ELSEVIER

Plant-produced vaccines: promise and reality

Edward P. Rybicki

Department of Molecular and Cell Biology and Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa

Plant-produced vaccines are a much-hyped development of the past two decades, whose time to embrace reality may have finally come. Vaccines have been developed against viral, bacterial, parasite and allergenic antigens, for humans and for animals; a wide variety of plants have been used for stable transgenic expression as well as for transient expression via *Agrobacterium tumefaciens* and plant viral vectors. A great many products have shown significant immunogenicity; several have shown efficacy in target animals or in animal models. The realised potential of plant-produced vaccines is discussed, together with future prospects for production and registration.

Introduction

Plant-produced vaccines are at first sight an exciting and at the same time a controversial concept: exciting because of the much-touted possibility of being able to produce vast amounts of vaccine protein or even edible vaccines at low cost; controversial because of the perception of the possibilities of contamination of the food supply, the potential for the development of immunological tolerance to orally dosed or edible vaccines and of potential regulatory and production problems.

The central justifications for the development of the technology – the promises – have been that vaccine antigen production in plants is safe and potentially very cheap and infinitely scalable; that plants can often be used to produce biologically active proteins far more easily than can bacteria or yeast; and that the use of food plants could allow edible vaccines to be locally and cheaply produced where they are needed most – in the developing world – and for vaccines that are presently not available and which may never be made conventionally.

The reality is that, despite nearly 20 years of development, there are only two plant-produced vaccine-related products that have gone all the way through all production and regulatory hurdles – and one will not be sold, and the other is an antibody that will be used in the purification of a vaccine, rather than a vaccine itself, and both are produced in sophisticated facilities. There are still major hurdles in the way of routine vaccine production via plants, not the least of which are low yields, the weak antigenicity of many products, and the lack of buy-in from major pharmaceutical companies and governments.

Rybicki Biosketch I have been at the University of Cape Town (UCT) since I came to Cape Town on holiday from Zambia in 1974, and fell in love with the place. I obtained BSc, Hons, MSc and PhD degrees in Virology/Microbiology, and became a lecturer in Virology in 1981, and a professor in Microbiology in 2003. I am also a founder member of the Institute of Infectious Disease and Molecular Medicine (IIDMM).

My main research interests are presently in making human and animal vaccine candidates in plants and insect cells: these include vaccines for mucosal human papillomaviruses (HPV) and human immunodeficiency virus type 1 (HIV-1) subtype C. We also work on the characterisation and molecular biology of the parrot beak and feather disease virus and the possibility of making vaccines and therapeutics for the viral disease. I also have an interest in the diversity of southern African *Mastreviruses* (family *Geminiviridae*), the use of geminiviruses as vectors of foreign genes in plants and the engineering of viral resistance, especially in maize.

E-mail address: ed.rybicki@uct.ac.za.

1359-6446/06/$ - see front matter doi:10.1016/j.drudis.2008.10.002

This review will explore the ways of producing foreign proteins in plants, the historical development of plant-produced vaccines, and the development of robust, stable plant production systems for vaccine antigens, the potential of plant-produced vaccines to advance human and animal health, as well as the issues surrounding the delivery of these products.

Plant production of foreign proteins

The types of plants and the types of plant tissues used for the production of protein and other vaccine antigens include the following:

- leaf and stem tissues of tobaccos of various species and varieties, *Arabidopsis thaliana*, alfalfa, spinach and potatoes;
- aquatic weeds such as *Lemna* spp. (duckweed);
- seeds of rice, beans, maize and tobacco;
- fruits like tomatoes and strawberries;
- root vegetables like carrots;
- single-cell cultures of the algae *Chlorella* and *Chlamydomonas*;
- suspension cell cultures of tobacco and other plants;
- hairy root cultures derived from various plants via *Agrobacterium rhizogenes* transformation;
- transformed chloroplasts of a variety of plant species.

Antigens may be expressed in the cytoplasm and remain there, or be localised to any of the plant organelles or compartments (nucleus, mitochondria, chloroplasts, vacuole, endoplasmic reticulum or apoplast) by means of specific signal peptides [1]. The magic number for protein yield for economic extraction is often given as 1% of the total soluble protein; however, this is seldom reached.

Transgenic plants represent a potentially stable and cheap propagation source; however, the development and selection of a suitable transgenic line can take many months, and the production at high yield is often not attainable or stable, often owing to the phenomenon of post-transcriptional or siRNA-dependent gene silencing. This may be triggered by the high concentrations of any particular mRNA, and in any case is reset at meiosis, leading to both immediate and potential long-term production instability [2,3]. This notwithstanding, Li *et al.* [4] reported that a transgenic potato line expressing a human rotavirus VP7 protein was stable over 50 generations and maintained immunogenicity when fed to mice, indicating the potential of the system. However, this is a sole example, and the stability of vaccine expression in transgenic plants is an under-investigated phenomenon. Whilst constitutive or 'green leaf' expression is the easiest to engineer, it can lead to problems if the protein interferes with plant development, and it is often difficult to purify proteins away from leaf constituents such as pigments, alkaloids and polyphenols. Perhaps the simplest commercial whole-plant transgenic expression system is that using the common duckweed, *Lemna*: Biolex Therapeutics claims that their 'LEX System^{SM}' allows rapid product development owing to the ease of regeneration and rapid growth, and they can produce under GMP conditions. They have produced over 35 proteins, many of which could not be produced in any other system [5]. The system is apparently very well suited to monoclonal antibody (MAb) production, because it is possible to engineer glycosylation relatively easily [6].

Expression and accumulation of proteins in seeds – via seed-specific promoters – is generally seen as preferable to expression in green tissue, because of the far easier purification and higher accumulation levels [7–10]. Seed-stored proteins are also generally stable for long-term storage, even at room temperature, owing to the drying process which accompanies seed maturation. A disadvantage is that transgenic expression can only begin to be assessed at seed set, which can take many months of plant growth.

Transgenic single-cell cultures – whether of algae or of suspension-cultured plant cell lines – offer the advantages over whole-plant systems of a high level of containment and the possibility of producing proteins in bioreactors under 'good manufacturing practice' (GMP) conditions, as is currently the case with conventional fermentation or cell culture techniques. However, yields are generally not high [1]. There is usually little perceived advantage in these systems over animals or humans or especially yeast cell culture production systems, other than the generally simpler culture media for cell lines, and the possibility of using simply ponds or flow-through transparent 'reactors', salts and sunlight for algae. However, Protalix Therapeutics have claimed that the therapeutic enzyme glucocerebrosidase produced in cultured carrot cells has a significant advantage over conventionally produced protein made in CHO cells: it has terminal mannose residues on its complex glycans, which have to be chemically added to the CHO cell product, because it is vital for uptake by macrophages to combat a lysosomal storage disorder [11].

Expression of proteins from transformed chloroplasts often gives high yields, and avoids many of the problems associated with nuclear transformation [12,13]. However, this system is often not suitable for glycosylated proteins or secreted proteins, given the lack of suitable eukaryote-type machinery in what is essentially an intracellular prokaryote expression system. The transformation system is also difficult, as is selection, and there is not as wide a choice of plant types as for nuclear transformation.

