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Review article

Plant-made vaccines for humans and animals

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Summary

The concept of using plants to produce high-value pharmaceuticals such as vaccines is 20 years old this year and is only now on the brink of realisation as an established technology. The original reliance on transgenic plants has largely given way to transient expression; proofs of concept for human and animal vaccines and of efficacy for animal vaccines have been established; several plant-produced vaccines have been through Phase I clinical trials in humans and more are scheduled; regulatory requirements are more clear than ever, and more facilities exist for manufacture of clinic-grade materials. The original concept of cheap edible vaccines has given way to a realisation that formulated products are required, which may well be injectable. The technology has proven its worth as a means of cheap, easily scalable production of materials: it now needs to find its niche in competition with established technologies. The realised achievements in the field as well as promising new developments will be reviewed, such as rapid-response vaccines for emerging viruses with pandemic potential and bioterror agents.

Keywords: vaccine, plant, transgenic plant, transient expression, plant biotechnology, pharmaceutical.

Introduction

The routine production of inexpensive vaccines in plants has for nearly two decades been an idealistic and largely unrealised dream. The romantic notion of 'cheap, edible vaccines' of the early years [see (Fooks, 2000; Giddings *et al.*, 2000; Rybicki, 2009a)] has not been realised, and indeed, may never be. While the idea of using edible plants or fruits to deliver vaccines is still very attractive—largely because it means that vaccines could be 'pharmed' where they are needed, obviating transport and cold chains, and because oral delivery is seen as being easier and safer than injection—there are simply too many practical and potentially ethical problems with the concept for it to be implemented as envisioned. In the first place, quality control issues would largely preclude on-site production of a vaccine in a food plant: unless every single plant or even every dosage portion of an engineered food was to be assayed for vaccine content, for example, it would be impossible to guarantee that there would be enough of a dose of vaccine for efficacy—or that the plants were even still producing vaccine, or enough of it. Second, the physical administration of such vaccines needs to be regulated, as otherwise too much or too little could be given, and too frequently: the latter prospect especially raises the spectre of immune tolerance to the chosen antigen being elicited, which would negate the whole raison d'être for the vaccine. Thus, the way the field is now developing recognises that even though oral dosing is still a desirable feature, the product itself will have to be processed to some extent, formulated in a reproducible way, and given under supervision so as to ensure reproducible effects.

However, recent research and commercial developments, as well as increased clarity as to just what regulatory hurdles exist, have brought at least part of the dream far closer to realisation. While many plant models have been tried, there are now a relatively limited number of proven systems relevant for industrial-scale production of vaccines. Expression systems include stable transgenic or transplastomic plants or plant cell lines, with inducible or constitutive expression, seed-specific expression, and

plant virus-based and *Agrobacterium tumefaciens*-based transient expression systems. There are now registered products, produced according to Good Manufacturing Practice (GMP) protocols, entering clinical trials. There are presently several independent facilities capable of manufacturing clinical-grade material for human trial; products have been in, and there are present and planned, clinical trials. Candidate vaccines have been produced for human and animal viruses and bacteria and other parasites; for allergens; and as cancer therapeutics. This review will accordingly summarise some important historical developments in the field, as well as covering future prospects, and discuss aspects of vaccine antigen production relevant to plant biotechnologists.

As the history of this research area has been recently and extensively covered [see (Rybicki, 2009a; Tacket, 2009; Tiwari *et al.*, 2009)], I will briefly detail here only the most important proofs of principle and of concept relevant to plant biotechnology. I will also cover hosts and expression systems used, as background to latest developments and trends.

Expression hosts

An extensive list of plant types and systems have been used for expression of vaccine antigens (Rybicki, 2009a): these include (but are not limited to) various *Nicotiana* spp., *Arabidopsis thaliana*, alfalfa, spinach, potatoes, duckweed, strawberries, carrots, tomatoes, aloe and single-celled algae. Proteins have been expressed in seeds of maize, rice, beans and tobacco, in potatoes, tomatoes and strawberries, in suspension cell cultures of tobacco and maize, in hairy root cultures and in transformed chloroplasts of a variety of plant species.

Initially, considerations of what type of host to use were often governed by what was edible by humans and animals, or had 'generally regarded as safe' (GRAS) status, given that the presumption was that vaccines would be eaten without further processing. In the absence of viable large-scale transient expression technologies pre-late 1990s, this also usually meant stable transgenic plant lines, producing edible leaves, fruits, tubers or seeds. Other considerations were biomass that could be generated, yield and ease of processing. Thus, while vaccine-relevant proteins have been successfully produced in *A. thaliana*—including antigens from transmissible gastroenteritis virus of swine (Gomez *et al.*, 1998), *Shigella flexneri* invasion plasmid antigen IpaC (MacRae *et al.*, 2004), infectious bursal disease of chickens (Wu *et al.*, 2004), *Mycobacterium tuberculosis* (TB) ESAT-6 antigen (Rigano *et al.*, 2006), recombinant hepatitis B/human immunodeficiency virus [HBV/HIV particles and HIV-1 p24 protein (Greco *et al.*, 2007; Lindh *et al.*, 2008) and human papillomavirus L1 protein (Kohl *et al.*, 2007)]—its agronomic properties are such that it would be almost useless as a commercially relevant production system. While leaves and seeds are edible, individual plants simply do not produce enough biomass or enough seed mass to allow an economic production model.

Fruits and tubers

In terms of edible fruits and tubers, tomatoes have been engineered to express a variety of antigens, including rabies virus glycoprotein G (McGarvey *et al.*, 1995), respiratory syncytial virus F glycoprotein (Sandhu *et al.*, 2000), a hepatitis E virus surface protein (Ma *et al.*, 2003), a *Yersinia pestis* F1-V antigen (Alvarez *et al.*, 2006), a synthetic HBV/HIV antigen (Shchelkunov *et al.*, 2006), Norwalk virus capsid antigen (Zhang *et al.*, 2006), hepatitis B virus surface antigen (HBsAg) (Lou *et al.*, 2007) and a synthetic polypeptide containing epitopes of the diphtheria, pertussis and tetanus (DPT) exotoxins (Soria-Guerra *et al.*, 2007), among others. Paz de la Rosa *et al.* (Paz de la Rosa *et al.*, 2009) expressed a chimaeric HPV-16 L1 protein in transgenic tomatoes, with a string of epitopes from HPV 16 E6 and E7 proteins, to demonstrate that a combination prophylactic/therapeutic human papillomavirus (HPV) vaccine could be made in plants: however, while the VLPs produced stimulated both antibody and T-cell responses, yields were not high (0.05%–0.1% of total soluble protein).

