History and Promise of Plant-Made Vaccines for Animals

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Abstract Plant-made vaccines are now a well-established and well-tested concept in veterinary medicine—yet the only product so far licenced was never produced commercially. This is puzzling, given the breadth of exploration of plant-made animal vaccines, and their immunogenicity and efficacy, over more than twenty years of research. The range of candidate vaccines that have been tested in laboratory animal models includes vaccines for *E. coli*, *Salmonella, Yersinia pestis*, foot and mouth disease virus, rabbit haemorrhagic disease virus, rabbit and canine and bovine papillomaviruses, mink enteritis and porcine circovirus, and lately also bluetongue virus, among many others. There are many proofs of efficacy of such vaccines, and regulatory pathways appear to have been explored for their licencing. This review will briefly explore the history of plant-made vaccines for use in animals, and will discuss the unique advantages of plant-made vaccines for use in a veterinary medicine setting in detail, with a proposal of their relevance within the "One Health" paradigm.

Keywords Plant-made vaccines · Therapeutic vaccine · One health
Transient expression · *Agrobacterium tumefaciens* · *Nicotiana benthamiana*
Regulatory · cGMP · FDA · EMEA

1 Introduction

Plant-produced vaccines for veterinary medicine are an exciting prospect, largely because of the possibilities of producing protein-based vaccines' including edible vaccines' at low cost, at almost any scale, and potentially locally and on demand. They have also been controversial because of the very real possibilities of contamination of the human food supply with vaccine-producing transgenic plants, and

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because of concerns around the possibility of immunological tolerance developing to oral or edible vaccines. However, one set of problems that many foresaw—regulatory and production problems—has not eventuated, and in fact the environment now seems primed very favourably for their introduction.

The main justifications for plant-made vaccines are that vaccine antigen production in plants is safe; that it is both cheap and highly scalable; that plants produce and process eukaryote-derived proteins much better than can bacteria or even yeasts; that use of plants would allow for production of vaccines in the developing world where they are needed most; and of vaccines or therapeutics that will never be produced economically by other technologies.

However, despite more than twenty years of development, there are still no plant-produced vaccines or biologics available for animals—although there are in fact products licenced for and in use in humans.

This review will explore the early history of plant-produced vaccines with an emphasis on proofs of principle and of efficacy, what the recent development of robust, stable transient plant production systems for vaccine antigens could mean for veterinary medicine, and the potential of plant-produced vaccines to advance both animal and potentially human health' under the banner of the One Health Movement.

2 Early History of Plant-Made Animal Vaccines

While viral proteins have probably been the most common vaccine candidates made in plants (reviewed in Rybicki 2014), it was expression of a bacterial protein—*Escherichia coli* heat labile enterotoxin (LT-B)—that first proved that veterinary-relevant antigens could be produced in plants, and provided the first proof of principle for edible vaccines. LT-B produced in transgenic tobacco or potatoes (Haq et al. 1995) was functionally equivalent to *E coli*-produced protein in specific assays, and immunisation of mice by oral gavage with plant material elicited systemic and mucosal toxin neutralising antibodies. Moreover, fresh potato containing LT-B was immunogenic in mice when eaten.

An early virus vaccine candidate was one against mink enteritis virus (MEV) disease: this was novel in that it comprised chimaeric Cowpea mosaic virus (CPMV) virions incorporating a short linear epitope from MEV VP2 capsid protein and displaying it on the surface of virions, produced by inoculation of bean plants with an infectious cDNA clone of rCPMV (Dalsgaard et al. 1997). This conferred protection against clinical disease and virtually abolished virus shedding—and given that the epitope sequence used is found in MEV, canine parvovirus, and feline panleukopenia virus, the same vaccine could potentially also protect against these viruses.

Another early virus vaccine candidate was against rabbit haemorrhagic disease virus (RHDV): this was made by expressing the whole RHDV VP60 capsid protein in transgenic potatoes; parenteral immunisation with plant extracts was protective in rabbits (Castanon et al. 1999). Subsequently, another study demonstrated that an

edible vaccine consisting of leaves of transgenic plants containing presumably partially-assembled VP60 subunits, was an effective priming vaccine for later baculovirus-derived parenterally-delivered vaccine (Gil et al. 2006).

The first report of a Foot and mouth disease virus (FMDV) plant-made antigen was of expression in plant protoplasts of a VP1-derived peptide of FMDV as an insertion into the minor coat protein of a replicating CPMV as a demonstration of antigen presentation (Usha et al. 1993). However, the first proof of efficacy was done using transgenic *Arabidopsis thaliana* expressing whole VP1: parenteral immunisation of mice with leaf extracts elicited antibodies that bound to VP1 and to intact FMDV particles, and all immunised mice were protected against virulent FMDV challenge (Carrillo et al. 1998). The Wigdorovitz group went on to demonstrate that mice could be protected against FMDV challenge after oral or parenteral vaccination with extracts of transgenic alfalfa expressing VP1 (Wigdorovitz et al. 1999), or immunisation with leaf extracts of tobacco plants expressing VP1 via a recombinant Tobacco mosaic virus vector (Wigdorovitz et al. 1999). A refinement of these achievements included transgenic expression in alfalfa of amino acid residues 135–160 of VP1 (VP135-160) fused to glucuronidase (GUS), which both allowed selection of strongest expressers by assay of enzyme activity, and was protective in mice (Dus Santos et al. 2002). Another novel application of carrier technology was the insertion of VP1 amino acids 140–160 (G-H loop) in an interior region of the hepatitis B virus core antigen gene (HBcAg), and expression of the chimaera in transgenic *Nicotiana tabacum*. The chimaeric protein formed virus-like particles (VLPs) in the tobacco leaves, and mice immunised intraperitoneally with a soluble extract were protected against viral challenge (Huang et al. 2005).

An early attempt at showing the feasibility of making an anthrax vaccine was the expression in transgenic *N. tabacum* of the protective antigen (PA) protein of *Bacillus anthracis*, possibly the best target for a subunit vaccine because it alone is protective (Aziz et al. 2002), although it went no further than showing cytolytic activity of the protein. Soon after, the same group went on to express PA in transplastomic *N. tabacum,* with significant yield increases but still no efficacy trial (Aziz et al. 2005). Another investigation of transplastomic tobacco by Henry Daniell's group was more thorough: yields were high (2.5 g/kg in fresh leaf tissue), the protein was protected in chloroplasts from protease cleavage and was stable when stored in leaves or as crude extracts, and was biologically active (Watson et al. 2004). While they did not show immunogenicity or protection, the authors speculated that "With an average yield of 172 mg of PA per plant using an experimental transgenic cultivar grown in a greenhouse, 400 million doses of vaccine (free of contaminants) could be produced per acre". The Daniell group subsequently showed that chloroplast-derived PA was equal in potency to the natural product from *B anthracis*, and that mice immunised subcutaneously with partially purified chloroplast-derived PA with adjuvant produced high IgG titres and survived challenge with lethal doses of toxin (Koya et al. 2005).

A different sort of approach to anthrax, and one of the first attempts at making a therapeutic antibody in plants, was taken by Vidadi Yusibov's group, who used the technique of transient *Agrobacterium* infiltration-mediated expression in

N. benthamiana to produce a human-derived PA-specific monoclonal antibody (Hull et al. 2005). The antibody neutralised toxin activity both in vitro and in vivo at a comparable level to hybridoma-produced antibodies. The Yusibov group at what became Fraunhofer USA Center for Molecular Biotechnology later used the same transient expression technology to separately express artificial antigens comprising domain 4 of PA or domain 1 of *B anthracis* lethal factor (LF), fused in-frame with lichenase (LicKM), a thermostable enzyme from *Clostridium thermocellum* (Chichester et al. 2007). Mice immunised with a combination of the two antigens produced high titres of mainly IgG1, and sera could neutralise the effects of anthrax lethal toxin (LeTx) in vitro.

Rabies vaccines made in plants included an early yet highly sophisticated candidate that was composed of the Alfalfa mosaic virus (AMV) CP fused to an artificial polypeptide containing rabies virus G protein amino acids 253–275, and N protein amino acids 404–418, and expressed either in *N. tabacum* plants transgenic for AMV replicase, or via rTMV in either *N. benthamiana* or spinach (Yusibov et al. 2002). The plants made particles containing AMV-derived RNA, encapsidated with chimaeric CP: raw spinach leaves were orally immunogenic in mice and in human volunteers. A simpler candidate was the G protein alone, with plant signal peptide and ER retention signal, made in transgenic *N. tabacum* (Ashraf et al. 2005). While yields were relatively low (0.38% of total soluble leaf protein), purified protein injected peritoneally in mice elicited protective immunity against lethal intracerebral challenge with live rabies virus—an excellent proof of both principle and efficacy.

