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Current status of plant-made vaccines for veterinary purposes

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Interest is growing for the use of plant-made vaccines for veterinary purposes since the regulatory landscape still enables delivery of either crude extracts or minimally processed plant materials to animals for medicinal purposes. In this article, we highlight the current research directions taken with four diseases considered as important constraints to international trade in animals: avian influenza, Newcastle disease, foot-and-mouth disease and diarrheal disease caused by enterotoxigenic *Escherichia coli*. We also discuss appropriate plant production platforms with regards to plant species and transformation methodologies, possible areas of development, and the remaining challenges for plant-made vaccines for veterinary purposes.

Keywords: mucosal delivery • plant vaccines • regulations • veterinary

Plant-made vaccines (PMVs) are a type of subunit vaccine where the bioreactor is a plant or plant cell. There are many methods of producing PMVs, each with their advantages and disadvantages dependent on the end application. The advantages of producing vaccines in plants often include: the ability of plants to produce complex proteins; ability to target expression of the antigen of interest to different plant subcellular locations, thereby increasing expression, stability and/or enabling different degrees of post-translational modifications such as glycosylation; reduced chance of contamination with animal or human disease-causing agents; the plant cell offering increased protection from digestion, thereby enabling use of less expensive, partially processed material for oral delivery; reduced cold chain reliance and cost; reduced complexity in vaccine delivery; and increased ease of vaccine production scale-up. The expression platform of recombinant proteins in transgenic plants has been actively researched in the past 20 years, resulting in a fast and flexible system. Regulations associated with veterinary vaccines are significant yet still less burdensome than those associated with human pharmaceuticals, therefore, crude or partially purified formulations and oral delivery are still possible. Therefore, it is understandable that PMV development for veterinary purposes has gained momentum.

Animal populations can be loosely divided into the three categories: companion animals, domesticated animals and free-ranging animal populations. Although successful vaccination of these three categories has different requirements, it is undeniable that the ability to produce a vaccine in plant material that could then be delivered by feeding would have tremendous value. However, guidelines for human health have hindered progress of mucosal delivery of PMVs for delivery to humans such that the emphasis is now on purification [1], in-depth antigen characterization and parenteral delivery. Following this trend, the first PMV to be commercially licensed, a veterinary vaccine to protect poultry from Newcastle disease virus (NDV) [101], was injectable. We discuss the PMV technology for veterinary applications (vaccines intended for animal end users as opposed to merely a test system), highlighting the current directions taken by research, possible areas of development and veterinary PMV platform challenges.

Current veterinary PMV research

Vaccination of animals decreases pharmaceutical treatment (particularly antibiotic use), prevents long-term suffering and death due to disease, decreases economic burden of disease through loss of livestock or well-being, and safeguards agricultural industries. We focus on four veterinary PMVs against diseases classified

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as List A by l'Office International des Épizooties (OIE; World Organization for Animal Health) and recognized as important constraints to international trade in animals with substantial economical impact (Figure 1).

Avian influenza

Being a respiratory, zoonotic virus, the influenza virus causes regular seasonal epidemics with the recent avian influenza (AI) H5N1 and swine influenza threats highlighting how human health can benefit from the control of disease in animals. Influenza virus is a negative-stranded, segmented RNA virus that belongs to the family *Orthomyxoviridae*. This highly contagious virus replicates rapidly in the respiratory mucosa and intestinal tract of infected animals, and transmits to others on contact with contaminated secretions or excretion. There are three influenza viral types A, B and C with distinct pathogenicity and genome properties. Influenza type A virus is endemic in aquatic birds. It is contagious not only to avian species but also to a variety of mammals. Influenza types B and C infect mainly humans and are generally less lethal. We focus on influenza A viruses owing to the strain's importance to animal and human populations.

Influenza A viruses are differentiated into subtypes based on the surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). Currently, 16 subtypes of HA [H1–H16] and nine subtypes of NA [N1–N9] have been identified in influenza A viruses. These glycoproteins are found in numerous combinations thus making up numerous subtypes and strains [2]. AI infection among birds is endemic. Although these influenza A viruses do not commonly infect humans, they can transmit to humans in close and direct contact with infected birds. AI epidemics occur almost annually, especially in Asia where trading of live poultry in open areas is common. Highly virulent forms of the virus evolve by antigenic drift (random accumulations of mutations) and genetic reassortment (rearrangement and recombination among subtypes of HA and NA surface antigens), facilitating the evasion of host immunity owing to the mismatch between antigens present in the circulating viral strains and antigens already presented to the host immune system through natural encounters or vaccination [3,4]. There are several subtypes that have mutated to form highly pathogenic AI viruses. These strains have caused significant economic losses due to reoccurring cases and continue to affect domestic birds in several countries.

Development of vaccines for poultry is a sound strategy for controlling highly pathogenic AI since, in addition to protecting the poultry industry, it would reduce the likelihood of transmission of AI from birds to humans. Several reports of plant-made influenza vaccines for animals and humans have been published, with the HA glycoprotein being the main target immunogen. *Agrobacterium*-mediated transient expression of HA in *Nicotiana benthamiana* was demonstrated by D'Aoust and colleagues [5]. High accumulation (50 mg/kg) of virus-like particles (VLPs) made from HA antigen was observed. Mice immunized intramuscularly with two doses of 0.5 µg of the purified H5 VLPs were protected against H5N1 influenza virus challenge [5]. Another report of transient expression of HA used agroinfiltration of a tobacco mosaic virus (TMV)-based viral system in *N. benthamiana* [6]. In this study, the purified plant codon-optimized HA elicited strong H5-specific immune responses in mice and displayed high hemagglutination inhibition and virus-neutralizing antibody titers. A subsequent study with this plant-made HA showed full protection against challenge in immunized ferrets [7].

