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
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 Ni²⁺ 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 \textit{N. tabacum} or \textit{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 \textit{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 \textit{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 \(~50 \mu g\) 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 \( \sim 2 \text{ mg} \) 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) haemagglutinin-based vaccine for poultry, that had been made in a suspension cultured \( \text{N. tabacum} \) 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\textsuperscript{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 influenza virus 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 ∼3× 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 (SICDI) significantly lowered A1 and S1 protease activities in the *N. benthamiana* apoplast, while increasing recombinant protein content by ∼45% (Goulet et al. 2012). It was found that co-expression of tomato cystatin SICYS8, 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 transgensics 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 influenza virus 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 GcGn 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.

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