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Plant-made immunogens and effective delivery strategies

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Plant systems for the production of recombinant immunogens have the potential to overcome obstacles currently impeding the delivery of vaccines to poorer, more remote populations by localizing production and reducing associated costs. The nature of the future plant-derived vaccine candidates will have an important impact on the extent to which universal access to vaccines can be achieved using these technologies. In this article, we examine approaches taken to design immunogens, expression systems and delivery strategies that are medically feasible and immunologically effective while retaining key benefits of a plant production platform. We identify three 'target areas' in which plant-made immunogens may offer particular advantages over conventional production systems.

KEYWORDS: adjuvant • molecular pharming • plant-made pharmaceuticals • PMP • post-translational modification • vaccine • virus-like particles • VLP

Plant-made immunogens

There is a pronounced need for vaccines to combat prevalent infectious diseases that currently account for more than 25% of deaths worldwide [1]. Many of these deaths occur in countries with poor infrastructure and low income per capita, factors that can challenge traditional vaccine production and delivery concepts. Transgenic plants represent an attractive production platform, as they offer reduced manufacturing costs and the potential for local production through established agricultural methods and technology transfer practice [2,3].

Transgenic plants have been used as bioreactors for the production of many proteins derived from diverse sources (recently reviewed in [4,5]). Within the plant cell, transgenes can be expressed either from the nucleus via the cytoplasm or endomembrane system, from the genome of the plastids, or (in theory) from the mitochondria. The vast majority of transplastomic lines report protein production within the chloroplasts in the green parts of the plant, rather than the leukoplasts, whereas nuclear expression permits the accumulation of the gene product in tissues such as leaves, fruits, seeds and tubers.

The traditional approach of integrating transgenes into the genome of the plant has the advantage of contributing to the germplasm, but has been associated with poor yields and long generation times. Numerous strategies have been described to minimize these drawbacks. The yield of a recombinant protein may be improved through codon optimization [6,7], coexpression of companion protease inhibitors [8], targeting to different subcellular compartments [9], and genetic fusions with matrix attachment regions [10], oleosins [11], elements of storage proteins [12] and immunoglobulins [13]. Long generation times can also be offset through the use of nonconventional breeding techniques such as the induction of doubled haploids. Nuclear transformation has been used to express a wide range of recombinant proteins in plants, including viral sequences [14], mycobacterial proteins [15] and heteropolymeric complexes such as a secretory SIgA antibody [16]. More recently, the use of additional genetic elements derived from plant viruses, such as enhancer untranslated regions and RNA-dependent RNA polymerases, in combination with *Agrobacterium tumefaciens*-mediated gene delivery into somatic cells, has provided an alternative approach to increasing recombinant protein yields. Such systems have the potential to produce small to mid-scale quantities of proteins over a shorter time scale than other technologies. This technology may have found particular relevance to the production of products that require a very rapid development time, such as seasonal vaccines to highly polymorphic viruses [17] and 'designer drugs' such as patient-specific anti-idiotype vaccines [18].

The chloroplast contains a distinct genome and gene expression machinery from the nucleus that can also be utilized to produce heterologous

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proteins. The chloroplast is effectively partitioned from the rest of the cell, and no report of protein export from the stroma to the cytosol has been made [19]. This compartmentalization offers the potential advantage of two independent protein factories within the same cell, which could be employed to produce separate protein subunits concurrently and lead to a potentially greater final yield. This could find application in the production of multisubunit vaccines such as the recombinant diptheria–pertussis–tetanus (DPT) immunogen, the production of which was recently demonstrated in plants [20]. Typically, chloroplast expression is associated with high yields (between 4 and 40% of the total soluble protein [21]). Although some eukaryote proteins produced in the chloroplast, such as human somatotrophin, have been demonstrated to fold and form disulphide bridges [22], the chloroplast lacks the capacity to glycosylate proteins and is therefore only suitable for a subset of potential protein targets.

Despite the appeal of plant-based vaccine manufacture, the only licensed product to come from these technologies to date is a veterinary vaccine against Newcastle disease in chickens [201]. This vaccine was produced in plant cells under containment and leveraged existing guidelines for animal vaccine production in cell culture. In this article, we present strategies currently under development to broaden the repertoire and potential appeal of plant-produced vaccines.

Improving the immunogenic potential of plant-produced vaccines

Transit through the secretory pathway

To act as an efficient immunogen, any recombinant protein must contain appropriate epitopes recognized by the B- and T-cell populations of the immune system. T-cell epitopes are subjected to intracellular processing and are recognized when bound to the MHC, whereas B-cell epitopes are recognized by cognate B-cell receptors in their native context. The display of B-cell epitopes in a manner that resembles the pathogen is of key importance in inducing an appropriate humoral response, which is usually the correlate of protective immunity in most, if not all, effective vaccine preparations.

Nuclear transcription of transgenes and cotranslational import into the plant secretory pathway leads to interactions with an array of chaperones and enzymes that largely parallel the functions of the equivalent molecules in the animal cell. As a result, proteins expressed in this manner are able to adopt complex tertiary and quaternary structures, and acquire glycans and other post-translational modifications. Such an environment is potentially advantageous for the production of recombinant vaccine candidates, as carbohydrate modification and subunit assembly serve to enhance the half-life of proteins both within the plant production platform and the vaccinated individual [23], and may tailor the immune response by contributing to epitopes or by masking immunodominant, but nonneutralizing, B-cell epitopes.

