Overview of Plant-based Vaccines

1Salehe Naderi and 2Baratali Fakheri

ABSTRACT

Plants are reviewed with regard to their ability to express and produce vaccines. Advancements in the field of plant biotechnology have brought optimism that vaccines and therapeutics produced in plant systems may be able to address numerous animal and human disease issues. Plant-produced vaccines are a much-hyped development of the last 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. A great many products have shown significant immunogenicity; several have shown efficacy in target animals or in animal models. This review article includes historical development, promise vs reality, production, safety, efficacy and future of plant vaccines.

KEY WORDS: Plant vaccine, Biopharmaceutical, immunology, Transgenic plant

INTRODUCTION

According to WHO, various diseases are responsible for 80% of illness worldwide and cause more than 20 million deaths annually (Jogdand 2000). Vaccines represent an invaluable contribution in the field of biotechnology as they provide protection against various diseases. Conventional vaccines are made up of live/attenuated vaccine and killed vaccine. Newer approaches have also been made with regard to the use of purified antigen vaccines and recombinant vaccines. Despite the successful immunization program, the mortality rates are increasing every year (Land ridge 2000). This may be constraints on vaccine production, distribution and delivery. Moreover, the administration of vaccine is cost effective method of combating spread of infections and epidemics (Lal et al., 2007). So it is necessary to produce new vaccine that have economic and other advantages over the existing vaccines. An alternative approach is the use of edible vaccines. It has given new and dramatic hope for improved life. Edible vaccines are emerging innovations in medical science and plant biology for the efficacious and affordable pharmaceuticals (Morr et al., 1998; Daniell et al., 2001; Shah et al., 2011).

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 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 (Rybicki, 2009). Expression of recombinant proteins in plants has become a well-established practice in science and industry. Advances in recombinant DNA technology and plant cell transformation, allowing the introduction of novel genes into plants, has provided the basis for new technology to improve vaccination in poultry production. Plant-cell-produced vaccines, a new category of...
vaccines, have the potential to eliminate the risks associated with extraneous agents. They also are uniquely suited to produce subunit vaccines in a well-defined media and controlled environment, which introduce only the specific antigen, which is not live, and therefore cannot revert to virulence, shed or replicate in the poultry production environment (Mihaliak et al., 2005). Plant-based expression systems represent an interesting production platform due to their reduced manufacturing costs and high scalability. In addition, plants have the ability to generate complex recombinant proteins with desired structures, maintaining biological functions and offerings greater safety because plants do not harbor mammalian pathogens or microbial toxins (Davoodi-Semiromi et al., 2009; Seon et al., 2002; Goldstein & Thomas, 2004). In addition to their use as bioreactors, plants can be used as potential delivery systems for oral vaccines (Paul & Ma, 2010). In particular, plant tissues provide protection and prevent degradation of the antigen when it passes through the gut (Streatfield2006; Haydenet al., 2012). It has been demonstrated that plant-made vaccines applied to mucosal surfaces in the absence of adjuvant are able to induce a protective immune response, suggesting that some phytochemicals could synergistically affect the immunogenicity of plant-expressed antigens acting as endogenous adjuvants (Kapusta 1999; Soria-Guerra et al., 2011). In addition, plants are known for their natural immune-stimulating or antimicrobial activity due to secondary metabolites as lecithins, sapsins, alkaloids, phenolic compounds, and flavonoids (Granell, 2010). Moreover, some commonly occurring plant components, such as methylated Cog’s motifs of DNA, carotenoids and immunogenic proteins, have adjuvant properties (Wang et al., 2002; Liceardi & Underwood, 2011). Hence, it might be assumed that plants can be used in the short term as oral or injectable vaccine producers and as a source of endogenous adjuvants as well (Clementeant Corigliano, 2012).