Plant-made animal rotavirus vaccines were an early target, with a stand-out study by Yu and Langridge (2001) providing evidence that transgenic potato could produce fusion proteins consisting of cholera toxin (CT) B and A2 subunits fused with murine rotavirus enterotoxin and enterotoxigenic *E coli* fimbrial antigen, respectively. Fusion antigens assembled in potato tubers into cholera holotoxin-like structures that bound enterocytes, and elicited serum and intestinal antibodies after oral immunisation in mice. Moreover, passively immunised mouse neonates were partially protected against diarrhoea after rotavirus challenge, demonstrating that combination vaccines for viral and bacterial pathogens may be made in plants. A simpler approach to rotavirus prevention was expression of a His-tagged $VP8^{*}$ fragment of bovine rotavirus (BRV) VP4 in *N. benthamiana* via recombinant TMV, purification of the antigen by Ni^{2+} chromatography, and intraperitoneal immunisation of adult female mice (Perez Filgueira et al. 2004). Eighty-five percent of suckling mice born from these mothers were protected from BRV challenge, compared to 35% immunised with an irrelevant control antigen. The same group also showed that a fusion protein made in transgenic alfalfa consisting of a short peptide derived from BRV VP4 fused to GUS was immunogenic both when given intraperitoneally and orally to adult female mice, and their sucklings were protected against challenge (Wigdorovitz et al. 2004). Another group used transgenic alfalfa to produce human rotavirus VP6, and showed that female mice gavaged with alfalfa extract containing oligoCpG as an adjuvant developed high titres of antibodies both systemically and mucosally, and their pups were partially protected against simian rotavirus challenge.

The same animal model first used to show the efficacy of insect cell-made papillomavirus virus-like particle (VLP)-based vaccines (Breitburd et al. 1995) was also used to demonstrate the efficacy of two very different plant-made papillomavirus vaccines, a few years after the demonstration that Human papillomavirus L1 major capsid protein virus-like particles could be produced in transgenic tobacco or potato (Biemelt et al. 2003; Varsani et al. 2003; Warzecha et al. 2003). Cottontail rabbit papillomavirus (CRPV), the cause of the famous "jackalope" sightings in the USA, provides an excellent model system in domestic rabbits for investigation of prophylactic and therapeutic papillomavirus vaccines (Breitburd et al. 1997). Accordingly, in the first study CRPV L1 major capsid protein-containing extracts were prepared either from transgenic *N. tabacum* or *N. benthamiana* infected with recombinant TMV, and used with Freund's incomplete adjuvant to immunise rabbits that were subsequently challenged with live virus (Kohl et al. 2006). Although the vaccines appeared to contain small aggregates of CRPV L1 rather than intact VLPs, and immune rabbit sera failed to neutralise CRPV infectivity in an in vitro assay, the rabbits were protected from wart development (Kohl et al. 2006). In the second study, infectious recombinant TMV was used to surface display, via fusion to the capsid protein, a peptide consisting of amino acids 94–122 of the L2 minor capsid protein from either CRPV or the Rabbit oral papillomavirus (ROPV) (Palmer et al. 2006). Groups of rabbits received either or both vaccines, and were challenged with live CRPV or ROPV. Immune rabbit sera reacted with whole L2 protein, and CRPV-specific sera neutralised CRPV pseudovirion infectivity. Rabbits receiving the CRPV or CRPV + ROPV vaccines were completely protected against CRPV infection, and those receiving ROPV alone were weakly protected against CRPV. These studies demonstrated for the first time that plant-made papillomavirus vaccines based on L1 protein or L2-derived peptide had real potential as prophylactic vaccines, for use in animals as well as in humans. Strangely, given that Bovine papillomaviruses (BPV) had been used for many years as model systems for anti-wart vaccination, it was not until 2012, with transient agroinfiltration-mediated expression of BPV-1 VLPs in *N. benthamiana*, that a candidate plant-made BPV L1 VLP-based vaccine was successfully made, although no efficacy trials were done (Love et al. 2012).

Expression of animal vaccine components in seeds of transgenic plants was attempted quite early on, with Lamphear et al. (2002) in 2002 reviewing their own earlier work on maize seed expression of the B subunit of *E coli* heat-labile enterotoxin and the TGEV S protein, with data on the potency, efficacy, and stability of these vaccines. Another report followed in 2002 on the expression in maize seed of the S envelope protein of transmissible gastroenteritis coronavirus (TGEV) of swine, and its protective efficacy in piglets fed with the seed (Jilka 2002). This followed an earlier demonstration of oral immunogenicity of the S protein N-terminal domain in transgenic potato tubers (Gomez et al. 2000). Rabies too was a target for maize seed expression, with a report of G protein expression in transgenic maize seed to 1% of total soluble protein, and complete protection in a heterologous rabies strain challenge of mice orally immunized with one dose of ~50 μg of G protein in seed extract (Loza-Rubio et al. 2008). The same group later

showed that sheep orally given a single dose of the transgenic maize seed containing ~2 mg of the G protein were protected to the same extent as those immunized with a commercial inactivated vaccine (Loza-Rubio et al. 2012). The authors claimed that "this is the first study in which an orally administered edible vaccine showed efficacy in a polygastric model", which was an important development.

Maize was a popular target for both production and storage of recombinant proteins in early molecular farming times (see Streatfield et al. 2003); however, other hosts were used too. For instance, the haemagglutinin (H) protein of Rinderpest virus was expressed in transgenic pigeon pea to 0.49% of total soluble protein (Satyavathi et al. 2003), and also in peanuts for a product that was both orally and parenterally immunogenic in mice (Khandelwal et al. 2004); so too was glycoprotein B (gB) of human cytomegalovirus in seeds of transgenic tobacco (Tackaberry et al. 2003), the fusion (F) glycoprotein of Newcastle disease virus in transgenic rice seed (Yang et al. 2007), and the serotype-specific VP2 protein of Bluetongue virus in transgenic peanuts (Athmaram et al. 2006).

Most of these efforts were negated, however, by the one big scandal to have hit molecular farming as far as the use of food plants for vaccine production is concerned. In 2002, APHIS inspectors found volunteer TGEV CP-expressing maize growing in soybean fields in two locations that were used to grow ProdiGene Inc's TGEV transgenic maize in the previous season (APHIS 2008)—and in one, the soybeans were harvested with the maize plants still standing and sent to a storage facility, where they were mixed with a large volume of other seeds. The company was fined and paid substantial cleanup costs, had to develop a new compliance implementation programme, and the US Dept of Agriculture issued new guidelines for trials of such products. This had an unfortunate knock-on effect for molecular farming, in that it resulted in an effectively voluntary moratorium on the use of food crops for recombinant protein production worldwide.

The one major success story of early work on veterinary vaccines was the approval by the US Department of Agriculture's Center for Veterinary Biologics of Dow AgroSciences' injectable Newcastle disease virus (NDV) haemagglutin-based vaccine for poultry, that had been made in a suspension cultured *N. tabacum* cell line. Sadly, the product was never sold: the company only wanted '… to demonstrate that our Concert™ Plant-Cell-Produced system is capable of producing a vaccine that is safe and effective and to demonstrate that it meets the requirements for approval under the rigorous USDA regulatory system. NDV is well known and understood by the regulatory agency, so it served as an excellent model to prove this new technology' (Rybicki 2009).

3 New Developments in Plant Expression Technology

The early historical account of molecular farming for veterinary vaccines given above gives an idea of the array of technologies available and used up to the mid-2000s: transgenic and transplastomic expression of subunit proteins; recombinant plant viruses either used to express whole vaccine candidate genes, or to

display chosen peptides fused to their capsid proteins; fusion of vaccine protein genes to carrier proteins to improve immunogenicity, including by inherent adjuvant properties; candidate parenteral and oral vaccines to both viruses and bacteria; therapeutics for animals made in plants; use of plant cell cultures to make antigens. Many proofs of principle were obtained, for candidate vaccines against a wide range of viral and bacterial disease agents; and proofs of efficacy for vaccines delivered orally or parenterally, in whole plant material or as extracts.

While all of these aspects are still currently used in molecular farming, developments that have revolutionised the field were first, the widespread adoption of *Agrobacterium*-mediated transient expression (agroinfiltration) of recombinant proteins; and second, the use of "deconstructed" plant virus-derived vectors delivered via *Agrobacterium* to amplify expression (reviewed in Rybicki 2010). These innovations enabled the advent of high-throughput testing of expression constructs, coupled with very rapid and generally higher yield production of vaccine antigens once optimal construct design had been determined. For example, our group investigated, via agroinfiltration techniques, three different codon usage schemes and three different intracellular localization strategies for optimization of Human papillomavirus type 16 L1 protein expression in *N. benthamiana*, in one large experiment over only 7 days (Maclean et al. 2007).

Use of deconstructed TMV-based vectors delivered by *Agrobacterium* routinely has allowed significant increases of antigen yield, up to grams per kilogram fresh tissue weight (Gleba et al. 2014; Klimyuk et al. 2014). The so-called TMV-based "launch vectors" of Fraunhofer USA have also allowed significant yield increases and rapid production of antigens (Chichester et al. 2013; Shamloul et al. 2014). Improved non-replicating hyper-translational (HT) expression vectors derived from Cowpea mosaic virus RNA2 have also allowed significantly higher yields via agroinfiltration (Sainsbury et al. 2008, 2009) and the possibility of multiple genes from the same vector (Saxena et al. 2016); so too has the use of a ssDNA geminivirus-derived set of vectors by different groups (Huang et al. 2009; Regnard et al. 2010), and other ssDNA plant (or other host) virus-derived vectors (Rybicki and Martin 2014).