Transgenic potato tubers have been used to produce *Escherichia coli* heat-labile enterotoxin (LT-B) (Haq *et al.*, 1995), Norwalk virus coat protein (Mason *et al.*, 1996), rabbit haemorrhagic disease virus (RHDV) VP60 (Castanon *et al.*, 1999), HBsAg (Richter *et al.*, 2000), a combination cholera/*E. coli*/rotavirus vaccine (Yu and Langridge, 2001), human papillomavirus E7 and L1 proteins (Franconi *et al.*, 2002; Biemelt *et al.*, 2003; Warzecha *et al.*, 2003), and Newcastle disease virus envelope proteins (Berinstein *et al.*, 2005), among many others. The RHDV vaccine—administered as injections of leaf extracts—was both strongly immunogenic and protective in rabbits and is an excellent proof of efficacy. Possibly the most important proof of concept of a potato-produced vaccines for humans, however, was the demonstration via clinical trial of the immunogenicity of potato-delivered HBsAg as a booster immunisation in conventionally vaccinated human

volunteers (Thanavala *et al.*, 2005). However, yields were low (~9 mg/kg) and even three doses of 100 g of raw potatoes worked in only 63% of volunteers.

An unusual animal model using transgenic potato tubers was investigated by Companjen *et al.* (Companjen *et al.*, 2005, 2006), who demonstrated that various peptides and green fluorescent protein (GFP) translationally fused to LT-B were efficiently taken up in the gut of carp after incorporation in a crude extract in standard feed pellets, and the model vaccines elicited a specific systemic humoral immune response.

Edible leafy crops

Edible leafy crops such as alfalfa, spinach, lupins and lettuce have been used for antigen production as well. One early and notable success was the protection of mice against challenge with foot and mouth disease virus (FMDV) after immunisation either via oral or parenteral routes with leaf or leaf homogenates of transgenic alfalfa expressing the structural protein VP1 of FMDV (Wigdorovitz *et al.*, 1999a). Mice fed on transgenic alfalfa expressing a highly immunogenic epitope from FMDV fused to the glucuronidase (gus A) reporter gene were protected from experimental challenge (Dus Santos *et al.*, 2002); mice could be protected against a lethal dose of rabies virus after being fed with transgenic spinach (Modelska *et al.*, 1998); HBsAg produced in both transgenic lupins and lettuce elicited specific antibodies in mice and humans, respectively (Kapusta *et al.*, 1999); sunflower seed albumin produced in lupin has been tested as an anti-allergen vaccine (Smart *et al.*, 2003); the small surface antigen of hepatitis B virus (S-HBsAg) has been produced in transgenic yellow lupin calli or tumours (Pniewski *et al.*, 2006). The most notable achievement for this sort of expression for human vaccines, however, was probably the demonstration that measles virus haemagglutinin (MV-H) protein could successfully expressed in transgenic lettuce and was immunogenic in mice (Webster *et al.*, 2006): this followed other work from this group demonstrating first, that plant-derived MV-H induced neutralising antibodies in mice (Huang *et al.*, 2001); and second, that DNA-mediated measles immunisation could be successfully boosted with an oral plant-derived measles virus vaccine (Webster *et al.*, 2002).

Seeds

The use of edible or easily processable seeds was also initially popular, given that it is both easier to process dried seeds than green leaf tissue for extraction of relevant antigens, and seeds tend to preserve proteins very well for long periods, even under ambient conditions, because of 'glassification' and other desiccation-dependent stabilisation conditions [e.g. see (Giddings *et al.*, 2000; Hiroi and Takaiwa, 2006; Lamphear *et al.*, 2002; Streatfield *et al.*, 2003)]. Maize or corn seed has been especially popular, because of both the high potential yield of material and the very well-established milling/processing technology and a number of viral and bacterial antigens and antibodies have been successfully produced. Chikwamba *et al.* (Chikwamba *et al.*, 2002), for example, demonstrated that *E. coli* LT-B toxin subunit assembled correctly, was functional, and protected orally-immunised mice against bacterial challenge. Indeed, ProdiGene Inc. (TX, USA) built a business portfolio around successful expression of various vaccine and other proteins in maize seed (Lamphear *et al.*, 2002; Horn *et al.*, 2003; Streatfield *et al.*, 2003) and were able to show that maize seed containing a transmissible gastroenteritis virus (TGEV) subunit vaccine was an effective booster vaccine for systemic and mucosal immunity in pigs (Lamphear *et al.*, 2004).

Rice too has been investigated as a production and delivery vehicle, for the same reasons as maize, and probably has a significant advantage over maize in that plants are self-fertilised, which limits potential escape of vaccine genes. One recent and innovative finding was that feeding mice rice seed-expressed Cry j 1 and Cry j 2 allergen proteins of Japanese cedar reduced allergen-specific IgE, T-cell proliferative reaction and histamine responses (Hiroi and Takaiwa, 2006): this vaccine has been improved to include further T-cell epitopes and will be trialled in humans (Takaiwa, 2007). Oszvald *et al.* (Oszvald *et al.*, 2007) described the production of a synthetic fusion gene comprising *E. coli* LT-B and an epitope of porcine epidemic diarrhoea virus; oral vaccination of chickens with transgenic rice seed containing infectious bursal disease virus (IBDV) VP2 protein resulted in protection from virulent IBDV challenge (Wu *et al.*, 2007). More recently, an antigen protective against the roundworm *Ascaris suum* (s16) produced as a fusion chimaera with cholera toxin (CT) B subunit (CTB) elicited an As16-specific serum antibody response when orally administered to mice in combination with CT and resulted in a lower lung worm burden after challenge than in control mice (Matsumoto *et al.*, 2009).

Other seed systems such as legumes have been investigated [see (Tiwari *et al.*, 2009) for a review]; however, cereal seeds seem to be distinctly favoured, probably because of yield and processing advantages. An

interesting recent article, however, demonstrated that a pea (*Pisum sativum*)-RHDV CTB::VP60 chimaeric protein protected rabbits against lethal challenge (Mikschofsky *et al.*, 2009).

In 1997, a Science World article opened with 'Getting vaccinated against diseases could soon be as easy as eating a banana' (Haq *et al.*, 1995; Goldstein, 1997). However, production of vaccines in edible plants and especially in seed plants has fallen into disfavour in recent years—-mostly because of perceived problems with 'contamination' of food crops, due mainly to two incidents involving one company. In the triggering incident in 2002, soya bean and maize harvests in two states in the USA were contaminated with transgenic volunteer maize plant seeds expressing TGEV capsid protein: the company was fined and forced to clean up the seed by the US Department of Agriculture. However, a subsequent similar incident in 2004, where volunteer transgenic maize contaminated an oat crop, which was harvested and baled for use as on-farm animal feed, and was also found growing and flowering in a nearby sorghum field, led to an agreement that the company and its successors would never again apply for a notification or permit to introduce transgenic plants (APHIS, 2008).