**Historical development:**

The first vaccine-relevant protein produced in plants was in fact not an antigen, but an antibody: (Hiatt et al., 1989) produced monoclonal antibodies (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 manifold since: it appears that the one class of proteins that are reliably produced in plants, to reasonable yield, are immunoglobulins or Ig fragments (Fischer et al., 2000). 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 (Mason et al., 1992). The group showed that the plant-produced HBsAg formed 22 nm particles that were antigenic ally 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 (Rybicki, 2009). 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 antitoxin (LT-B) - that provided the first proof of principle for edible plant-produced vaccines. Haq et al. (1995) showed that LT-B produced in transgenic tobacco or potatoes appeared functionally equivalent to E coli-produced protein, and mice immunized by oral gavage produced systemic and mucosal neutralizing antibodies. Moreover, fresh potato containing LT-B was orally immunogenic in mice (Rybicki, 2009). 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 mutansadhesis protein produced in a transgenic plant could prevent microbial colonization in the human oral cavity (Ma, 1999). However, the first real proof of concept for orally-delivered plant-produced vaccines was by Mason et al. (1998), who demonstrated the efficacy of a potato-produced E coil-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 cholera enterotoxin subunit (CTB), in mice and in humans (Mason et al., 1996; Tacketet al., 1998; Arakawa et al., 1998; Tackett et al., 2000; Kongetal., 2001). 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 (1997), who showed that mink could be protected against disease caused by Mink enteritis virus (MEV) by subcutaneous injection of chimeric Cowpea mosaic virus (CPMV) visions expressing an MEV peptide on their surfaces. Cast anon et al. (1999) showed that parenteral immunization with potato-produced VP60 protein protected rabbits against infection Rabbit hemorrhagic disease virus (RHDV), and Wigdorovitz et al. (1999a; 1999b) later demonstrated that oral immunization or injection of mice with Foot and mouth disease virus (FMDV) VP1 coat protein precursor polyprotein derived either from transgenic alfalfa or from Nicotine benthamianaplans transiently infected with recombinant Tobacco mosaic virus (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 immunization using only crude extracts (Rybicki, 2009). 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) see (Hathaway & Kraehenbuhl, 2000; Ogra et al., 2001). In particular, the concept of oral vaccination
with non-replicating or subunit vaccines was being vigorously explored, as were the mysteries of “tolerisation”, or induction of immune tolerance at mucosal surfaces (Mestecky et al., 1997; Czerkinsky et al., 1999; Zivnyet al., 2001). 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 endeavors (Rybicki, 2009). 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 outnumbered the scientific articles describing new developments in the field: Rybicki (2009) said that in my database in 2000 alone there were 11 reviews among 20 papers dealing mainly with vaccines from plants (Rybicki, 2009). 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 (Rybicki, 2009). Efforts to produce viable vaccines continued, however, and a number of landmark advances have occurred in the last six years despite the decreasing public interest. One important one was the demonstration by Rose et al. (1999) and Gerber et al. (2001) 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 Chg.-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 (Varsani et al., 2003; Biemelt et al., 2003; Warzecha et al., 2003). The first group tested only parenteral immunization of mice; the latter two both attempted immunization 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 in by Merck in yeast (Gardasil™) and in insect cells via recombinant baculovirus infection by GlaxoSmithKline (Cervarix™) (Rybicki, 2009). Other salient advances in recent years include the use of a partially-purified tobacco-produced measles virus haem agglutinin as an oral boost to a DNA vaccine in mice, with the production of neutralizing antibodies (Webster et al., 2002); the induction of high titres of mucosal neutralizing IgA in mice by oral immunization with rotavirus VP7 in transgenic potatoes (Wuet et al., 2003), 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 (Huang & Mason, 2004); the proof that porcine TGEV capsid protein in maize seeds can orally boost lactogenic immunity in swine (Lamphear et al., 2004), the use of a plant-produced HIV gp41-CTB fusion protein to elicit transcytosis-blocking antibodies in intranasally-primed and intraperitoneal-boosted mice (Matoba et al., 2004), and the protection of mice against tetanus toxin by a single intranasal dose of soluble transgenic tobacco leaf protein (Tregoning et al., 2005). A novel strategy described by Chargeleugue et al. (2005) involved the expression of a fusion between a Moab 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 (Rybicki, 2009). In what was probably - in retrospect - the swansong of classical edible vaccines (Coghlan, 2005), Thanavala et al. (2005) described the use of raw potatoes expressing Haig to orally boost preexisting immunity in conventionally-immunized human volunteers: while 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 (Rybicki, 2009). “Magnification”, 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 (Gleba et al., 2005). 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 (Giritch et al., 2006), and of Yersinia pestisantigens F1, V, and fusion protein F1-V, which protected guinea pigs against aerosol challenge with virulent Y. pestis. They followed this with the report of intramuscular vaccination of mice and minify with magnification-derived Vaccine virus B5 protein-derived antigen and subsequent protection against lethal challenge doses of vaccine (Golovkin et al., 2007). The use of chloroplasts to express antigens received a boost with the demonstrations that chloroplast-produced anthrax protective antigen were protected against lethal toxin challenge (Koay et al., 2005), and that Gal/Galan lecition of Ent amoeba histolytic was highly immunogenic in mice (Chebolu & Daniell, 2007). The latter represents a true “orphan disease” vaccine: E histolyticainfacts50 million people annually, causing about 100 000 deaths (Rybicki, 2009). 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 (Hiroi & Takaiwa, 2006; Takaiwa, 2007). This and other important work in allergy therapy involving recombinant antigens was reviewed by Valenta & Niederberger (2007). 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 Bio source 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 (McCormick et al., 1999). These proteins were shown to induce appropriate anti-idiotype humoral responses in mice (McCormick et al., 2003), 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 6 times each (Rybicki, 2009) 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 (Verchet et al., 2004). Papillomavirus-induced disease has been a popular target with a number of studies targeting HPV oncogenic proteins: Franconia et al. (2002; 2006) and Massa et al. (2007) report that immunization with plant-produced antigen protected of mice from tumor challenge with an E7-expressing tumor cell line, and in the latter two cases, that tumor regression could also be seen. A plant-derived tumor-associated colorectal cancer antigen Encamp (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 (Brodziket al., 2008). Sera from immunized Balb/C mice efficiently inhibited growth of SW948 colorectal carcinoma cells engrafted in nude mice, compared to a EpCAM-specific mAb(Rybicki, 2009).One of the newest hype factors around plant-produced vaccines has been in their supposed potential for rapid-response vaccines for bio threat 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 hemorrhagic fever viruses (Santiet al., 2006; Mettet al., 2007). Indeed, as of July 2004, legislation dubbed “Bio Shield” provides US industry with incentives to research and develop bioterrorism countermeasures, including vaccines, and speeds the approval process (Pappalardo, 2004).A useful historical case study in the evolution of the potential of plant-produced vaccines is provided by papillomaviruses. In 1997, Rybicki’s laboratory first began investigating 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 immunized several times with thousand-fold concentrated sap extracts developed low titers of IgG against baculovirus-produced L1 (A Varsani, M Gehringer, A-L Williamson, EP Rybicki, unpublished). By 2003 the situation had improved, with HPV-16 L1 yields up to the tens of milligrams per kg of transgenic plant (Biemelt et al., 2003), 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 chimeric TMV particles carrying a CRPV L2 peptide (Palmer et al., 2006), or partially-purified extracts containing whole CRPV L1 protein, produced either in transgenic tobacco or via recombinant TMV (Kohlet al., 2006). 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 up/kg (Varsani et al., 2003), while native CRPV L1 expressed up to 1mg/kg (Kohl et al., 2006), and HPV-11 L1 expressed at 11 mg/kg (Kohlet al., 2007). A significant breakthrough occurred in 2007, nuclear transformation - whether transient or stable - and chloroplastic rather than cytoplasmic or ER localization of a human, and not a plant codon-optimized 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 (Varsaniet al., 2006). This was also an object lesson in the necessity for empirical valuation of a number of parameters, including intracellular targeting and codon optimization, for maximization of expression (Figure 1). This protein was also the first plant-produced papillomavirus L1 to be shown to elicit high-titer neutralizing antibodies - the gold standard of for a HPV vaccine candidate. The production record for HPV L1 protein has now been claimed by Fernandez-San Ricky (2009), 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 optimization must be empirically determined rather than predicted, and so should intracellular targeting.

