nuclear or chloroplast genome recombination is routinely

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used to obtain transgenic plants. The choice of the plant species and technology determines the vaccine administration route because some plants can be consumed only when processed, whereas heat or pressure treatments may destroy the antigen. Cereal crops are attractive for subunit vaccine production because vaccines produced in seeds are stable over long storage periods [Hefferon, 2013].

There are two options for vaccine administration: injection (intramuscular or subcutaneous) and mucosal (oral or nasal) administration. Injection-type vaccines elicit strong protective immunity by preferentially inducing IgG production. They are most suitable against pathogens that infect via a systemic or respiratory route; however, the antigens have to be purified before administration. These vaccines are often produced in tobacco plants using transient expression.

Oral- or nasal-type vaccines induce mucosal and systemic immunity [Azegami *et al.* 2014; Lamichhane *et al.* 2014]. In a conceptual sense, oral plant-based vaccines are ideal because the manufacturing process is simple; no additional medical devices are needed for injection; and the antigen immunogenicity and biological activities are preserved in the gastrointestinal tract due to their natural bioencapsulation in a plant cell organelle. Oral plant-based vaccines have been developed in edible plants, including rice, maize, potato, lettuce, and carrot. Once these vaccines pass through the gastric environment and reach the small intestine, antigens are incorporated into M cells in the follicle-associated epithelium (FAE) for the induction of mucosal and systemic immune responses [Azegami *et al.* 2014; Holmgren and Czerkinsky, 2005].

This review discusses technologies and regulations in the development of plant-based vaccines and recent achievements in the production of vaccines that are already or expected to be under clinical trials and are intended for worldwide distribution in the near future.

Recombinant technologies

To use plants as bioreactors for commercial vaccines, one needs to (a) attain a high expression level of recombinant genes, (b) be able to quickly and easily design and produce new antigens in response to new pathogen subtypes, and (c) identify the genes to be transfected and ensure the safety of produced proteins for use in humans or animals.

Agrobacterium-*based nucleus transformation*

Plant recombinant technologies are being constantly improved and diversified [Hefferon, 2014] (Figure 1). Genes can be introduced into plants either directly or by using the gram-negative bacterium *Agrobacterium tumefaciens*. Plant transformation with polyethyleneglycol or by electroporation usually includes protoplast preparation by removing the cell wall, which requires time and skill. Almost half of plant transformation technologies use *A. tumefaciens*, which infects plants naturally. The T-DNA region between the left and right borders of the *A. tumefaciens* Ti plasmid is introduced into plant genome and transcribed in the plant cell; this process induces abnormal plant hormone production, resulting in crown gall disease. The T-DNA region can be replaced with a gene of interest, and the Ti plasmid has been modified into a binary vector that can be manipulated in *Escherichia coli* [Bevan, 1984]. Selection pressure is used to establish stable integration of the gene of interest in the nuclear genome. Once the transgenic line stably producing the target protein is established, it can be used as a permanent source of vaccine and established as a master seed bank. A stable and characterized line can be adopted as Good Manufacturing Practice (GMP)-compliant production. Yet, the development of such lines is time-consuming and can be complicated by gene silencing, host genome damage, or hybridization with non-transgenic crops cultivated without strict regulations.

Plastid transformation

A new technology, which targets the chloroplast genome instead of the nuclear genome, is now available. Chloroplasts originate from cyanobacteria that were incorporated into algae. Chloroplast and nuclear genomes coevolved, and now chloroplast genomes possess only 100–250 genes, which is smaller than nuclear genomes. The chloroplast genome is maternally inherited, and plants can stably produce protein without transgene outcrossing via pollen. Multiple chloroplasts with a high transgene copy number can accumulate large amounts of recombinant protein (as much as 70% of total leaf protein [Oey *et al.* 2009]). Foreign genes are usually transformed into chloroplast DNA by biolistic process or polyethylene glycol treatment of protoplasts [Cardi *et al.* 2010]. For plastid transformation, a target gene and selectable marker genes are placed between the two flanking sequences originated