The number of peptide display vectors/chimaeric protein fusion partners has multiplied: while self-replicating rTMV was once state of the art, now one may choose between TMV- and Potato virus X (PVX)-based vectors (Lico et al. 2015), Cucumber mosaic virus (CMV) CP (Nemchinov and Natilla 2007; Zhao and Hammond 2005), Bamboo mosaic virus (Yang et al. 2007), PVX-vectored Alternanthera mosaic virus (AltMV) CP gene (Tyulkina et al. 2011), lichenase (LickM), cholera toxin B subunit (CTB), AMV CP, and GUS, as mentioned earlier. Plant virus virions in particular are now seen as easily-made nanoparticles suitable for a number of vaccine-relevant purposes (Steele et al. 2017), including as self-adjuvanting peptide-based vaccine display vehicles (Lebel et al. 2015; Leclerc 2014), and excellent inducers of cross-presentation by MHC receptors (Hanafi et al. 2010).

The use of tags or small peptide fusion partners is now also considerably more sophisticated, with a variety of specialized tags to choose from. These include the now-ubiquitous 6xHis tag, used for Ni^{2+} or other immobilised metal affinity

chromatography (IMAC) protein purification technique; a new "Cysta-tag" for the same purpose (Sainsbury et al. 2016); the N-terminal proline-rich domain of maize seed gamma zein (Zera) that induces the formation of ER-located protein bodies (Torrent et al. 2009); elastin-like polypeptides (ELPs) with repeating pentapeptide 'VPGXG' sequences, or hydrophobins—small fungal proteins which alter the hydrophobicity of the fusion partner—both of which also form protein bodies (Conley et al. 2011). As examples, our group has recently successfully used ELP fusion to the CP of Beak and feather disease virus (BFDV) of parrots to aid in both accumulation and purification of the protein as a candidate vaccine (Duvenage et al. 2013). We have also used the Zera tag as a protein body display vehicle for an ectopic M2e moiety common to all influenzavirus A types, which could serve as a universal vaccine for these viruses (Mbewana et al. 2015). Another potentially veterinary use of Zera was in the enhancement of *Yersinia pestis* F1-V antigen fusion protein accumulation: this was $\sim 3\times$ higher than F1-V alone in three different host plant systems—namely, *N. benthamiana*, alfalfa and *N. tabacum* NT1 suspension-cultured cells (Alvarez et al. 2010).

The expression vehicles themselves have also been subject to engineering: it is now possible to precisely control glycosylation of plant-made proteins. This can be done by knock-out modification via RNA interference (RNAi) technology of the plant glycosyltransferases beta1,2-xylosyltransferase (XylT) and core alpha1,3-fucosyltransferase (FucT). These enzymes are responsible for the transfer of beta1,2-linked xylose and core alpha1,3-linked fucose residues to glycoprotein N-glycans, which are plant-specific modifications not found in mammalian glycoproteins (Strasser et al. 2008). It is also possible to use transient co-expression technologies to modify glycosylation (Castilho and Steinkellner 2016), as well as to achieve almost completely native sialylated recombinant proteins by expression of whole mammalian glycosylation pathways in plants (Castilho et al. 2010; Steinkellner and Castilho 2015). It is possible to abolish N-glycosylation entirely, by co-expression of bacterial PNGase F (Mamedov and Yusibov 2013). One can also control endogenous plant proteases that may limit recombinant protein accumulation: for example, transient co-expression of secreted A1/S1 protease inhibitor tomato cathepsin D inhibitor (SlCDI) significantly lowered A1 and S1 protease activities in the *N. benthamiana* apoplast, while increasing recombinant protein content by $\sim 45\%$ (Goulet et al. 2012). It was found that co-expression of tomato cystatin SlCYS8, which inhibits C1A proteases, increased the transient expression yield of a monoclonal antibody in *N. benthamiana* by nearly 40% (Robert et al. 2013). It is also possible to reduce protease activity in cell suspension cultures by expression of specific antisense RNAs, resulting in significantly increased accumulation of recombinant antibodies (Mandal et al. 2014).

While suspension-cultured plant cells have been used for many years for molecular farming—and in fact were used for the only USDA-licenced plant-produced animal vaccine, against NDV—new developments have made them an even more attractive prospect for low-cost vaccine production. Use of flow cytometry with cell sorting, formerly the province of mammalian cell culture work only, has allowed high-expressing MAb-producing tobacco BY-2 cell lines from a

heterogeneous population of cells by selecting the co-expressed fluorescent marker protein DsRed (Kirchhoff et al. 2012). However, one of the most exciting recent developments with this technology is the advent of the "cell pack": this is a technique for getting highly efficient (up to 100%) *Agrobacterium*-mediated transient transformation of suspension-cultured cells that have been captured by suction onto a filter (Rademacher 2014). Cell packs can be tiny (Eppendorf tube tips) or large (e.g.: centimetres deep in a 20 cm Buchner funnel); protein expression occurs in immobilised cells in the presence of minimal liquid media, and can continue for days (https://tinyurl.com/k22da6q). The technology is ideal for rapid and high-throughput screening of expression—and the possibility exists for taking cells back into culture and selecting for permanent transfection. These are important developments, because of the acceptability of the products of plant cell cultures for production of biologics to regulatory bodies (see below). Another production host highly suited to industrial-scale production is microalgae: they are easier to establish and use than plant cell cultures, and share all the same advantages of scalability, contained growth, and consistent transgene expression levels (Specht and Mayfield 2014).

A very important development for molecular farming has been the development of protocols for increasing yields and implementing industrial-scale production and downstream processing of vaccines and biologics, without which no large-scale trials could take place, or routine manufacturing occur. A useful development was use of a transgenic *N. tabacum*/*N. glauca* hybrid that does not synthesize alkaloids, is highly vigorous, can easily be propagated by vegetative cuttings and does not produce viable pollen, which greatly aids biocontainment (Ling et al. 2012). The application of techniques more familiar to chemical engineers is also advantageous: for example, it proved possible, by sequential use of fractional factorial designs and response surface methodology, to optimize culture media for MAb production in transgenic tobacco BY-2 cells, and to increase MAb yields up to 31-fold after 10 days of culture compared to use of standard media (Vasilev et al. 2013). The Fraunhofer IME group have described generic chromatography-based strategies focusing on the binding behaviours of host cell proteins to chromatography resins under varying conditions of pH and conductivity (Buyel and Fischer 2014). Another useful technique from that group is a comprehensive description of the use of heat treatment of either intact leaves or of plant extracts to facilitate the industrial-scale removal of host cell proteins, optimised by a design-of-experiments approach that will also be familiar to engineers (Buyel et al. 2016). Many of these and other strategies used to optimise yields in molecular farming are reviewed here (Twyman et al. 2013).

The establishment by various companies and institutes of facilities suitable for manufacture of animal and clinical trial material is also a very welcome development. As examples, the long-established Kentucky BioProcessing Inc (KBP) is a contract manufacturer capable of production from transgenic plants or transiently transfected plants, using the U.S. Food and Drug Administration's current Good Manufacturing Practices (cGMP) for pharmaceuticals, at scales up to thousands of kilograms of plants per week (https://www.kentuckybioprocessing.com/). They have recently produced and stockpiled Mabs against Ebolaviruses.

Another contract manufacturing firm with large production capacity is iBio Inc: like KBP, they have a wide range of patents on their proprietary gene expression technology (Holtz et al. 2015). They are also partnering with a range of agencies and companies, including with the Brazilian Oswaldo Cruz Foundation for plant-made yellow fever vaccine, and the US Dept of Defense and the Bill & Melinda Gates Foundation for influenza vaccines (http://www.ibioinc.com/).

The Fraunhofer USA Center for Molecular Biotechnology (http://www.fhcmb.org/) is a not-for-profit research and development organisation, that offers "… plant-based protein production, purification, scale-up and GMP manufacturing to support the development of vaccines, therapeutics and diagnostics", also with proprietary expression platforms, and can take products right through to fill and finish. The Fraunhofer IME in Aachen also has a state-of-the-art mechanised plant production facility still under construction as of 2017.

4 Regulatory Approvals

The regulatory environment has changed for the better, even though it was not in truth as inimical as first supposed: this was borne out by the fact that as early as 2006, the Cuban regulatory agencies and the USDA had approved plant-made MAbs for the purification of an already-licenced yeast-made hepatitis B vaccine, and the tobacco cell-made NDV vaccines, respectively (Rybicki 2009).

As another early example, the Fraunhofer IME molecular farming group published in 2004 that use of whole plants for biologics production lacks intrinsic benefits of cell culture techniques, such as precise control over growth conditions, batch-to-batch product consistency, sterile containment, and it being much harder to be in compliance with good manufacturing practice (GMP) (Hellwig et al. 2004). They pointed out that plant cell suspension cultures have all the merits of microbial and animal cell cultures, have an established track record for secondary metabolite production, and are far cheaper to use.