Spitsin *et al.* demonstrated that tobacco could express polypeptide variants of the HA1 domain alone or fused to His/c-myc tags, or with mouse or human heavy-chain IgG Fc fragments. The fusions were designed to assist purification, and did indeed improve yield using a single-step purification [8]. However, despite the fact that mice immunized intramuscularly with the purified HA1 domain from leaf extracts developed H5-specific IgG responses, the sera of immunized mice was not active in virus-neutralizing or -inhibition assays.

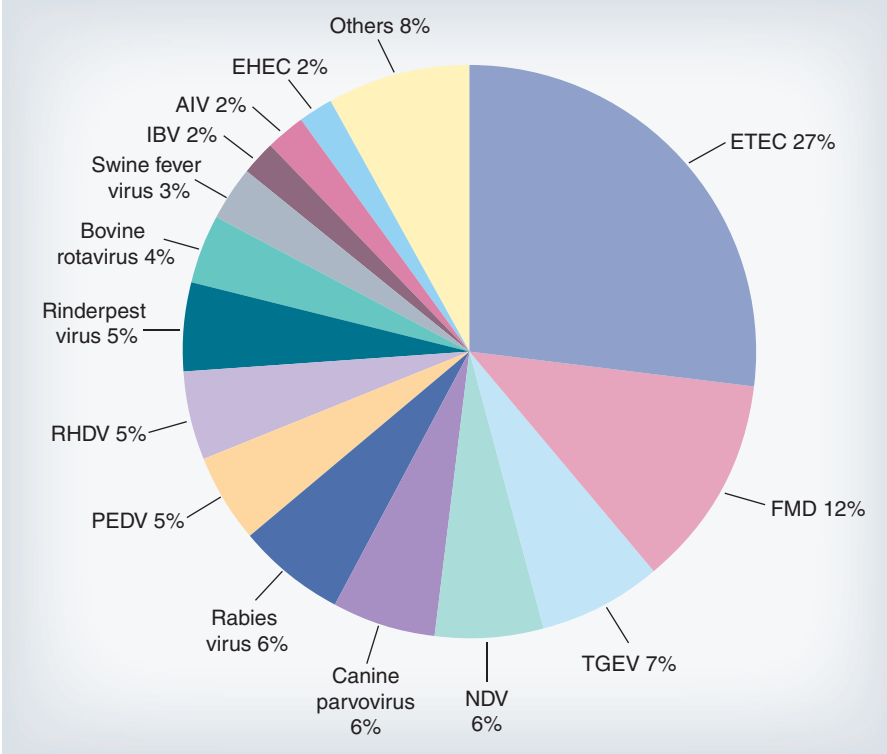

Figure 1. Percentage of published manuscripts on antigens from animal pathogens expressed in plants.
AIV: Avian influenza virus; EHEC: Enterohemorrhagic *Escherichia coli*; ETEC: Enterotoxigenic *E. coli*; FMD: Foot-and-mouth disease; IBV: Infectious bronchitis virus; NDV: Newcastle disease virus; PEDV: Porcine epidemic diarrhea virus; RHDV: Rabbit hemorrhagic disease virus; TGEV: Transmissible gastroenteritis virus.

An alternative vaccine candidate was based on conserved ectodomain M2 protein (M2e) of influenza A [9]. A M2e epitope was integrated into the βB–βC loop of cowpea mosaic virus (CPMV) S capsid protein and agroinfiltrated into *Vigna unguiculate*. VLPs were produced; however, when crude leaf extracts were administered subcutaneously to mice, only partial protection was displayed in challenged mice [9].

Newcastle disease

Newcastle disease virus belongs to the family *Paramyxoviridae*. NDV has a nonsegmented, single-stranded, negative-sense RNA genome [10], which encodes a nucleocaspid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin–neuraminidase protein (HN), and a large RNA-dependent RNA polymerase protein (L) [11]. NDV is highly infectious, affecting domestic poultry and wild birds. Virulent forms of NDV cause devastating disease of poultry and are, therefore, economically important avian viruses. Hardly one commercial poultry flock is reared that is not influenced by measures aimed at controlling NDV [12]. NDV transmission occurs through direct contact with secretions or discharge of infected birds, and contact with fomites such as vehicles, equipment, clothing or animal by-products. Several PMV studies report the use of the HN or F surface glycoprotein alone or in fusion as immunogens towards a subunit vaccine against Newcastle disease. The world's first regulatory approval for a PMV was against NDV [101]. This PMV was made using a contained, plant cell production system. The HN protein from NDV was expressed in a tobacco cell system and found to retain the size and immunoreactivity of the native antigen. There was no degradation of the HN in the plant cells and it was bioactive in red blood cell hemagglutination assays. A master seed stock established from the cell line was used to produce a vaccine batch that was used in a disease challenge study in chickens [13]. Nontransgenic, negative controls plus four different formulations of the recombinant vaccine were prepared from plant cells. On days 0 and 14, specific pathogen-free chicks were vaccinated subcutaneously with the partially purified, plant cell material. Individual blood samples were collected from each bird on day 24, and the chicks (except unchallenged controls) were challenged with NDV Texas GB strain on day 28. Birds vaccinated subcutaneously with the plant-made, subunit HN antigen from NDV proved to be protected against lethal challenge to NDV. The dose response capable of greater than 90% protection ranged between 3 and 33 µg/dose with overall protection of 95% [13]. Thus, a plant cell-produced vaccine serologically converted birds and provided protective immunity against NDV. A formulation was advanced through the US Department of Agriculture Center for Veterinary Biologics' regulatory approval process in a feat that demonstrated PMVs could be developed within the existing regulatory framework.