Although the structures of *N*-linked glycans produced in the plant endoplasmic reticulum are indistinguishable from their mammalian counterparts, the subsequent modifications made as the protein progresses through the plant Golgi differ substantially (reviewed in [24]). The potential interaction of these glycans with the immune system in the context of an immunogen has prompted some concern. It is reported that IgE and IgG_4 antibodies present in the sera of allergic humans can cross-react with plant *N*-glycans [24]. Furthermore, an inappropriate immune reaction to the $\beta1\rightarrow2$ xylose and fucose in the plant-specific $\alpha1\rightarrow3$ linkage was observed to trigger the production of glycan-specific antibodies to a plant-produced antibody in rabbits [25], but not in mice [26]. Efforts are ongoing to adapt the plant glycosylation machinery to produce structures more consistent with mammalian glycans, and considerable progress has been made [27,28]. The extent to which this is necessary for each recombinant protein needs to be established on a case-by-case basis, with appropriate testing in human trials, given the lack of a reliable animal model for glycan immunogenicity.

Some aspects of plant glycosylation may prove beneficial in the development of new vaccines. Plant glycans lack fucose in the mammalian $\alpha1\rightarrow6$ linkage, a moiety demonstrated to limit the effector functions of antibodies [29], although data regarding immune system receptor affinity to common plant complex *N*-glycans has not yet been reported. The absence of terminal sialic acid residues in plant glycans may also increase binding to cellular immune receptors [30]. Perhaps more significant is the observation that in contrast to mammalian cells, plant cells can yield a very homogeneous population of glycans [31]. In combination with an engineered glycosylation pathway, plant cells could therefore represent an attractive platform for the production of glycoproteins with improved consistency and possibly effector functions (in the case of antibodies) and thus more effective glycoprotein prophylactics and therapeutics.

Many proteins undergo further post- or cotranslational modifications when synthesised in their native environment. The enzymatic addition of C8 and C12 saturated acids myristate and palmitate, isoprenoid groups and the glycosylphosphatidylinositol anchor contributes to membrane association and may influence the immune response to recombinant proteins, including potential vaccine and immunotherapeutic targets, such as oncogenes and cellular signaling components [32], as well as antigens from parasitic protozoa [33]. Plants share similar pathways for the addition of these components to endogenous proteins (for recent publications see [34–36]). Early indications suggest recombinant proteins are also correctly modified, for example, it was observed that the myristoylated full-length nef protein of HIV became associated with membranes when expressed in tobacco protoplasts [37]. In some cases, it may be necessary to engineer a native signal sequence towards the plant consensus sequence in order to achieve efficient processing of these moieties.

The requirements of each recombinant protein to adopt an appropriate immunogenic structure must be determined empirically. While it is clear that heavily glycosylated proteins such as viral envelope glycoproteins require the environment of the endomembrane system to fold correctly and hence retain immunogenicity, some proteins that normally receive secretory pathway specific modifications remain immunogenic when synthesized in other compartments, such as the chloroplast [22]. Plants therefore offer a flexible platform for investigating the relevance of these modifications.

Adjuvants & carrier proteins

The development of adjuvants is a major hurdle in vaccine development, as the modulation of the immune response must be appropriate and predictable. Inevitably, there will be no single adjuvant suitable for the delivery of each candidate plant produced vaccine; indeed, the only US FDA-licensed adjuvants are salts of aluminium and calcium, which do not induce strong mucosal responses appropriate for an orally delivered vaccine.

In recent years, several promising adjuvants developed in other systems have been produced as recombinant proteins *in planta*. Fibronectin extradomain A (EDA), expressed in chloroplasts [38], and chicken IL-12 from tobacco leaf [39] both hold promise as parenteral adjuvants due to their immunostimulatory activities. Much interest has focused on the pentameric B subunits of the toxins produced by certain pathogenic bacteria. These proteins are readily synthesized in a range of plant tissues including maize [40], potato [41] and rice [42]. Cholera toxin B subunit (CTB; from *Vibrio cholerae*) and the heat-labile toxin B subunit (LTB; from enterotoxigenic *E. coli* [ETEC]) have been investigated as both separate mucosal adjuvants and as auto-adjuvanting fusion proteins with the target antigen. CTB has been successfully used to boost the immunogenicity of several plant-produced oral vaccines tested in animals [43–45], but some doubts have been raised over its efficacy when delivered intranasally [46,47]. LTB produced in both potato and corn has been tested as an oral vaccine in its own right against both ETEC and cholera in humans [41,48], and has also shown promising adjuvanticity when delivered nasally to mice [49]. However, the intranasal administration of enterotoxin B subunits in humans has been associated with unpleasant side effects, including facial palsy [50].