The incidents, and the ensuing media and public concern, have led to an effective moratorium on vaccine pharming in edible crops in the USA and indeed worldwide. This trend, together with increasing concern over regulatory compliance of pharmed products, has probably stimulated the increasing use of non-food crop plants for especially vaccine production. Paradoxically, perhaps the most successful use of an engineered potato as a vaccine—the demonstration in 2005 that HBsAg-producing potatoes could be used as a booster vaccine in humans (Thanavala *et al.*, 2005)—was also possibly one of the last times this would be carried out. In a contemporary review, Andy Coghlan of New Scientist commented that 'the newly published study missed a moving target—drug developers are now abandoning their quest for vaccines contained in staple foods like bananas, tomatoes or potatoes' (Coghlan, 2005).

Non-food crop plants

Thus it is that cultivated tobacco and other high-bulk plant varieties, formerly generally seen as only useful proof of principle model plants, are increasingly being seen as the production vehicle of the future. Another contributory factor is the increasing realisation that 'cheap edible' vaccines composed of raw plant material are not easily realisable and that the first products will probably be extensively processed and maybe even injectable—and therefore could be produced in hosts that at first sight did not fit the edible paradigm (Rybicki, 2009a).

The use of *Nicotiana* spp. and mainly *N. tabacum* for production of a variety of recombinant proteins has been well documented [see (Rybicki, 2009a; Sharma and Sharma, 2009; Tiwari *et al.*, 2009) for reviews] and will not be covered in any depth here. However, it was the use of tobacco, which led to many of the early proofs of concept and of principle. Thus, the first proof of concept for production of a vaccine-relevant protein in plants was published exactly 20 years ago: Hiatt *et al.* (Hiatt *et al.*, 1989) described the successful production of a mouse hybridoma-derived monoclonal antibody (mAb) in transgenic tobacco (*Nicotiana tabacum* L.). The first vaccine protein to be produced in any plants was the hepatitis B virus (HBV) surface antigen (HBsAg), just 3 years later: Hugh Mason *et al.* in Charles W Arntzen's group demonstrated that transgenic tobacco containing the relevant gene under the control of a constitutive promoter produced HBsAg that could, moreover, assemble *in planta* into the characteristic 22-nm particles first described from the blood of HBV-infected people, and which constitute the basis of the commercial recombinant vaccines Recombivax HB and Engerix B, licenced in 1986 and 1989, respectively (Mason *et al.*, 1992).

The same research group were responsible for the first proof that a plant-produced recombinant toxin protein was functionally equivalent to the natural product and that orally vaccinated animals produced antibodies that neutralised the native toxin (Haq *et al.*, 1995). They made transgenic tobacco and potato plants containing genes encoding *E. coli* heat-labile enterotoxin (LT-B): plants expressed protein that spontaneously oligomerised into pentamers, and which bound the natural ligand. Mice that were immunised by gavage produced circulating and gut mucosal anti-LT-B immunoglobulins that could neutralise the native enterotoxin in standard cell protection assays.

In another investigation of the potential of plants to produce equivalent vaccines to those produced in conventional systems—Gardasil by Merck and Cervarix by Glaxo-SmithKline, in yeast and insect cell cultures, respectively—three groups published results of transgenic expression in tobacco and/or potato from two different human papillomaviruses (Biemelt *et al.*, 2003; Varsani *et al.*, 2003; Warzecha *et al.*, 2003). This has been

reviewed elsewhere (Rybicki, 2009a); however, while yields were quite poor in all cases (maximum ~30 mg/kg), proofs of principle were that protein-only virus-like particles resembling those of the established vaccines could be made in plants and that these were immunogenic by injection or gavage.

Chloroplast/plastid expression

While most early work and much present work too are focussed on the use of classical transgenesis or nuclear transformation for expression, transplastomic and especially transformed chloroplast expression have had its champions from early years. Indeed, a recent review touts the technology as useful in terms of combating bioterrorism, given the high yields obtainable (Streatfield, 2006). Singh *et al.* (Singh *et al.*, 2009) have recently reviewed protocols for transformation, expression and oral administration; the group of Henry Daniell in particular has pioneered much in this regard (Daniell, 2006; Dhingra and Daniell, 2006). An early demonstration from this group of the potential of chloroplast expression was the production in tobacco of viable *E. coli* LT-B and *Cholera vibrio* CT-B antigens (Daniell *et al.*, 2001): at the time they made the point, repeated frequently since, that even very high-level recombinant protein expression by chloroplasts often does not seem to affect plant phenotype as much as nuclear-mediated expression. The high-level expression of *Clostridium tetanii* toxin Fragment C followed (Tregoning *et al.*, 2003); so too demonstrations of the production and efficacy of chloroplast-produced *Bacillus anthracis* (anthrax) protective antigen (PA) (Watson *et al.*, 2004; Aziz *et al.*, 2005; Koya *et al.*, 2005).

Unsurprisingly, bacterial-derived antigens express well in chloroplasts, given their origin and similar mechanisms for folding proteins—and a lack of downstream processing such as glycosylation. However, possibly the first animal vaccine was the protective 2L21 peptide from virulent canine parvovirus (CPV), expressed in tobacco chloroplasts as a C-terminal translational fusion with the cholera toxin B subunit (CTB) (Molina *et al.*, 2004), which could elicit neutralising antibodies in mice and rabbits—although not via the oral route of administration (Molina *et al.*, 2005). It is interesting that the severe acute respiratory syndrome coronavirus (SARS-CoV) spike protein—a membrane-associated glycoprotein—apparently accumulates well in transformed chloroplasts (Li *et al.*, 2006). Another recent development was the demonstration of the high-level production in tobacco chloroplasts and very effective immunogenicity of the LacA lectin fragment of the eukaryote protist pathogen *Entamoeba histolytica,* which causes ~100 000 deaths annually: subcutaneous immunisation of mice produced high IgG titres, and—in the words of the authors—'An average yield of 24 mg of LecA per plant should produce 29 million doses of vaccine antigen per acre of transgenic plants' (Chebolu and Daniell, 2007). Other recent successes include the highest expression level yet achieved (~3 g/kg) for HPV-16 L1 protein in transplastomic tobacco, which both assembled correctly and elicited neutralising antibodies (Fernandez-San Millan *et al.*, 2008), despite another claim that HPV-16 L1 expressed well in chloroplasts only when fused to a chloroplast protein fragment (Lenzi *et al.*, 2008). The HIV-1 Pr^{55}Gag protein has also been successfully produced at relatively high levels in transplastomic tobacco (to ~360 mg/kg) and assembled into VLPs similar to those produced in animal cells, which makes this the first vaccine-relevant HIV antigen to be produced in reasonable quantities in plants (Scotti *et al.*, 2009).

It is also possible that the glycosylation barrier may be breached soon as well: novel pathways have been identified, which could allow in chloroplasts the kinds of protein maturation that occur in the ER and Golgi compartments (Faye and Daniell, 2006). However, this topic is covered in detail elsewhere in this volume and will not be discussed further here (Gomord *et al.*, 2009).