Transient expression systems – wherein whole plants are induced to express foreign proteins by means of recombinant virus infection or other means – are rapidly gaining popularity as a means of both rapidly exploring viable promoter-protein-localisation options and the actual production of recombinant proteins. The first of these expression systems to see the light was a recombinant tobacco mosaic virus (TMV) with its capsid protein fused to a malarial peptide [14]; by the late 1990s a HIV-1 gp41 peptide fused to Cowpea mosaic virus (CPMV) capsids had been tested in mice both parenterally and orally [15]. Similar expression systems – as in the fusion of antigenic peptides to plant virus CPs – include alfalfa mosaic virus (AMV) and potato virus X (PVX), among others [16–18]. Other useful fusion partners for chimaeric protein expression include *Escherichia coli* heat-labile enterotoxin (LT-B), *Cholera vibrio* toxin B subunit protein (CTB) and *Clostridium thermocellum* lichenase (LickM), the last of which is expressed via a TMV-based system [19–21]. Whole gene expression in plants is also possible via recombinant TMV and other viral vectors: however, the choice of appropriate vectors is limited, as is the choice of host plants, and vectors are often unstable and do not express large proteins very well. An interesting new development is the use of plant-produced TMV coat protein to encapsidate an antigen-expressing alphavirus-derived RNA, to make a pseudovirus that is taken up by antigen-presenting cells [22].

Another important transient expression system is *Agrobacterium tumefaciens*-based. This technique relies on the infiltration of

whole-plant tissue with a suspension of *Agrobacterium*: T-DNA is transferred to a very high proportion of cells, where it can integrate or remain as an episome, and in either case will express its payload. This transient somatic transformation or 'agroinfection' allows very high levels of expression without the uncertainties inherent in the regeneration and propagation of transgenic plants, and, like viral vectors, at a time of the experimenter's choosing. Whilst early application of the technology was mainly in the study of plant–pathogen interactions and in the investigation of endogenous gene expression, by 1999 it was being used successfully to produce antibody molecules, and was being touted as a major advance in plant expression technology [23]. Its main advantage lies in the fact that the simultaneous expression of a large number of constructs can very rapidly be investigated, unlike the case with plant viruses, which can exclude each other from infected cells. It is also possible to do large-scale production via vacuum-mediated agroinfiltration [1].

A very promising transient expression technique is the 'MagniFection' system of Icon Genetics: this uses agroinfiltration to systemically deliver a TMV-based transient expression vector, which significantly amplifies the mRNA expression level compared to agroinfiltration, whilst maintaining the advantage of the systemic delivery owing to the latter [24]. Another possibly under-investigated system is the use of transgenic or transiently infected plants or cell cultures which can constitutively or inducibly express geminivirus replicons, resulting in amplified gene expression [25,26].

The main experience gained from these various systems is that there is no reliable way of predicting:

- if any given host plant will work;
- whether or not a given DNA sequence will express protein at a reasonable level;
- if that the protein will be stable;
- if it will assemble correctly;
- if it will be antigenically appropriate for the purpose.

Thus, empirical determinations – generally using transient expression systems for the sake of speed and convenience – are the only safe way of determining whether a given antigen can be expressed in a given system, and immunogenicity and preferably efficacy trials are the only way to determine whether they may work.

Historical development

The first vaccine-relevant protein produced in plants was in fact not an antigen, but an antibody: Hiatt *et al.* [27] produced MAbs derived from a set of mouse genes expressed in transgenic tobacco. The authors' prescient comment that 'The results demonstrate that production of immunoglobulins and assembly of functional antibodies occurs very efficiently in tobacco' has been vindicated manyfold since: it appears that the one class of proteins that are reliably produced in plants, to reasonable yield, are immunoglobulins or Ig fragments [28,29].

The person who has become the godfather of the plant-produced vaccine research world, Charles J. Arntzen, entered the field in 1992 with a paper on the production of hepatitis B virus surface antigen (HBsAg) in transgenic tobacco [30]. The group showed that the plant-produced HBsAg formed 22 nm particles that were antigenically and physically similar to HBsAg particles derived from human serum and recombinant yeast, and concluded that transgenic plants held promise as low-cost vaccine production systems.

Despite the fact that it appears that mainly viral proteins have been expressed in plants as potential vaccines, it was a bacterial protein – *Escherichia coli* heat-labile enteroxin (LT-B) – that provided the first proof of principle for edible plant-produced vaccines. Haq *et al.* [31] showed that LT-B produced in transgenic tobacco or potatoes appeared functionally equivalent to *E. coli*-produced protein, and mice immunised by oral gavage produced systemic and mucosal neutralising antibodies. Moreover, fresh potato containing LT-B was orally immunogenic in mice.

Possibly the next most effective demonstration of the power of a plant-produced protein was in the demonstration that a high affinity, monoclonal secretory antibody against *Streptococcus mutans* adhesion protein produced in a transgenic plant could prevent microbial colonisation in the human oral cavity [32]. However, the first real proof of concept for the orally delivered plant-produced vaccines was by Mason *et al.* [33], who demonstrated the efficacy of a potato-produced *E coli* LT-B vaccine in mice. There was also around this time – mainly from the Mason-Arntzen group – a series of demonstrations of the vaccine efficacy of plant-produced antigens for as varied a selection of pathogens as Norwalk virus capsid protein, HBsAg and a *Vibrio cholerae* enterotoxin subunit (CTB), in mice and in humans [34–39].

However, it was in the animal vaccine arena that the most effective first proofs of concept of plant-produced vaccines came, given the possibility of doing challenge experiments in animal model systems. One of the first such demonstrations was that of Dalsgaard *et al.* [40], who showed that mink could be protected against disease caused by Mink enteritis virus (MEV) by subcutaneous injection of chimaeric CPMV virions expressing an MEV peptide on their surfaces. Castanon *et al.* [41] showed that parenteral immunisation with potato-produced VP60 protein protected rabbits against infection rabbit haemorrhagic disease virus (RHDV), and Wigdorovitz *et al.* [42,43] later demonstrated that oral immunisation or the injection of mice with Foot and mouth disease virus (FMDV) VP1 coat protein precursor polyprotein derived either from transgenic alfalfa or from *Nicotiana benthamiana* plants transiently infected with recombinant TMV protected mice against viral challenge with live FMDV. The latter work provided the first evidence that full-length foreign proteins could successfully be produced using a plant virus, in amounts that were sufficient to allow immunisation using only crude extracts.

An important development happening alongside the flowering of plant-produced vaccines and antibodies research was a major advance in our understanding of the working of mucosa- and in particular gut-associated lymphoid tissue (MALT and GALT) [44,45]. In particular, the concept of oral vaccination with non-replicating or subunit vaccines was being vigorously explored, as were the mysteries of 'tolerisation', or the induction of immune tolerance at mucosal surfaces [46–48]. This undoubtedly stimulated the strong conviction among workers in the plant-produced vaccine field at the time that 'cheap oral vaccines' were the goal of their endeavours.