Using stable transformation, Berinstein and colleagues demonstrated NDV F or HN protein accumulation in transgenic potato leaves [14]. Mice subsequently fed with the transgenic potato leaves developed NDV-specific IgG and IgA antibodies [15]. In addition, stable transgenic maize plants expressing the *F* gene of NDV were produced [16]. Chickens orally administered ground, transgenic corn displayed antigen-specific immune responses and were protected against NDV challenge [16]. A similar attempt using the F antigen as an NDV plant-made subunit vaccine was reported in rice. High accumulation of F antigen was obtained in leaf and seed tissues using the *Ubi* and *Gt1* promoter, respectively. Mice immunized intraperitoneally with crude protein extracts of transgenic rice showed antigen-specific immune responses [17]. Stable transgenic tobacco expressing HN of NDV was also produced [18]. Immunogenicity of the transgenic tobacco was confirmed when chickens fed with lyophilized tobacco leaves displayed low HN-specific IgG immune responses [18].

An alternative strategy for high expression reported by Gómez and colleagues was to target subcellular expression of the HN protein within *N. benthamiana* cells using signal peptides and the endoplasmic reticulum (ER) retention sequence [19]. The highest accumulation of HN antigen was obtained with the construct harboring *HN* gene with its native signaling peptide, fused to the KDEL retention peptide [19].

Foot-and-mouth disease

Foot-and-mouth disease (FMD) is one of the most contagious viral diseases of wild ruminant and domestic cloven-hoofed animals, including economically important cattle, sheep and swine [20]. The causative pathogen, FMD virus (FMDV), belongs to the family *Picornaviridae* and is the type species of the genus *Aphthovirus* genus. FMDV is a single-stranded, positive-sense RNA virus, possessing four capsid proteins (VP1 [1D], VP2 [1B], VP3 [1C] and VP4 [1A]). The VP1 protein is the critical determinant for vaccination against FMD with the induction of VP1-neutralizing antibodies required for immunity.

Foot-and-mouth disease virus has a wide host range and is transmitted rapidly between animals by fomites, including vehicles, animal by-products and aerosols. Animals infected with FMDV show vesicular lesions on the snout, teats, hooves and tongue, as well as fever and lameness. The signs of disease appear within 2–14 days after exposure to FMDV with persistent infection of the virus observed in carrier animals for up to 3.5 years in cattle, 9 months in sheep and 4 months in goat [21]. Although the mortality rate in adult animals is low, FMD decreases animal productivity, afflicting dairy and meat-producing industries and as a consequence results in decreased animal well-being and economic burden [102].

A FMD outbreak can also cause catastrophic impact to tourist-related businesses since containment procedures such as limiting public access to or through regions adversely impact on their popularity. Moreover, a general aversion is created by mass animal slaughter and burning carcases. This was highlighted in 2001 when a FMD epidemic in the UK led to the loss of approximately 20% of the estimated total annual farming income [22] and 21% of the estimated total tourism revenue [23].

Foot-and-mouth disease viruses have seven serotypes defined as types O, A, C, Southern African Territories (SAT) 1, SAT 2, SAT 3 and Asia 1. Although vaccines are available against FMDV,

similar to the case of influenza A viruses, high antigenic variation leads to lack of cross-protection between the different FMD viral serotypes, and vaccination with killed virus thwarts diagnostic testing for disease presence. There is a strategy to differentiate infected from vaccinated animals (DIVA). While unlikely to work with whole-virus-based vaccines, it may work with highly processed viral vaccine or subunit vaccines that lack nonstructural proteins [24]. PMVs being subunit vaccines hold yet another key advantage as they could be used as a DIVA vaccine, and allow differentiation between vaccinated and infected individuals.

Studies have shown the potential of using VP1 capsid protein as a subunit PMV candidate [25,26]. Several early studies on PMVs against FMDV were discussed by Hammond and Nemchinov [27]. The reviewing authors provide an extensive report on plants as a production system for FMDV. Strategies include expressing the gene encoding VP1 from modified viral vectors including CPMV [28], TMV [29] or bamboo mosaic virus [17]; or a stable binary vector system using the cauliflower mosaic virus (CaMV) 35S promoter in foliage of *Arabidopsis* [30], alfalfa [31], potato [32], tobacco [33] and tomato [34].

In order to achieve high accumulation of plant-made antigen *in planta*, transplastomic technology has been attempted. VP1 recombinant protein accumulated in tobacco chloroplasts to 3% of the total soluble protein (TSP) [35]. More recently, high accumulation of recombinant VP1 protein was produced in tobacco chloroplasts (51% of the TSP) after fusion of the VP1 epitope to β-glucuronidase (GUS). This fusion was also found to be immunogenic in mice [36].