Cell culture systems

Single-cell or tissue culture systems for production of vaccine proteins have always been a minor part of the total effort, probably because of the greater level of technical expertise required—and because, as noted elsewhere (Fischer *et al.*, 2004; Rybicki, 2009a), cell culture does not offer a great deal of advantage in terms of cost over conventional bacterial and eukaryotic cell culture systems because of requirements for sterility and containment, and yields are generally not very high. A number of microalgae systems (e.g. *Chlorella* and particularly *Chlamydomonas* spp.) have been investigated [see (Fuhrmann, 2004; Siripornadulsil *et al.*, 2007; Surzycki *et al.*, 2009)]; however, vaccines are not prominent among the products. Two examples are human IgA single-chain antibodies produced in transgenic *Chlamydomonas reinhardtii* (Mayfield and Franklin, 2005), and a fusion of the FMDV VP1 gene and the cholera toxin B subunit (CTB) produced in chloroplast of the same organism (Sun *et al.*, 2003)—both without evidence of efficacy, however.

Terrestrial plant-derived cultures have also been investigated for their potential as production systems, largely because of the perception that the high level of containment and the possibility of producing proteins in bioreactors under 'good manufacturing practice' (GMP) conditions would be more acceptable to regulators. The successful production of antibodies and antibody fragments in tobacco suspension-cultured cells has been reported (Fischer *et al.*, 1999a,b); however, this is not being pursued by this group. Mass-produced transgenic somatic embryos of transgenic Siberian ginseng have been used as a production system for *E. coli* LT-B (Kang *et al.*, 2006), but this does not seem to have been commercialised. An interesting variant on cell culture techniques was the demonstration that clonal root cultures established by *Agrobacterium rhizogenes* transformation of rTMV-infected *N benthamiana* could express foreign proteins at an elevated level in the absence of selection for 3 years (Skarjinskaia *et al.*, 2008).

The potential success of this route of production is illustrated by two examples: one therapeutic protein presently in human trial worldwide is a recombinant human glucocerebrosidase (rGCD) produced via a contained disposable bioreactor system with suspension-cultured carrot or tobacco cells (Shaaltiel *et al.*, 2007; Baum, in press)—the only other licenced product is a purified injectable Newcastle disease virus vaccine for chickens produced in a suspension-cultured tobacco cell line by Dow AgroSciences, which is not intended for sale (Dow AgroSciences, 2008). While plant cell culture is at first sight not much less technically demanding than—for example—mammalian cell culture such as of the Chinese hamster ovary (CHO) cells now used routinely for production of many human therapeutics, one very obvious advantage was shown up in 2009—which is that plant-based production is not susceptible to the kinds of contamination experienced by conventional systems. A June 16th 2009 press release on the Genzyme web site (http://www.genzyme.com/corp/media/GENZ%20PR-061609.asp) mentions interference in their CHO cell production of Cerezyme®, the commercial Gaucher disease therapeutic, by infection with a calicivirus (Vesivirus 2117) '…likely introduced through a nutrient used in the manufacturing process'. A later report (Bethencourt, 2009) states that 'The US Food and Drug Administration (FDA) has contacted rival manufacturers Shire of Basingstoke, UK, and Carmiel, Israel-based Protalix, who have enzyme replacement therapies for Gaucher disease in clinical trials, to file treatment protocols, which would allow physicians to use their drugs ahead of approval'.

Transient expression systems

While much of the early work was carried out with stably transformed plants or cells, there has been an increasing trend towards the use of transient expression systems in recent years. The major reason for this is sheer convenience and speed: both virus vector-based and *Agrobacterium* infiltration-based systems offer the chance of getting large amounts of protein in days after the initial molecular cloning event, rather than the months necessary for transgenic expression (Fischer *et al.*, 1999c).

Transient expression systems are practically limited to virus-based and *Agrobacterium*-mediated somatic expression, usually in whole plants: while it is possible to include such systems as transfection of protoplasted cells with DNA or RNA, this is of very limited utility other than for laboratory investigation.

Simple virus-based vectors

Viral vectors have been used for some time for the expression of foreign proteins or of chimaeric coat proteins in plants [for reviews, see (Gleba *et al.*, 2007; Steinmetz *et al.*, 2009; Yusibov *et al.*, 2006)]; however, their utility, variety and application have expanded greatly since the early use of cDNA-derived *in vitro*-synthesised RNA to infect plants with recombinant tobacco mosaic virus (TMV) (Yusibov *et al.*, 1999). Indeed, one of the first plant-based vaccines described was recombinant TMV with malarial epitopes exposed on virion surfaces (Turpen *et al.*, 1995). Biosource Technologies, which later became Large Scale Biology Corp. (Vacaville, CA, USA), had the recombinant TMV technology (Geneware™) as one of their main products, and it was successfully used to express their flagship patient-specific non-Hodgkin lymphoma tumour-derived single-chain Fv epitope vaccines for FDA-approved clinical trial (McCormick *et al.*, 1999, 2003, 2006, 2008). A significant proof of principle in an animal model was the successful use of rTMV expressing surface-located epitopes derived from cottontail rabbit papillomavirus (CRPV) and rabbit oral papillomavirus (ROPV) L2 minor capsid proteins as a protective injectable vaccine (Palmer *et al.*, 2006). Our group also showed that CRPV major capsid protein L1 produced either via transgenesis or via rTMV expression, protected rabbits against CRPV challenge (Kohl *et al.*, 2006). Significant findings for animal vaccines were the demonstrations of protection against lethal FMDV infection in mice immunised by injection of foliar extracts of *N. benthamiana* infected with rTMV expressing the whole

FMDV VP1 (Wigdorovitz *et al.*, 1999b) and passive immunisation via maternal antibodies of mouse pups born to dams immunised with purified VP8* fragment of the VP4 protein from bovine rotavirus (BRV) produced similarly in *N. benthamiana* (Perez Filgueira *et al.*, 2004). Our group was able to improve the previously very poor expression levels of HPV-16 L1 capsid protein in transgenic *N. tabacum* (Varsani *et al.*, 2003) by 10-fold in *N. benthamiana* by use of Geneware™ TMV (Varsani *et al.*, 2006): however, expression levels at ~40 μg/kg were still well below what was achieved later. The possible uses of TMV-based expression and/or presentation are also described in a recent review (McCormick and Palmer, 2008).