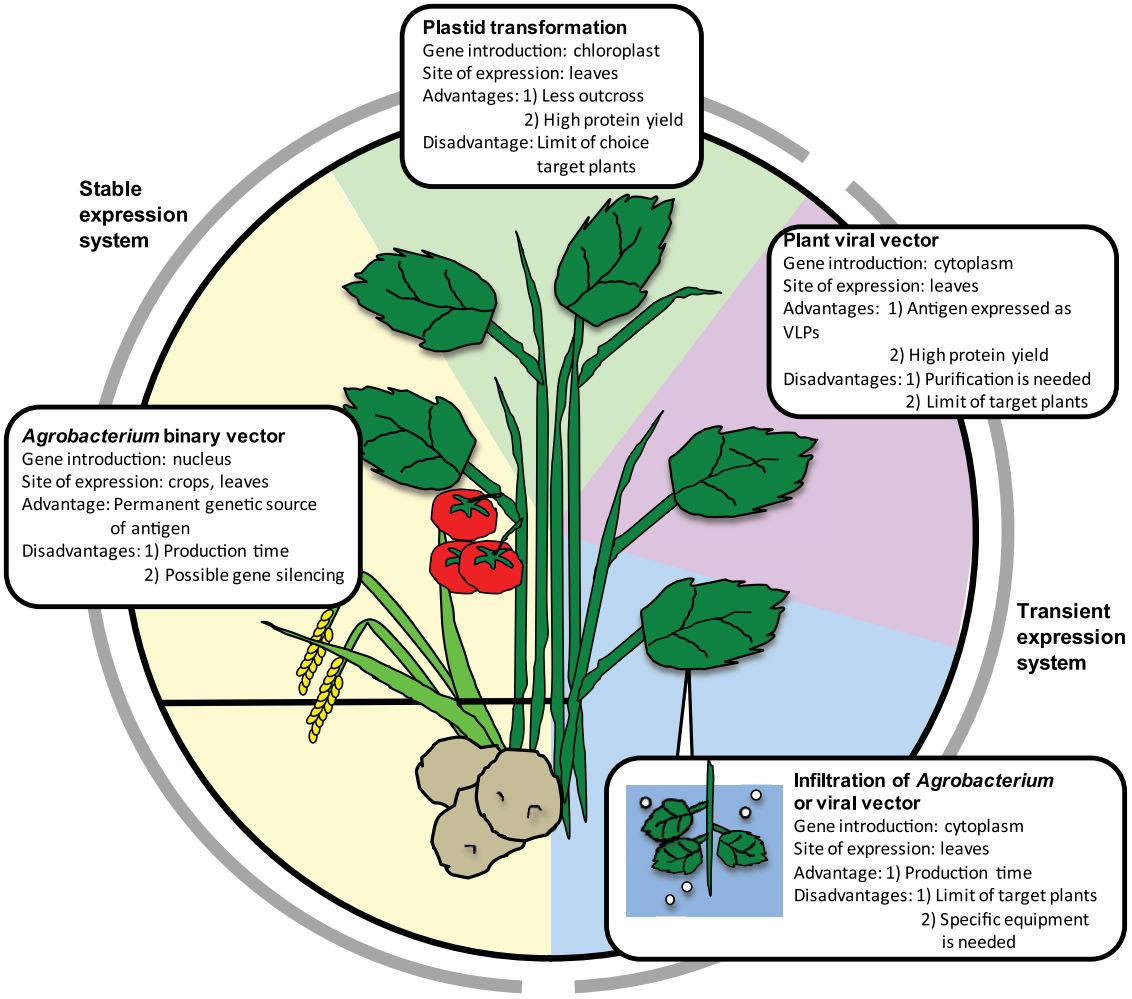

Figure 1. Plant transgenic technologies and their advantages and disadvantages.
VLP, virus-like particle.

from the chloroplast genome to induce homologous recombination between the vector and plastid genome [Verma and Daniell, 2007; Scotti *et al.* 2012]. The chloroplasts of tobacco and other leafy plants such as carrot, petunia, and lettuce have mainly been used; nonphotosynthetic plant organs are less efficient in producing target proteins [Rigano and Walsley, 2005; Verma and Daniell, 2007].

Transient expression with plant virus expression vectors

The benefit of transient expression using plant viral vectors is their high replication ability in the target plant, resulting in high vaccine yield. Several plant viruses are used for this purpose: tobacco mosaic virus (TMV), cowpea mosaic virus (CPMV), potato virus (PVX), alfalfa mosaic virus, and plum pox virus [Salazar-González *et al.* 2015]. First-generation plant viral expression vectors encode almost all viral complements; the gene encoding the protein or peptide of interest is placed downstream of viral polymerase, movement protein, and coat protein genes. This technology has been employed to produce various plant-made vaccines, such as those against human papilloma virus [Cerovska *et al.* 2012; Noris *et al.* 2011] and influenza virus [Ravin *et al.* 2012; Shoji *et al.* 2011], by modifying PVX or TMV and a vaccine against norovirus [Lai and Chen, 2012] by modifying TMV. These recombinant viruses retain infectivity to plants and then shed the transgene, which may spread to other plants, thus prompting safety concerns. Second-generation viral vectors, which are safe in natural environments, have been developed. These vectors rely on an integrated system that has the minimal

Table 1. GMP-compliant plant factories for biopharmaceuticals.

Company	Location	Plant	Bioproduct
Kentucky BioProcessing LLC (KBP)	Owensboro, KY, USA	Tobacco, potato	Norovirus VP1 Ebola virus antibody (ZMapp)
Sigma-Aldrich Fine Chemicals	St. Louis, MO, USA	Maize	Trypsin
Medicago Inc.	Quebec, Canada	*Nicotiana benthamiana*	Influenza HA-VLP
Protalix	Carmiel, Israel	Carrot cells, tobacco cells	Alphataliglicerase
Caliber Biotherapeutics LLC	Byran, TX, USA	Tobacco	Influenza HA
Fraunhofer CMB USA	Newark, DE, USA	*Nicotiana benthamiana*	Influenza HA
Fraunhofer IME	Aachen, Germany	Tobacco	Antibody (for HIV)
National Institute of Advanced Industrial Science and Technology	Hokkaido, Japan	Strawberry	Canine interferon alpha
Institute of Medical Science, The University of Tokyo	Tokyo, Japan	Rice	Cholera toxin B subunit

number of viral elements required for vector replication, whereas other functions, such as DNA delivery, are provided by nonviral elements. These 'deconstructed' expression vectors usually provide higher yields than those attained with full-virus vectors [Peyret and Lomonossoff, 2013; Salazar-González *et al.* 2015].