These justifications notwithstanding, the same group later noted, in a review on GMP issues for plant-made proteins in whole plants, that: "When [plant-derived] recombinant proteins are intended for medical use… they fall under the same regulatory guidelines for manufacturing that cover drugs from all other sources, and when such proteins enter clinical development this includes the requirement for production according to [GMP]. In principle, the well-characterized GMP regulations that apply to pharmaceutical proteins produced in bacteria and mammalian cells are directly transferrable to plants" (Fischer et al. 2012). They subsequently were able to get GMP manufacturing authorisation from German authorities for making MAbs from transgenic *N. tabacum* for a phase I clinical trial (Ma et al. 2015).

Other entities have also scaled and regularised production to allow production of materials for animal and clinical trial—and one of the most successful has been Medicago Inc., who presently has routine large-scale production of influenzavirus A haemagglutinin (HA)-based VLPs for use in advanced human clinical trial

(D'Aoust et al. 2010). In 2012, Medicago Inc. succeeded in manufacturing 10 million doses of an H1N1 VLP-based influenza vaccine candidate in one month, by Phase 1-appropriate cGMP, as part of the US Defense Advanced Research Projects Agency (DARPA)-funded challenge (DARPA 2012).

A group in Japan has also recently developed a GMP-compliant production process for a transgenic rice seed-based cholera vaccine—MucoRice-CTB—which is simply polished, powdered seed, now in clinical trial (Kashima et al. 2016).

As evidence of the increasing maturity of veterinary molecular farming, one of the editors of this book has co-authored a recent article on regulatory and commercial hurdles hampering the advance to market of plant-produced veterinary vaccines, covering developing business plans, assessing market opportunities, manufacturing scale-up, financing, protecting and using intellectual property, and regulatory approval (MacDonald et al. 2015).

5 Future of Plant Expression to Make Biologics for Veterinary Use

At first sight, molecular farming appears the ideal way to make recombinant protein-based veterinary vaccines: production of active ingredients is markedly cheaper per unit mass than by use of any animal tissue-culture system, and generally cheaper than yeast or bacterial culture (Rybicki 2010); partially-purified or unprocessed extracts are highly unlikely to contain any animal pathogens; edible and oral vaccines appear highly feasible; the financial barrier to entry for manufacture appears far lower than for conventionally-made vaccines. It is possible to efficiently make bacterial proteins using bacterial-derived translational machinery in chloroplasts in transplastomic plants, as well as to make other proteins at very high yield; conventional transgenics have been used to make many vaccine candidates, with many proofs of efficacy; transient expression technologies have revolutionised the field in terms of providing high yields and very rapid development times from concept to product. And yet, only one product—Dow's NDV vaccine—is registered for use, and that is not sold.

It is possible that heavy investment by big industry players in conventional manufacturing technologies has stalled their uptake of molecular farming technology for veterinary vaccines and biologics: this has certainly been true for human biologics. However, perhaps developments from the human field could be used as a spur for uptake of veterinary vaccines and biologics: an example here is the licencing of Protalix Biotherapeutics' Elelyso® or glucocerebrosidase, a therapeutic for a genetic mitochondrial enzyme deficit called Gaucher disease, made using transgenic carrot cell lines in 800 litre plastic bag fermenters (http://protalix.com/about/elelyso/). A contamination of Genzyme's mammalian cell production facilities in 2009 with a mammalian calicivirus led to the FDA allowing Protalix to supply the drug to patients who needed it, and to accelerated licensure (Bethencourt 2009).

The company has also successfully tested oral administration of drugs in plant cells, which would be a highly welcome development: they claim that "Oral delivery of protein therapies [is] possible due to the unique cellulose wall of plant cells that makes them resistant to degradation when passing through the digestive tract" (Protalix 2017). Another apposite example was the fortuitous availability of a plant-made anti-Zaire ebolavirus MAb cocktail known as ZMapp™, at the height of the recent West African Ebola disease outbreak (reviewed in Rybicki 2014). This was made by transient expression in *N. benthamiana*, and only a few clinical trial doses were available: these were used under the humanitarian principle, and later the MAbs were cleared for use by the FDA in an efficacy trial just before the end of the epidemic (LeafBio 2016).

Both these examples are of niche products that were not being made at large scale or for a large market by conventional techniques, and for which there was a sudden, pressing need that could not be supplied by other means. This could provide motivation for small companies to either develop inexpensive vaccines for emerging diseases, or to target niche vaccines or niche therapeutics, in the knowledge that large established entities are unwilling to take the risk.

One example for the former possibility comes from the recent emergence of bluetongue virus (BTV) disease in sheep and small ruminants in Europe, due to northward spread of the insect vector with climate change (Purse et al. 2008): while attenuated live vaccines are available—South Africa presently uses a cocktail of 24 such viruses—concerns in Europe about reassortment of virus dsRNA genome components between vaccinated and naturally diseased animals, as well as of the safety of the vaccines in terms of possible under-attenuation which may result in disease development in certain sheep breeds (Niedbalski 2011), mean these are not being used. The irregular occurrence of outbreaks, and the limited number of strains involved, mean that stockpiling vaccines is desirable. However, killed vaccines still require growing potentially dangerous viruses, and while it is possible to make VLPs in cell cultures and these are effective (Pearson and Roy 1993; Roy et al. 1994), the technology is too expensive for farm animal use. It is fortunate, therefore, that it is also possible to make BTV-8 VLPs via transient expression in *N. benthamiana*, and these are as effective in a single injected dose as the commercial vaccine (Thuenemann et al. 2013). There are currently no plans to manufacture this or other plant-produced BTV vaccines for the European or other markets; however, this may soon change.

An example for a niche vaccine product comes from ours and others' work on beak and feather disease virus (BFDV) vaccines: psittacines are highly valued companion animals; however, there are very few vaccines for their diseases, and none yet available for BFDV. While some recent work in this area has shown that recombinant CP can be made in *E coli* and in insect cells (Heath et al. 2006; Patterson et al. 2013; Stewart et al. 2007), that it appears to be protective (Bonne et al. 2009) and that this can apparently form VLPs (Sarker et al. 2015), it still appears that the protein is too expensive to produce for use as a vaccine. While initial work with plant production of BFDV CP was disappointing due to low yields, recent work from our group (Duvenage et al. 2013) showed a significant

increase in BFDV yield due to fusion with elastin-like polypeptide (ELP), and good immunogenicity in mice. This, coupled with a very simple purification protocol enabled by ELPylation (Conley et al. 2009), could allow scalable, cheap production of BFDV vaccines.

While therapeutics such as MAbs or other biologics for veterinary use are generally limited to high-value companion animals, plant production could open up a hitherto neglected market niche. One excellent example is the manufacture in Japan of canine interferon-α (Tabayashi and Matsumura 2014): this is done via transgenic strawberries in a completely enclosed GMP-compliant facility, and the product is powdered strawberry extract given orally, to combat canine periodontal disease. Another very recent example in dogs, albeit with them being used as a model for human disease, was the proof that lyophilised transplastomic lettuce leaves expressing CTB fusions of coagulation factor IX (FIX) could be used orally in feed for >300 days in haemophilia B dogs with no ill effects—and that this treatment resulted in robust suppression of IgG/inhibitor and IgE formation against intravenously-provided FIX, and a marked shortening of blood coagulation times (Herzog et al. 2017).

An example for agricultural use is the oral dosing of pigs with transgenic *Arabidopsis thaliana* seeds containing designer IgAs against enterotoxigenic *E coli* (ETEC) (Virdi et al. 2013): this product consisted of dimeric llama-derived heavy chain variable region fused to the Fc portion of a porcine IgA and the porcine IgA J chain and secretory component, which allowed production of dimeric secretory IgA-like antibodies (VHH-IgA). In a piglet feed-challenge experiment with ETEC, dosing piglets with 20 mg/d per pig VHH-IgA produced a progressive decline in bacterial shedding and a significantly higher weight gain than seen in control or other experimental pigs.

A highly novel plant-made therapeutic product was the receptor binding domain of the tailspike protein Gp9 from the P22 bacteriophage: this is known to reduce *Salmonella* colonisation in the chicken gut (Miletic et al. 2015). Purified ELP-fused Gp9 bound to *Salmonella enterica* serovar Typhimurium in vitro, and feeding lyophilized leaves containing Gp9-ELP to newly hatched chickens showed that it has the potential to control *Salmonella* contamination in commercially-raised fowl. These and other experiments are reviewed here (Juarez et al. 2016; Topp et al. 2016), in articles that make an excellent case for plant-made immunotherapeutics for veterinary use.