Other plant viruses that have successfully been used for either peptide display or expression of whole antigens include the potexviruses potato virus X (PVX), bamboo mosaic virus (BaMV) and papaya mosaic virus (PapMV), cowpea mosaic comovirus (CPMV) [reviewed by (Brennan *et al.*, 2001; Canizares *et al.*, 2005)], bean yellow dwarf mastrevirus (BeYDV), the related alfalfa mosaic (AlMV) and cucumber mosaic viruses (CMV), and tomato bushy stunt tombusvirus, among others. Important proofs of concept using these vehicles include the use of chimaeric CPMV particles displaying a peptide derived from outer membrane protein F of *Pseudomonas aeruginosa* to protect mice in a model of human chronic pulmonary infection (Brennan *et al.*, 1999); CPMV carrying a peptide derived from the VP2 capsid protein of CPV protecting against lethal challenge (Langeveld *et al.*, 2001); PVX-mediated expression of a human papillomavirus type 16 E7 oncoprotein in *N benthamiana*, and the use of crude foliar extracts as a vaccine to protect mice against E7-producing tumour cell challenge (Franconi *et al.*, 2006); the use of chimaeric BaMV virions displaying a partial sequence of FMDV VP1 to induce both humoral and cell-mediated immune responses, and full protection against FMDV in pigs (Yang *et al.*, 2007); the proof that PapMV particles possess intrinsic adjuvant-like properties enhancing T-cell responses to displayed peptides (Lacasse *et al.*, 2008), and the related finding that PVX engineered to display a T-cell epitope of influenza A virus nucleoprotein (NP) activates specific CD8+ T cells in mice (Lico *et al.*, 2009).

Geminiviruses in particular have been the subject of some interest for many years for their supposed potential as expression vectors: in 1999, it was shown that maize streak mastrevirus (MSV)-derived vectors could express significantly elevated levels of a number of test proteins in suspension-cultured maize cells for years in the absence of selection, but that it was not a viable infectious vector (Palmer *et al.*, 1999; Palmer and Rybicki, 2001); there have also been several reviews on geminiviruses as expression vectors (e.g. Palmer and Rybicki, 1997; Mor *et al.*, 2003). A vaccine application was described in 2004, with the use of BeYDV-derived system to express a *Staphlococcus* endotoxin B (SEB) gene: a 20-fold increase in expression in tobacco NT-1 cells bombarded with a replicating vector was noted, compared to the control (Hefferon and Fan, 2004).

While filamentous or rod-like plant viruses are probably better for expression of whole genes—their particles can easily accommodate a larger genome, unlike isometric virions—a major problem with all autonomously replicating plant virus vectors constructs, which spread via natural means in plants, is loss of the transgene. This is a problem whether the vectors are delivered via inoculation with *in vitro*-generated ssRNA or cDNA clones capable of generating infectious transcripts and is because of the increased replicative fitness of the deleted recombinant. An example from our work was the demonstration that the CRPV L1 protein gene expressed in *N. benthamiana* via rTMV was progressively lost as infected plants developed, leading to lower yields of protein than were obtained in transgenic tobacco (Kohl *et al.*, 2006). An elegant means of getting around the problem and of completely redesigning plant viruses involves the use of *Agrobacterium tumefaciens* for systemic delivery of viral genomes, described in the following paragraphs.

Agroinfiltration vectors

Agrobacterium tumefaciens-mediated transient expression exploits the fact that infiltration of the intercellular spaces in plant leaves with a suspension of the bacterium—agroinfiltration, via syringe or vacuum—can result in mobilisation of T-DNA into the nuclei of a large proportion of the cells so exposed and subsequent expression of any transgene from integrated or episomal DNA [reviewed by (Fischer *et al.*, 1999c)]. The technique has essentially become the gold standard for determining whether or not any given gene can be expressed in plants, and if so, in which cell compartments the protein accumulates best, given a wide variety of vectors available for intracellular targetting of expressed proteins. Optimisation of expression of transgenes became simple when many constructs could be tested in parallel by syringe infiltration, using different versions of the same basic gene, different promoters and different leader or organelle targetting sequences.

One of the first vaccine-related applications of the technology was the proof that transiently expressed HBsAg and an GFP::HBsAg fusion formed virus-like particles (VLPs) similar to yeast-derived vaccine HBsAg and that the system could be used for high-throughput conformational screening of vaccine proteins (Huang and Mason, 2004). An example from our laboratory was the high-level (0.8 g/kg) expression of HPV-16 L1 protein in *N benthamiana*, with the demonstration that protein accumulated better when exported to chloroplasts than in the cytoplasm, assembled into VLPs, and elicited neutralising antibodies in mice after injection with or without adjuvant (Maclean *et al.*, 2007). While several groups have been successful in expressing HIV antigen peptides as chimaeric proteins (Yusibov *et al.*, 1997; Durrani *et al.*, 1998; Matoba *et al.*, 2004) or whole virion capsid p24 protein (Zhang *et al.*, 2002; Obregon *et al.*, 2006; Lindh *et al.*, 2008), none of these antigens is particularly relevant in the wider search for HIV vaccines compared to the HIV Env or Gag proteins, for example (Baker *et al.*, 2009). We were successful in expressing whole Pr55Gag but only at very low levels; however, a Gag-derived p17/p24 fusion protein could be expressed to 5 mg/kg if localised to chloroplasts and was a successful booster vaccine from both T-cell and humoral responses in mice primed by a *gag* DNA vaccine (Meyers *et al.*, 2008).

Another very impressive recent investigation by D'Aoust *et al.* (D'Aoust *et al.*, 2008) reported a similar investigation of the potential of plant agroinfiltration to produce an influenza vaccine. They expressed haemagglutinin (HA) from H5N1 (A/Indonesia/5/05) and H1N1 (A/New Caledonia/20/99) viruses by agroinfiltration of *N. benthamiana* and showed that the HAs yielded up to 50 mg/kg, assembled correctly into trimers, and moreover budded at the plasma membrane at apoplastic indentations into VLPs largely devoid of host proteins. The purified H5-derived VLPs were highly immunogenic: two doses of 0.5 μg of H5-VLPs conferred complete protection against a lethal challenge with a heterologous (A/Vietnam/1194/04) virus.

Agroinfiltrated viral vectors

One of the most significant developments in this field in recent times was the coupling of agroinfiltration with delivery of cDNA encoding a 'deconstructed' TMV-based vector—a technology termed 'Magnifection' (Gleba *et al.*, 2005), and which became the centrepiece of production technology of the new Icon Genetics (Halle, Germany). The system was soon used in a landmark vaccine model exercise to produce recombinant *Yersinia pestis* antigens F1, V and fusion protein F1-V: purified antigens injected into guinea pigs were protective against an aerosol challenge of virulent *Y. pestis* (Santi *et al.*, 2006). The first really acceptable production level of HBsAg in a plant system was also achieved using MagnICON™ viral vectors (~300 mg/kg leaf fresh weight): the product was full-length, had disulphide-linked dimers and assembled into VLPs (Huang *et al.*, 2008). A very welcome development reported recently was that Icon Genetics have rescued the concept of personalised non-Hodgkin lymphoma vaccines pioneered by the now defunct LSBC Corp. and are using MagnICON™ vectors to produce lifelong supplies of single-patient vaccines from as little as 5 kg of *N. benthamiana* (Rybicki, 2009b). Another landmark recent report in the popular media was that human Norwalk norovirus capsid vaccines could be easily and quickly produced with Icon technology (Dylewski, 2009; Rybicki, 2009b).