Optimization of expression of Human papillomavirus type 16 L1 protein in tobacco by codon optimization and intracellular targeting. The vertical bars represent amounts of L1 protein in mg per kg 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 = expression from wild-type L1 gene; PL = expression from plant codon-optimized gene; HUM = expression from human codon optimised gene; plant control = normal plant extract. Cytoplasm = no targeting signal in expression construct; ER = endoplasmic reticulum targeting and retention; chloroplast = chloroplast import signal included. Modified from (Rybicki, 2009).

Promise vs 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 parenteral-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 last nineteen years. Big companies are also entering the fray: in 2006 Bayer Innovation GmbH bought out Icon Genetics, whose Magnification technology has increasingly been seen as the front-runner in viable plant expression systems; Dow Agro Sciences made a surprise entry into the field with a new Newcastle disease virus vaccine (Rybicki, 2009).

Fig. 1:

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 (Pujol et al., 2005): this is produced in transgenic tobacco, and completely replaces about the ~300 000 mice formerly used per year to produce mAbs via ascetic fluid (C Borroto, personal communication). The second - in January 2006 - was a Newcastle disease virus (NDV) vaccine for poultry, produced in a suspension-cultured tobacco cell line by Dow Agro Sciences, and successfully tested as a purified injectable product in chickens (Dow AgroSciences, 2008). 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 ConcertTMPlant-Cell-Produced system is capable of producing a vaccine that is safe and effective and to demonstrate that it meets the requirements for approval under the rigorous USDA regulatory system. NDV is well known and understood by the regulatory agency, so it served as an excellent model to prove this new technology (Rybicki, 2009).