Subunit vaccines often require adjuvanted delivery systems for immune response stimulation. Alternative strategies that coexpress FMDV antigens with adjuvants have produced chimeric recombinant proteins or multicomponent subunit vaccines. One approach used tobacco to express a fusion between VP1 and the hepatitis B core protein. This study found the fusion protein to protect mice from FMDV challenge [33]. An alternative strategy expressed the FMDV structural polyprotein (P1) and protease (3C) in alfalfa [37] and in tomato [34]. Both studies found animals parenterally immunized with transgenic leaf extracts (mice and guinea pigs, respectively) were protected from FMDV challenge [23,26].

Enterotoxigenic *Escherichia coli*

Escherichia coli is frequently the causative agent of diarrheal disease in animals and humans. There are several infectious pathotypes of *E. coli*, including enterotoxigenic (ETEC), enterohemorrhagic (EHEC), Vero- or Shiga-like toxin-producing (VTEC or STEC), necrotoxigenic (NTEC), enteropathogenic (EPEC), enteroaggregative (EAggEC) and enteroinvasive (EIEC) [38]. ETEC causes diarrhea by colonizing the hosts' small intestine and producing enterotoxins and potent cytotoxins that stimulate the efflux of fluids into the enterocyte. ETEC infection may be fatal in young animals and significantly reduces agriculture productivity. Adhesins (fimbriae – the hair-like appendages) and enterotoxins (heat-labile or heat-stable enterotoxins) are the main virulence factors in ETEC.

The heat-labile toxin (LT) of ETEC consists of a single, active ADP-ribosylation domain (LTA) and a nontoxic homopentamer (LTB). The B subunit targets the holotoxin to the gut wall by binding to the GM1 ganglioside receptors present on the surface of mucosal epithelial cells [39]. LTB is a favored vaccine candidate as it is strongly immunogenic and nontoxic [40]. Strategies of using LTB alone as a subunit vaccine or as a partner in a fusion protein as carrier protein have been proposed [41,42]. Initial attempts to produce LTB in plants used a codon-optimized, synthetic *LTB* gene to produce antigen that would ultimately lead to an immune response capable of neutralizing toxin activity. *Agrobacterium*-mediated stable transformation of tobacco and potato was performed using the synthetic gene under the control of the CaMV 35S constitutive promoter [43,44] or the tuber-specific patatin [45]. The authors demonstrated that oral immunization of mice raised neutralizing serum.

Several seed-based PMVs for ETEC stimulated antigen-specific immune responses in mice after oral immunization. Studies to date include corn and soybean [46–51]. Production of antigen in seeds has the advantages of long-term, stable, room-temperature storage [52] and condensing of the antigen in corn germ [48]. Lamphear and colleagues expressed LTB in corn seed and investigated expression of the recombinant protein across different fractions of the seed (germ, grits and bran) and long-term stability [46]. Although LTB was expressed across all fractions, the germ was enriched with antigen that was found to persist in pentamer form for over 1 year. The LTB-expressing seed was orally delivered to mice and piglets, and resulted in antigen-specific, mucosal IgA and protection in piglets.

Production of LTB has also been reported in tobacco chloroplasts, ginseng embryos and lettuce leaves [53–56]. However, while the plant-made LTB showed *in vitro* binding to GM1 ganglioside in these instances, immunogenicity of these vaccines is yet to be confirmed [53–56].

Rosales-Mendoza and colleagues demonstrated the possibility of using carrot for LTB production [57]. Subsequent studies on the efficacy of the carrot-made vaccine were performed [58]. Mice orally immunized with rehydrated, freeze-dried, carrot-made LTB showed antigen-specific immune responses and less accumulation of intestinal fluid after cholera toxin challenge. Transient, plant-virus-mediated plant expression has also been employed to obtain high expression of LTB. The LTB antigen was expressed in *N. benthamiana* after agroinfiltration of a potato virus X-based vector into the leaves [59]. Expression of LTB of up to 2% of the TSP was observed in leaves, with the LTB showing pentameric assembly.

Heat-labile toxin B subunit can also be used as a carrier protein or protein adjuvant to enhance immunogenicity of a fused antigen. Walmsley and colleagues demonstrated high expression of LTB fused to an immunocontraceptive epitope in tomato plants [60]. The fusion protein displayed autoassembly of pentamers with 354.7 µg/g accumulation of antigen in freeze-dried tomato fruit [60]; however, its immunogenicity was not tested. Using *Arabidopsis* as a production system, Rigano *et al.* developed a plant-made subunit fusion protein of LTB and the TB antigen, ESAT-6 [61]. The fusion protein displayed GM1-ganglioside

binding and demonstrated oral immunogenicity in a subsequent challenge trial in mice [62]. However, despite eliciting an antigen-specific Th1 response, mice were not protected against challenge with the *Mycobacterium tuberculosis* H37Rv strain.