A novel version of an agroinfiltration-delivered TMV-based vector—entitled 'launch vector' (Musiychuk *et al.*, 2007)—has also been used to produce, among other antigens, HPV E7 protein and H5N1 influenza virus haemagglutinin and neuraminidase domains fused to a thermostable lichenase (LickM): the fusions are apparently able to activate both innate and adaptive antigen-specific immune responses. Both vaccines were protective, the E7 against tumours in a mouse model and the H5-derived vaccine against viral challenge in ferrets (Massa *et al.*, 2007; Mett *et al.*, 2008). The same group recently used the same vector and fusion system to produce Fraction 1 (F1) and V antigens of *Y. pestis*, and demonstrated protection of cynomolgus macaques against aerosolised bacteria (Chichester *et al.*, 2009b).

Both the classical MagnICON™ and launch vectors have a host range problem, with amplified expression being limited mainly to *N benthamiana*: this has been addressed both by Icon Genetics and Fraunhofer, USA, with different plant viruses being brought in to play. Thus, Icon now has improved TMV, turnip vein-clearing potyvirus and PVX vectors (Rybicki, 2009b), and Fraunhofer USA has investigated launch vectors comprising cucumber mosaic and alfalfa mosaic virus-derived constructs in *Pisum sativum* varieties (Green *et al.*, 2009).

Other new vector systems of note for amplified protein expression in plants include those derived from the plant single-stranded DNA geminiviruses and nanoviruses—as well as from CPMV. An improvement on the geminivirus systems described earlier, with a two-component vector (Rep/RepA and replicon-payload components) delivered via agroinfiltration with the silencing suppressor protein

P19, was used to produce hepatitis B core antigen (HBc) and Norwalk virus capsid protein (NVCP) at high levels: a replicon vector with built-in Rep/RepA cassette without P19 worked as well (Huang *et al.*, 2009). Our group has also used a BeYDV-based agroinfiltration-delivered single plasmid replicon system to significantly boost expression of HPV-16 L1 and HIV p24 and other vaccine proteins in *N. benthamiana*, compared to nonreplicating controls (Regnard *et al.*, 2010). A novel gemini- and nanovirus-based technology that has previously been shown to be very effective in the inducible expression of proteins is the 'In-Plant Activation technology' (INPACT) of Farmacule Bio-Industries (Vic., Australia): this is an inducible system that allows amplified replicon-based transgenic expression of even highly toxic molecules (Farmacule, 2009; Rybicki, 2009b)—but should prove equally useful in a transient expression setting.

The CPMV-based technology derives from an extremely potent translational enhancer—a modified 5′-untranslated region (UTR) and the 3′-UTR from CPMV RNA-2 (CPMV-HT)—which allows agroinfiltration-mediated high-level simultaneous expression of multiple polypeptides from a single plasmid within a few days (Sainsbury *et al.*, 2009).

Optimisation of expression

As many have discovered over 20 years, the process of taking a gene encoding a candidate vaccine protein and expressing it in plants at a level that is acceptable for economic production (e.g. >50 mg/kg for antibodies) is far from being a trivial process. Indeed, in many cases yields have been very low, especially in transgenic plants, and proofs of efficacy have suffered as a result. This is of concern for oral vaccination schemes, as experiments have shown that orders of magnitude more protein is required via oral intake than parenterally for the same level of immune response to the same protein, even with the use of potent adjuvants. The landmark 2005 potato-produced HBsAg human clinical trial (Thanavala *et al.*, 2005) is a case in point: while parenteral vaccination requires only 40 μg of HBsAg, oral boosting with three 100-g doses of potato containing HBsAg doses of around 1 mg was only partially effective. It is worth noting that much higher levels of HBsAg expression have since been obtained (Huang *et al.*, 2008), which may yet make the dream of an oral HBV vaccine a reality. In a direct comparison of oral vs. parenteral dosing with the same HPV vaccine, it was determined that 10 μg/dose of insect cell-produced HPV VLPs with adjuvant were required to orally immunise mice for the same response elicited by injection of 1 μg (Rose *et al.*, 1999; Gerber *et al.*, 2001). Others have shown that oral administration of crude extracts of L1-expressing insect cells could induce neutralising antibodies and L1-specific cytotoxic T-lymphocytes, indicating that similar plant preparations might work—especially since yields of between 1–3 g/kg have been obtained.

As an example of an attempt at optimisation of expression of the cancer-associated human pathogen HPV-16 major capsid protein L1, which we had previously only managed to produce to a level of 40 μg/kg via TMV expression (Varsani *et al.*, 2006), our group first investigated the effects of changing the type of tobacco used for transgenic expression; then, by using agroinfiltration, the effects of using L1 genes with three different codon optimisations and targetting the protein to cytoplasm, ER and chloroplasts (Maclean *et al.*, 2007). Simply changing host from *N tabacum* cv. Xanthi to SR1 allowed a 100-fold increase in expression of the native viral gene (A Varsani, J Maclean, EP Rybicki, unpublished), to ~0.5 mg/kg. Via agroinfiltration work, we reiterated a surprising earlier finding that a human codon-use optimised gene worked best (Biemelt *et al.*, 2003) and a plant codon-optimised version least well, compared to the native gene sequence, despite using completely different optimisations than the other group. The removal of 24 amino acids at the C-terminus of L1—which dramatically improves expression in yeast and insect cells—had a negative effect in agroinfiltrated plants. We also showed that chloroplast export allowed significantly greater protein accumulation than cytoplasmic and that subsequent generation of transgenic SR1 tobacco with the relevant constructs reiterated the cytoplasmic vs. chloroplast accumulation differences and increased by a factor of ~20-fold the previous best transgenic HPV-16 L1 yield, to around 500 mg/kg. However, this was achieved only in the T1 generation of plants regenerated from transformed callus: expression in all subsequent generations was either much lower or completely absent (J Maclean, M Koekemoer, EP Rybicki, unpublished). This points up another problem with attempts to maximise constitutive nuclear gene transgenic expression: the fact that gene silencing may occur! Thus, while high-level HPV-16 L1 protein has been achieved in plants, to levels that could allow oral vaccines to be made, it appears that this will be from agroinfiltrated plants or transplastomic plants rather than from stably transformed conventional transgenics.