It is not as if the regulatory environment has not been explored. For example, Kirk et al. (2005) discussed the risk analysis for plant-made vaccines, and concluded that “Risks to human health include oral tolerance, allergen city, 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 Organization (WHO) convened an expert panel in 2005 to discuss the scientific basis for regulatory evaluation of candidate human vaccines from plants (van der Laanet al., 2005) - 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”(Rybicki, 2009).

This last point has been emphasized 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 (Rybicki, 2009).

Why, then, are there so few products in the regulatory pipeline? Kirk et al. (2005) make 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 (2005) make the point that 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: while 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 (Rybicki, 2009).

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 standardization of plant-produced antigens will have to be done, even for oral use, in order 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 (Figure 2). Thus, even though the cost of raw material may be very significantly reduced 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 (Fischer et al., 2004) 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 (Figure 2) - 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 in order to achieve the same magnitude of immune responses (Gerber et al., 2001): 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.

Fig. 2:

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 = research & development costs; Site Costs = production plant; Batch Cost = cost of production of raw vaccine material; Sales & Dist. = sales and distribution; Vialing etc. = final vialing and labelling and packaging. Reduced components shown in red are the only probably savings due 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 figures given by Dr Ian Gust, University of Melbourne, at the Plant based Vaccines and Antibodies Conference, Prague, 2005 (Rybicki, 2009).

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 Institute in Aachen, Germany, has another pilot plant; there is one being built at the Arizona State University’s Bio design Institute and a number of smaller facilities in-house in various companies such as Icon Genetics. This represents a severe bottleneck for any aspirant vaccine manufacturer, who would effectively have to duplicate one of the larger facilities in order just to process sufficient quantities of material to make enough vaccine for Phase II/III human trial (Rybicki, 2009).

Production, safety and efficacy:

Drug research is a unique multi-disciplinary process leading to the development of novel therapeutic agents for disease states that have unmet needs (Panchagnula et al., 2000). The search for new biopharmaceuticals is
driven by a medical need and by the perceived likelihood of technological success, as determined by both therapeutic efficacy and safety parameters. There are several factors to consider for the safety testing of a new biopharmaceuticals (Thomas et al., 1995). Because of the protein nature of most biopharmaceutical products; few non-allergic adverse reactions other than those attributable to the primary pharmacological activity are anticipated. Nevertheless, both Good Laboratory Practice and Good Manufacturing Practice, as established for other modes of pharmaceutical production, are essential to plant made pharmaceuticals. Before experimental or clinical use is initiated, it is critical to have fully-characterized, contaminant-free materials, as well as appropriate quality assurance so that both the product itself and the therapeutic results will be reproducible. New pharmaceutical agents derived through plant biotechnology must be subjected to the same purity, quality-control, and safety standards as materials derived from bacterial or mammalian cell systems or from other traditional sources such as vaccine production. Sites used for the cultivation of genetically modified plants have in some cases been disrupted or destroyed by individuals opposed to the use of plant biotechnology, raising additional security concerns. In part, these concerns can be addressed via increased field site monitoring and security, and the use of enclosed environments (greenhouses) for small scale operations. The relatively small scale and favorable economics of biopharmaceutical operations allows the placement of field operations in geopolitical locations selected for optimal security, with subsequent shipping of raw or processed materials. Transgenic plants have the added safety feature of freedom from human or animal pathogens (Ma & Drake, 2003). Additionally, plant cells are capable of producing complex proteins while largely avoiding the presence of endotoxins in bacterial systems. Endotoxins are often difficult to remove and can contaminate final product. Thus, there is intrinsic safety and value in using plants as a source of recombinant protein (Fischer et al., 2000). However, as with all plant-derived pharmaceuticals, appropriate measures must be taken to eliminate undesirable plant-derived proteins or other biomolecules and to control the presence of fungal toxins or of pesticides used in plant production (Russell, 1999). Safety evaluations must consider possible no target organ responses as well as the entire gamut of anticipated and unanticipated side-effects as with any biopharmaceutical product. Somewhat unique to plant-produced pharmaceuticals are potential effects on non-target species such as butterflies, honeybee, and other wildlife at or near the growing sites. Fortunately, in most instances, the effect on non-target species is limited by the fact that proteins are a normal part of the diet; are readily digested, and are degraded in the environment. Further, many biopharmaceuticals proteins, especially antibodies, are highly species-specific in their effects. Pharmaceutical production in plants may create the potential for the flow of pharmaceutical materials into the human food chain, especially when food crops are used. This could occur as a result of inadvertent cross-contamination of foodstuffs, through spontaneous growth of genetically engineered plants where they are not desired, or by virtue of pollen flow with some plants (e.g. corn), but not others (e.g. potato). While some have therefore suggested restricting pharmaceutical production to non-food crops such as tobacco, it is the food crops that present the greatest opportunities for efficient production of biopharmaceuticals and that will be most useful for the production of edible vaccines. Because of the potential for adventitious presence in food, care must be exercised in the production of biopharmaceuticals in food crops. Fortunately, acreage requirements for pharmaceutical production are limited, with metric ton protein production being feasible with >5000 acres of corn (Humphreys & Glover, 2001). This allows for production under tightly controlled conditions which include production in areas of the country where the crop in question is not routinely grown, the use of physical isolation distances and temporal separation to prevent cross-pollination with food crops, the use of de-tasseling and/or male-sterile traits to control pollen flow, dedicated harvest and storage equipment, and controlled processing separate from all food crops. Unlike commodity crops, plant production of pharmaceuticals should be performed only under tightly controlled conditions similar to those of other pharmaceutical manufacturing; and production standards have been developed jointly by industry, USDA, FDA, and international organizations (BIO, 2003). These standards are enforced in thus through USDA and FDA, and compliance is further encouraged by the desire of producers to avoid potential liability and infractions. FDA required Good Manufacturing Practice necessitates extensive control of field access, harvest, and product disposition. While production controls are necessary and appropriate, it should be kept in mind that the majority of therapeutic proteins are not anticipated to have any pharmacological activity when ingested, and are thus unlikely to present a safety issue in the event of accidental contamination of foodstuffs. For example, antibodies, insulin, growth hormone, and most other proteins produce few, if any, systemic pharmacological affects by oral route. This does not preclude the possibility of local effects on the gastro-intestinal tract or the possibility of immunological effects, as seen in the context of oral vaccines, where such an effects introduced by design. In fact, one plant-derived antibody directed against epithelial cellular adhesion molecules was withdrawn from clinical development as a result of gastro-intestinal side-effects believed to be due to binding to the relevant antigen, which is expressed in the GI tract (Ma & Drake, 2003). This is a result of the antigenic specificity of the antibody, and is not attributable to the plant-derived nature of the molecule. While a case-by-case determination of risk will be necessary when considering proteins for food crop applications, it appears that the majority of proteins would present no great hazard to the public in the event that control technologies should fail to be fully effective (Goldstein and Thomas, 2004).
**Vaccines:**