An interesting phenomenon in retrospect was the fact that reviews and opinion pieces in scientific and especially popular literature on 'vaccine pharming' from around this time probably

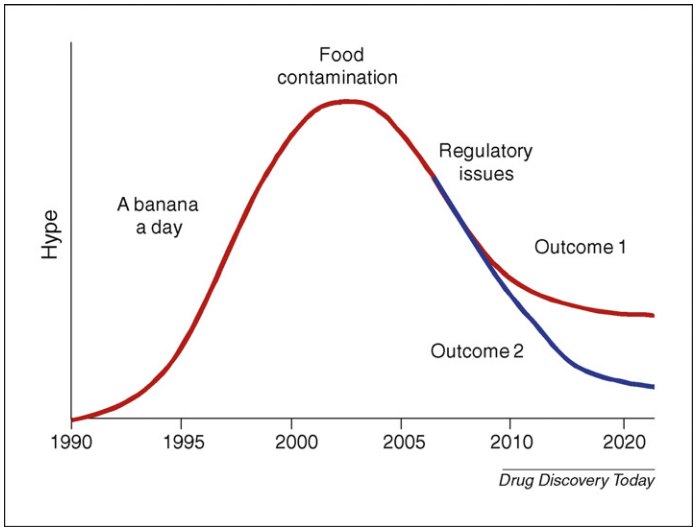

FIGURE 1

The 'hype curve' – tracking public perception and sentiment. The curve depicts the publicity generated around plant-derived vaccines as a function of time: favourable publicity up until around 2002 was fuelled largely by the idea of edible vaccines; this was soured with by fears of contamination of the food chain, and died away further with worries about regulatory problems, and the lack of success and further development. Two outcomes are shown: Outcome 1 represents my view of the future (see 'The future'). Outcome 2 represents what may happen if industry and governments do not take up the technology: the hype will die away to levels that may make it very difficult in terms of public sentiment and negativity to fund any plant-based production vehicles, however useful these may be.

outnumbered the scientific articles describing new developments in the field: in my database in 2000 alone there were 11 reviews among 20 papers dealing mainly with vaccines from plants. This was probably near the peak of the 'hype' phase of the publicity around the field (see Fig. 1), when the prospects of growing your own vaccine were being seriously discussed in public media (see http://whyfiles.org/166plant_vaccines/, http://www.acfnewsource.org/science/edible_vaccines.html).

A decline from the height of the hype certainly started sometime after the most publicised incident to cause public concern; however, a doubt had been growing for some time concerning several issues. The biggest blow to the field was an incident involving ProdiGene Corp: they had proprietary technology allowing high levels of accumulation of foreign proteins especially maize seeds, and several potential oral vaccine products including a hepatitis B vaccine, a LT-B vaccine to treat *E. coli* infections in humans. In 2002 soybean and maize harvests in two states in the USA were found to contain maize seeds from 'volunteer' plants engineered to express transmissible gastroenteritis virus (TGEV) capsid protein, intended as an experimental pig vaccine. ProdiGene was fined and forced to clean up the seed by the US Department of Agriculture, which has since issued guidelines to prevent a recurrence [49]. This has led to an effective moratorium on the development of 'pharmed' products in food crops in the USA and worldwide – including vaccine pharming.

Other issues starting to cause growing concern were doubts about whether regulatory bodies would or could licence plant-produced products for use in humans or even in animals, and persistent concerns about whether edible vaccines would cause inappropriate antigenic tolerance (Fig. 1).

Efforts to produce viable vaccines continued, however, and several landmark advances have occurred in the past six years despite the decreasing public interest. An important one was the demonstration by Rose *et al.* [50] and Gerber *et al.* [51] of the excellent humoral and cellular immunogenicity of baculovirus-produced human papillomavirus (HPV) L1 major capsid protein (L1) virus-like particles (VLPs) given by gavage to mice, especially when adjuvanted with CpG-containing DNA or *E. coli* heat-labile enterotoxin mutant R192G. This was followed by the essentially simultaneous publication by three groups of accounts of the plant production and immunogenicity in mice of VLPs made from L1 proteins of HPV types 11 and 16 [52–54]. The first group tested only parenteral immunisation of mice; the latter two attempted immunisation of mice with HPV-16 and HPV-11 L1 VLPs, respectively, by feeding them with transgenic potato. Yields were low in all cases, and immune responses were relatively weak: however, the successful equivalence in terms of VLP assembly and antigenicity had been established for plant production of a major new human vaccine, which is commercially produced by Merck in yeast (Gardasil™) and in insect cells via recombinant baculovirus infection by GlaxoSmithKline (Cervarix™).

Other salient advances in the recent years include the use of a partially purified tobacco-produced measles virus haemagglutinin as an oral boost to a DNA vaccine in mice, with the production of neutralising antibodies [55]; the induction of high titres of mucosal neutralising IgA in mice by oral immunisation with rotavirus VP7 in transgenic potatoes [56]; the use of transient *Agrobacterium*-based expression system in tobacco to quickly evaluate the production potential and conformation of HBsAg, as an exemplar of a potentially high-throughput evaluation system [57]; the proof that porcine TGEV capsid protein in maize seeds can orally boost lactogenic immunity in swine [58]; the use of a plant-produced HIV gp41–CTB fusion protein to elicit transcytosis-blocking antibodies in intranasally primed and intraperitoneally boosted mice [59]; and the protection of mice against tetanus toxin by a single intranasal dose of soluble transgenic tobacco leaf protein [60]. A novel strategy described by Chargelegue *et al.* [61] involved the expression of a fusion between a MAb specific for tetanus toxin C fragment with the fragment itself and its successful use as a self-adjuvanting cross-linked complex for the single-injection subcutaneous vaccination of mice.

In what was probably – in retrospect – the swansong of classical edible vaccines [62], Thanavala *et al.* [63] described the use of raw potatoes expressing HBsAg to orally boost pre-existing immunity in conventionally immunised human volunteers: whilst this was nominally successful, in that most volunteers showed increased serum responses, immunogenicity was low notwithstanding up to 3 doses of nearly 1 mg of protein in potato tissue – 25 times the routinely administered parenteral dose.

'MagniFection', or the use of reconstructed TMV-based vectors delivered via *Agrobacterium* infiltration of whole plants for high-level vaccine protein expression, was first described in 2005 [24]. By a year later the group had reported the use of multiple vectors for the highest level expression yet achieved of full sized IgG in plants [64], and of *Yersinia pestis* antigens F1, V and fusion protein F1–V, which protected guinea pigs against aerosol challenge with virulent *Y. pestis* [65]. They followed this with the report of intramuscular vaccination of mice and minipigs with MagniFection-derived

vaccinia virus B5 protein-derived antigen, and subsequent protection against lethal challenge doses of vaccinia [66].

The use of chloroplasts to express antigens received a boost with the demonstrations that chloroplast-produced anthrax protective antigen was protected against lethal toxin challenge [67], and that Gal/GalNAc lectin of *Entamoeba histolytica* was highly immunogenic in mice [68]. The latter represents a true 'orphan disease' vaccine: *E. histolytica* infects 50 million people annually, causing about 100,000 deaths.