6 The One Health Approach and Its Relevance for Modern Veterinary Vaccines

The One Health concept has as one of its central themes the integration of opportunities for vaccine-based approaches for the prevention of zoonotic and emerging diseases across veterinary and human medicine (Monath 2013), and three

different frameworks for the use of vaccines in these contexts have been formulated. Framework I vaccines are used to protect humans and economically valuable animals, where neither are central to the transmission cycle: a good example here would be West Nile virus, which is a mosquito-borne flavivirus spread around the world by birds, and which incidentally infects animals like horses as well as humans. Framework II vaccines, on the other hand, are intended for use in domesticated animals as a means of preventing disease in both animals and humans: examples of disease agents here would be *Brucella abortis, E coli* O157, and rabies, influenza, Rift Valley fever, and Hendra viruses. Framework III vaccines are for immunising non-domesticated animals in order to prevent transmission of disease agents to humans and domesticated animals: examples here are the use of oral bait rabies, and *Mycobacterium bovis* and Lyme disease vaccines.

A set of disease agents which exemplify the potential strength of the One Health approach are influenza viruses, and they have in fact been the focus of a number of international meetings and planning sessions (Chien 2013; Dwyer and Kirkland 2011; Kahn et al. 2014; Ludwig et al. 2014; Powdrill et al. 2010; Short et al. 2015). The unique mix of hosts that occurs in intensive agricultural environments that could give rise to pandemics—swine, birds and humans—is a major cause of international concern; so too is the development of suitable vaccines for the prevention of infection in domesticated birds, farmed swine, and humans. Plants have been shown to be highly useful for the production of influenza vaccines, and indeed possibly the fastest ever production at scale of an influenzavirus A strain vaccine—1 month for 10 million doses—was done by Medicago Inc. for H1N1pdm 2009 HA VLPs in 2012 (Rybicki 2014). Medicago also managed in 2013, as an exercise to demonstrate preparedness, to produce grams of cGMP-grade plant-made H7 HA-only VLPs only 19 days after accessing the H7 HA gene cDNA sequence, in response to an outbreak in China in the same year. The fact that plant-made influenza vaccines have worked very well in animal models means that they should be trialled extensively in domestic fowl and swine, to see if the maintenance of the viruses in these hosts can be curbed. As for companion animals, there is even a canine influenza vaccine candidate: following a 2004 H3N8 outbreak in the US, a group in Canada used the plant-derived filamentous Malva mosaic virus (MaMV) nanoparticles as a vaccine platform to display the highly conserved ectopic M2e peptide and to increase its immunogenicity. Together with the adjuvant OmpC derived from *Salmonella typhi,* the vaccine was protective against both the homologous virus and a heterosubtypic strain of influenza in mice, as well as eliciting antibodies reactive with M2e peptides derived from H9N2, H5N1 and H1N1 strains and being immunogenic in dogs (Leclerc et al. 2013).

Given that brucellosis is listed as a One Health priority, it is worth noting that a transgenic plant-produced *B abortus* outer membrane protein (U-Omp19) was an effective oral vaccine in mice against a systemic challenge, eliciting an adaptive IL-17 immune response (Pasquevich et al. 2011)—and that the protein has significant adjuvant activity, and oral vaccination of mice with U-Omp19 plus *Salmonella* antigens was protective against virulent challenge with *S typhimurium* (Risso et al. 2017).

It is important to realise that, while vaccines are the target of this review, One Health products can also be reagents to be used in more effective or cheaper diagnostic kits, and in particular for point-of-care devices, or for research laboratory use—and especially proteins that could be both a reagent and used as a candidate vaccine in animals and possibly humans. A few of the best potential One Health targets for plant-made dual-function proteins would be proteins from Middle Eastern respiratory syndrome (MERS) coronavirus (Wirblich et al. 2017), Nipah and Hendra viruses (Landford and Nunn 2012; Mackenzie et al. 2003), diagnostic/vaccine candidate proteins from Rift Valley Fever and Crimean-Congo haemorrhagic fever viruses (Kortekaas 2014; Monath 2013). Inexpensive and abundant proteins made from these agents could first serve as reagents in the development of cheap point-of-care diagnostics, and then as vaccine candidates in animals, if appropriate, and then possibly in humans.

A useful example here is of the expression both by agroinfiltration in *N. benthamiana* as a reagent, and in transgenic *N. tabacum* roots and leaves as a vaccine, of a fused GcGn envelope glycoprotein-encoding gene from Crimean-Congo haemorrhagic fever virus (Ghiasi et al. 2011). The protein yield was 1–2 mg/kg fresh plant weight. Transgenic material was orally immunogenic, and elicited humoral and mucosal antibody responses, and antibodies bound inactivated virus used as a vaccine booster in some experiments. Agroinfiltration-produced GnGc was used as a reagent in ELISA to detect immune responses. Another study from our group was of the production of CCHFV N protein in *N. benthamiana* by agroinfiltration specifically as a reagent for use in diagnostic tests (Atkinson et al. 2016): a plant codon-optimised and 6xHis tagged N protein gene was found to accumulate best as a soluble protein in the cytoplasm, from which it could be easily purified by ammonium sulphate fractionation and immobilised Ni^{2+} column chromatography. Purified NP was used in a validated indirect ELISA to detect anti-CCHFV IgG in sera from convalescent human patients: this was successful for 13/13 samples, with no readings for samples from patients with no history of CCHFV infection. The results were 100% concordant with those from a commercially available immunofluorescent assay. Given that soluble N protein is hard to produce and difficult to purify from insect cell cultures, the plant-made product would seem to be a desirable replacement.

7 Conclusions

While the same has been said in many venues over more than twenty years now, the field of molecular farming really does seem to be near to meeting its initial promise for veterinary use. All of the technology that is required for efficient, high-yield production of biologics is in place; downstream processing modalities have been well worked out by a number of near- and cGMP-compliant facilities; many candidate vaccines for a wide variety of pathogens have been tested; therapeutic biologics too for veterinary use are now feasible; regulatory agencies seem agreeable to

considering plant-made products. The generally shorter regulatory path, the possibility of using less stringently purified products, and the very real possibility of using oral vaccines and therapeutics, should also be highly attractive for product developers. I sincerely hope, then, that realisation of the promise comes very soon.

References

Alvarez ML, Topal E, Martin F, Cardineau GA (2010) Higher accumulation of F1-V fusion recombinant protein in plants after induction of protein body formation. Plant Mol Biol 72:75–89

APHIS (2008) Noncompliance history

Ashraf S, Singh PK, Yadav DK, Shahnawaz M, Mishra S, Sawant SV, Tuli R (2005) High level expression of surface glycoprotein of rabies virus in tobacco leaves and its immunoprotective activity in mice. J Biotechnol 119:1–14

Athmaram TN, Bali G, Devaiah KM (2006) Integration and expression of Bluetongue VP2 gene in somatic embryos of peanut through particle bombardment method. Vaccine 24:2994–3000

Atkinson R, Burt F, Rybicki EP, Meyers AE (2016) Plant-produced Crimean-Congo haemorrhagic fever virus nucleoprotein for use in indirect ELISA. J Virol Methods 236:170–177

Aziz MA, Singh S, Anand Kumar P, Bhatnagar R (2002) Expression of protective antigen in transgenic plants: a step towards edible vaccine against anthrax. Biochem Biophys Res Commun 299:345–351

Aziz MA, Sikriwal D, Singh S, Jarugula S, Kumar PA, Bhatnagar R (2005) Transformation of an edible crop with the pagA gene of Bacillus anthracis. FASEB J 19:1501–1503

Bethencourt V (2009) Virus stalls Genzyme plant. Nat Biotechnol 27:681

Biemelt S, Sonnewald U, Galmbacher P, Willmitzer L, Muller M (2003) Production of human papillomavirus type 16 virus-like particles in transgenic plants. J Virol 77:9211–9220

Bonne N, Shearer P, Sharp M, Clark P, Raidal S (2009) Assessment of recombinant beak and feather disease virus capsid protein as a vaccine for psittacine beak and feather disease. J Gen Virol 90:640–647

Breitburd F, Kirnbauer R, Hubbert NL, Nonnenmacher B, Trin-Dinh-Desmarquet C, Orth G, Schiller JT, Lowy DR (1995) Immunization with viruslike particles from cottontail rabbit papillomavirus (CRPV) can protect against experimental CRPV infection. J Virol 69:3959–3963

Breitburd F, Salmon J, Orth G (1997) The rabbit viral skin papillomas and carcinomas: a model for the immunogenetics of HPV-associated carcinogenesis. Clin Dermatol 15:237–247

Buyel JF, Fischer R (2014) Generic chromatography-based purification strategies accelerate the development of downstream processes for biopharmaceutical proteins produced in plants. Biotechnol J 9:566–577

Buyel JF, Hubbuch J, Fischer R (2016) Comparison of tobacco host cell protein removal methods by blanching intact plants or by heat treatment of extracts. J Visualized Exp: JoVE

Carrillo C, Wigdorovitz A, Oliveros JC, Zamorano PI, Sadir AM, Gomez N, Salinas J, Escribano JM, Borca MV (1998) Protective immune response to foot-and-mouth disease virus with VP1 expressed in transgenic plants. J Virol 72:1688–1690

Castanon S, Marin MS, Martin-Alonso JM, Boga JA, Casais R, Humara JM, Ordas RJ, Parra F (1999) Immunization with potato plants expressing VP60 protein protects against rabbit hemorrhagic disease virus. J Virol 73:4452–4455