There has been considerable interest in developing low-cost, edible (i.e., oral) vaccines. Traditional edible vaccines, as for polio, use whole, attenuated organisms or semi-purified materials to induce both systemic (Ig-G-mediated) and local membrane (Ig-A-mediated) immunity. Plant vaccines can express entire selected proteins, but the use of DNA encoding only desired antigenic sequences from pathogenic viruses, bacteria and parasites has received considerable attention (Giddings et al., 2000). Key immunogenic proteins or antigenic sequences can be synthesized in plant tissues and subsequently ingested as edible subunit vaccines (Korban et al., 2002; Tacket & Mason, 1992; Mahon et al., 1998). The mucosal immune system can induce protective immune responses against pathogens or toxins, and may also be useful to induce tolerance to ingested or inhaled antigens (Mason, 1999; Korban et al., 2002). The production of secretory Ig-A (sIg-A) and provocation of specific immune lymphocytes can occur in mucosal regions, and these regions take on special importance in the development of edible vaccines. Aside from intrinsic low production cost, plant-based vaccines offer a number of unique advantages, including increased safety, stability, versatility, and efficacy (Streatfield et al., 2001). Plant produced vaccines can be grown locally where needed, avoiding storage and transportation costs. Relevant antigens are naturally stored in plant tissue, and oral vaccines can be effectively administered directly in the food product in which they are grown, eliminating purification costs (Streatfield et al., 2001; Korban et al., 2002). In many instances, it appears that refrigeration will not be needed to preserve vaccine efficacy, removing major impediment to international vaccination efforts of the past (Mahon et al., 1998; Korban et al., 2002). Plants engineered to express only a select antigenic portions of the relevant pathogen may reduce immunotoxicity and other adverse effects, and plant-derived vaccines are free of contamination with mammalian viruses. Finally, the development of multi-component vaccines is possible by insertion of multiple genetic elements or through cross-breeding of transgenic lines expressing antigens from various pathogenic organisms. There are, however, some limitations associated with the use of transgenic plants for vaccine production (Chargelegue et al., 2001). A major limitation of the expression of recombinant antigens in transgenic plants is obtaining a protein concentration adequate to confer total immunity, given varying protein expression among and within the various plant species. Tight control of expression yields will likely be necessary to reduce variability and assure consistent, effective immunization (Chargelegue et al., 2001).