Allergy therapy too received a boost, with the news that a transgenic rice-produced allergy vaccine for Japanese cedar pollen, consisting of the T-cell epitopes only, resulting in a tolerance which caused reduction in allergen-specific IgE, T-cell proliferative reaction and histamine responses [10,69]. This and other important work in allergy therapy involving recombinant antigens was reviewed by Valenta and Niederberger [70].

Cancer therapeutics too have been both historically and recently targeted by plant-derived vaccines. Perhaps the highest profile of these was the production by Large Sale Biology Corp – formerly Biosource Technologies – of Vacaville, CA, USA, of individually tailored monoclonal single-chain variable region antibody fragments derived from individual patients' non-Hodgkin lymphomas, in plants via recombinant TMV [71]. These proteins were shown to induce appropriate anti-idiotype humoral responses in mice [72], and so to be suitable for use as vaccines in humans. The company got Food and Drug Administration (FDA) approval for their manufacturing and formulation processes, and were able to do a successful Phase I clinical trial: the vaccines were well tolerated in 16 patients, vaccinated six times each [73] and Phase II trials were planned – unfortunately, the company filed for bankruptcy soon afterward. Another significant study was the comparison of plant- and baculovirus-produced colorectal cancer antigen GA733-2, which found similar humoral and only slightly different cellular responses to the antigens in mice [74]. Papillomavirus-induced disease has been a popular target with several studies targeting HPV oncogenic proteins: Franconi *et al*. [75,76] and Massa *et al*. [77] reported that immunisation with plant-produced antigen protected mice from tumour challenge with an E7-expressing tumour cell line, and in the latter two cases, that tumour regression could also be seen. A plant-derived tumour-associated colorectal cancer antigen EpCAM (pGA733) was recently purified at high yields with two plant expression systems: this was purified and its antigenic and immunogenic properties were compared to baculovirus-produced protein [78]. Sera from immunised Balb/C mice efficiently inhibited the growth of SW948 colorectal carcinoma cells xenografted in nude mice, compared to a EpCAM-specific mAb.

One of the newest hype factors around plant-produced vaccines has been in their supposed potential for rapid-response vaccines for biothreat agents: thus, the US Department of Homeland Security-influenced initiatives involving 'biodefence' have provided a lot of funding for work on vaccines for everything from anthrax, through plague, to ricin and haemorrhagic fever viruses [21,65]. Indeed, as of July 2004, legislation dubbed 'BioShield' provides US industry with incentives to research and develop bioterrorism countermeasures, including vaccines, and speeds the approval process [79]. This may well affect the future of plant-derived vaccines, allowing Outcome 1 rather than 2 (Fig. 1).

A useful historical case study in the evolution of the potential of plant-produced vaccines is provided by papillomaviruses. In 1997, my laboratory first began investigating the expression of the HPV-16 native L1 protein gene in transgenic tobacco, with very little success: yields were too low to be measured; the only indication of expression was that rabbits immunised several times with 1000-fold concentrated sap extracts developed low titres of IgG against baculovirus-produced L1 (A. Varsani *et al*., unpublished). By 2003 the situation had improved, with HPV-16 L1 yields up to tens of milligrams per kilogram of transgenic plant [52], but still giving only low immunogenicity upon oral dosing with transgenic potato. The first proofs of efficacy of plant-produced papillomavirus vaccines followed, with demonstrations of the protection of rabbits against warts caused by cottontail rabbit papillomavirus (CRPV) by injection of either chimaeric TMV particles carrying a CRPV L2 peptide [80], or partially purified extracts containing whole CRPV L1 protein, produced in either transgenic tobacco or via recombinant TMV [81]. Incidentally, our group has shown that different papillomavirus (PV) L1 genes express at very different levels in the same expression system: thus, in our hands in transgenic tobacco the native HPV-16 L1 gene expressed no higher than 4 μg/kg [53], whilst native CRPV L1 expressed up to 1 mg/kg [81], and HPV-11 L1 expressed at 11 mg/kg [82]. A significant breakthrough occurred in 2007, with my group's demonstration that nuclear transformation – whether transient or stable – and chloroplast rather than cytoplasmic or ER localisation of a human, and not a plant codon-optimised HPV-16 L1 gene, led to yields as high as 17% of total soluble protein (TSP) or 0.8 g/kg of L1 protein, an order of magnitude higher than the best previous attempt and 25,000-fold better than our original best, achieved via rTMV [83,84]. This was also an object lesson in the necessity for empirical evaluation of several parameters, including intracellular targetting and codon optimisation, for maximisation of expression (see Fig. 2). This protein was also the first plant-produced papillomavirus L1 to be shown to elicit high-titre neutralising antibodies – the gold standard for a HPV vaccine candidate. The production record for HPV L1 protein has now been claimed by Fernandez-San Millan *et al*. [85], who used expression in transplastomic tobacco to achieve 3 g/kg or ~24% of TSP. The salient lessons learned in this process were that even cognate L1 genes of different PVs may express at very different levels in the same system; that codon optimisation must be empirically determined rather than predicted, and so should intracellular targeting.

Promise versus reality

The promise of plant-produced vaccines has been amply demonstrated in the historical account above: thus, the potential of human and animal vaccines, of oral and parenterally delivered vaccines, or viral, amoebic and bacterial vaccines, of prophylactic and therapeutic vaccines, of transgenic and transient expression systems, have all been amply demonstrated in the past 19 years. Big companies are also entering the fray: in 2006 Bayer Innovation GmbH bought out Icon Genetics, whose MagniFection technology has increasingly been seen as the front-runner in viable plant expression systems; Dow AgroSciences made a surprise entry into the field with a new Newcastle disease virus (NDV) vaccine (see below).

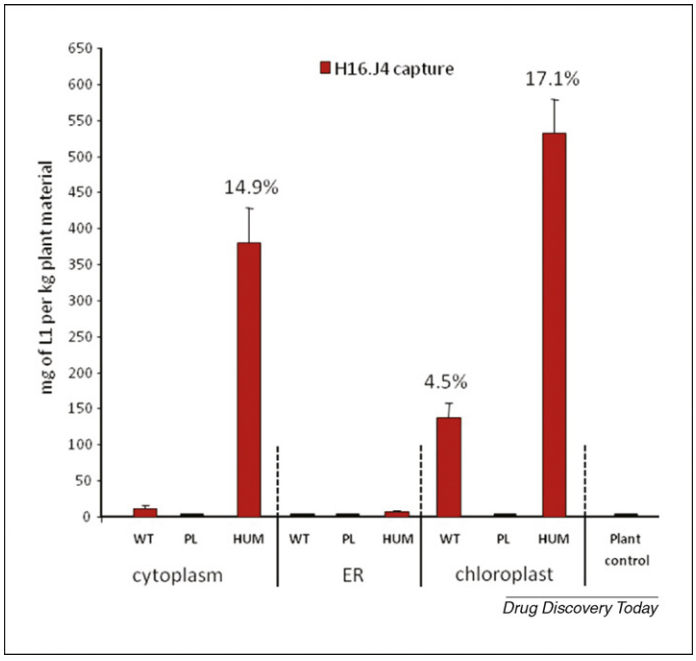

FIGURE 2

Optimisation of expression of Human papillomavirus type 16 L1 protein in tobacco by codon optimisation and intracellular targetting. The vertical bars represent the amounts of L1 protein in milligram per kilogram of plant, assayed in plant extracts using L1-specific enzyme-linked immunosorbent assay. Error bars indicate variation in triplicated samples. Percentages shown above the bars represent L1 protein as a fraction of total soluble protein (TSP). WT is the expression from wild-type L1 gene; PL is the expression from plant codon-optimised gene; HUM is the expression from human codon-optimised gene; plant control is the normal plant extract. Cytoplasm is the no targetting signal in expression construct; ER is the endoplasmic reticulum targetting and retention; chloroplast is the chloroplast import signal included. Modified from Maclean *et al.* [83].