Although CTB and LTB are both strong adjuvants of a mucosal humoral response, preparations including them are associated with rapid systemic tolerance and have been employed in potential immunotherapeutics for autoimmune disease (recently reviewed in [51]). The immunomodulatory properties of CTB and LTB have been attributed to the ability of these molecules to bind ganglioside lipids on the surface of M cells and several populations of leukocytes in the mucosa, particularly B cells [51]. This principle can be expanded to target other cell surface markers and receptors on antigen-presenting cells in order to induce uptake and presentation. The production in plants of chimeric molecules consisting of an antigen and self-specific antibody, known as a recombinant immune complex, has been reported [52]. These molecules target the Fc receptors expressed on most antigen-presenting cells by mimicking heavily opsonized antigens, promoting an immune response to the antigen moiety. Accordingly, it was shown that a recombinant immune complex directed against tetanus toxin fragment C (TetC) induced a strong and protective immune response in mice when delivered systemically without adjuvant [52].

Virus-like particles

When expressed in cells, viral capsid proteins or envelope glycoproteins frequently exhibit self-assembly into empty capsids or viral envelopes in the absence of additional gene products. These virus-like particles (VLPs) are convenient immunogens owing to their faithful reproduction of B-cell epitopes, and some preparations have also been shown to induce good cytotoxic T-lymphocyte responses [53]. VLP-based vaccines against hepatitis B and human papillomavirus produced in yeast (Engerix-B® [GlaxoSmithKline, Brentford, UK], Recombivax HB® and Gardasil® [Merck, NJ, USA]) and insect cells (Cervarix® [GlaxoSmithKline]) are now licenced [54]. Plants are also an appropriate production platform for these particles as VLPs based on animal viruses are unlikely to interact with plant cells once they are formed. Given that nearly all described plant viruses are nonenveloped, VLP based on nonenveloped pathogenic viruses such as norovirus (Norwalk virus capsid protein [NVCP]) [55,56] and human papillomavirus (L1) [57] have been attractive targets for production *in planta*, and the production and immunogenicity of these particles has been described. The formation of enveloped VLPs based on the surface antigen of HBV (HBsAg) has further established the versatility of the plant cell for VLP production [54]. Most recently, influenza hemagglutin (HA) was shown to bud as VLPs from the plant plasma membrane when expressed in the context of a signal peptide [17]. This pathway of production mirrors the budding of the virion in infected host cells and these enveloped particles were subsequently found to be capable of eliciting a strain-specific protective immune response against influenza.

Recombinant plant viruses engineered to display antigens either as epitopes integrated into the viral capsid or as covalently conjugated proteins have also been developed. Various model plant viruses have been investigated as potential vaccine vectors; tobacco mosaic virus (TMV), alfalfa mosaic virus [58], cytomegalovirus (CMV), cowpea mosaic virus (CPMV) [59], tomato bushy stunt virus (TBSV) [60] and potato virus X (PVX) [61]. The capsid protein of TMV is an effective peptide carrier for an epitope up to 25 amino acid residues long, and has been used to induce immune responses against numerous target pathogens [62]. Smith *et al.* report the engineering of a recombinant TMV in which each capsid subunit is associated with the canine oral papillomavirus L2 protein via an inserted lysine residue [63]. This vector significantly improved the poor immunogenicity of the papillomavirus protein when delivered subcutaneously in mice.

Plant-made VLPs and recombinant plant viruses may address two drawbacks of the oral immunization route for plant-made vaccines. First, a viral scaffold can shield associated immunogens from the harsh conditions of the lower GI tract [64]. Second, the ordered display of epitopes on the surface of the virus can serve to enhance the immune response by co-ordinating the cross-linking of various receptors of the immune system [65]. In human volunteers, oral immunization with plant-derived HBV VLPs results in significant, but inconsistent immune responses [66,67]. The response to plant NVCP VLPs in a similar trial was also modest, but significant in 19 out of 20 volunteers [56].

The interaction of certain plant viruses with components of the immune system may also contribute to successful vaccination. Empty CPMV capsids produced in plants have recently been demonstrated to interact with antigen-presenting cells expressing surface vimentin [68], including those found in the Payer's patches [59]. Tuning this interaction may provide a valuable

platform for future vaccine design when potent mucosal responses are desired. Furthermore, the excellent systemic bioavailability of CPMV particles after oral delivery to mice via infected cowpea leaves suggests that this virus also has the potential to induce systemic responses to associated antigens [69].

Immunization strategies for plant-based vaccines

Parenteral delivery

Systemic immune responses elicited by intramuscular or subcutaneous delivery of vaccines are frequently robust due to the exposure of antigen to the important lymphoid compartments of the spleen and multiple lymph nodes, as well the apparent lack of a tolerogenic pathway. Well-established formulations are available to optimize the stability, pharmacokinetics and adjuvant properties of vaccines delivered by these routes. Consequently, most vaccinations commonly administered to humans today are formulated for parenteral delivery.