Infiltration technologies for transient expression

New time-saving technologies to introduce recombinant genes into plants have also been developed. Protocols called 'agroinfiltration' and 'magnifection' use vacuum or syringe to infiltrate leaves of 6-week-old plants such as *Nicotiana benthamiana* or *Arabidopsis* with *Agrobacterium* containing either binary vectors or deconstructed viral vectors [Leuzinger *et al.* 2013]. The use of agroinfiltration in vaccine production was pioneered by the Canadian biotechnology company Medicago, which developed virus-like particle (VLP) vaccines for influenza HA antigens, and these vaccines were used in a clinical trial [Landry *et al.* 2010]. Antigenicity of human and animal viruses is often determined by the conformation of their surface proteins, and to acquire protective immunity, it is desirable to express antigens in a VLP form [Kushnir *et al.* 2012; Vacher *et al.* 2013]. Agroinfiltration uses suspensions of *A. tumefaciens*; this method allows the production of large amounts of vaccine proteins within a few days to a couple of weeks, which is much faster than in stable expression systems [Leuzinger *et al.* 2013]. Another advantage of this protocol is that a variety of T-DNA vectors can be used. Agroinfiltration has also been applied to other leafy plants, such as lettuce [Chen *et al.* 2013].

'Magnifection' has been developed to address various safety concerns, namely the use of intact viral expression vectors and possible transgene loss during systemic spreading. It combines the agroinfiltration method with the delivery of cDNA encoding a 'deconstructed' TMV-based vector [Gleba *et al.* 2004, 2005, 2014; Marillonnet *et al.* 2004]. The magnifection system is restricted to *N. benthamiana*. Icon Genetics, a German plant biotechnology company, has developed and adapted this technology as MagnICON™ for the manufacturing of various plant-based vaccines, including high amounts of hepatitis B virus (HBV) surface antigen (HBsAg; up to 300 mg/kg *N. benthamiana* fresh leaves) in the form of VLP [Huang *et al.* 2006], norovirus capsid proteins [Scotti and Rybicki, 2013; Rybicki, 2014], and non-Hodgkin lymphoma vaccines, which proceeded to a phase I clinical trial (Table 1) [McCormick *et al.* 2008].

A group from Fraunhofer USA Center for Molecular Biotechnology (CMB) has developed 'launch vector', an advanced gene infiltration system that combines the elements of TMV vector and *A. tumefaciens* binary plasmids [Musiychuk *et al.* 2007]. The hybrid launch vector pBID4 contains the 35S promoter from cauliflower mosaic virus (35S CaMV) that drives transcription of the viral genome, the nopaline synthase

(*nos*) terminator, genes for virus replication and cell-to-cell movement proteins, and the target gene cloned under the transcriptional control of the coat protein subgenomic mRNA promoter. Following infiltration, primary transcripts produced in the nucleus are transported into the cytoplasm, resulting in robust protein production [Musiychuk *et al.* 2007].

The pEAQ system is based on full-length or truncated versions of CPMV RNA-2 and permits efficient and rapid protein production without viral replication [Peyret and Lomonossoff, 2013; Sainsbury *et al.* 2009]. In pEAQ system, a series of small binary vectors, which may be used for production of a wide variety of proteins in both transient and stable expression systems, was engineered. These vectors contain the 35S CaMV promoter, *nos* terminator, the P19 sequence encoding a suppressor of silencing, and 5′- and 3′-UTRs from CPMV RNA-2. The gene of interest is inserted between the UTRs. A new-generation vector, pCPMV-HT, provides extremely high translational efficiency and, consequently, a high level of the recombinant protein [Peyret and Lomonossoff, 2013].

Other methods with improved performance of transfection

Engineering of genetically modified plants without the use of antibiotic resistance genes as selection markers eliminates the potential risk of transfer of these genes to gut microbes when the vaccine is orally administered or to the environment. Using *Agrobacterium*-mediated nucleus transformation, Mejima and colleagues cotransformed the selection marker hygromycin phosphotransferase gene and cholera toxin B subunit gene encoded by separate T-DNA vectors and selected marker-free candidate plants by segregation in the seed progeny [Mejima *et al.* 2015]. Daniell and colleagues used betaine aldehyde dehydrogenase (BADH) gene from spinach as a selectable marker for plastid transformation [Daniell *et al.* 2001]. This enzyme is naturally produced in the chloroplast and converts toxic betaine aldehyde into non-toxic glycine betaine. BADH transgenic tobacco plant showed higher BADH activity than did nontransgenic plants, indicating that transgenic plants could be selected by the level of enzyme activity.

Knocking down mRNAs for rice storage proteins, glutelin and 13K prolamin, by RNA interference (RNAi) using the same T-DNA vector enhanced the production of botulinum neurotoxin A or cholera toxin B subunit in rice seeds [Kuroda *et al.* 2010; Kurokawa *et al.* 2013; Yuki *et al.* 2012]. Other options to increase protein production in plants include modification of codon usage from that of the host to that of the plant [Hiwasa-Tanase *et al.* 2011; Jackson *et al.* 2014], intron introduction in TMV before the target gene [Chakravarthi *et al.* 2015], and co-expression of a suppressor of gene silencing [Garabagi *et al.* 2012; de Ronde *et al.* 2014].