Given all this, it is surprising that only two products have made it through the regulatory processes to be licenced. The first was a plant-made scFv mAb used in the production of a recombinant HBV vaccine in Cuba [86]: this is produced in transgenic tobacco, and has the potential to completely replace the ~300,000 mice formerly used per year to produce mAbs via ascitic fluid (G. Guillen, CIGB, pers. commun.). The second – in January 2006 – was a NDV vaccine for poultry, produced in a suspension-cultured tobacco cell line by Dow AgroSciences, and successfully tested as a purified injectable product in chickens [87]. This has been registered and approved by the US Department of Agriculture (USDA) – the final authority for veterinary vaccines – but is not for sale. The reason is apparently that the company 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.'

It is not as if the regulatory environment has not been explored. For example, Kirk *et al.* [88] discussed the risk analysis for plant-made vaccines, and concluded that 'Risks to human health include oral tolerance, allergenicity, inconsistent dosage, worker exposure and unintended exposure to antigens or selectable marker proteins in the food chain.', but also that 'These risks are controllable through appropriate regulatory measures at all stages of production and distribution of a potential plant-made vaccine....'. The World Health Organisation (WHO) convened an expert panel in 2005 to discuss the scientific basis for regulatory evaluation of candidate human vaccines from plants [89] – and '...concluded that existing guidelines for the development, evaluation and use of vaccines made by traditional methods can be applied to plant-derived vaccines. For plant-derived vaccines some specific issues will have to be addressed. These include, but are not restricted to, containment of the plants including disposal of waste materials. It was noted that plant-derived vaccines have been produced and clinically tested under US investigational new drug application, and all applicable regulatory and good manufacturing practice requirements are in place for this type of product.'

This last point has been emphasised repeatedly by personnel from the USDA, the US Food and Drug Administration (FDA), the European EMEA and the Cuban regulatory authority, at conferences devoted to the plant production of antibodies and vaccines in Annecy, France, in 2004, and Prague, Czech Republic, in 2005 (personal observations).

Why, then, are there so few products in the regulatory pipeline? Kirk *et al.* [88] made a case for balancing the value of new or replacement vaccines produced in plants, against potential risks in their production and use, and the cost of not deploying this technology – in other words, the risk of continuing with the status quo. Further, Kirk and Webb [90] made the point that the use of plant-based technology for especially human vaccines requires significant investment – but that the research sector is not currently supported by big pharma to any significant degree. This is apparently the sticking point: whilst the potential may be huge, and many of the proofs of concept and even of efficacy that could be asked for have been done, there remains very significant investment in manufacturing plant and especially human trials and potentially in regulatory aspects before the potential can be realised – and big pharmaceutical companies already have plants that make vaccines, using accepted technologies, and see very little incentive to change. The industry is by nature conservative, as it has to balance big long-term investments against the relatively slim prospect of success for any given *conventional* product – which, incidentally, accounts for most of the price for any new vaccine upon introduction.

A possible factor in the observed reluctance of established industry to come to the plant party is that the field is the victim of its own enthusiasm: the hugely enthusiastic initial predictions for cheap or even free edible vaccines and pharmaceuticals were made as a result of both naivety and idealism, neither of which feature to any great extent in the commercial world. The reality is that edible vaccines are probably as far away now as they were in the 1990s, given the very slim likelihood that any will pass regulatory requirements any time soon, especially with the moratorium on the use of edible crop plants. This means that a significant degree of processing and standardisation of plant-produced antigens will have to be done, even for oral use, to satisfy requirements for regular composition and antigen content and stability and non-toxicity. This will present a significant cost factor, on top of the very much under-appreciated costs of packaging, distribution and marketing (see Fig. 3a). Thus, even though the cost of raw material may be very significantly reduced

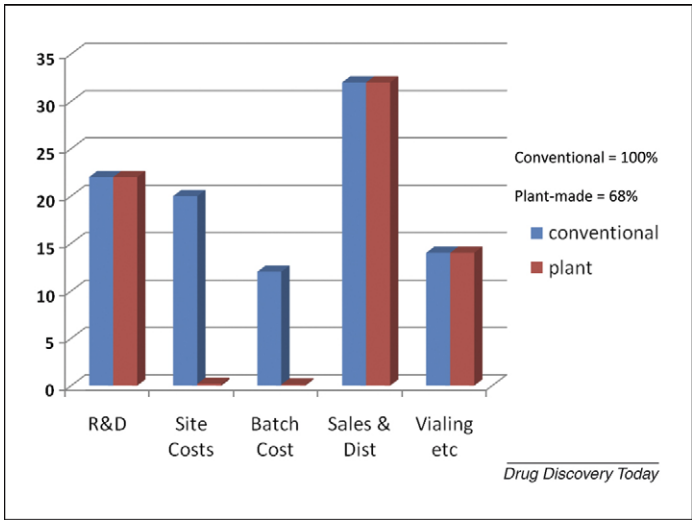

FIGURE 3

Probable effect of plant production of vaccine antigen compared to conventional production on the pricing of a vaccine. Bars represent the percentage contribution of the different components shown to the final wholesale price of a vaccine: R&D is the research & development costs; Site Costs is the production plant; Batch Cost is the cost of production of raw vaccine material; Sales & Dist is the sales and distribution; Vialing etc is the final vialing and labelling and packaging. Reduced components shown in red are the only probable savings owing to plant production (taken as 1% of conventional costs), as all other components are considered as fixed costs. Note that the total costing for plant production is 68% of the costing for conventional production. From the figures given by Dr Ian Gust, University of Melbourne, at the Plant-based Vaccines and Antibodies Conference, Prague, 2005.

compared to conventionally produced vaccine antigens – possibly as much as 1000-fold less than animal cell production, and 100-fold less than bacterial or yeast cell production [1] – downstream processing would be unchanged, as would all other costs. Thus, a 99% reduction in both batch and site costs would result in only a 31% reduction in price (Fig. 3b) – hardly the stuff of dreams. Moreover, it has been empirically shown that oral versus parenteral administration of the same antigen requires at least ten times as much material for oral dosing to achieve the same magnitude of immune responses [51]: this means that major cost savings need to be found elsewhere before any non-replicating oral vaccine becomes an economic reality – and may in fact not be possible at all for most antigens. It is possible, then, that the needle-free vaccine delivery vision for plant-produced vaccines should be tempered with reality – and that the first vaccines produced this way should be injectable products that are directly comparable with cell-culture-produced offerings.