The LTB antigen has also been expressed in transgenic rice as a fusion antigen with the major outer membrane protein (MOMP) of *Chlamydophila psittaci* [63]. Although very low expression of the LTB–MOMP fusion protein was detected (0.0033–0.0054% of the TSP) an antigen-specific immune response was elicited in mice immunized orally with the fusion protein, and this produced partial protection from *C. psittaci* 6BC strain challenge [64]. A fusion has also been produced between NDV-neutralizing epitope and LTB in chloroplasts. The fusion protein accumulated to 0.5% of the TSP in the chloroplasts of tobacco leaves [65].

In addition to LTs, the adhesion of the bacteria to host tissue at the site of infection plays a key role in the pathogenesis. This adhesion is mediated by proteinaceous filamentous surface structures called fimbriae, which contain major virulence factors of *E. coli* [66]. Six types of fimbria have been identified in ETEC strains isolated from pigs and calves: F4 (K88), F5 (K99), F6 (987P), F17 (Fy/Att25), F41 and F18 [38]. F4 (K88) is the most prevalent fimbrial antigen found in strains that are pathogenic in swine [55,56]. Efforts have been made to express the major antigen for F4 fimbriae, FaeG, in plants. Tobacco-made FaeG accumulated to 0.15% of the TSP. The authors showed that the sera of mice immunized intraperitoneally with leaf extracts neutralized K88ad fimbrial-expressing ETEC in rabbit and that in an *in vitro* intestinal villious adhesion assay, the sera of orally immunized mice prevented adhesion of ETEC bacteria [57,58]. Joensuu and colleagues also demonstrated expression of FaeG in several plant species including tobacco (up to 1% of the TSP in leaves [67]), barley seeds [68] and alfalfa leaves [69]. Post-weaning piglets were partially protected after oral immunization with an alfalfa-derived FaeG subunit vaccine.

Another study using F5 K99 fimbrial subunit expressed the antigen in soybean plants [62,63]. Parenteral administration of the foliar extract to mice induced antigen-specific $CD4^+$ T lymphocytes [70].

Plant production platforms

Plant-made vaccines have been produced in cells and tissues from a diverse range of plants including monocotyledonous and dicotyledonous species. In each case, the therapeutic protein accumulates in the host tissue or is engineered for secretion into the surrounding hydroponic or growth medium [71]. Veterinary PMVs have been produced and delivered from tissues including fruit (tomato), leaves (alfalfa, peanut and lettuce), tubers (potato), seeds (maize, rice and barley) and cell suspensions (reviewed by Floss *et al.* [72] and Joensuu *et al.* [1]). A previously stated advantage of PMVs, particularly for delivery to animals, is the potential for oral immunization. The anticipated benefit of PMVs is that by encapsulating the antigen in plant material, it can afford protection against early degradation in the stomach to ensure safe passage and targeted delivery to immune-responsive sites in the GI tract of the target animal. This is especially important when the target animal has a more complex gut physiology than experimental mouse models.

The plant tissue utilized for veterinary vaccine production must fill two essential criteria. First, it must produce sufficient quantities of recombinant antigen to permit delivery of preferably a single dose. Sheep and cattle, for example, could ideally receive oral vaccination at the time of drenching to avoid excessive handling and herding. Second, the plant material should require limited processing into a form suitable for oral administration, whether it be drenching or feedlots. A technical challenge facing oral delivery of PMVs for veterinary purposes is that larger quantities of antigen and therefore plant material are typically required to attain a level of immunogenic and protective efficacy that is comparable with their injected counterparts [73]. In preparing an oral dose, one must consider the potential for loss of some or all of the administered vaccine due to degradation in the GI tract before reaching the gut-associated lymphoid tissue where it then needs to be taken up efficiently at the mucosal epithelium. As such, the choice of plant production platform is critical to ensure sufficient vaccine is produced in an appropriate mass easily consumable by the intended animal. Perhaps this is the reason there are limited examples of immunogenic trials of oral delivery in larger farmed animals such as cattle or pigs (Table 1), and no examples in sheep. It is anticipated that food crops typically used in animal feed such as alfalfa, white clover or grains may be good candidates for vaccine production because they should require limited processing to maintain functional integrity of the antigen. The concern is that such vaccines could potentially enter the food chain if crops are not tightly regulated.

Recombinant protein accumulation can vary depending on the antigen of interest, the plant tissue/species, the strength, inducibility or specificity of promoters, codon usage, subcellular organelle targeting and nuclear or plastid transformation (reviewed in [73]. Plants can be engineered to accumulate recombinant proteins in either stable or transient expression systems (Table 2).

Stable expression systems

Stable transgenic plant systems are generated by incorporation of foreign DNA encoding the recombinant protein of interest into nuclear or plastid genome by either *Agrobacterium*-mediated transformation or particle bombardment of DNA-coated gold/tungsten beads. Whole plants can be regenerated, eventually producing a seed stock, or plant tissues maintained in aseptic culture. The advantage of this system is that the transgene is heritable, permitting the establishment of a seed stock or vaccine bank that can be stored for a long term. While establishment and characterization of stable lines can be costly and time consuming, the seed stock generated can be utilized to rapidly produce vaccine when demand is high [74]. Unfortunately, expression of recombinant proteins can vary over generations, enforcing a need to test levels during plant growth, which for some crops may be months depending on the plant type and target tissue. The output of vaccine produced by this system can be severely

Table 1. Efficacy of candidate veterinary vaccines orally delivered in cattle and pigs.