Castilho A, Strasser R, Stadlmann J, Grass J, Jez J, Gattinger P, Kunert R, Quendler H, Pabst M, Leonard R, Altmann F, Steinkellner H (2010) In planta protein sialylation through overexpression of the respective mammalian pathway. J Biol Chem 285:15923–15930

Castilho A, Steinkellner H (2016) Transient Expression of Mammalian Genes in N. benthamiana to Modulate N-Glycosylation. Methods Mol Biol 1385:99–113

Chichester JA, Musiychuk K, de la Rosa P, Horsey A, Stevenson N, Ugulava N, Rabindran S, Palmer GA, Mett V, Yusibov V (2007) Immunogenicity of a subunit vaccine against *Bacillus anthracis*. Vaccine 25:3111–3114

Chichester JA, Manceva SD, Rhee A, Coffin MV, Musiychuk K, Mett V, Shamloul M, Norikane J, Streatfield SJ, Yusibov V (2013) A plant-produced protective antigen vaccine confers protection in rabbits against a lethal aerosolized challenge with Bacillus anthracis Ames spores. Human Vaccines Immunotherapeutics 9:544–552

Chien YJ (2013) How did international agencies perceive the avian influenza problem? The adoption and manufacture of the 'One World, One Health' framework. Sociol Health Illn 35:213–226

Conley AJ, Joensuu JJ, Jevnikar AM, Menassa R, Brandle JE (2009) Optimization of elastin-like polypeptide fusions for expression and purification of recombinant proteins in plants. Biotechnol Bioeng 103:562–573

Conley AJ, Joensuu JJ, Richman A, Menassa R (2011) Protein body-inducing fusions for high-level production and purification of recombinant proteins in plants. Plant Biotechnol J 9:419–433

D'Aoust MA, Couture MM, Charland N, Trepanier S, Landry N, Ors F, Vezina LP (2010) The production of hemagglutinin-based virus-like particles in plants: a rapid, efficient and safe response to pandemic influenza. Plant Biotechnol J 8:607–619

Dalsgaard K, Uttenthal A, Jones TD, Xu F, Merryweather A, Hamilton WD, Langeveld JP, Boshuizen RS, Kamstrup S, Lomonossoff GP, Porta C, Vela C, Casal JI, Meloen RH, Rodgers PB (1997) Plant-derived vaccine protects target animals against a viral disease. Nat Biotechnol 15:248–252

DARPA (2012) DARPA makes 10 million strides in the race to contain a hypothetical pandemic. US Defense Advanced Research Projects Agency

Dus Santos MJ, Wigdorovitz A, Trono K, Rios RD, Franzone PM, Gil F, Moreno J, Carrillo C, Escribano JM, Borca MV (2002) A novel methodology to develop a foot and mouth disease virus (FMDV) peptide-based vaccine in transgenic plants. Vaccine 20:1141–1147

Duvenage L, Hitzeroth II, Meyers AE, Rybicki EP (2013) Expression in tobacco and purification of beak and feather disease virus capsid protein fused to elastin-like polypeptides. J Virol Methods 191:55–62

Dwyer DE, Kirkland PD (2011) Influenza: one health in action. N S W Public Health Bull 22:123–126

Fischer R, Schillberg S, Hellwig S, Twyman RM, Drossard J (2012) GMP issues for recombinant plant-derived pharmaceutical proteins. Biotechnol Adv 30:434–439

Ghiasi SM, Salmanian AH, Chinikar S, Zakeri S (2011) Mice orally immunized with a transgenic plant expressing the glycoprotein of Crimean-Congo hemorrhagic fever virus. Clin Vaccine Immunol: CVI 18:2031–2037

Gil F, Titarenko E, Terrada E, Arcalis E, Escribano JM (2006) Successful oral prime-immunization with VP60 from rabbit haemorrhagic disease virus produced in transgenic plants using different fusion strategies. Plant Biotechnol J 4:135–143

Gleba YY, Tuse D, Giritch A (2014) Plant viral vectors for delivery by Agrobacterium. Curr Top Microbiol Immunol 375:155–192

Gomez N, Wigdorovitz A, Castanon S, Gil F, Ordas R, Borca MV, Escribano JM (2000) Oral immunogenicity of the plant derived spike protein from swine-transmissible gastroenteritis coronavirus. Arch Virol 145:1725–1732

Goulet C, Khalf M, Sainsbury F, D'Aoust MA, Michaud D (2012) A protease activity-depleted environment for heterologous proteins migrating towards the leaf cell apoplast. Plant Biotechnol J 10:83–94

Hanafi LA, Bolduc M, Gagne ME, Dufour F, Langelier Y, Boulassel MR, Routy JP, Leclerc D, Lapointe R (2010) Two distinct chimeric potexviruses share antigenic cross-presentation properties of MHC class I epitopes. Vaccine 28:5617–5626

Haq TA, Mason HS, Clements JD, Arntzen CJ (1995) Oral immunization with a recombinant bacterial antigen produced in transgenic plants. Science 268:714–716

Heath L, Williamson AL, Rybicki EP (2006) The capsid protein of beak and feather disease virus binds to the viral DNA and is responsible for transporting the replication-associated protein into the nucleus. J Virol 80:7219–7225

Hellwig S, Drossard J, Twyman RM, Fischer R (2004) Plant cell cultures for the production of recombinant proteins. Nat Biotechnol 22:1415–1422

Herzog RW, Nichols TC, Su J, Zhang B, Sherman A, Merricks EP, Raymer R, Perrin GQ, Häger M, Winberg B, Daniell H (2017) Oral tolerance induction in hemophilia B dogs fed with transplastomic lettuce. Mol Ther 25:512–522

Holtz BR, Berquist BR, Bennett LD, Kommineni VJ, Munigunti RK, White EL, Wilkerson DC, Wong KY, Ly LH, Marcel S (2015) Commercial-scale biotherapeutics manufacturing facility for plant-made pharmaceuticals. Plant Biotechnol J 13:1180–1190

Huang Y, Liang W, Wang Y, Zhou Z, Pan A, Yang X, Huang C, Chen J, Zhang D (2005) Immunogenicity of the epitope of the foot-and-mouth disease virus fused with a hepatitis B core protein as expressed in transgenic tobacco. Viral Immunol 18:668–677

Huang Z, Chen Q, Hjelm B, Arntzen C, Mason H (2009) A DNA replicon system for rapid high-level production of virus-like particles in plants. Biotechnol Bioeng 103:706–714

Hull AK, Criscuolo CJ, Mett V, Groen H, Steeman W, Westra H, Chapman G, Legutki B, Baillie L, Yusibov V (2005) Human-derived, plant-produced monoclonal antibody for the treatment of anthrax. Vaccine 23:2082–2086

Jilka J (2002) An oral vaccine in maize protects against transmissible gastroenteritis virus in swine. In: Erickson L, Yu W-J, Brandle J, Rymerson R (eds) Molecular farming of plants and animals for human and veterinary medicine. Springer, Netherlands, pp 223–236

Juarez P, Virdi V, Depicker A, Orzaez D (2016) Biomanufacturing of protective antibodies and other therapeutics in edible plant tissues for oral applications. Plant Biotechnol J 14:1791–1799

Kahn RE, Ma W, Richt JA (2014) Swine and influenza: a challenge to one health research. Curr Top Microbiol Immunol 385:205–218

Kashima K, Yuki Y, Mejima M, Kurokawa S, Suzuki Y, Minakawa S, Takeyama N, Fukuyama Y, Azegami T, Tanimoto T, Kuroda M, Tamura M, Gomi Y, Kiyono H (2016) Good manufacturing practices production of a purification-free oral cholera vaccine expressed in transgenic rice plants. Plant Cell Rep 35:667–679

Khandelwal A, Renukaradhya GJ, Rajasekhar M, Sita GL, Shaila MS (2004) Systemic and oral immunogenicity of hemagglutinin protein of rinderpest virus expressed by transgenic peanut plants in a mouse model. Virology 323:284–291

Kirchhoff J, Raven N, Boes A, Roberts JL, Russell S, Treffenfeldt W, Fischer R, Schinkel H, Schiermeyer A, Schillberg S (2012) Monoclonal tobacco cell lines with enhanced recombinant protein yields can be generated from heterogeneous cell suspension cultures by flow sorting. Plant Biotechnol J 10:936–944

Klimyuk V, Pogue G, Herz S, Butler J, Haydon H (2014) Production of recombinant antigens and antibodies in Nicotiana benthamiana using 'magnifection' technology: GMP-compliant facilities for small- and large-scale manufacturing. Curr Top Microbiol Immunol 375:127–154

Kohl T, Hitzeroth II, Stewart D, Varsani A, Govan VA, Christensen ND, Williamson AL, Rybicki EP (2006) Plant-produced cottontail rabbit papillomavirus L1 protein protects against tumor challenge: a proof-of-concept study. Clin Vaccine Immunol 13:845–853

Kortekaas J (2014) One Health approach to Rift Valley fever vaccine development. Antiviral Res 106:24–32