Another under-appreciated reality in the world of plant-made vaccines is that there are very few facilities on the planet that can process bulk plant material to an acceptable degree of purity for human vaccine use. The best known is the facility built by Large Scale Biology Corp in Owensboro, KY, USA: this is now Kentucky Bioprocessing LLC, and is owned by Owensboro Medical Health System. The Fraunhofer Institut in Aachen, Germany, has another pilot plant; there is one being built at the Arizona State University's Biodesign Institute, and several smaller facilities in-house in various companies such as Icon Genetics, and Biolex and Protalix Therapeutics (see Plant Production of Foreign Proteins, above). This represents a severe bottleneck for any aspirant vaccine manufacturer, who would effectively have to duplicate one of the larger facilities just to process sufficient quantities of material to make enough vaccine for Phase IIB/III human trial.

The future

A cynical view of the future of plant-produced vaccines would be that there is not one – pretty much what Outcome 2 would be as shown in Fig. 1. A possibly more realistic view would be that they do have a future – albeit a less rosy and more circumscribed than the one that was hyped in the pre-2002 era, for Outcome 1. For a start, cheap edible vaccines are out, and – in the words of Charles J. Arntzen – 'We don't say 'edible' vaccine any more – we say 'heat-stable oral vaccines'.' [62]. There is widespread acceptance in the community that at least some degree of good manufacturing process (GMP) will be required for their vaccines; increasingly, researchers and developers are also coming to see that injectable vaccines may be no bad thing. Given that the first licenced pharmaceutical recombinant proteins products from plants are an antibody and a poultry vaccine, it is probable that more of the same will follow soon: the regulatory pathway is after all far shorter for products not intended for use in humans. In fact, the most easily realisable products for this technology are presently high-value proteins for use in diagnostics or as reagents for use in kits, because there would be no hurdles such as clinical trials to hinder commercialisation, and volumes would probably be far lower. For example, Farmacule BioIndustries in Queensland, Australia, has recently started producing the protein vitronectin in plants: the protein is presently derived from animal serum and currently costs up to US$5 million per gram – and Farmacule can produce up to a gram a month, enough to satisfy world demand [91]. Thus, the idealistic vision of a plant-provisioned arsenal of vaccines for poor people may still be far away: it may take commercial exploitation of the lower-hanging fruit to bring in both the production/processing base, and industry and public acceptance of the technology. It is encouraging, therefore, to see the recent news of a big tobacco company investing in a company – Medicago Inc., of Canada – which is using tobacco to produce seasonal and H5N1 influenza vaccines [92].

My vision of the future for these vaccines – corresponding to Outcome 1 shown in Fig. 1 – is the delivery in the next few years of an effective set of vaccines and therapeutics, produced in non-food plants, approved by FDA/USDA/EMEA, as being equivalent to similar products made by conventional means. I think these will be primarily for animal use initially – but that increased use and acceptance of these products, and demonstration of their safety, will lead to increased interest for human use. I also think that big pharma may not be involved at all at first, or only peripherally: I believe this will be driven by small companies and independent institutes, possibly with the involvement of the large philanthropic organisations such as the Bill and Melinda Gates Foundation, maybe via vehicles such as the Global HIV Vaccine Enterprise – or maybe even governments. Despite the fact that vaccines such as HBV have been so heavily targeted, it is improbable that these will be the first products: existing vaccines are already too cheap, are already licenced as generics and are already included in many countries in the Extended Programme of Immunisation (EPI) bundle which is provided free. It is more probable that new

versions of blockbuster products such as the new HPV or possibly rotavirus vaccines will be the first ones targeted, given their current very high pricing. After this may come the inexpensive heat-stable oral vaccines, possibly formulated on site as suspensions to be drunk under supervision. Finally, then, it may be possible to provide high-grade pharmaceutical products at very low-cost in resource-poor settings:

- Where there is a high burden of preventable disease.
- Where existing vaccines are expensive.
- Where there are no vaccines for 'orphan diseases'.
- Where antibody therapy could be very effective in treating disease.

I thank Anna-Lise Williamson, Charles Arntzen, Hugh Mason, Rainer Fischer, François Arcand, Kenneth Palmer and my research group past and present, for helping to try and achieve the dream.

Conflicts of interest

Research cooperation with Era Biotech, Spain.

Acknowledgements

I acknowledge the funding from the following South African sources: National Research Foundation, the South African AIDS Vaccine Initiative, the Poliomyelitis Research Foundation and the Medical Research Council.