Study (year)	Pathogen	Antigen	Production system	Dose regime	Efficacy	Ref.
Khandelwal *et al.* (2003)	Rinderpest virus	Hemagglutinin	Peanut leaves	Three doses of 0.5% of the TSP	Immunogenic and neutralized virus in cattle	[94]
Lamphear *et al.* (2002)	TGEV	TGEV-S	Maize seed	Four to 16 doses of 2 mg Ag/50 g transgenic plant material	Immunogenic and protective in piglets	[46]
Joensuu *et al.* (2006)	Enterotoxigenic *Escherichia coli*	F4 fimbrial adhesin FaeG	Alfalfa leaves	One dose of 20 mg Ag per 30 g dwt transgenic plant material	Immunogenic and protective in pigs	[68]
Shewen *et al.* (2009)	Pneumonia (*Mannheimia haemolytica*)	Leukotoxin	Alfalfa	Ten doses of 27 mg Ag in 300 g dwt transgenic plant material	Enhanced immune response following challenge only in calves	[95]

Ag: Antigen; dwt: Dry weight; TGEV: Transmissible gastroenteritis virus; TSP: Total soluble protein.

hampered by insertion of multiple copies of the transgene into the host's nuclear genome, resulting in gene silencing and position effects [75]. Consequently, large numbers of transgenic lines need to be screened and analyzed before a single optimal line can be selected for vaccine production.

Typically, antigen yields derived from nuclear transformation are low (<1% of the TSP [76]) with up to 12% of the TSP observed for LTB when targeted to the vacuole of maize [48]. Higher levels of antigen accumulation have been achieved from tobacco chloroplast transformation with yields for cholera toxin B subunit (CTB)–2L21 peptide fusion observed at 31.1% of the TSP [77] and a VP1–GUS fusion at 51% of the TSP [36]. This is in part due to the capacity for a high gene copy number in the chloroplast genome (up to 10,000 copies) [78]. Production of chloroplast-derived vaccines is no longer limited to tobacco, with a recent example also described in lettuce where malarial antigens fused to CTB accumulated up to 7.3% of the TSP in lettuce compared with 13.2% in tobacco [79]. Other advantages include stable maternal heritability of the transgene, absence of gene silencing and position effects, and no obvious deleterious effects to morphology [70,72,73]. The major limitation of this technology is that proteins do not always undergo full post-translational modification in the chloroplasts and therefore may be limited to antigens that do not require glycosylation or complicated assembly for immunogenicity [76]. Nonetheless, chloroplast-derived veterinary vaccine antigens, including 2L21 peptide from canine parvovirus [77] and LTB from ETEC [80], have shown functional efficacy in animal models.

Transient expression system

Recombinant proteins can be more rapidly generated by plants using a transient expression system. Transient transformation of differentiated tissues may use particle bombardment and/or engineered plant viruses (reviewed in [81,82]) but we focus on *Agrobacterium*-mediated or agroinfiltration applications. Agroinfiltration involves the permeation of plant intercellular spaces with *Agrobacterium* that consequently transforms the surrounding cells and results in the extrachromosomal expression or incorporation and expression of the transgenes of interest. Permeation of the intercellular spaces may result from two methods: injection of the culture into the leaves via a syringe applied to the stomata or small knick in the abaxial leaf surface (optimal for small sample sizes and low repetitions); or vacuum infiltration of the aerial portion of plants. Vacuum infiltration is quickly growing in popularity as a means for quickly producing bulk, transiently expressing plant materials. In general, the aerial portion of the plant is submerged in *Agrobacterium* culture and a vacuum is then pulled to remove the air from the plant intercellular spaces. When the vacuum is released, the *Agrobacterium* culture floods into the leaves via the stomata. *Agrobacterium*-mediated transient transformation may be unassisted [5,83], use suppressors of post-transcriptional gene silencing, or use deconstructed viral vectors that replicate autonomously. In each case, recombinant protein is transiently produced within 14 days, without stable genomic transformation (reviewed by Gleba *et al.* 2007 [84] and Joensuu *et al.* 2008 [1]).

Unassisted, *Agrobacterium*-mediated transient transformation of plants usually entails permeating the intracellular spaces of fully differentiated plant tissues (usually leaves) with *Agrobacterium* carrying basic binary vector(s) that do not replicate in plant cells and that code for proteins that do not moderate cellular function, such as gene silencing. Several transgenes of interest can be infiltrated into the same cells by infiltration, mixing several appropriately transformed *Agrobacterium* strains carrying the desired genes of interest. Transgene expression usually peaks at 60–72 h post-infiltration and declines rapidly afterwards [85] due to post-transcriptional gene silencing (PTGS) [86]. The success of this technique therefore partly relies on PTGS not being invoked in addition to actual infiltration parameters.

Agrobacterium-mediated transient transformation may be assisted by suppression of gene silencing by a viral-encoded protein such as P19 of tomato bushy stunt virus. PTGS occurs using a sequence-specific, RNA-mediated mechanism. In brief, dsRNA is processed to small interfering RNA (siRNA), 21–25 nucleotides in length. The siRNAs made through the digestion of the dsRNA convey sequence specificity to a nuclease that degrades RNA homologous to the siRNA and hence initiating dsRNA sequences [85]. In the arms race between plant cells and plant

Table 2. Production platforms for oral delivery of plant-made vaccines.