Although the additional burden of purification associated with drug production for parenteral delivery to humans requires full processing of the plant biomass, some aspects of purification could be simplified through the use of a plant production system, due to the absence of animal pathogens such as viruses and pathogenic mycobacteria. However, any product entering clinical trials must adhere to the accepted current good manufacturing practice in both production and analysis, as enforced by the relevant local agencies. The only plant-produced vaccine candidate delivered by a parenteral route to enter clinical trials to date is the anti-idiotype vaccine against non-Hodgkin's lymphoma [18]. Vaccine production was achieved through a transient production system based on TMV infection of *Nicotiana benthamiana* leaves, and the products were subjected to a panel of analytical tests before quality assurance release (supplemental data in [18]). A detailed study of the immune response mounted by the 16 subjects to subcutaneous vaccination revealed sustained antigen-specific responses in seven subjects, demonstrating the potential of the approach. Importantly, no specific reactions to plant glycans were revealed by comparative immunoassays supported by a detailed glycoanalysis of each vaccine single-chain variable fragment. Similar anti-idiotype antibodies are now produced using the transient MagnICON® system in *N. benthamiana* leaves and are entering clinical trials undertaken by Bayer Innovation (Dusseldorf, Germany) [202].

Parenteral administration is often the first delivery route chosen by researchers to establish immunogenicity of a plant-derived antigen in a model animal system. In an early study of the immunogenicity of HBsAg, the investigators showed that a crude preparation of plant-derived HBsAg delivered qualitatively similar immune responses when compared with a purified HBsAg preparation from yeast [70]. Another study compared the neutralizing mean serum titer of mice immunized parenterally with cell extracts from NT1 cells expressing Shiga holotoxin (Stx2) with a bacterial preparation of the same immunogen [71]. Despite lower average serum titers than the bacterial group, all mice receiving the plant cell preparation were protected against lethal Stx challenge. These promising results, along with the licensing of a veterinary vaccine for Newcastle disease in chickens [202], have established parenteral dosing as a target route of administration for several plant produced vaccine candidates currently under development. These include influenza virus HA [17], an approach that also benefits from the rapid turn around of transient plant expression systems to quickly react to outbreaks, and the merozoite surface proteins of protist genus *Plasmodium*, which includes the causative agents of malaria [72].

Oral delivery

An attractive format for a potential plant-based vaccine is the crude, partially processed tissue of the plant itself. These 'edible vaccines' would have a unique potential to reach areas of high disease prevalence by avoiding the need for a cold chain and sterility both before and during administration. These practical advantages are supported by evidence from existing oral vaccination regimes indicating that the oral route is appropriate for the induction of strong, local mucosal antibody responses to certain antigens. Two barriers exist to the induction of a strong mucosal response: the epithelium itself and the tolerance of the local lymphoid tissue to the constant antigenic challenge of harmless particles. Despite these barriers, a successful recombinant mucosal vaccine from plants may trigger a mucosal immune response by mimicking a potential pathogen. Delivery of an antigen as a VLP or on the surface of a microparticle has been shown to stimulate uptake via M cells present in gut lymphoid follicle-associated epithelium [73]. Second, coadministration of an enterotoxin adjuvant such as CTB, or bacterial Toll-like receptor ligand such as flagellin [74], may prime an immune response to the vaccine by triggering the release of immunomodulatory cytokines into the lymphoid tissue.

Preliminary immunological data exists for many plant-based vaccines when delivered orally to animals (Table 1). However, to date, only four oral vaccine candidates derived from plants have been tested in preliminary human studies. They target hepatitis B (HBsAg) [54,70], ETEC (LTB) [41,48], norovirus (NVCP) [56] or rabies (G protein) [75]. In each case, crude or lightly processed plant material was given as food to volunteers; lettuce [54] or spinach leaves [75], corn [48] and potato tubers [41,56,70]. In each case, these preparations were delivered without adjuvant, and a significant systemic and mucosal humoral immune response was observed in the majority of subjects. Despite these promising results, it is unlikely that the oral route will be appropriate for all plant-made vaccine candidates. Currently, the only licensed oral vaccines in the UK are for diseases where the natural route of infection is through the gut mucosa: poliovirus (live-attenuated oral polio vaccine), rotavirus (Rotarix® [GlaxoSmithKline] and Rotateq® [Merck]), typhoid (Ty21a) and cholera (Dukoral® [Crucell, Leiden, The Netherlands]). Although oral immunization has been demonstrated to induce systemic as well as mucosal responses [66], these are often far weaker than those induced by parenteral dosing. Furthermore, the nature of the antigen itself, along with the dose, frequency, and the nature of any adjuvant present, may lead to the induction of systemic immune tolerance (recently reviewed in [76]). In this scenario, systemic cell-mediated

Table 1. Plant-made vaccine candidates reporting immunogenicity following oral delivery.