**Table 1:** Recombinant vaccines expressed in plants.

<table>
<thead>
<tr>
<th>Year</th>
<th>Vaccine antigen</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1992</td>
<td>Hepatitis B surface antigen</td>
<td>Tobacco</td>
</tr>
<tr>
<td>1995</td>
<td>Malaria parasite antigen</td>
<td>Virus particle*</td>
</tr>
<tr>
<td>1995</td>
<td>Rabies virus glycoprotein</td>
<td>Tomato</td>
</tr>
<tr>
<td>1995</td>
<td>Escherichia coli heat-labile</td>
<td>Tomato</td>
</tr>
<tr>
<td>1996</td>
<td>Human rhinovirus 14 (HRV-14) and human immunodeficiency virus type (HIV-1) epitopes</td>
<td>Virus particle*</td>
</tr>
<tr>
<td>1996</td>
<td>Norwalk virus capsid protein</td>
<td>Tobacco</td>
</tr>
<tr>
<td>1997</td>
<td>Diabetes-associated auto antigen</td>
<td>Tomato</td>
</tr>
<tr>
<td>1997</td>
<td>Hepatitis B surface proteins</td>
<td>Potato</td>
</tr>
<tr>
<td>1997</td>
<td>Mink Enteritis Virus epitope</td>
<td>Virus particle*</td>
</tr>
<tr>
<td>1997</td>
<td>Rabies and HIV epitopes</td>
<td>Virus particle*</td>
</tr>
<tr>
<td>1998</td>
<td>Foot and mouth disease virus VP1 structural protein</td>
<td>Arabidopsis</td>
</tr>
<tr>
<td>1998</td>
<td>Escherichia coli heat-labile enterotoxin</td>
<td>Potato</td>
</tr>
<tr>
<td>1998</td>
<td>Escherichia coli heat-labile enterotoxin</td>
<td>Potato</td>
</tr>
<tr>
<td>1998</td>
<td>Rabies virus</td>
<td>Virus particle*</td>
</tr>
<tr>
<td>1998</td>
<td>Cholera toxin B subunit</td>
<td>Potato</td>
</tr>
<tr>
<td>1998</td>
<td>Human insulin-Cholera toxin B subunit fusion protein</td>
<td>Potato</td>
</tr>
<tr>
<td>1999</td>
<td>Foot and mouth disease virus VP1 structural protein</td>
<td>Alfalfa</td>
</tr>
<tr>
<td>1999</td>
<td>Hepatitis B virus surface antigen</td>
<td>Yellow lupin, lettuce</td>
</tr>
<tr>
<td>1999</td>
<td>Human cytomegalovirus glycoprotein B</td>
<td>Tobacco</td>
</tr>
<tr>
<td>1999</td>
<td>Dental caries (S. mutans)</td>
<td>Tobacco</td>
</tr>
<tr>
<td>1999</td>
<td>Diabetes-associated auto antigen</td>
<td>Tobacco</td>
</tr>
<tr>
<td>2002</td>
<td>Respiratory syncytial virus</td>
<td>Tobacco</td>
</tr>
</tbody>
</table>

*Plant virus—can be expressed in multiple plant species.
Modified from (Goldstein and Thomas, 2004)
During the last decade, nearly a dozen vaccine antigens have been expressed in plants (Table 1) (Fischer & Means 2000). Transgenic potatoes can produce antigens of enterotoxigenic E. coli heat labile enterotoxin B subunit, and is effective in immunizing against viruses and bacteria that cause diarrhea. Still other “edible vaccines” are under development for rabies, foot and mouth disease (veterinary), cholera, and autoimmune diabetes. Transgenic lupine and lettuce plants can express hepatitis B surface antigen. Efforts are underway to develop an “edible vaccine” against the measles virus using the tobacco plant. A plant based oral subunit vaccine for the respiratory syncytial virus (RSV) using either the apple or the tomato is under development (Korban et al., 2002). The plant species to be used for the production and delivery of an oral vaccine can be specifically selected to achieve desired goals. A large number of food plants (e.g. alfalfa, apple, asparagus, banana, barley, cabbage, canola, cantaloupe, carrots, cauliflower, cranberry, cucumber, eggplant, flax, grape, kiwi, lettuce, lupines, maize, melon, papaya, pea, peanut, pepper, plum, potato, raspberry, rice, service berry, soybean, squash, strawberry, sugar beet, sugarcane, sunflower, sweet potato, tomato, walnut, and wheat) have been transformed (Richter & Kipp, 1999). Many of the high volume, high acreage plants such as corn, soybeans, rice, and wheat may offer advantages. Corn, since it is a major component in the diet of the domestic animal, is a good candidate for vaccine production. In humans, particularly infants, the plant of choice to produce the vaccine might be the banana. Bananas are a common component of many infant diets and can be consumed uncooked, thus eliminating the possibility of protein denaturation due to high temperatures. Unfortunately, it is relatively difficult to create transgenic bananas and the production time is longer than for certain other food crops. Cereals and other edible plants are advantageous for vaccine production over plant species such as tobacco because of the lower levels of toxic metabolites. It is evident that there are numerous opportunities to identify and develop low-cost plant derived vaccine materials, including edible plant-based vaccines (Goldstein and Thomas, 2004).