References

1 Fischer, R. *et al.* (2004) Plant-based production of biopharmaceuticals. *Curr. Opin. Plant Biol.* 7, 152–158
2 Baulcombe, D. (2004) RNA silencing in plants. *Nature* 431, 356–363
3 Baulcombe, D. (2005) RNA silencing. *Trends Biochem. Sci.* 30, 290–293
4 Li, J.T. *et al.* (2006) Immunogenicity of a plant-derived edible rotavirus subunit vaccine transformed over fifty generations. *Virology* 356, 171–178
5 Biolex Therapeutics. Strengths of the LEX System. http://www.biolex.com/lexsystemstrengths.htm (2008)
6 Cox, K.M. *et al.* (2006) Glycan optimization of a human monoclonal antibody in the aquatic plant Lemna minor. *Nat. Biotechnol.* 24, 1591–1597
7 Horn, M.E. *et al.* (2003) Advantageous features of plant-based systems for the development of HIV vaccines. *J. Drug Target* 11, 539–545
8 Lamphear, B.J. *et al.* (2002) Delivery of subunit vaccines in maize seed. *J. Control Release* 85, 169–180
9 Ma, J.K. and Vine, N.D. (1999) Plant expression systems for the production of vaccines. *Curr. Top. Microbiol. Immunol.* 236, 275–292
10 Takaiwa, F. (2007) A rice-based edible vaccine expressing multiple T-cell epitopes to induce oral tolerance and inhibit allergy. *Immunol. Allergy Clin. North Am.* 27, 129–139
11 Shaaltiel, Y. *et al.* (2007) Production of glucocerebrosidase with terminal mannose glycans for enzyme replacement therapy of Gaucher's disease using a plant cell system. *Plant Biotechnol. J.* 5, 579–590
12 Daniell, H. *et al.* (2005) Chloroplast-derived vaccine antigens and other therapeutic proteins. *Vaccine* 23, 1779–1783
13 Daniell, H. (2006) Production of biopharmaceuticals and vaccines in plants via the chloroplast genome. *Biotechnol. J.* 1, 1071–1079
14 Turpen, T.H. *et al.* (1995) Malarial epitopes expressed on the surface of recombinant tobacco mosaic virus. *Biotechnology (N.Y.)* 13, 53–57
15 Durrani, Z. *et al.* (1998) Intranasal immunization with a plant virus expressing a peptide from HIV-1 gp41 stimulates better mucosal and systemic HIV-1-specific IgA and IgG than oral immunization. *J. Immunol. Methods* 220, 93–103
16 Gleba, Y. *et al.* (2007) Viral vectors for the expression of proteins in plants. *Curr. Opin. Biotechnol.* 18, 134–141
17 Yusibov, V. *et al.* (1999) Plant viral vectors based on tobamoviruses. *Curr. Top. Microbiol. Immunol.* 240, 81–94
18 Yusibov, V. *et al.* (2006) The potential of plant virus vectors for vaccine production. *Drugs R. D.* 7, 203–217
19 Choi, N.W. *et al.* (2005) Synthesis and assembly of a cholera toxin B subunit-rotavirus VP7 fusion protein in transgenic potato. *Mol. Biotechnol.* 31, 193–202
20 Companjen, A.R. *et al.* (2006) Improved uptake of plant-derived LTB-linked proteins in carp gut and induction of specific humoral immune responses upon infeed delivery. *Fish Shellfish Immunol.* 21, 251–260
21 Mett, V. *et al.* (2007) A plant-produced plague vaccine candidate confers protection to monkeys. *Vaccine* 25, 3014–3017
22 McCormick, A.A. and Palmer, K.E. (2008) Genetically engineered tobacco mosaic virus as nanoparticle vaccines. *Expert Rev. Vaccines* 7, 33–41
23 Fischer, R. *et al.* (1999) Towards molecular farming in the future: transient protein expression in plants. *Biotechnol. Appl. Biochem.* 30 (Pt 2), 113–116
24 Gleba, Y. *et al.* (2005) Magnifection – a new platform for expressing recombinant vaccines in plants. *Vaccine* 23, 2042–2048
25 Hefferon, K.L. and Fan, Y. (2004) Expression of a vaccine protein in a plant cell line using a geminivirus-based replicon system. *Vaccine* 23, 404–410
26 Palmer, K.E. and Rybicki, E.P. (2001) Investigation of the potential of maize streak virus to act as an infectious gene vector in maize plants. *Arch. Virol.* 146, 1089–1104
27 Hiatt, A. *et al.* (1989) Production of antibodies in transgenic plants. *Nature* 342, 76–78
28 Fischer, R. *et al.* (1999) Molecular farming of recombinant antibodies in plants. *Biol. Chem.* 380, 825–839
29 Fischer, R. *et al.* (1999) Towards molecular farming in the future: moving from diagnostic protein and antibody production in microbes to plants. *Biotechnol. Appl. Biochem.* 30 (Pt 2), 101–108
30 Mason, H.S. *et al.* (1992) Expression of hepatitis B surface antigen in transgenic plants. *Proc. Natl. Acad. Sci. U. S. A.* 89, 11745–11749
31 Haq, T.A. *et al.* (1995) Oral immunization with a recombinant bacterial antigen produced in transgenic plants. *Science* 268, 714–716
32 Ma, J.K. (1999) The caries vaccine: a growing prospect. *Dent. Update* 26, 374–380
33 Mason, H.S. *et al.* (1998) Edible vaccine protects mice against *Escherichia coli* heat-labile enterotoxin (LT): potatoes expressing a synthetic LT-B gene. *Vaccine* 16, 1336–1343
34 Arakawa, T. *et al.* (1998) Efficacy of a food plant-based oral cholera toxin B subunit vaccine. *Nat. Biotechnol.* 16, 292–297
35 Arakawa, T. *et al.* (1999) Food plant-delivered cholera toxin B subunit for vaccination and immunotolerization. *Adv. Exp. Med. Biol.* 464, 161–178
36 Kong, Q. *et al.* (2001) Oral immunization with hepatitis B surface antigen expressed in transgenic plants. *Proc. Natl. Acad. Sci. U. S. A.* 98, 11539–11544
37 Mason, H.S. *et al.* (1996) Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. *Proc. Natl. Acad. Sci. U. S. A.* 93, 5335–5340
38 Tacket, C.O. *et al.* (1998) Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. *Nat. Med.* 4, 607–609
39 Tacket, C.O. *et al.* (2000) Human immune responses to a novel norwalk virus vaccine delivered in transgenic potatoes. *J. Infect. Dis.* 182, 302–305
40 Dalsgaard, K. *et al.* (1997) Plant-derived vaccine protects target animals against a viral disease. *Nat. Biotechnol.* 15, 248–252
41 Castanon, S. *et al.* (1999) Immunization with potato plants expressing VP60 protein protects against rabbit hemorrhagic disease virus. *J. Virol.* 73, 4452–4455
42 Wigdorovitz, A. *et al.* (1999) Protection of mice against challenge with foot and mouth disease virus (FMDV) by immunization with foliar extracts from plants infected with recombinant tobacco mosaic virus expressing the FMDV structural protein VP1. *Virology* 264, 85–91
43 Wigdorovitz, A. *et al.* (1999) Induction of a protective antibody response to foot and mouth disease virus in mice following oral or parenteral immunization with alfalfa transgenic plants expressing the viral structural protein VP1. *Virology* 255, 347–353
44 Hathaway, L.J. and Kraehenbuhl, J.P. (2000) The role of M cells in mucosal immunity. *Cell Mol. Life Sci.* 57, 323–332
45 Ogra, P.L. *et al.* (2001) Vaccination strategies for mucosal immune responses. *Clin. Microbiol. Rev.* 14, 430–445
46 Czerkinsky, C. *et al.* (1999) Mucosal immunity and tolerance: relevance to vaccine development. *Immunol. Rev.* 170, 197–222
47 Mestecky, J. *et al.* (1997) Routes of immunization and antigen delivery systems for optimal mucosal immune responses in humans. *Behring Inst. Mitt.* 33–43
48 Zivny, J.H. *et al.* (2001) Mechanisms of immune tolerance to food antigens in humans. *Clin. Immunol.* 101, 158–168
49 APHIS. Noncompliance History. http://www.aphis.usda.gov/biotechnology/compliance_history.shtml (2008)
50 Rose, R.C. *et al.* (1999) Oral vaccination of mice with human papillomavirus virus-like particles induces systemic virus-neutralizing antibodies. *Vaccine* 17, 2129–2135

51 Gerber, S. *et al.* (2001) Human papillomavirus virus-like particles are efficient oral immunogens when coadministered with *Escherichia coli* heat-labile enterotoxin mutant R192G or CpG DNA. *J. Virol.* 75, 4752–4760

52 Biemelt, S. *et al.* (2003) Production of human papillomavirus type 16 virus-like particles in transgenic plants. *J. Virol.* 77, 9211–9220

53 Varsani, A. *et al.* (2003) Expression of Human papillomavirus type 16 major capsid protein in transgenic *Nicotiana tabacum* cv. Xanthi. *Arch. Virol.* 148, 1771–1786

54 Warzecha, H. *et al.* (2003) Oral immunogenicity of human papillomavirus-like particles expressed in potato. *J. Virol.* 77, 8702–8711

55 Webster, D.E. *et al.* (2002) Successful boosting of a DNA measles immunization with an oral plant-derived measles virus vaccine. *J. Virol.* 76, 7910–7912

56 Wu, Y.Z. *et al.* (2003) Oral immunization with rotavirus VP7 expressed in transgenic potatoes induced high titers of mucosal neutralizing IgA. *Virology* 313, 337–342