Antigen/immunogen	Production system/ purification	Recipient organism	Humoral or cellular responses	Ref.
Norwalk virus recombinant nucleocapsid protein rNV (nonenveloped VLP)	*Nicotiana benthamiana* magnifection (ICON)/sucrose gradient	Mouse	Systemic and mucosal IgA production	[56]
Enterotoxigenic *Escherichia coli* LTB	*Oryza sativum* calli/lyophilization	Mouse	Systemic IgG and mucosal IgA production	[95]
Rabies lyssavirus chimeric N and CP epitope displayed on AIMV CP	*Spinacia oleracea,* infected leaves	Mouse Human	Systemic IgA and IgG, gastric secretory IgA Protection persisted for at least 120 days Low levels of serum IgG and IgA in some subjects	[96] [75]
Rabies lyssavirus GP	*Zea mays* transgenic	Mouse	Single oral dose (50 µg GP) conferred 100% protection through a specific antibody response	[97]
CTB	*Oryza sativa* endosperm/ lyophilization	Mouse Macaque	Systemic IgG and mucosal IgA production (correlate of protection) Enhanced serum IgG even in the presence of intestinal secretory IgA	[41,98] [98]
Yersinia pestis LcrV-F1 chimeric antigen fused to lichenase	*N. benthamiana* transient	Macaque	High serum IgG_1, 100% protection from aerosol challenge	[99]
Orthopoxviridae B5 membrane glycoprotein	*N. benthamiana* magnifection (ICON)/lyophilization	Mouse	None detected	[100,101]
HBsAg	Potato tubers	Human	19 out of 33 volunteers respond with serum IgG	[67]
Tetanus (TetC)	*Nicotiana tabacum* chloroplasts	Mouse	Systemic IgG and mucosal IgA to protective levels when administered with CT	[47]
Diphtheria, pertussis, tetanus protective antigens	*Daucus carota* cells and *N. tabacum* leaf	Mouse	Protective levels of systemic antibody production	[20]
Measles hemagglutinin	*N. tabacum* transgenic/ lyophilization *Lactuca sativa* transgenic/ lyophilization	Mouse	DNA intraperitoneal prime required, significant boosting of serum IgG with subsequent oral doses Th2 bias observed for antigen	[92,93,102]
PRRSV/envelope GP5	*N. tabacum* transgenic	Pig	Systemic IgA and mucosal IgG with some neutralization capacity	[103]
Influenza (plasma-membrane derived VLP containing HA)	*N. benthamiana* magnifection (tobacco etch virus)	Mouse	Significantly higher HI titers than 'naked' HA particles	[17]
FMDV epitopes fused to TMV CP (naked VLP)	*Medicago sativa* (alfalfa) transgenic *N. tabacum*	Mouse Guinea pig	Serum immunoglobulin response to levels associated with protection follow parenteral vaccination Partial protection (three out of eight animals)	[104] [105]
ARV σ C protein	*Arabidopsis thaliana* transgenic	Chicken	'Significant protection' observed	[106]
β-APP cleaving enzyme (Alzheimers)	*N. tabacum* chloroplasts	Mouse	Serum IgG response	[107]
Cholera/malaria chimeric antigens (CTB-AMA1 or CTB-MSP1)	*L. sativa* transgenic and *N. tabacum* plastid transgenic	Mouse	Intestinal secretory IgA, systemic IgA and IgG production IL-10 T cells raised, FOX-P3 cells unchanged, IFN-γ reduced, IL-17 not detected	[108]

AIMV: Alfalfa mosaic virus; AMA1: Apical membrane antigen 1; ARV: Avian reovirus; β-APP: β-amyloid precursor protein; CP: Capsid protein; CPMV: Cowpea mosaic virus; CT: Cholera toxin; CTB: *Vibrio cholerae* toxin B subunit; E: Envelope; FMDV: Foot-and-mouth disease virus; FnBP-D2: Fibronectin-binding protein B D2 peptide; GP: Glycoprotein; HA: Hemagglutinin; HBsAg: Hepatitis B surface antigen; HI: Hemagglutination inhibition; HPV: Human papillomavirus; LTB: Heat-labile toxin B subunit; MSP: Merozoite surface protein; PRRSV: Porcine respiratory syndrome virus; TetC: Fragment C from tetanus toxin; Th: T helper; TMV: Tobacco mosaic virus; VLP: Virus-like particle.

Table 1. Plant-made vaccine candidates reporting immunogenicity following oral delivery (cont.).

Antigen/immunogen	Production system/ purification	Recipient organism	Humoral or cellular responses	Ref.
Staphylococcus aureus FnBP-D2 fusion with CPMV S coat protein (naked VLP)	*Vigna unguiculata* (cowpea), infected leaves	Mouse	Unadjuvanted VLP elicit weak D2-specific IgG in the sera of most animals with very weak to undetectable mucosal IgA responses Antigen-specific Th1 bias. Poor splenocyte proliferation in response to CPMV	[109]
Helicobacter pylori UreB	*D. carota* transgenic	Mouse	Serum IgG, intestinal secretory IgA when administered with CTB	[110]
Japanese encephalitis E protein	*O. sativa* transgenic	Mouse	Subprotective levels of serum IgG and intestinal secretory IgA detected using an unadjuvanted formulation	[111]
Dermatophagoides pteronyssinus group 1 antigen Der p 1	*O. sativa* transgenic	Mouse	Reduction in allergen-specific IgG and IgE responses compared with nonimmunized animals on challenge with adjuvanted Der p 1 Reduction in Th2 cytokine production and leukocyte infiltration into the airways	[112]
HPV-11 L1 capsid protein VLP	*Symphytum tuberosum* transgenic	Mouse	None detected (unless boosted with an adjuvanted L1 preparation from insect cells)	[113]
Malaria *Plasmodium* MSP4/5	*N. benthamiana* magnifection (ICON)/freeze dried	Mouse	DNA intraperitoneal prime required for protective levels of serum IgG	[114]
HIV gp41 731–752 (cytosolic domain) fused to CPMV S coat protein	*Vigna unguiculata*, infected leaves	Mouse	No fecal antibody responses Serum responses in two out of five mice	[115]