N-glycosylation is relatively well-conserved in eukaryotes; however, there are several sugar-modification enzymes specific to plants. Knockout of genes for plant-specific N-glycan-processing enzymes and the introduction of the enzymatic machinery catalyzing synthesis, transport, and addition of mammalian sugars have been reported [Gomord *et al.* 2010]. Whereas this strategy is effective for producing native forms of viral antigens, unexpected N-glycosylation of vaccine antigens of bacterial origin produced in plants may result in molecular heterogeneity and difficulties in recognition by immune cells. Because N-glycosylation occurs at asparagine residues, the substitution of asparagine with aspartic acid or another amino acid can solve this problem [Yuki *et al.* 2013].

Risk analysis and regulations

Potential risks

Several considerable risks are associated with plant-based pharmaceuticals [Kirk *et al.* 2005]. Plant-made vaccines, particularly the oral ones, might induce either allergenicity or oral tolerance, which are two conflicting phenomena. Post-translational modifications such as N-glycosylation might induce allergic responses, whereas co-administration with oral adjuvants to broadly stimulate the mucosal immune system might induce hypersensitive responses to other proteins in daily food [Guan *et al.* 2013]. Frequent administration of plant-made vaccines via the oral route can enhance regulatory T-cell activation against vaccine antigen [Fujihashi *et al.* 1999], as seen in successful hyposensitization therapy by oral antigen intake in cases of pollen allergy or food allergy [Cox *et al.* 2012; Sato *et al.* 2014].

Another potential risk of the use of plant-made vaccines is their influence on the environment. Small-scale production of genetically modified plants

(including their production for research purposes) must be managed. Even manufacturing in regulated facilities to control the quality and safety of the products may pose difficulties in the use of genetically modified plants. Another aspect is that open-field production of stably transfected plants increases the possibility of contamination of nontransgenic crops intended for human or animal consumption [Bawa and Anilakumar, 2013; Fischer *et al.* 2012]. Recombinant genes can spread to field crops via pollen and accidentally result in genetic modifications in nontarget plants. Genetically modified plants might be eaten by wild animals or accidentally harvested by humans. Contact with insects and the release of contaminated water to the environment are also possible mechanisms for DNA or antigen escape. The probability and severity of each risk will depend on the plant species and the antigen and has to be determined on a case-by-case basis for each plant-based vaccine.

Government regulations

In the United States, the FDA ensures the safety for both manufacturing and clinical use of plant-based biopharmaceuticals and vaccines; it also approves and licenses them. Similar to other biopharmaceuticals, plant-based vaccines should be free of impurities, including other transgenes and resistance marker products, which must all be evaluated under the same criteria. The FDA also approves GMP facilities for plant manufacturing. In 2005, the World Health Organization (WHO) conducted an 'informal consultation on scientific basis for regulatory evaluation of candidate human vaccines from plants' [van der Laan *et al.* 2006]. In its report, the WHO recommended that the guidelines on Good Agricultural and Collection Practices (GACP), which are typically applied to herbal plants, should also be applied to plants producing biopharmaceuticals. A report of quality-control methods for medicinal plant materials recommended tests to assess the identity, purity, and content of biopharmaceutical plant materials. The United States Department of Agriculture (USDA) also plays a key role in the introduction of plant-made pharmaceuticals. The USDA approves veterinary biologics such as vaccines. The USDA considers the nature of the plant, the probability of cross-contamination, and the genetic background of stably transfected plants. The USDA also considers the risk-management strategies, taking into account physical and geographical aspects and plant reproduction.

The European Medicines Agency (EMA) published draft guidance notes on 'the quality of biological active substances produced by stable transgene expression in higher plants' [EMA, 2008], which complement the FDA and USDA regulations. However, these notes cover only plants with stable expression of transgenes and exclude transiently transfected plants and plant cell cultures. The use of several transient expression technologies, which rely on plant viral vectors or agroinfiltration (or both), requires advanced regulation applicable to all plant transgenic technologies. An interesting point in the EMA guidelines is a statement of the importance of the establishment of master and working seed banks from the final transformant. Both banks should be well-characterized in respect to the transgene (sequence, integrity, site of insertion, copy number, and the fate of the marker sequence), recombinant protein expression (tissue and organ specificity, regulation, and expression level), and unintended changes in the levels of endogenous plant proteins. The storage properties (conditions, shelf-life, and closure criterion) of the master transgenic bank should be defined also. The authorities of European Food Safety Authority (EFSA) and EMA partially overlap. The former cares for the cultivation of transgenic plants, and the latter cares for biopharmaceutical products from transgenic plants. Importantly, EMA regulatory guidelines [EMA, 2008] indicate that all biopharmaceutical products intended for phase I trials should be manufactured according to GMP. Thus, GMP compliance appears to be a key point in developing plant-based vaccines for clinical use.