The optimisation of relevant HIV antigen expression is also an instructive lesson. While the 'conventional'

developers of HIV vaccines are utilising multi-antigen approaches and focussing on whole Gag and Env proteins in particular, it seems that no one has successfully expressed whole HIV Env gp160 protein or even the majority of the protein, in plants at reasonable yield—although expression of a SIV gp130 was reported in maize seed (Horn *et al.*, 2003). It has been speculated that this may be because of the misdirection of the new protein into the calreticulin pathway; however, there is no hard evidence for this (J. Ma, pers. commun.). It also appears to be very difficult to express more than portions of Gag in plants via stable or transient nuclear transformation, at reasonable yield (Meyers *et al.*, 2008). While Tat protein has been produced at quite high yields in spinach and tomato (Karasev *et al.*, 2005; Ramirez *et al.*, 2007), and Gag-derived p24 capsid protein in tobacco and *Arabidopsis* (Zhang *et al.*, 2002; Obregon *et al.*, 2006; Lindh *et al.*, 2008), these proteins are not serious vaccine candidates in the global HIV vaccine hunt. It is most heartening, therefore, that Scotti *et al.* (Scotti *et al.*, 2009) have recently reported that it is possible to produce whole Pr^{55}Gag protein, at levels up to 800 mg/kg, in transplastomic tobacco. The fact that the protein assembled into VLPs like those produced in animal cell systems, and which are increasingly seen as potential HIV vaccines (Speth *et al.*, 2008; Chege *et al.*, 2009), means that a viable plant-derived T-cell stimulating HIV vaccine may finally be possible—and could even be orally administered, given the kinds of yield that were demonstrated.

While it may be possible to some extent to predict ways of increasing recombinant protein expression in plants, I suspect that on the basis of our experience with proteins from HPVs, HIV proteins, antibody fragments, influenza virus HA proteins and genes from rota- and orbiviruses, and the documented experiences of others that the process is completely empirical. The automatic assumption that 'plant codon usage' will be optimal is naïve; so too are most assumptions based on experience in other non-plant cell culture systems. There is no one universally suitable host or production system, although the use of agroinfiltration and deconstructed plant viral vectors seems to be on the way to becoming an industry standard. It is advisable to explore all intracellular organelle and export targetting options, but also plastid transformation, as plastid targetting of proteins after cytoplasmic synthesis may not predict what happens with mRNA expression and translation in these organelles.

Some of these principles are illustrated in a simple example taken from our work (Figure 1). Here, the effects

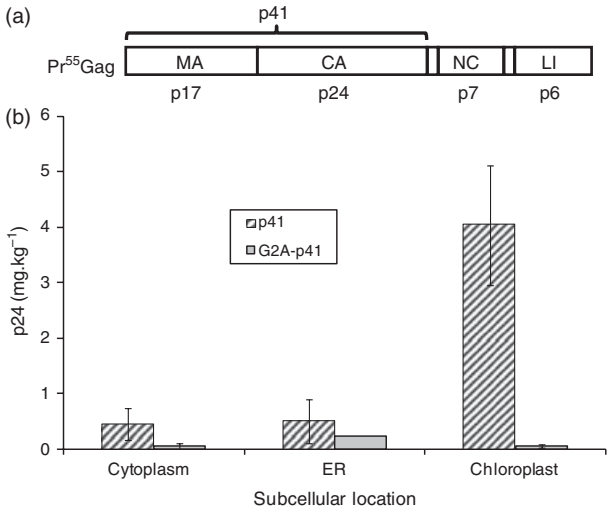

Figure 1 The effect of intracellular localisation and modification on the accumulation of HIV-1 Gag-derived p41. (a) Depiction of Pr^{55}Gag precursor polyprotein translated from the *gag* gene of HIV-1. MA or p17 is the matrix protein, myristylated at position 2; CA or p24 is the isometric capsid protein; NC or p7 is the nucleocapsid protein; p6 is an accessory protein, which mediates interactions between Pr^{55}Gag and Vpr. P41 is a truncated version of Pr^{55}Gag comprising only p17 and p24. (b) Transient expression of HIV-1 p41 (p17::p24) protein in *Agrobacterium*-infiltrated *N. benthamiana* was measured by commercial HIV-1 p24 ELISA test [described by (Meyers *et al.*, 2008)] 4 days after infiltration. G2A-p41 is p41 mutated by Gly->Ala replacement at position 2: this removes the myristylation signal. Figure adapted from Meyers *et al.* (2008).

of expression of two versions of a HIV-1 *gag* gene-derived construct by agroinfiltration in *N. benthamiana* using a range of vectors targeting proteins to the cytoplasm, the ER and the chloroplasts can be seen. These 3′-terminal truncated *gag* genes encode a 'p41' matrix (MA, p17) and capsid (CA, p24) protein fusion, with and without (G2A) a p17 N-terminal myristylation signal: myristic acid addition allows association of the Pr^{55}Gag precursor protein (and presumably p41) with the cell membrane and subsequent budding of a VLP. While only the myristylated version buds, there is no difference in total accumulation of either version of Pr^{55}Gag in insect cells (Halsey *et al.*, 2008). In this case, while native p41 accumulated significantly better in chloroplasts than cytoplasm or ER, changing a single amino acid and cancelling myristylation very significantly reduced accumulation of G2A-p41, especially in chloroplasts (Meyers *et al.*, 2008). Thus, expression of the same protein in insect and animal cells is very different: I have mentioned previously that nuclear expression of whole Pr^{55}Gag in plants is very low indeed, whatever the targeting; this contrasts with insect cell expression of the same protein in our hands, which gives reasonable yields (Jaffray

et al., 2004; Halsey *et al.*, 2008). We have now determined that myristylation may also be a significant factor to have to take into account in considering expression potential of a membrane-associating protein.

Future prospects

While the public profile of plant-produced vaccines has been much reduced since its heyday in the 'grow your own vaccine' times in the 1990s, the feasibility and potential efficacy of such vaccines has been well established, and it may be that their time is about to come at last. While many groups have experimented with expressing antigens from human pathogens in plants, it is people working with animal models and animal pathogens who have been able to demonstrate the most convincing proofs of efficacy for plant-produced vaccines in general and for both oral and injectable versions. Among the leaders in this area—possibly because of their working with several versions of the same vaccines, produced via different platforms—is the group at the Instituto de Virologia in Buenos Aires [see review (Dus Santos and Wigdorovitz, 2005)]. While they have worked extensively on plant-produced FMDV vaccines and shown efficacy in all cases (Wigdorovitz *et al.*, 1999a,b; Dus Santos *et al.*, 2002), they have also recently been able to produce a partially purified bovine viral diarrhoea virus E2 glycoprotein subunit vaccine from transgenic alfalfa, which injected in equivalent amounts (two doses of 1.5 μg of antigen) protected cattle better than the conventional vaccine [reported in (Rybicki, 2009b)]. It is quite possible that the first targets of large-scale production of plant-derived vaccines should be diseases of animals, given the easier route to demonstration of efficacy, and the potentially far shorter regulatory path for such vaccines—as has been demonstrated by Dow with their NDV vaccine (Dow AgroSciences, 2008).