Koya V, Moayeri M, Leppla SH, Daniell H (2005) Plant-based vaccine: mice immunized with chloroplast-derived anthrax protective antigen survive anthrax lethal toxin challenge. Infect Immun 73:8266–8274

Lamphear BJ, Streatfield SJ, Jilka JM, Brooks CA, Barker DK, Turner DD, Delaney DE, Garcia M, Wiggins B, Woodard SL, Hood EE, Tizard IR, Lawhorn B, Howard JA (2002) Delivery of subunit vaccines in maize seed. J Control Release 85:169–180

Landford J, Nunn M (2012) Good governance in 'one health' approaches. Rev Sci Tech 31:561–575
LeafBio (2016) LeafBio announces conclusion of ZMapp™ clinical trial
Lebel ME, Chartrand K, Leclerc D, Lamarre A (2015) Plant viruses as nanoparticle-based vaccines and adjuvants. Vaccines (Basel) 3:620–637
Leclerc D, Rivest M, Babin C, Lopez-Macias C, Savard P (2013) A novel M2e based flu vaccine formulation for dogs. PLoS ONE 8:e77084
Leclerc D (2014) Plant viral epitope display systems for vaccine development. Curr Top Microbiol Immunol 375:47–59
Lico C, Benvenuto E, Baschieri S (2015) The two-faced potato virus X: from plant pathogen to smart nanoparticle. Front Plant Sci 6:1009
Ling HY, Edwards AM, Gantier MP, Deboer KD, Neale AD, Hamill JD, Walmsley AM (2012) An interspecific Nicotiana hybrid as a useful and cost-effective platform for production of animal vaccines. PLoS ONE 7:e35688
Love AJ, Chapman SN, Matic S, Noris E, Lomonossoff GP, Taliansky M (2012) In planta production of a candidate vaccine against bovine papillomavirus type 1. Planta
Loza-Rubio E, Rojas E, Gomez L, Olivera MT, Gomez-Lim MA (2008) Development of an edible rabies vaccine in maize using the Vnukovo strain. Dev Biol 131:477–482
Loza-Rubio E, Rojas-Anaya E, Lopez J, Olivera-Flores MT, Gomez-Lim M, Tapia-Perez G (2012) Induction of a protective immune response to rabies virus in sheep after oral immunization with transgenic maize, expressing the rabies virus glycoprotein. Vaccine 30:5551–5556
Ludwig S, Zell R, Schwemmle M, Herold S (2014) Influenza, a One Health paradigm–novel therapeutic strategies to fight a zoonotic pathogen with pandemic potential. Int J Med Microbiol 304:894–901
Ma JK, Drossard J, Lewis D, Altmann F, Boyle J, Christou P, Cole T, Dale P, van Dolleweerd CJ, Isitt V, Katinger D, Lobedan M, Mertens H, Paul MJ, Rademacher T, Sack M, Hundleby PA, Stiegler G, Stoger E, Twyman RM, Vcelar B, Fischer R (2015) Regulatory approval and a first-in-human phase I clinical trial of a monoclonal antibody produced in transgenic tobacco plants. Plant Biotechnol J 13:1106–1120
MacDonald J, Doshi K, Dussault M, Hall JC, Holbrook L, Jones G, Kaldis A, Klima CL, Macdonald P, McAllister T, McLean MD, Potter A, Richman A, Shearer H, Yarosh O, Yoo HS, Topp E, Menassa R (2015) Bringing plant-based veterinary vaccines to market: managing regulatory and commercial hurdles. Biotechnol Adv 33:1572–1581
Mackenzie JS, Field HE, Guyatt KJ (2003) Managing emerging diseases borne by fruit bats (flying foxes), with particular reference to henipaviruses and Australian bat lyssavirus. J Appl Microbiol 94(Suppl):59S–69S
Maclean J, Koekemoer M, Olivier AJ, Stewart D, Hitzeroth II, Rademacher T, Fischer R, Williamson AL, Rybicki EP (2007) Optimization of human papillomavirus type 16 (HPV-16) L1 expression in plants: comparison of the suitability of different HPV-16 L1 gene variants and different cell-compartment localization. J Gen Virol 88:1460–1469
Mamedov T, Yusibov V (2013) In vivo deglycosylation of recombinant proteins in plants by co-expression with bacterial PNGase F. Bioengineered 4:338–342
Mandal MK, Fischer R, Schillberg S, Schiermeyer A (2014) Inhibition of protease activity by antisense RNA improves recombinant protein production in Nicotiana tabacum cv. Bright Yellow 2 (BY-2) suspension cells. Biotechnol J 9:1065–1073
Mbewana S, Mortimer E, Pera FF, Hitzeroth II, Rybicki EP (2015) Production of H5N1 influenza virus matrix protein 2 ectodomain protein bodies in tobacco plants and in insect cells as a candidate universal influenza vaccine. Front Bioeng Biotechnol 3:197
Miletic S, Simpson DJ, Szymanski CM, Deyholos MK, Menassa R (2015) A plant-produced bacteriophage tailspike protein for the control of salmonella. Front Plant Sci 6:1221
Monath TP (2013) Vaccines against diseases transmitted from animals to humans: a one health paradigm. Vaccine 31:5321–5338
Nemchinov LG, Natilla A (2007) Transient expression of the ectodomain of matrix protein 2 (M2e) of avian influenza A virus in plants. Protein Expr Purif 56:153–159

Niedbalski W (2011) Bluetongue vaccines in Europe. Pol J Vet Sci 14:299–304
Palmer KE, Benko A, Doucette SA, Cameron TI, Foster T, Hanley KM, McCormick AA, McCulloch M, Pogue GP, Smith ML, Christensen ND (2006) Protection of rabbits against cutaneous papillomavirus infection using recombinant tobacco mosaic virus containing L2 capsid epitopes. Vaccine 24:5516–5525
Pasquevich KA, Ibanez AE, Coria LM, Garcia Samartino C, Estein SM, Zwerdling A, Barrionuevo P, Oliveira FS, Seither C, Warzecha H, Oliveira SC, Giambartolomei GH, Cassataro J (2011) An oral vaccine based on U-Omp19 induces protection against B. abortus mucosal challenge by inducing an adaptive IL-17 immune response in mice. PLoS ONE 6: e16203
Patterson EI, Swarbrick CM, Roman N, Forwood JK, Raidal SR (2013) Differential expression of two isolates of beak and feather disease virus capsid protein in Escherichia coli. J Virol Methods 189:118–124
Pearson LD, Roy P (1993) Genetically engineered multi-component virus-like particles as veterinary vaccines. Immunol Cell Biol 71(Pt 5):381–389
Perez Filgueira DM, Mozgovoj M, Wigdorovitz A, Dus Santos MJ, Parreno V, Trono K, Fernandez FM, Carrillo C, Babiuk LA, Morris TJ, Borca MV (2004) Passive protection to bovine rotavirus (BRV) infection induced by a BRV VP8* produced in plants using a TMV-based vector. Arch Virol 149:2337–2348
Powdrill TF, Nipp TL, Rinderknecht JL (2010) One health approach to influenza: assessment of critical issues and options. Emerg Infect Dis 16:e1
Protalix (2017) Orally delivered proteins
Purse BV, Brown HE, Harrup L, Mertens PP, Rogers DJ (2008) Invasion of bluetongue and other orbivirus infections into Europe: the role of biological and climatic processes. Rev Sci Tech 27:427–442
Rademacher T (2014) Method for the generation and cultivation of a plant cell pack. Google Patents
Regnard GL, Halley-Stott RP, Tanzer FL, Hitzeroth II, Rybicki EP (2010) High level protein expression in plants through the use of a novel autonomously replicating geminivirus shuttle vector. Plant Biotechnol J 8:38–46
Risso GS, Carabajal MV, Bruno LA, Ibanez AE, Coria LM, Pasquevich KA, Lee SJ, McSorley SJ, Briones G, Cassataro J (2017) U-Omp19 from brucella abortus is a useful adjuvant for vaccine formulations against salmonella infection in mice. Front Immunol 8:171
Robert S, Khalf M, Goulet MC, D'Aoust MA, Sainsbury F, Michaud D (2013) Protection of recombinant mammalian antibodies from development-dependent proteolysis in leaves of Nicotiana benthamiana. PLoS ONE 8:e70203
Roy P, Bishop DH, LeBlois H, Erasmus BJ (1994) Long-lasting protection of sheep against bluetongue challenge after vaccination with virus-like particles: evidence for homologous and partial heterologous protection. Vaccine 12:805–811
Rybicki EP (2009) Plant-produced vaccines: promise and reality. Drug Discov Today 14:16–24
Rybicki EP (2010) Plant-made vaccines for humans and animals. Plant Biotechnol J 8:620–637
Rybicki EP (2014) Plant-based vaccines against viruses. Virol J 11:205
Rybicki EP, Martin DP (2014) Virus-derived ssDNA vectors for the expression of foreign proteins in plants. Curr Top Microbiol Immunol 375:19–45
Sainsbury F, Lavoie PO, D'Aoust MA, Vezina LP, Lomonossoff GP (2008) Expression of multiple proteins using full-length and deleted versions of cowpea mosaic virus RNA-2. Plant Biotechnol J 6:82–92
Sainsbury F, Thuenemann EC, Lomonossoff GP (2009) pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. Plant Biotechnol J 7:682–693
Sainsbury F, Jutras PV, Vorster J, Goulet MC, Michaud D (2016) A chimeric affinity tag for efficient expression and chromatographic purification of heterologous proteins from plants. Front Plant Sci 7:141