**Opportunities:**

Oral vaccines are potentially applicable to any vaccine formulation based on or including a subunit component. Theorell delivery of a subunit vaccine is particularly suited to protect against pathogens that infect via the intestinal surface, such as diarrhea causing agents. In addition, because of the linked nature of the mucosal immune system, these vaccines are very relevant to combating pathogens that infect other mucosal surfaces, prominent examples being hepatitis B and HIV. Early studies indicate that oral vaccines may also be viable options to combat pathogens that typically invade via the circulatory system, such as rabies (Modelska et al., 1998). Plants allow for the rapid bulk up of large supplies of subunit vaccines, for example a 40,000-fold increase per year is possible using corn. Therefore, oral vaccines are particularly applicable to combating diseases that affect very large populations. Furthermore, oral plant-based vaccines are stable during storage at ambient temperatures (Lamphear, et al., 2002) and do not require syringes, needles and trained personnel for administration. These features also favour the use of oral vaccines for large-scale immunization programmers, particularly in developing countries with limited resources to provide a cold chain and the equipment and personnel needed for injections. This ability to stackpile plant-based vaccines without expensive refrigerated storage and administer them without injections also favors the development of such vaccines to protect against sudden outbreaks of disease in developed countries as a result of, for example, terrorist actions. Perhaps, most significantly, the low cost of plant-based vaccines make them ideal for large-scale programmers in developing countries. Inexpensive raw material and processing costs together with the absence of a cold chain and reduced administration costs should serve to make oral plant-based vaccines accessible worldwide. The advantages listed above also favor the use of oral vaccines in veterinary medicine to combat pathogens of domestic animals, particularly large herds of farmed animals. The development of an oral vaccine against transmissible gastroenteritis virus for swine is one such example (Stratfield et al., 2001; Lamphear et al., 2002). An additional advantage of oral vaccines for livestock animal applications is that the carcass is not damaged by injections. Inexpensive vaccination programmers using oral plant vaccines for domestic animals could also combat human disease agents such as enterohemorrhagic E. Coli which infect cattle and can spread to humans through poorly processed meat. Oral plant vaccines may also allow the practical and inexpensive immunization of wild animal populations, which may act as reservoirs for disease, as with rabies. Most likely, the first application of oral plant vaccines will be in the area of animal health, since, the trials to demonstrate safety and efficacy are much shorter than for human vaccines. They may also demonstrate their value in combination with established components, just as the B subunit of cholera toxin produced in recombinant bacteria has been tested in combination with chemically killed strains (Qadri et al., 2000). Alternatively, plant vaccines may first be used as a single oral component in immunisation course also involving injections, for example, as a hepatitis B booster vaccine, where it may serve to widen compliance or as a measles vaccine to protect infants prior to the time at which it is efficacious to administer the established live attenuated vaccine. Ultimately, oral plant combination vaccines should be developed that can protect against multiple pathogens. Furthermore, their inexpensive costs of production and administration advantages make oral vaccines excellent candidates for...
situations where many doses are needed for protection, as may be the case with HIV (Streatfield and Howard, 2003).