AIMV: Alfalfa mosaic virus; AMA1: Apical membrane antigen 1; ARV: Avian reovirus; β-APP: β-amyloid precursor protein; CP: Capsid protein; CPMV: Cowpea mosaic virus; CT: Cholera toxin; CTB: *Vibrio cholerae* toxin B subunit; E: Envelope; FMDV: Foot-and-mouth disease virus; FnBP-D2: Fibronectin-binding protein B D2 peptide; GP: Glycoprotein; HA: Hemagglutinin; HBsAg: Hepatitis B surface antigen; HI: Hemagglutination inhibition; HPV: Human papillomavirus; LTB: Heat-labile toxin B subunit; MSP: Merozoite surface protein; PRRSV: Porcine respiratory syndrome virus; TetC: Fragment C from tetanus toxin; Th: T helper; TMV: Tobacco mosaic virus; VLP: Virus-like particle.

immunity is rendered unresponsive to the delivered antigen due to an upregulation of certain regulatory T-cell populations [77], or by antigen-presenting cell-directed deletion of antigen-specific $CD8^+$ cells [78]. It is apparent that most vaccines in current use induce protective humoral responses, rather than increased cytotoxic T-lymphocyte populations. However, the possibility of induced tolerance has informed the design of two plant vaccine trials; an oral HBV vaccine trial [70], where all participants were screened for prior immunity to HBV and only participants previously immunized systemically were used, and a rabies trial [75], where a naive group was recruited only after promising results on preimmunized individuals.

While the immunogenic potential of edible vaccines is now well documented, the approach suffers from a number of potential medical and environmental issues. The rigid, fibrous plant cell wall may in itself affect the bioavailability and pharmokinetics of recombinant antigens when delivered in an unprocessed form or as a lightly processed foodstuff. The encapsulation of proteins trapped within the periplasmic space by the cell wall may provide a degree of protection against the harsh conditions of the stomach, leading to high levels of antigen presentation to the lymphoid tissues of the intestine. On the other hand, proteins accumulating within intracellular compartments, such as the plastids, may not be fully released by normal digestive transit. Dose standardization is further complicated by possible fluctuations in antigen expression levels due to environmental factors or through breeding of the pharmaceutical crop. Part processing of the plant tissue may allow for greater control over the dose administered to, and adsorbed by, patients, as well as improving the shelf life of the vaccine. Freeze-drying [79–81] and batch processing of maize has been used to produce a vaccine against ETEC in a digestible form with a defined recombinant protein content [48]. From a regulatory point-of-view, full purification of a plant-derived therapeutic or vaccine prophylatic provides the greatest control over the administered product. For this reason, it remains likely that the first plant-produced vaccines to reach the market will be fully defined formulations.

Although genetically modified plant technology has long been viewed with considerable scepticism by environmental campaigners as well as the general public, the political and environmental ramifications of vaccine crop production are perhaps mitigated by the potential health benefits of wider vaccine availability. Pharmaceutical crops are unlikely to adversely affect biodiversity as they are often less fit than the corresponding wild-type cultivar. However, the potential of unwanted vaccines to enter the food chain remains a significant concern. The expression of a visible reporter

gene closely linked to the antigenic locus may provide some degree of control over vaccine crops [82], particularly if combined with an appropriate genetic use restriction technology.

Other delivery routes for plant-derived vaccines

Compared with the upper GI tract, the nasal mucosa is an important inductive site for the mucosal immune system, characterized by a relatively low concentration of enzymes, a moderate pH and relatively high permeability (reviewed in [83]). The delivery of immunogens to the nasal mucosa may therefore represent an appropriate vaccination strategy, particularly against pathogens that infect via interaction with cellular receptors in the upper respiratory tract or lungs. Chicken egg-produced FluMist® (AstraZeneca, London, UK), a live-attenuated nasal vaccine against seasonal and pandemic influenza, is however the only nasal vaccine currently on the market.

In plants, there have been several reports detailing responses to nasally administered immunogens in animals (Table 2). It has been reported that TetC, formulated as a total soluble extract from tobacco chloroplasts, elicited protective systemic immune responses in the absence of adjuvant when administered to mice intranasally but not orally [47,84]. The induction of both strong systemic and mucosal immune responses is a desirable feature of a mucosal vaccination regime as it provides for multiple layers of immune defense and may reflect the formation of specific memory B- and T-cell populations [85]. As with other mucosal inductive sites, systemic tolerance following nasal immunization has been demonstrated [86]. However, a recent publication reports that tolerance induced through nasal immunization may be effected through a different mechanism than oral systemic tolerance [78]. In ongoing clinical trials in the USA, NVCP particles produced in the endomembrane system of tobacco have been applied to the nasal mucosa (clinical trials references NCT00806962 [203] and NCT00973284 [204], further details in [87]). US FDA current good manufacturing practice-compliant production, purification and quality control procedures for plant-produced NVCP-based VLP vaccine candidates destined for nasal instillation have also been published [88]. In combination with promising immunological results, these data provide a base for the development of plant-derived nasal vaccines.