57 Huang, Z. and Mason, H.S. (2004) Conformational analysis of hepatitis B surface antigen fusions in an Agrobacterium-mediated transient expression system. *Plant Biotechnol. J.* 2, 241–249

58 Lamphear, B.J. *et al.* (2004) A corn-based delivery system for animal vaccines: an oral transmissible gastroenteritis virus vaccine boosts lactogenic immunity in swine. *Vaccine* 22, 2420–2424

59 Matoba, N. *et al.* (2004) A mucosally targeted subunit vaccine candidate eliciting HIV-1 transcytosis-blocking Abs. *Proc. Natl. Acad. Sci. U. S. A.* 101, 13584–13589

60 Tregoning, J.S. *et al.* (2005) Protection against tetanus toxin using a plant-based vaccine. *Eur. J. Immunol.* 35, 1320–1326

61 Chargelegue, D. *et al.* (2005) Highly immunogenic and protective recombinant vaccine candidate expressed in transgenic plants. *Infect. Immun.* 73, 5915–5922

62 A. Coghlan, Breaking News: Potato-based vaccine success comes too late, NewScientist. com news service (2005)

63 Thanavala, Y. *et al.* (2005) Immunogenicity in humans of an edible vaccine for hepatitis B. *Proc. Natl. Acad. Sci. U. S. A.* 102, 3378–3382

64 Giritch, A. *et al.* (2006) Rapid high-yield expression of full-size IgG antibodies in plants coinfected with noncompeting viral vectors. *Proc. Natl. Acad. Sci. U. S. A.* 103, 14701–14706

65 Santi, L. *et al.* (2006) Protection conferred by recombinant *Yersinia pestis* antigens produced by a rapid and highly scalable plant expression system. *Proc. Natl. Acad. Sci. U. S. A.* 103, 861–866

66 Golovkin, M. *et al.* (2007) Smallpox subunit vaccine produced in Planta confers protection in mice. *Proc. Natl. Acad. Sci. U. S. A.* 104, 6864–6869

67 Koya, V. *et al.* (2005) Plant-based vaccine: mice immunized with chloroplast-derived anthrax protective antigen survive anthrax lethal toxin challenge. *Infect. Immun.* 73, 8266–8274

68 Chebolu, S. and Daniell, H. (2007) Stable expression of Gal/GalNAc lectin of *Entamoeba histolytica* in transgenic chloroplasts and immunogenicity in mice towards vaccine development for amoebiasis. *Plant Biotechnol. J.* 5, 230–239

69 Hiroi, T. and Takaiwa, F. (2006) Peptide immunotherapy for allergic diseases using a rice-based edible vaccine. *Curr. Opin. Allergy Clin. Immunol.* 6, 455–460

70 Valenta, R. and Niederberger, V. (2007) Recombinant allergens for immunotherapy. *J. Allergy Clin. Immunol.* 119, 826–830

71 McCormick, A.A. *et al.* (1999) Rapid production of specific vaccines for lymphoma by expression of the tumor-derived single-chain Fv epitopes in tobacco plants. *Proc. Natl. Acad. Sci. U. S. A.* 96, 703–708

72 McCormick, A.A. *et al.* (2003) Individualized human scFv vaccines produced in plants: humoral anti-idiotype responses in vaccinated mice confirm relevance to the tumor Ig. *J. Immunol. Methods* 278, 95–104

73 McCormick, A.A. *et al.* (2008) Plant-produced idiotype vaccines for the treatment of non-Hodgkin's lymphoma: safety and immunogenicity in a phase I clinical study. *Proc. Natl. Acad. Sci. U. S. A.* 105, 10131–10136

74 Verch, T. *et al.* (2004) Immunization with a plant-produced colorectal cancer antigen. *Cancer Immunol. Immunother.* 53, 92–99

75 Franconi, R. *et al.* (2002) Plant-derived human papillomavirus 16 E7 oncoprotein induces immune response and specific tumor protection. *Cancer Res.* 62, 3654–3658

76 Franconi, R. *et al.* (2006) Exploiting the plant secretory pathway to improve the anticancer activity of a plant-derived HPV16 E7 vaccine. *Int. J. Immunopathol. Pharmacol.* 19, 187–197

77 Massa, S. *et al.* (2007) Anti-cancer activity of plant-produced HPV16 E7 vaccine. *Vaccine* 25, 3018–3021

78 Brodzik, R. *et al.* (2008) Plant-derived EpCAM antigen induces protective anti-cancer response. *Cancer Immunol. Immunother.* 57, 317–323

79 Pappalardo, J. Vaccine stockpiles now required by law. http://goliath.ecnext.com/coms2/gi_0199-775174/Vaccine-stockpiles-now-required-by.html (2004)

80 Palmer, K.E. *et al.* (2006) Protection of rabbits against cutaneous papillomavirus infection using recombinant tobacco mosaic virus containing L2 capsid epitopes. *Vaccine* 24, 5516–5525

81 Kohl, T. *et al.* (2006) Plant-produced cottontail rabbit papillomavirus L1 protein protects against tumor challenge: a proof-of-concept study. *Clin. Vaccine Immunol.* 13, 845–853

82 Kohl, T.O. *et al.* (2007) Expression of HPV-11 L1 protein in transgenic *Arabidopsis thaliana* and *Nicotiana tabacum*. *BMC Biotechnol.* 7, 56

83 Maclean, J. *et al.* (2007) Optimization of human papillomavirus type 16 (HPV-16) L1 expression in plants: comparison of the suitability of different HPV-16 L1 gene variants and different cell-compartment localization. *J. Gen. Virol.* 88, 1460–1469

84 Varsani, A. *et al.* (2006) Transient expression of human papillomavirus type 16 L1 protein in *Nicotiana benthamiana* using an infectious tobamovirus vector. *Virus Res.* 120, 91–96

85 Fernandez-San Millan, A. *et al.* (2008) Human papillomavirus L1 protein expressed in tobacco chloroplasts self-assembles into virus-like particles that are highly immunogenic. *Plant Biotechnol. J.* 6, 427–441

86 Pujol, M. *et al.* (2005) An integral approach towards a practical application for a plant-made monoclonal antibody in vaccine purification. *Vaccine* 23, 1833–1837

87 Dow AgroSciences. Animal Health FAQs No.11. http://www.dowagro.com/animalhealth/resources/faq.htm#faq11 (2008)

88 Kirk, D.D. *et al.* (2005) Risk analysis for plant-made vaccines. *Transgenic Res.* 14, 449–462

89 van der Laan, J.W. *et al.* (2006) WHO informal consultation on scientific basis for regulatory evaluation of candidate human vaccines from plants, Geneva, Switzerland, 24–25 January 2005. *Vaccine* 24, 4271–4278

90 Kirk, D.D. and Webb, S.R. (2005) The next 15 years: taking plant-made vaccines beyond proof of concept. *Immunol. Cell Biol.* 83, 248–256

91 O'Neill, G. Farmacule grows proteins in tobacco. http://www.biotechnews.com.au/index.php?id=33469691 (2005)

92 Medicago Inc. Product development. http://www.medicago.com/en/product/ (2008)