**Unintended consequences:**

While there are many potential advantages for plant made vaccines, there also exist the potential for unintended consequences. The two most frequently cited are the potential to induce tolerance and the potential for the vaccines to inadvertently enter the food chain. Tolerance to specific antigens can be induced through repeated exposures regardless of how the antigen is administered (Challacombe and Tomasi, 1980; Tomasi, 1980). Oral tolerance mechanisms have been studied as they relate to food allergens and autoimmune diseases (Weiner, 1997; Hachiman, 2000; Stanley, 2002; Strobel, 2002). There is no reason to believe that plant-based vaccines will be any different in respect to inducing tolerance compared to vaccines produced in other systems. However, all vaccines undergo extensive testing, with oversight from regulatory agencies, to define the correct dosage and the appropriate schedule of boosting. Therefore, tolerance from prescribed doses is highly unlikely. Plant-based vaccines do have the potential to induce tolerance if the vaccine inadvertently enters the food chain and repeated exposures are experienced without our knowledge. Currently, many vaccines are made in animals, yeast and eggs. These production systems are also used to produce food. The reason we do not see vaccines currently in our food supply is that in generating vaccines, a completely different production system is followed. There exists an array of procedures to ensure that the vaccines are contained throughout the entire production phase with regulatory oversight. These procedures ensure the safety of commodity food, and at the same time, the quality of vaccines. This situation is not unlike that proposed for the use of plant-based vaccines. If the plants were grown as commodity crops, then there would be potential for exposure leading to tolerance. However, plant-based vaccines are grown under contained conditions with regulatory oversight, similar to vaccines produced in other production systems. Plant-based vaccines represent only a very small percentage of any total food crop (~0.1%) and would be produced in a closed loop system, thereby, keeping them separate from the food chain. While it is unlikely that there would be any vaccine in a food crop, we cannot set a zero tolerance standard, which is both theoretically and practically impossible. We can, however, set a limit for exposure in food crops based on safety models. Risk models can be used to show that the amount of vaccine that could potentially end up in a food crop would be orders of magnitude lower than that shown to be needed to induce oral tolerance, if, in some rare case, an individual is exposed (Streatfield and Howard, 2003).

**Challenges for the technology:**

At the Plant Made Pharmaceutical Conference held in Montreal, Canada in 2005, the potential limitation of plant-made vaccines was discussed. While the focus was on orally delivered vaccines targeted for human health, many of the same conclusions are similar for animal health vaccines (Arntzen et al., 2005). There are clear potential benefits of oral delivery of plant-made vaccines; however, there are still questions of expression levels and efficacy in general. For example, will adjuvants be required to obtain the desired immune response or will VLPs be a major platform due to their expanded immune regulatory potential? Regulatory approval for a plant-made vaccine has not yet been achieved and, for some production systems, remains uncharted (Arntzen et al., 2005). In fact, for field-grown materials, multiple requirements will be demanded, both for efficacy of the products and proper stewardship and containment of the plants. To our knowledge, no animal health product derived from field-produced plants has been advanced significantly toward product licensure. Additionally, dependent on the type of system utilized to express the vaccine antigen, the cost of registration and the potential cost of containment can have a significant impact on the commercial viability of the end product. One issue of oral delivery of transgenic proteins that is commonly discussed is the potential for oral tolerance. Infect, oral delivery of auto-antigens may lead to oral tolerance and reversal or reduction of autoimmunity (Arakawa et al., 1998; Carter and Lang ridge, 2002; Snowden and Lang ridge, 2003; Meat al., 2004). These reports involve animal models and have yet to be fully evaluated in the target host. In the research that has focused on oral delivery of plant-expressed antigens as vaccines, there have been no examples of oral tolerance reported (Arntzen et al., 2005). The difference in biological outcome of oral administration of transgenic proteins may be dependent on the specific regimen used for the treatment. For full adoption of a vaccine dependent on oral delivery, optimization of vaccine delivery and a more thorough understanding of the immune response to vaccination will be necessary (Rice et al., 2005).

**Future directions:**

The use of plants as factories for the production of novel vaccines, antibodies and other therapeutic proteins will undoubtedly continue to develop. Molecular farming may become the premier expression system for a wide variety of new biopharmaceuticals and 'plant bodies’. Important economic advantages will likely be realized as the technology continues to evolve and improve. Efforts will need to focus on increasing yields, on scale-up of production, on distribution and handling of transgenic plant material, and on the development and validation of production techniques which effectively isolate pharmaceutical production from human and animal food. Plant-
derived biopharmaceuticals will need to meet the same safety and efficacy standards as those products obtained from non-plant sources. There will be a need for continued vigilance to safeguard the environment, ensuring that errant substances do not affect non-target organisms. Gene containment methodologies will continue to develop, and there must be safeguards against the over-expression of potentially harmful proteins in transgenic pollen. Undoubtedly, there will be a continuing debate about the use of transgenic food plants, as opposed to non-food plants, for producing new pharmaceuticals. The advantages of recombinant plant DNA technology for the production of antibodies, vaccines, other pharmaceuticals, and even high-volume plasma proteins are becoming increasingly apparent. As the technology involves, it appears highly likely that plant-derived pharmaceuticals will play a significant role in the future of clinical therapeutics.

REFERENCES