GMP facilities for plant bioreactors

Production of recombinant proteins used in pharmaceutical applications requires certain quality standards. The GMP grade is compulsory for clinical applications. At least two large biomanufacturing facilities are capable of producing plant-derived HA proteins under GMP conditions, Fraunhofer CMB [Shoji *et al.* 2011, 2015] in the United States and Medicago Inc. in Canada [Yusibov *et al.* 2014]. Fraunhofer CMB is situated in Delaware. It possesses a GMP pilot plant that is capable of dealing with regulatory and clinical affairs and technology transfer. The key processing areas in the GMP plant are equipped with complete processing cycles for transient expression in plants, such as plant and bacterium cultivation, infiltration, plant harvest, and protein purification. The major product in this facility is

Table 2. Plant-based human vaccines in clinical trials.

Pathogen or disease	Antigen	Plant	Expression system	Administration route	Clinical trial	Reference
Enterotoxigenic *E. coli*	LTB	Potato	Transgenic	Oral	Phase I	Tacket *et al.* [1998]
Enterotoxigenic *E. coli*	LTB	Maize	Transgenic	Oral	Phase I	Tacket *et al.* [2004]
Norovirus	Capsid protein	Potato	Transgenic	Oral	Phase I	Tacket *et al.* [2000]
Hepatitis B virus	Viral major surface protein	Lettuce	Transgenic	Oral	Phase I	Kapusta *et al.* [1999]
Hepatitis B virus	Viral major surface protein	Potato	Transgenic	Oral	Phase I	Thanavala *et al.* [2005]
Rabies virus	Glycoprotein and nucleoprotein (fusion)	Spinach	Viral vector (transient)	Oral	Phase I	Yusibov *et al.* [2002]
Influenza virus (H5N1)	HA	*Nicotiana benthamiana*	Launch vector (transient)	Intramuscular	Phase I	Chichester *et al.* [2012]
Influenza virus (H1N1; 2009 pandemic)	HA	*Nicotiana benthamiana*	Launch vector (transient)	Intramuscular	Phase I	Cummings *et al.* [2014]
Influenza virus (H5N1)	HA (H5; VLP)	*Nicotiana benthamiana*	Agrobacterial binary vector (transient)	Intramuscular	Phase I Phase II	D'Aoust *et al.* [2008] Landry *et al.* [2010]
Influenza virus (H7N9)	HA (H7; VLP)	*Nicotiana benthamiana*	Agrobacterial binary vector (transient)	Intramuscular	Phase I	Medicago Inc. (http://www.medicago.com)
Influenza virus	HA (VLP) (seasonal; quadrivalent)	*Nicotiana benthamiana*	Agrobacterial binary vector (transient)	Intramuscular	Phase I	Medicago Inc. (http://www.medicago.com)
Cholera	CTB	Rice	Transgenic	Oral	Phase I	Nochi *et al.* [2009] Yuki *et al.* [2013]

influenza HA antigen, which is produced by using plant-based Proficia™ technology, VLPs, and the VLPExpress™ platform for transient expression in *N. benthamiana*. Medicago Inc., located in Québec, is now proceeding to phase II clinical trial with influenza VLP (H5) produced in *N. benthamiana* by using the agroinfiltration method (see http://www.medicago.com). Rabies and rotavirus antigens produced by Medicago Inc. are at a preclinical stage. Some university-launched plant-made vaccines that reached phase I clinical trials have been produced in collaboration with GMP facilities. Norovirus capsid protein subunit vaccine produced in potato tubers was developed in Arizona State University and manufactured at Kentucky BioProcessing GMP plant [Tacket *et al.* 2000]. In Japan, rice-based cholera vaccine has been developed at the Institute of Medical Science, The University of Tokyo (IMSUT); test vaccine produced at the GMP facility at IMSUT is now in a phase I clinical trial [Yuki *et al.* 2013; Mejima *et al.* 2015] (Table 1).

Human vaccines in clinical trials

Only a few plant-based human vaccines have reached clinical trials (Table 2). Non-toxic B subunit of heat-labile enterotoxin (LTB) of enterotoxigenic *E. coli* (ETEC) produced either in potato or maize was administered orally to healthy volunteers to examine its safety and immunogenicity. Raw, diced, transgenic potato tubers containing LTB (0.4 or 1.1 mg) were given to volunteers on days 0, 7, and 21 [Tacket *et al.* 1998, 2007; Yusibov *et al.* 2011]. For maize-derived LTB, the clinical study was placebo-controlled, and each group received 2.1 g of either transgenic or wild-type maize germ meal suspended in water on days 0, 7, and 21 [Tacket *et al.* 2004, 2007]. No adverse effects of vaccination were noticed in comparison with the placebo control. LTB-specific IgA-secreting cells were detected in peripheral blood 1 week after the first vaccination. Serological survey revealed that vaccinated volunteers had increased levels of LTB-specific serum IgG (91% of the volunteers) and IgA (55%) on day 59.

Norovirus capsid protein VP1 was produced in potato tubers in the same way as LTB. As norovirus is a nonenveloped virus, VP1 is the only protein of the capsid. Vaccination with approximately 500 μg of recombinant VP1 was done on days 0, 7, and 21 (three doses) or on days 0 and 21 (two doses). Some volunteers had VP1-specific antibodies before the study because norovirus is highly infectious, and repeated epidemics are frequent, especially in winter [Campos and Lees, 2014; Teunis *et al.* 2008]. However, 20% of vaccinated volunteers developed VP1-specific serum IgG titers. The geometric mean of IgG titers (four responders) was 1:67 before vaccination and 1:757 after vaccination [Tacket *et al.* 2000].