Sarker S, Ghorashi SA, Swarbrick CM, Khandokar YB, Himiari Z, Forwood JK, Raidal SR (2015) An efficient approach for recombinant expression and purification of the viral capsid protein from beak and feather disease virus (BFDV) in Escherichia coli. J Virol Methods 215–216:1–8

Satyavathi VV, Prasad V, Khandelwal A, Shaila MS, Sita GL (2003) Expression of hemagglutinin protein of Rinderpest virus in transgenic pigeon pea [*Cajanus cajan* (L.) Millsp.] plants. Plant Cell Rep 21:651–658

Saxena P, Thuenemann EC, Sainsbury F, Lomonossoff GP (2016) Virus-derived vectors for the expression of multiple proteins in plants. Methods Mol Biol 1385:39–54

Shamloul M, Trusa J, Mett V, Yusibov V (2014) Optimization and utilization of agrobacterium-mediated transient protein production in nicotiana. J Vis Exp: JoVE

Short KR, Richard M, Verhagen JH, van Riel D, Schrauwen EJ, van den Brand JM, Manz B, Bodewes R, Herfst S (2015) One health, multiple challenges: the inter-species transmission of influenza A virus. One Health 1:1–13

Specht EA, Mayfield SP (2014) Algae-based oral recombinant vaccines. Front Microbiol 5:60

Steele JF, Peyret H, Saunders K, Castells-Graells R, Marsian J, Meshcheriakova Y, Lomonossoff GP (2017) Synthetic plant virology for nanobiotechnology and nanomedicine. Wiley Interdiscip Rev Nanomed Nanobiotechnol

Steinkellner H, Castilho A (2015) N-Glyco-engineering in plants: update on strategies and major achievements. Methods Mol Biol 1321:195–212

Stewart ME, Bonne N, Shearer P, Khalesi B, Sharp M, Raidal S (2007) Baculovirus expression of beak and feather disease virus (BFDV) capsid protein capable of self-assembly and haemagglutination. J Virol Methods 141:181–187

Strasser R, Stadlmann J, Schahs M, Stiegler G, Quendler H, Mach L, Glossl J, Weterings K, Pabst M, Steinkellner H (2008) Generation of glyco-engineered Nicotiana benthamiana for the production of monoclonal antibodies with a homogeneous human-like N-glycan structure. Plant Biotechnol J 6:392–402

Streatfield SJ, Lane JR, Brooks CA, Barker DK, Poage ML, Mayor JM, Lamphear BJ, Drees CF, Jilka JM, Hood EE, Howard JA (2003) Corn as a production system for human and animal vaccines. Vaccine 21:812–815

Tabayashi N, Matsumura T (2014) Forefront study of plant biotechnology for practical use: development of oral drug for animal derived from transgenic strawberry. Soc Biotechnol J Japn 92:537–539

Tackaberry ES, Prior F, Bell M, Tocchi M, Porter S, Mehic J, Ganz PR, Sardana R, Altosaar I, Dudani A (2003) Increased yield of heterologous viral glycoprotein in the seeds of homozygous transgenic tobacco plants cultivated underground. Genome 46:521–526

Thuenemann EC, Meyers AE, Verwey J, Rybicki EP, Lomonossoff GP (2013) A method for rapid production of heteromultimeric protein complexes in plants: assembly of protective bluetongue virus-like particles. Plant Biotechnol J 11:839–846

Topp E, Irwin R, McAllister T, Lessard M, Joensuu JJ, Kolotilin I, Conrad U, Stoger E, Mor T, Warzecha H, Hall JC, McLean MD, Cox E, Devriendt B, Potter A, Depicker A, Virdi V, Holbrook L, Doshi K, Dussault M, Friendship R, Yarosh O, Yoo HS, MacDonald J, Menassa R (2016) The case for plant-made veterinary immunotherapeutics. Biotechnol Adv 34:597–604

Torrent M, Llop-Tous I, Ludevid MD (2009) Protein body induction: a new tool to produce and recover recombinant proteins in plants. Methods Mol Biol 483:193–208

Twyman RM, Schillberg S, Fischer R (2013) Optimizing the yield of recombinant pharmaceutical proteins in plants. Curr Pharm Des 19:5486–5494

Tyulkina LG, Skurat EV, Frolova OY, Komarova TV, Karger EM, Atabekov IG (2011) New viral vector for superproduction of epitopes of vaccine proteins in plants. Acta Naturae 3:73–82

Usha R, Rohll JB, Spall VE, Shanks M, Maule AJ, Johnson JE, Lomonossoff GP (1993) Expression of an animal virus antigenic site on the surface of a plant virus particle. Virology 197:366–374

Varsani A, Williamson AL, Rose RC, Jaffer M, Rybicki EP (2003) Expression of Human papillomavirus type 16 major capsid protein in transgenic Nicotiana tabacum cv. Xanthi. Arch Virol 148:1771–1786

Vasilev N, Gromping U, Lipperts A, Raven N, Fischer R, Schillberg S (2013) Optimization of BY-2 cell suspension culture medium for the production of a human antibody using a combination of fractional factorial designs and the response surface method. Plant Biotechnol J 11:867–874

Virdi V, Coddens A, De Buck S, Millet S, Goddeeris BM, Cox E, De Greve H, Depicker A (2013) Orally fed seeds producing designer IgAs protect weaned piglets against enterotoxigenic Escherichia coli infection. Proc Natl Acad Sci U S A 110:11809–11814

Warzecha H, Mason HS, Lane C, Tryggvesson A, Rybicki E, Williamson AL, Clements JD, Rose RC (2003) Oral immunogenicity of human papillomavirus-like particles expressed in potato. J Virol 77:8702–8711

Watson J, Koya V, Leppla SH, Daniell H (2004) Expression of Bacillus anthracis protective antigen in transgenic chloroplasts of tobacco, a non-food/feed crop. Vaccine 22:4374–4384

Wigdorovitz A, Carrillo C, Dus Santos MJ, Trono K, Peralta A, Gomez MC, Rios RD, Franzone PM, Sadir AM, Escribano JM, Borca MV (1999a) Induction of a protective antibody response to foot and mouth disease virus in mice following oral or parenteral immunization with alfalfa transgenic plants expressing the viral structural protein VP1. Virology 255:347–353

Wigdorovitz A, Perez Filgueira DM, Robertson N, Carrillo C, Sadir AM, Morris TJ, Borca MV (1999b) Protection of mice against challenge with foot and mouth disease virus (FMDV) by immunization with foliar extracts from plants infected with recombinant tobacco mosaic virus expressing the FMDV structural protein VP1. Virology 264:85–91

Wigdorovitz A, Mozgovoj M, Santos MJ, Parreno V, Gomez C, Perez-Filgueira DM, Trono KG, Rios RD, Franzone PM, Fernandez F, Carrillo C, Babiuk LA, Escribano JM, Borca MV (2004) Protective lactogenic immunity conferred by an edible peptide vaccine to bovine rotavirus produced in transgenic plants. J Gen Virol 85:1825–1832

Wirblich C, Coleman CM, Kurup D, Abraham TS, Bernbaum JG, Jahrling PB, Hensley LE, Johnson RF, Frieman MB, Schnell MJ (2017) One-Health: a safe, efficient, dual-use vaccine for humans and animals against middle east respiratory syndrome coronavirus and rabies virus. J Virol 91

Yang C-D, Liao J-T, Lai C-Y, Jong M-H, Liang C-M, Lin Y-L, Lin N-S, Hsu Y-H, Liang S-M (2007a) Induction of protective immunity in swine by recombinant bamboo mosaic virus expressing foot-and-mouth disease virus epitopes. BMC Biotechnol 7:62

Yang ZQ, Liu QQ, Pan ZM, Yu HX, Jiao XA (2007b) Expression of the fusion glycoprotein of Newcastle disease virus in transgenic rice and its immunogenicity in mice. Vaccine 25: 591–598

Yu J, Langridge WH (2001) A plant-based multicomponent vaccine protects mice from enteric diseases. Nat Biotechnol 19:548–552

Yusibov V, Hooper DC, Spitsin SV, Fleysh N, Kean RB, Mikheeva T, Deka D, Karasev A, Cox S, Randall J, Koprowski H (2002) Expression in plants and immunogenicity of plant virus-based experimental rabies vaccine. Vaccine 20:3155–3164

Zhao Y, Hammond RW (2005) Development of a candidate vaccine for Newcastle disease virus by epitope display in the Cucumber mosaic virus capsid protein. Biotechnol Lett 27:375–382