Transformation	Type	Target cells	Recombinant protein expression	Development and production time	Development and production costs	Processing	Processing costs	Comments	Ref.
Stable	Nuclear	Plant culture	Typically ≤1% of the TSP	0.5–1 year	++++	Batch processing	+	Extended time required to select appropriate lines (low copy number, high expression of authentic protein) but produced through continuous batch culture, not affected by climate	[54]
Stable	Nuclear	Whole plants	Typically ≤1% of the TSP, up to 12% of the TSP	1–2 years	+++	Batch processing, partial purification to reduce toxins (alkaloids)	++	Extended time required to select appropriate lines (low copy number, high expression of authentic protein) but once selected, vaccine sustained as seed or cloned plant stock	[48]
Stable	Chloroplast	Whole plants	≤51% of the TSP	1–2 years	++++	Batch processing, partial purification to reduce toxins (alkaloids)	++	Extended time required to select appropriate lines (low copy number, high expression of authentic protein) but once selected, vaccine sustained as seed or cloned stock, gene escape through pollen more difficult	[36]
Transient		Unassisted	50 mg Ag/kg	5–14 days	+	Batch processing, removal of bacterium	+++	Fast production of recombinant protein but results vary according to degree of post-transcriptional gene silencing occurring, constant plant transformation in required	[5]
Transient		P19 assisted	≤51% of the TSP	5–14 days	+	Batch processing, removal of bacterium	+++	Fast production of recombinant protein to large percentage of the TSP, but results vary according to degree of PTGS occurring, constant plant transformation in required	[87]
Transient		Viral replication	≤80% of the TSP	5–14 days	+	Batch processing, removal of bacterium	+++	Fast production of recombinant protein to large percentage of the TSP. Most suitable for production of large quantities of recombinant protein but constant plant transformation is required	[88]

+: Minimal cost; ++: Standard cost; +++: High cost; ++++: Considerable cost; Ag: Antigen; PTGS: Post-transcriptional gene silencing; TSP: Total soluble protein.

viruses, viruses have evolved protein supressors of PTGS that act by soaking up siRNAs, the triggers of PTGS. By codelivering a viral PTGS suppressor with the construct of interest, the host plant PTGS response is quenched, and transient expression is increased up to 50-fold, or up to 51% of the TSP [87]. The degree of expression enhancement varies and relies on the degree PTGS plays in quenching transgene expression (i.e., if PTGS acts strongly on the transgene then transient expression assisted by a viral suppressor is greatly enhanced, but if PTGS plays a small role, then expression may not increase to a significant degree).

When agroinfiltration is used to deliver TMV-based expression vectors, such as the 'magnifection' system developed by Icon Genetics in 2004 [88], recombinant protein can reach up to 80% of the TSP. This technology utilizes different lines of *Agrobacterium* each carrying transfer DNA (T-DNA) engineered to encode deconstructed components of the viral expression machinery and the gene of interest, such that upon infection the components are reassembled within the plant cell to produce a functional RNA replicon [89]. Magnifection is rapidly scalable with predicted capabilities of producing up to 500 kg of recombinant protein per annum in a contained 1-ha greenhouse fitted with vacuum infiltration devices that can facilitate simultaneous infiltration of numerous plants [89]. If these estimates can be realized for antigens of interest, then magnifection is potentially the most feasible system for producing large enough quantities of PMVs for oral delivery to large animals.

Antigen fate & release kinetics

We recently compared the timing of antigen release from different plant material's fruit (tomato), hairy roots (petunia) and leaves (*N. benthamiana*) in the GI tract of mice using a stable model antigen, LTB, as a reporter [Pelosi *et al.* Unpublished Data]. The plant materials were formulated in either peanut butter and peanut oil (1:3) or apple juice and honey (3:1) to assess the affect lipid and aqueous formulations contribute to antigen release.

In the first of two animal trials, mice were fed enough formulated freeze-dried plant materials to deliver a single dose of 50 µg LTB. Control mice were fed corresponding LTB-negative material. After 16 h, ingesta was sampled from the stomach, duodenum, ileum and large intestine of the mouse GI tract to determine the proportion of released and plant encapsulated antigen at each site by LTB-specific ELISA. In a second trial, mice were fed a single dose of the same formulated materials at days 0, 7 and 14 of a 28-day trial to assess induction of systemic and mucosal immune responses.

Our study shows that while encapsulation in plant material alone offers a certain level of antigen protection, the timing of antigen release can be manipulated by the plant tissue type and the formulation (either apple juice and honey or peanut butter) used to prepare the oral dose. Hairy root tissue offered the highest level of protection, breaking open in the small intestine (duodenum and ileum) to release sufficient antigen to induce moderate systemic and mucosal responses in mice, regardless of formulation used. For other tissue types tested, the timing of release was determined by the formulation used. When formulated in apple juice and honey, tomato and leaf vaccines released their antigen cargo at predominantly early sites of the GI tract, namely the stomach and duodenum. By contrast, when formulated in peanut butter, these same plant materials delayed release, delivering LTB to the ileum. The presence of bystander protein in the PB formulations may have protected LTB from proteolysis by acting as a decoy, and in doing so, safeguarding delivery of the antigen to the small intestine. In fact, resuspending recombinant LTB with untransformed tobacco cell suspension was enough to protect and deliver sufficient antigen to immune responsive sites. Interestingly, the leaf vaccine material elicited the strongest systemic and immune responses, and tomato the weakest.