According to the WHO, almost 780 000 people die every year from HBV infections worldwide. In different regions, 1–10% of the adult population are chronically infected with HBV, whereas 80–90% of infants infected under the age of 6 months develop chronic infections that lead to cirrhosis or liver cancer [MacLachlan and Cowie, 2015; Norkrans, 1990]. HBV surface antigen (HBsAg) was produced in plants. HBsAg transgenic lettuce leaves (0.1–0.5 μg of HBsAg per 100 g of fresh tissue) were given to adult volunteers (initially 200 g, then 150 g within 2 months). Two of three vaccinated volunteers showed transient protective levels of HBsAg-specific IgG (above 10 IU/l) 2 weeks after the second vaccination, but no HBsAg-specific serum IgA was detected [Kapusta *et al.* 1999].

Another clinical study was conducted with oral HBsAg produced in transgenic potato [Thanavala *et al.* 2005]. All volunteers enrolled in this study had received three doses of HBV injection-type vaccine within 15 years. The placebo group was given nontransgenic potato, the two-dose group was vaccinated at 0 and 28 days with 100 g of transgenic potato (850 ± 210 μg of antigen), and the three-dose group was vaccinated with the same doses at 0, 14, and 28 days. The authors found that 52.9% of participants in the two-dose group and 62.5% of participants in the three-dose group had elevated serum HBsAg antibody titers over the 70-day follow-up period after the first immunization.

Current human rabies vaccines are efficacious both pre- and post-exposure to rabies virus [Toovey, 2007]. Endemic rabies is spreading all over the world except in a few countries. To reduce mortality caused by rabies, a regular stock of safe rabies vaccine in endemic areas is needed. Antigenic determinants of rabies virus G and N proteins have been mapped, and a synthetic chimeric peptide (G5–24-N31D) containing a linear epitope of G protein and an epitope of N protein was found to be immunogenic in mice [Dietzschold *et al.* 1990]. Yusibov *et al.* [2002] fused fragments encoding a chimeric protein of G protein (amino acids 253–275) and N protein (amino acids 414–418) with that of alfalfa mosaic virus coat protein and introduced this fusion construct into TMV lacking native coat protein. Spinach was infected with recombinant virus to obtain transient expression of the chimeric rabies peptide. Three of 5 volunteers previously vaccinated with a commercial injection-type vaccine had elevated rabies-specific IgG after having ingested 3 doses of spinach (20 g; 84 μg of chimeric rabies peptide each) at 2-week intervals. Another protocol involved volunteers with no history of rabies vaccination; five of nine participants responded to the rabies antigen. An additional single dose of commercial vaccine enhanced rabies virus-neutralizing antibody production (three out of nine participants). Regardless of the vaccination order, spinach oral rabies vaccine in combination with currently available vaccines might enhance immunity against rabies virus [Yusibov *et al.* 2002].

Influenza virus has 16 hemagglutinin (HA) subtypes, and even in the strains with the same subtype, antigenic shift often occurs to abolish cross-protective immunity of the host. For example, in 2009, H1N1-type influenza virus became pandemic [Itoh *et al.* 2009; Neumann *et al.* 2009]. To control its spread by vaccination, fast production of a new HA antigen was required. As mentioned above, Medicago Inc. developed the technology to produce VLPs of influenza virus HA in *N. benthamiana* with the *A. tumefaciens*-based transient expression system [D'Aoust *et al.* 2008; Yusibov *et al.* 2011; US patent application number 20130183341]. VLPs of the expected size were found between the plasma membrane and the cell wall of *N. benthamiana* cells [D'Aoust *et al.* 2008]. Phase I and II clinical trial of the VLP composed of HA protein of H5N1 influenza virus (A/Indonesia/5/05) (H5-VLP) has been completed. In a phase I clinical trial, 5, 10, or 20 μg of H5-VLP was subcutaneously injected twice with alum adjuvant [Landry *et al.* 2010]. The vaccine-induced hemagglutinin inhibition titer at all tested doses. In addition, a phase II clinical trial of H5-VLP was conducted as a randomized, placebo-controlled, dose-ranging study

that used 20, 30, or 45 μg of H5-VLP [Landry *et al.* 2014]. After 6 months of vaccination with H5-VLP, the volunteer group showed cross-protective $CD4^+$ T-cell responses, which were not observed in the placebo group, indicating strong induction of long-term cell-mediated immunity by plant-made H5-VLP. The immunogenicity of plant-specific glycans has also been studied in this clinical trial; some vaccine recipients developed plant N-glycan-specific allergic or hypersensitivity symptoms. Some volunteers (34%) developed transient IgG and, in some cases, IgE to plant glyco-epitopes, but no IgE responses to mannose residues (MMXF motifs) were observed. The levels of antibodies returned to baseline by 6 months in most participants [Ward *et al.* 2014]. Medicago Inc. also completed phase I clinical trial using 5, 13, or 28 μg of H1N1 influenza (A/California/7/09) VLP (H1-VLP) vaccine [Landry *et al.* 2010]. All doses tested were safe and well-tolerated and induced immune response to the virus, including cell-mediated immunity. The company intends to proceed phase IIa trial of its seasonal trivalent vaccine with antigens from the recommended pandemic H1N1, H3N2, and B influenza strains [Redkiewicz *et al.* 2014].