The prospects for human vaccines are not as clear: while there has been much activity in producing alternatives to established vaccines—such as those to HBV and HPV and rotaviruses, for example—the simple fact is that there is either already enormous established conventional capacity for production of generics, as in the case of HBV, which is now cheap enough to be included in the Extended Programme on Immunisation (EPI) universal infant vaccination bundle or so much capital involved in development and production plants for the new HPV and rotavirus vaccines, for Big Pharma to have no interest in diversifying their means of production. As pointed out elsewhere, even reduction of cost of material and production plant to negligible amounts would only lower the cost of a plant-produced biosimilar or generic version of an established vaccine by a fraction (32%) of the retail price, all other costs (including downstream processing) remaining the same (Rybicki, 2009a). Thus, it is probably not an option to attempt to compete with established products, but to use the unique advantage of the technology—that is, the huge range in scalability and speed of response—to leverage a niche in 'orphan' vaccines or emerging disease vaccines or even bioterror threats, where other means of production are simply too slow to respond. It is worth stressing the point made by Charles Arntzen and reported elsewhere (Rybicki, 2009b) that 'The plant advantage over older technologies [is] speed to new production and platform flexibility in terms of several ways to produce antigen'. In this regard, the deconstructed viral vectors and even simple agroinfiltration are extremely potent tools for both pilot production and for scaling to full-scale production, in rapid-response scenarios.

Indeed, in this time of the new influenza A H1N1 pandemic, possibly the most impressive demonstration of the potential of plant-produced vaccines is the fact that two groups of researchers—at Medicago Inc. in Canada and at the Fraunhofer USA—very recently managed to go from PubMed-accessed H1 HA sequence, through to purification of grams of protein in less than a month, via transient expression in tobacco (Rybicki, 2009b). This is almost certainly the fastest production ever for any current pandemic or even seasonal influenza vaccine and represents a thorough vindication of the approach. The influenza pandemic response potential of the technology is explored elsewhere in this volume (D'Aoust *et al.*, 2010). The subject of new technologies for influenza seasonal and pandemic vaccines, and in particular the use of plants, has been well and comprehensively reviewed recently (Chichester *et al.*, 2009a); suffice it to say here that there is ample evidence that plants may be a very viable vehicle for rapid-response or even conventional vaccine production for the prevention of influenza. The recent demonstration of the ability of VAXX Inc. to quickly produce gram quantities of a diarrhoea-causing norovirus subunit vaccine (Dylewski, 2009) is also very promising, given that this virus too is subject to rapid mutational change.

Recent news on the regulatory front is favourable too: the first European guidelines for growing plants producing pharmaceuticals in genetically modified plants—drawn up by the European Food Safety Authority (EFSA)—were published on 7th August, 2009 (Gilbert, 2009). These are

apparently consistent with those of the US Food and Drug Administration and Department of Agriculture, meaning much of the northern hemisphere now has matching guidelines for PMPs.

A recent review on human trials of plant-based oral vaccines summarising human studies of oral transgenic plant-derived vaccines against enterotoxigenic *E. coli* infection, norovirus and HBV adds weight to the growing body of evidence that plant-made oral vaccines to these viruses are not only feasible, but effective (Tacket, 2009). There are now many proofs of concept and several of efficacy for oral immunisation in animal models—and the recent demonstrations that very high yields of human vaccine candidates can be obtained via plastid transformation or transient expression in plants makes the dream of 'heat-stable oral vaccines' [Charles Arntzen, quoted in (Rybicki, 2009a)] suddenly almost reachable. For example, while it may be true that 10–30× as much antigen is needed via the oral route as is required parenterally for the same immune response, if this much antigen can be reliably provided by one tobacco plant and can be suitably and cheaply purified by a simple process, then it becomes eminently possible to exploit the technology for the original goal.

On the regulatory front, the FDA approval of the plant-produced injectable purified antibody fragments used as individualised vaccines in human trials for non-Hodgkin lymphoma therapy (McCormick *et al.*, 2008) was the first for any injectable human vaccine derived from plants, meaning that this route of immunisation will open up as well. In fact, the insistence among many in this research field that oral administration is needed for mucosal immunity may need to rethink their assumptions: in a landmark 1998 article, the following quote may give pause for thought. 'Based on these observations [comparisons of mucosal and parenteral vaccination with conventional vaccines], it is suggested that development of serum immunological responses are effective in the prevention of systemic disease regardless of the types of vaccines or route of their administration. However, induction of pathogen-specific antibody or cellular immunity **at the mucosal sites** [my emphasis] is best elicited by mucosal application of the antigen' (Kaul and Ogra, 1998). Thus, plant-made vaccines may overlap far more than was formerly thought possible with conventional vaccines, in that injectable 'biosimilars' or even 'biobetters', as defined by Yuri Gleba (Rybicki, 2009b), could compete directly with those produced by fermentation or animal cell culture.

While the long-term future of plant-produced vaccines has always seemed bright, there has also been a disjunction between the present and this future, which has been hard to bridge. For example, it appears obvious that all subunit vaccines should be made in plants where this is feasible—yet none are, even for animals, despite all the proofs of efficacy over more than 10 years. There is also much made of the apparent reluctance of large pharmaceutical and other companies to become involved—yet when they do (e.g. Bayer and Dow), the impact is profound. Perhaps it will take the incremental successes of plant-made therapeutics such as Protalix's glucocerebrocidase and the Japanese AIST group's canine IFnα produced in strawberries for treatment of dog periodontal disease (Rybicki, 2009b), followed by vaccines for livestock such as the Argentinean BVDV vaccine, and human therapeutics such as rabies virus antibodies and even insulin and vaccines like ICON Genetics' NHL product, to finally tip the balance for broad-based acceptability of plant-based vaccines for human use. The apparent acceptance by the relevant funding agencies in the USA that rapid-response vaccines aimed at potential bioterror agents such as anthrax and haemorrhagic fever viruses could easily be made in plants may be a valuable lever in this regard. If acceptance does happen soon, then obvious first targets—other than the military options—should be the niche market of 'orphan disease vaccines' such as for Lassa fever and the South American haemorrhagic fever viruses, and other low volume markets, rather than competitors for blockbuster vaccines such as HPV and rotavirus. Increasing acceptability in the long term could mean a shift to the mainstream high volume/low cost generics market—which would appear to be ideally suited for this technology.

The regulatory landscape is also less intimidatory than it is often perceived—and recent developments such as the European Union's preparing for 'pharmed drugs' may mean it is changing to become even less so (Gilbert, 2009; Rybicki, 2009b). It is to be hoped that the barriers to application of this promising technology will soon be breached—and orphan vaccines will be orphans no more.

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