We proposed that while leaf material was optimal for delivery of enough intact antigen to stimulate strong immune responses in the mouse model, the hairy root material had an increased resistance to degradation in the monogastric digestive system of the mouse, but may be the plant tissue of choice when the target animal has a more complex GI tract such as ruminants, or the antigen is less stable and requires increased protection.

Possible areas of development

Mucosal delivery

Mucosal delivery of vaccines elicits mucosal and humoral immune responses. As most pathogens invade the host through the mucosal route, mucosal immune responses are often seen as being the most effective means of preventing infectious disease. Mucosal delivery of plant-made veterinary pharmaceuticals would also open the possibility of 'baiting' free-ranging populations (although the lack of control of dosage and exposure of nontargeted populations to the vaccine would need to be carefully modeled), and more crude preparations being used, therefore decreasing associated processing costs; and increasing ease and decreasing cost of delivery, particularly to poultry.

A challenge facing mucosal delivery of PMVs is preventing induction of peripheral immune tolerance (or oral tolerance) to antigens administered by either the oral, sublingual or nasal route. Oral tolerance is a mechanism employed by the immune system to avoid eliciting harmful immune responses to antigens presented at mucosal surfaces from an animal's normal diet or microflora. Repeated presentation of mucosal antigens can result in suppression of a humoral immune response in favor of oral tolerance. Kostrzak and colleagues showed that this is not an insurmountable problem when vaccination protocols and doses of a plant-made hepatitis B surface antigen are adjusted to skew the immune response towards induction of IgGs and IgAs, and minimize tolerance [90]. It is clear that efficacious vaccination protocols will need to strike a balance between oral tolerance and humoral immune responses. Veterinary PMVs will need to be evaluated on a case-by-case basis to ensure antigen dose, presence or absence of adjuvant, delivery route and immunization schedule will skew the immune response toward the desired outcome for the intended animal species.

Optimizing antigen delivery to target population

Many studies have reported antigenicity and immunogenicity of PMVs in laboratory animals but not many studies have been performed regarding protection from challenge; minimum and maximum doses of a PMV; length of immune response induced; protection

with candidate vaccines; or studies in the intended end user. This is perhaps understandable due to the narrow (but applicable) nature of these studies; however, in order for this biotechnology platform to be taken seriously by industry, proof that it works efficiently and effectively in end-user populations needs to be provided.

Expert commentary & five-year view on future challenges of plant-made veterinary vaccines

Although the regulatory processes for production and commercialization of a veterinary pharmaceutical produced by a transgenic plant are considerable, the success of Dow AgroSciences in gaining the first commercial license for a PMV established the feasibility of commercial, plant-made veterinary vaccines. However, it should be stressed that this process was simplified by using a fully contained production system (plant cell culture), and we propose that the use of contained systems (production in a plant cell or organ culture, or greenhouse) or at the very least, nonfood crops should be used at present. The ideal veterinary vaccine would preferably protect against multiple pathogens (multicomponent) but, in reality, protect against multiple strains or species of the same pathogen; be produced with ease and consistency according to current good manufacturing practices; be prepared in a formulation that retains potency for 1 year cold chain free (or at most 4°C); not require delivery by injection; be safe and free of side effects; induce an early onset of immunity; provide long-term protection against both disease and infection; allow serological discrimination between vaccination and infection; and be cheap enough to provide a cost–benefit advantage. While all these characteristics are not held by successful veterinary vaccines, the PMV technology has the potential to address many if not all of these wishes and they should be seen as challenges to be addressed in the not too distant future.

The evidence is quickly mounting to the potential and feasibility of this technology. Phase III human clinical trials are currently ongoing with a recombinant human glucocerebrosidase produced in stably transformed plant cells in culture [91]. Meanwhile, the ability of plants to act as quick-response vaccine production systems was proven by D'Aoust and colleagues [5,83]. As we previously described, the transient expressing plants produced large amounts of HA protein from H5N1 (avian) and H1N1 (human) influenza strains; however, this was performed in less than 3 weeks from release of viral sequence to purified vaccine product. Animal studies later confirmed the efficacy and safety of this vaccine and it is currently awaiting permission to go into Phase I human trials. Medicago Inc. and Fraunhoefer also used a transient, plant transformation system to go from recently released H1 HA sequence to grams of purified protein in less than 1 month [92,93]. Plants are quickly shaping up as quick and versatile recombinant protein producers and there is little, perhaps just the lack of financial backing of big pharma or agricultural companies, stopping these same techniques from rapidly producing vaccines for animal use. Such vaccines would be welcome in an AI outbreak in poultry or FMDV outbreak as recently occurred in Japan [102].

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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

- Research continues into the ability of plants to effectively produce different immunogenic vaccine candidates.
- Oral delivery of plant-made vaccines is still a much-desired trait, particularly for delivery to animals; however, studies need to optimize formulation and delivery.
- New transient transformation techniques have vastly overcome the previously limited ability of plants to produce recombinant proteins to competitive concentrations.
- Plants can produce recombinant proteins in a variety of ways, resulting in differing post-translational (namely glycosylation state) characteristics.
- Plants can produce safe and effective vaccines in less than 1 month (from release of the candidate antigen sequence to gram quantities).
- Plant-made vaccines have the potential to make the ideal veterinary vaccine should commercial entities become involved.

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