Another plant-based influenza vaccine that has completed phase I clinical trial is in production at Fraunhofer CMB USA. HA from A/California/04/2009 H1N1 (HAC1) and A/Indonesia/05/05 H5N1 (HAI-05) has been produced in *N. benthamiana* by using an infiltration method of *A. tumefaciens* in which genes are regulated by the 'launch vector' [Shoji *et al.* 2011, 2015]. Preclinical studies using mice and rabbits were conducted by the injection of HAC1 and HAI-05 twice with 3-week-intervals. Seropositive rate of serum HA antibody responses were 100% in mice with the dose of 5 μg for HAC1, and 45 μg for HAI-05 in a dose-dependence test with alum-adjuvant. Rabbits also showed seropositive for HA with the dose of 90 μg in both HAC1 and HAI-05 [Shoji *et al.* 2011]. A phase I clinical trial for HAC1 and HAI-05 was conducted as a randomized, double-blind, placebo-controlled study with healthy 18–49-year-old volunteers [Chichester *et al.* 2012; Cummings *et al.* 2014]. In both cases, three doses (15, 45, and 90 μg) of purified antigen with or without Alhydrogel® (as adjuvant) were administered twice intramuscularly. Nearly all adverse events were mild to moderate; the highest responses were detected by hemagglutining inhibition (HI) and viral-neutralizing (VN) antibody titers and were observed in the group immunized with the highest dose (90 μg) without adjuvant.

Development of veterinary vaccines

Antigens of farm-animal pathogens have been expressed in plants, and a few of them have been tested in host species. Plant-made vaccines for veterinary use are listed in Table 3. The first USDA-approved plant-made vaccine was for veterinary use: Newcastle disease vaccine for poultry from the USDA Center for Veterinary Biologics was approved in 2006 [reviewed in Floss *et al.* 2007; Kolotilin *et al.* 2014; Rybicki 2010]. Dow AgroSciences LCC (Indianapolis, IN, USA) produces hemagglutinin and neuraminidase of Newcastle disease virus in suspension-cultured tobacco cells [reviewed in Yusibov *et al.* 2011]. Two subcutaneous doses of this vaccine at a 2-week interval administered to neonatal chicks ensure 90% protection against Newcastle disease virus challenge. For farm animals, the cost of immunization tends to limit profit from selling products such as meat, milk, and eggs. Therefore, plant-based vaccines are an asset for the animal use if they can be manufactured at low cost. Moreover, edible vaccines require little effort for administration. In poultry, in addition to the approved vaccine mentioned above, glycoprotein of Newcastle disease virus has been expressed in potato, tobacco, maize, and rice [Guerrero-Andrade *et al.* 2006; Kolotilin *et al.* 2014; Zhou *et al.* 2004]. S1 glycoprotein gene of chicken infectious bronchitis virus (IBV) has been introduced into potato [Zhou *et al.* 2004]. Oral immunization with transgenic potato tubers (5 g; 12.45 μg of S protein) three times or intramuscular immunization with transgenic potato extracts two or three times elicited high neutralizing antibody titers against IBV in chicken serum. These levels were similar to those in chickens immunized with live attenuated intranasal vaccine and conferred 60–80% protection from a virulent IBV strain. Lymphocyte proliferation and IL-2 production by spleen cells were confirmed in vitro, indicating that the potato-based vaccine induced protective immunity. To protect chicken against infectious bursal disease virus (IBDV), the protective antigen VP2 was expressed in rice and tobacco [Gómez *et al.*, 2013; Wu *et al.* 2007], and efficiently protected chickens from a highly virulent IBDV strain.

Plant-based vaccines for pig protection from ETEC and foot and mouth disease virus (FMDV)

Table 3. Plant-based vaccines for veterinary use.

Host	Pathogen	Antigen	Plant	Administration route	Treated animal	Reference
Chicken	Newcastle disease	Hemagglutinin-neuraminidase	Tobacco suspension cells	Subcutaneous	Chicken	Vermij *et al.* [2006] Approved by USDA
Chicken	Newcastle disease	F protein	Maize	Oral	Chicken	Guerrero-Andrade *et al.* [2006]
Chicken	Newcastle disease	F protein	Rice	Oral	Mice	Yang *et al.* [2007]
Chicken	IBV	S1 glycoprotein	Potato	Oral	Chicken	Zhou *et al.* [2004]
Chicken	IBDV	VP2	Rice	Oral	Chicken	Wu *et al.* [2007]
Pig	ETEC	Fimbriae (F4)	Tobacco (chloroplast)	N/D	Pig (in vitro assay in intestines)	Kolotilin *et al.* [2012]
Pig	ETEC	Fimbriae (F4)	Alfalfa	Oral	Piglet	Joensuu *et al.* [2006]
Pig	ETEC	Cholera toxin B subunit	Rice	Oral	Pig	Takeyama et.al. [2015]
Pig	ETEC	Fimbriae (F4)	Barley	Subcutaneous	Mice	Joensuu *et al.* [2006]
Pig	Foot and mouth disease virus	VP1	*Nicotiana bentamiana*	Intramuscular	Pig	Yang *et al.* [2007]
Pig	TGEV	S protein	Tobacco	Intramuscular	Pig	Tuboly *et al.* [2000]
Cattle	Bovine Herpesvirus	gD protein	Tobacco	Intramuscular and subcutaneous	Cattle	Pérez Filgueira *et al.* [2003]
Cattle	Bovine Viral Diarrhea Virus	E2 protein	Alfalfa	Intramuscular	Cattle	Peréz Aguirreburualde *et al.* [2013]
Cattle	Rinderpest virus	Hemagglutinin	Peanut	Oral	Cattle	Khandelwal *et al.* [2003]