Other mucosal immunization sites such as the rectum and the vagina have been proposed as suitable routes for vaccination against sexually transmitted pathogens. The immunogenicity of several antigens from nonplant systems in these compartments has been investigated, and in most cases, local antibody production and cellular responses are detected (recently reviewed in [89]). However, immune responses in these compartments can be highly localized and, consequently, poor inducers of systemic immune memory. 2G12 IgG, a monoclonal antibody targeting the surface glycoprotein of HIV, has the potential to prevent HIV transmission as a topical microbicide. The production of 2G12 in transgenic tobacco, the purification and analysis of the antibody, and the formulation for vaginal delivery has been accomplished to current good manufacturing practice standards [205]. The successful entry of this molecule into clinical trials provides another set of benchmarks for plant-derived therapeutics.

Prime–boost strategies

Many immunization strategies employ the potent secondary response of the immune system to repeated doses of the same antigen or a related antigen to reinforce an immunizing response. Use of a different immunogen in the boost doses gives a vaccination regime the potential to target both humoral and cellular arms of the immune system through the careful selection of the

Table 2. Plant-made vaccine candidates reporting immunogenicity following nasal delivery.

Antigen/immunogen	Production system/ purification	Recipient organism	Humoral and cellular responses	Ref.
Staphylococcus aureus FnBP-D2 fusion with CPMV S coat protein (naked VLP)	*Vigna unguiculata* (cowpea), infected leaves	Mouse	Unadjuvanted VLP elicit high D2-specific IgG in sera and mucosal IgA responses in most animals Antigen-specific Th1 bias Strong splenocyte proliferation in response to CPMV	[109]
FMDV epitope fusions with tobacco necrosis virus A CP (naked VLP)	*Chenopodium amaranticolor* (goosefoot) leaves	Mouse	Serum IgG and some mucosal IgA in five out of five animals	[116]
HIV gp41 2F5 epitope, N-terminal fusion with PVX CP	*Nicotiana benthamiana*, infected leaves	Mouse	Unadjuvanted VLP elicit high serum IgG_{2a} and moderate IgA end point titers Human dendritic cell primed with immunogen induce immune response in SCID mice	[117]
HIV gp41 731–752 (cytosolic domain) fused to CPMV S coat protein	*V. unguiculata*, infected leaves	Mouse	CT-adjuvanted recombinant virus elicited specific fecal sIgA and IgG after prime or single boost Serum response primarily IgG_{2a}	[115]
Tetanus (TetC)	*Nicotiana tabacum* chloroplasts	Mouse	Serum IgG responses in all animals to protective levels when administered with CT Transient increase in $CD4^+CD45RB^{low}$ T cells in lung-associated lymphoid tissue	[47,84]

CP: Capsid protein; CPMV: Cowpea mosaic virus; CT: Cholera toxin; FMDV: Foot-and-mouth disease virus; FnBP-D2: Fibronection binding protein B D2 peptide; PVX: Potato virus X; SCID: Severe combined immunodeficiency; TetC: Tetanus toxin fragment C; VLP: Virus-like particle.

epitopes and formulation employed. In the case of many current vaccinations, 'boost' doses are administered parenterally. However, mucosal boosting can also be used to augment a systemic prime; an approach which appears to circumvent tolerogenic responses. These mucosal 'prime–boost' strategies frequently call for large quantities of oral booster vaccine, emphasising the role plants may play in reducing the cost of vaccine production.

A heterologous prime–boost strategy was recently employed in a preliminary study of the plant-based production and subsequent immunogenicity of a candidate HIV subunit vaccine [44]. The immunogen, consisting of CTB genetically fused to a peptide derived from the membrane-proximal ectodomain of viral envelope component gp41, was administered to mice in five nasal doses followed by a single intraperitoneal dose. The authors report a mucosal (vaginal) response in the form of elevated IgA to the nasal immunization, and that a significant systemic response was only observed after the intraperitoneal boost. A robust mucosal response, ideally in the form of secretory IgA capable of blocking HIV uptake, is considered to be an important correlate of immunity to HIV [90].

In another example, a chloroplast derived *Yersinia pestis* vaccine candidate, F1-V protein, was administered to mice using either a parenteral prime–boost, or a systemic prime–oral boost regime [91]. Although the researchers did not seek to quantitatively compare the immune response to the two regimes, it was noted that oral boosting without adjuvant appeared to confer greater protection against lethal *Y. pestis* challenge ($15 \times LD_{50}$), which correlated with robust serum IgG_1 responses. The suitability of this oral boost regime for the induction of IgA responses was more difficult to assess. Animals receiving oral boosts produced low, but significantly higher levels of serum IgA than the parenteral boost group, but no fecal secretory IgA could be detected in either group. The authors suggest that a mucosal response in the gut may be abrogated by the denaturing conditions of the stomach, and mucosal responses in upper GI tract were not investigated.

Systemic prime–oral boost immunization regimes also show promise in eliciting strong systemic antibody responses. In a study in mice, tobacco-derived measles MV-H protein was given four times over a 42-day period to boost an intramuscular priming dose of MV-H DNA [92]. This regime generated average serum neutralization titers many times greater than those considered protective in humans. However, it must be noted that a strong adjuvant combination of cholera toxoid and CTB was used in this study. In a follow-up study, lettuce-produced MV-H adjuvanted with saponins was administered to mice in a similar program, and was also found to be systemically immunogenic [93].

Expert commentary & five-year view

The prospect of vaccine production in plants is enormously appealing for many reasons. Perhaps the most important of these is that the concept and its advantages are immediately obvious to non-scientists and members of the public. However, to some extent plant production systems have been victims of their own potential because the field has generated multiple ideas and approaches and not focused on a single technology to take forward.

The introduction of any technology, particularly into a traditionally conservative area such as the pharmaceutical industry is inevitably slow and cautious. It is entirely predictable that the first plant-derived pharmaceuticals (Newcastle disease vaccine and glucocerebrosidase) would be produced using technologies that closely mimic existing approved production platforms. This incremental progress allows the introduction of new approaches within the context of an existing regulatory framework and does not pose major challenges to regulatory authorities. It is therefore a safe developmental strategy.

The vaccines produced by such plant systems do not differ significantly from conventional vaccines and while plants may offer some benefits in specific cases, there is no conceptual advance in vaccinology. The prospect of oral delivery has been linked with plants ever since plant-derived vaccines were first proposed, and it seems that the immunological and regulatory hurdles will continue to slow development efforts. Certainly, while the technical immunological difficulties of oral vaccination remain unsolved for conventional vaccines, it is unlikely that the vaccines produced in plants will make the breakthrough.

There are, however, three important areas where plant production platforms could contribute to a step change in vaccinology, and could all result in significant benefits for global health. The first is the administration of a plant-derived oral vaccine in the context of a heterologous prime–boost strategy. Oral boosting in systemically vaccinated individuals bypasses the issue of inducing oral tolerance. It is also a simple and convenient way to provide second, third and fourth booster immunizations, which will help to improve the success of any vaccine programs, particularly in developing countries. The approach has already been demonstrated for the hepatitis B and measles vaccines [67,92].

The second area where plant production platforms could change pharmaceutical thinking is by the introduction of pharmaceutically regulated products prepared by minimal downstream processing. Downstream processing contributes up to 80% of manufacturing costs [94] and the simplification of this step would greatly enhance the economic feasibility of many products. The regulatory issues relate to the consistency of the product, and this applies not only to the active pharmaceutical ingredient, but also to coadministered plant ingredients. The technical challenge, therefore, is to identify manufacturing processes that can comply with minimal and maximum specifications that are functionally acceptable and agreed by regulatory authorities. It is often observed that many plants have generally regarded as safe (GRAS) status, but this only applies to oral ingestion and the GRAS status may not apply to other routes of delivery. Thus, administration of minimally processed plant-derived pharmaceuticals may only be applicable to oral or topical applications.

The third important impact area for plants could be widening participation in pharmaceutical production, particularly in less developed countries with an emphasis on addressing local health issues. Any approach that offers a simplified and inexpensive route to pharmaceutical production would be of great interest to countries struggling with health issues that are of little or no interest

to the developed world. Rabies is a case in point. The upstream component of a transgenic plant production platform is attractively economic and would not necessitate a large infrastructure investment. The key to success would be the ability to integrate this with appropriate downstream processing technologies. In addition to developments such as minimal processing, the will of scientists in the West to collaborate with scientists from less developed countries, and the development of technology transfer programs at an early stage of produce development, will be the key to unlocking this step.

Plant production platforms offer wonderful prospects for improving global access to medicines. The urgency now is to accelerate development not only for conventional products, but also for new concepts. As with all fields of human advancement, an element of risk-taking must be included if we are to achieve and capitalize on the real potential of plant-derived medicines.

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Key issues

- Recombinant plant technology has become established as an appropriate production platform for a range of immunogens, including glycoproteins, enveloped and nonenveloped virus-like particles, and antibody-based complexes. This is reflected by the increasing involvement of big pharma industry (e.g., the recent purchase of ICON Genetics by Bayer) in the sector.
- The advent of enhanced transient production systems offer the potential to produce gram quantities of antigens within a short time frame and with low start-up costs. The advantages of this are twofold: patient-specific anti-idiotype vaccines become cost effective, and the reaction times to pandemic seasonal virus are reduced.
- Animal studies and early clinical trials with antigens produced in plants reveal appropriate immune reactions in both systemic and mucosal compartments.
- Previous, ongoing and future clinical trials of plant-produced vaccines have established the groundwork for the production of material to current good manufacturing practices for parenteral, oral, nasal and vaginal application, paving the way for new vaccine candidates from plants.
- The distribution of vaccines as minimally processed plant products offers the potential to drastically reduce substantial downsteam processing costs. However, issues regarding product consistency and the control of pharmaceutical crops and produce still present serious challenges to this approach.

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