have been well characterized. ETEC fimbriae (of type F4) were produced in alfalfa chloroplasts and remained stable for 2 years when alfalfa was dried and stored at room temperature [Joensuu *et al.* 2006]. Recombinant proteins in combination with cholera toxin as an adjuvant were introduced intragastrically into piglets, resulting in a reduction in ETEC excretion in their feces. Kolotilin and colleagues stably expressed F4 fimbrial adhesin FaeG in tobacco chloroplasts (1% of dry leaf weight or 11.3% of total soluble leaf protein) [Kolotilin *et al.* 2012]. Although the authors did not challenge vaccinated animals, they showed that recombinant F4 competitively inhibited the attachment of F4-positive ETEC to pig small-intestinal villi *in vitro* [Kolotilin *et al.* 2012]. We have produced rice-based cholera toxin B subunit (CTB) vaccine, which is efficient against pig ETEC heat-labile toxin because the amino acid sequence and conformation of this toxin are similar to those of cholera toxin. The use of CTB-producing rice, named MucoRice-CTB, ensured high antigen stability in the gastrointestinal tract and mucosal immune induction in a mouse model [Nochi *et al.* 2007; Tokuhara *et al.* 2010]. Pregnant sows and weaned minipigs produced antigen-specific IgG and IgA in their sera upon MucoRice-CTB immunization. MucoRice-CTB also induced maternal CTB-specific IgG and IgA in the colostrum and milk of sows after farrowing. These antigen-specific maternal antibodies offer newborn piglets passive immunization with ingested milk. CTB-specific antibodies also were secreted into the gut lumen of weaned piglets and reduced intestinal loop fluid accumulation upon ETEC challenge, indicating a protective effect of MucoRice-CTB against ETEC diarrhea. Therefore, MucoRice-CTB could be a

candidate oral vaccine for inducing both passive and active immunity to protect both suckling and weaned piglets from ETEC diarrhea [Takeyama *et al.* 2015].

FMDV infects pigs and cows but not humans and causes a major animal disease, the prevention of which requires international cooperation. Yang and colleagues expressed VP1 of FMDV on the surface of bamboo mosaic virus in *N. benthamiana* and *Chenopodium quinoa* [Yang *et al.* 2007]. Purified VP1-expressing bamboo mosaic virus was intramuscularly injected with an oil adjuvant into 2-month-old piglets twice with a 6-week interval. The neutralization titer was elevated in all piglets that received 0.5, 1, or 5 mg of purified antigen even after single vaccination. FMDV VP1 and the structural polyprotein P1 produced in tobacco, potato, alfalfa, or tomato protected mice and guinea pigs from FMDV challenge [Carrillo *et al.* 2001; Dus Santos *et al.* 2005].

Rabies virus causes a zoonotic disease transmitted from wild animals such as bats, raccoons, and foxes to pet animals and humans. To control rabies in humans, vaccination of wildlife and pets is needed [Fooks *et al.* 2014]. Capturing large numbers of wild animals for vaccine injection is almost impossible, and distribution of bait mixed with oral vaccine in the risk regions could be a major solution for rabies control [Yang *et al.* 2013]. Rabies G protein has been expressed in several species, including tobacco, tomato, spinach, carrot, and maize [Loza-Rubio *et al.* 2012; McGarvey *et al.* 1995; Rojas-Anaya *et al.* 2009]. Oral immunization with rabies G protein produced in maize (0.5–2 mg) protected sheep from rabies strain CASS-88; the mortality rate from virus challenge 120 days after vaccination was reduced in a dose-dependent manner [Loza-Rubio *et al.* 2012]. Plant-made rabies antigens may be used at different doses both in humans and animals.

Aquafarming relies on ocean water, and excessive use of antibiotics contaminates the environment. The use of oral vaccines for disease prevention in fisheries and aquaculture may ameliorate this problem [Clarke *et al.* 2013].

Conclusions

Several key points are essential for the development of a broadly effective GMP-compliant regulatory framework for clinical application of plant-based vaccines in humans and animals. First, we need to determine the eligible combinations of target plants and transgenic protocols. Unlike open-air farming, the production of transgenic plants for biotherapeutic use is strictly regulated. Plant selection would affect the whole procedure throughout commercialization. Transient expression systems enable the rapid production of high amounts of target proteins, but their implementation is complex because it requires infiltration and the large-scale use of *A. tumefaciens* or viral vectors. Second, we need to determine the most suitable cultivation system for transgenic plants (open-field or in-house cultivation). Open-field cultivation is less expense than greenhouse or in-house cultivation, but plant factories offer controllable, reproducible cultivation conditions suitable for GMP manufacturing. Finally, we need to define the procedures for manufacturing and processing of plant-based pharmaceuticals. The challenge is to facilitate the procedures without compromising quality, which is a prerequisite for manufacturing plant-based human and animal vaccines.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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