

Plant-made vaccine antigens and biopharmaceuticals

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Plant cells are ideal bioreactors for the production and oral delivery of vaccines and biopharmaceuticals, eliminating the need for expensive fermentation, purification, cold storage, transportation and sterile delivery. Plant-made vaccines have been developed for two decades but none has advanced beyond Phase I. However, two plant-made biopharmaceuticals are now advancing through Phase II and Phase III human clinical trials. In this review, we evaluate the advantages and disadvantages of different plant expression systems (stable nuclear and chloroplast or transient viral) and their current limitations or challenges. We provide suggestions for advancing this valuable concept for clinical applications and conclude that greater research emphasis is needed on large-scale production, purification, functional characterization, oral delivery and preclinical evaluation.

Need for a new platform for production of therapeutic proteins

Approximately 15 million (>25%) of 57 million deaths per year worldwide are estimated to be related directly to infectious diseases [1]. Vaccination is considered to be the most efficient and cost-effective means for health intervention to combat infectious diseases. However, the high cost of vaccination makes it unaffordable for most people living in developing countries, as the daily average income for nearly one billion people is <US\$1. A 14-fold increase in the cost of vaccines over the past decade [2] makes it necessary to investigate alternate strategies for their production and delivery. Also, with an emphasis on safety, there has been an increasing shift towards the development of subunit vaccines (that use one or two proteins instead of avirulent or killed pathogen strains), with reliance on recombinant expression systems. The high cost of current vaccines and biopharmaceuticals is largely as a result of their complex production and delivery methods, including the significant costs of fermentation and purification systems and additional expenses associated with adjuvant, cold storage, transportation and sterile delivery. No viable alternative to fermentation technology has yet emerged for their mass production, a void that could be addressed through the use of plants as bioreactors for the production and oral delivery of therapeutic proteins. In this review, we evaluate the advantages and disadvantages

of different plant expression systems (stable nuclear, transient viral and stable chloroplast) and their current limitations or challenges ahead. We further discuss directions for advancing this valuable concept for clinical applications.

Plant-made therapeutic proteins: current status and challenges ahead

Plant-made vaccines and therapeutics refer to protein products with clinical or veterinary applications produced in recombinant plant systems. These systems can be broadly divided into those using nuclear transgenic technology, chloroplast transplastomic technology and plant viral technology (Figure 1). In all cases, the antigens or therapeutics are expressed in plant tissues, from which they can be either purified or the plant tissue can be processed into a form that can be applied topically or, more uniquely, orally. The major advantage of plant expression systems over other vaccine production systems is reduced manufacturing cost. Fermenters and bioreactors can be replaced with contained plant growth rooms or greenhouses or plants can be grown in the field with appropriate biological containment of foreign genes, such as maternal inheritance or male sterility, or expression in vegetative tissues with harvest before appearance of any reproductive structure. This should lead to reduced upstream facility costs. In the case of oral plant-produced vaccines and therapeutics, downstream processing costs are also reduced. Instead of protein purification targeting up to ~99% purity, plant tissues can be inexpensively processed for oral delivery. The relevant technology has already been developed in the food and feed industries and can be adapted for plant-produced therapeutic proteins.

As with other eukaryotic systems, plant-produced vaccines enable the introduction of post-translational modifications. Proper folding and disulfide bond formation occur in chloroplasts [3] or the endoplasmic reticulum (ER) [4]. Proper lipid modifications of vaccine antigens have also been observed in chloroplasts [5]. Therapeutic proteins are not glycosylated in chloroplasts but are glycosylated when targeted to the ER, although glycosylation patterns are not identical to mammalian systems. Although numerous therapeutic proteins have been expressed in plant cells (Tables 1–3, Appendix B Tables S1–S3 in the supplementary material online), several challenges remain. Transgene silencing in nuclear transgenic plants requires further investigation and improvement. More tools are

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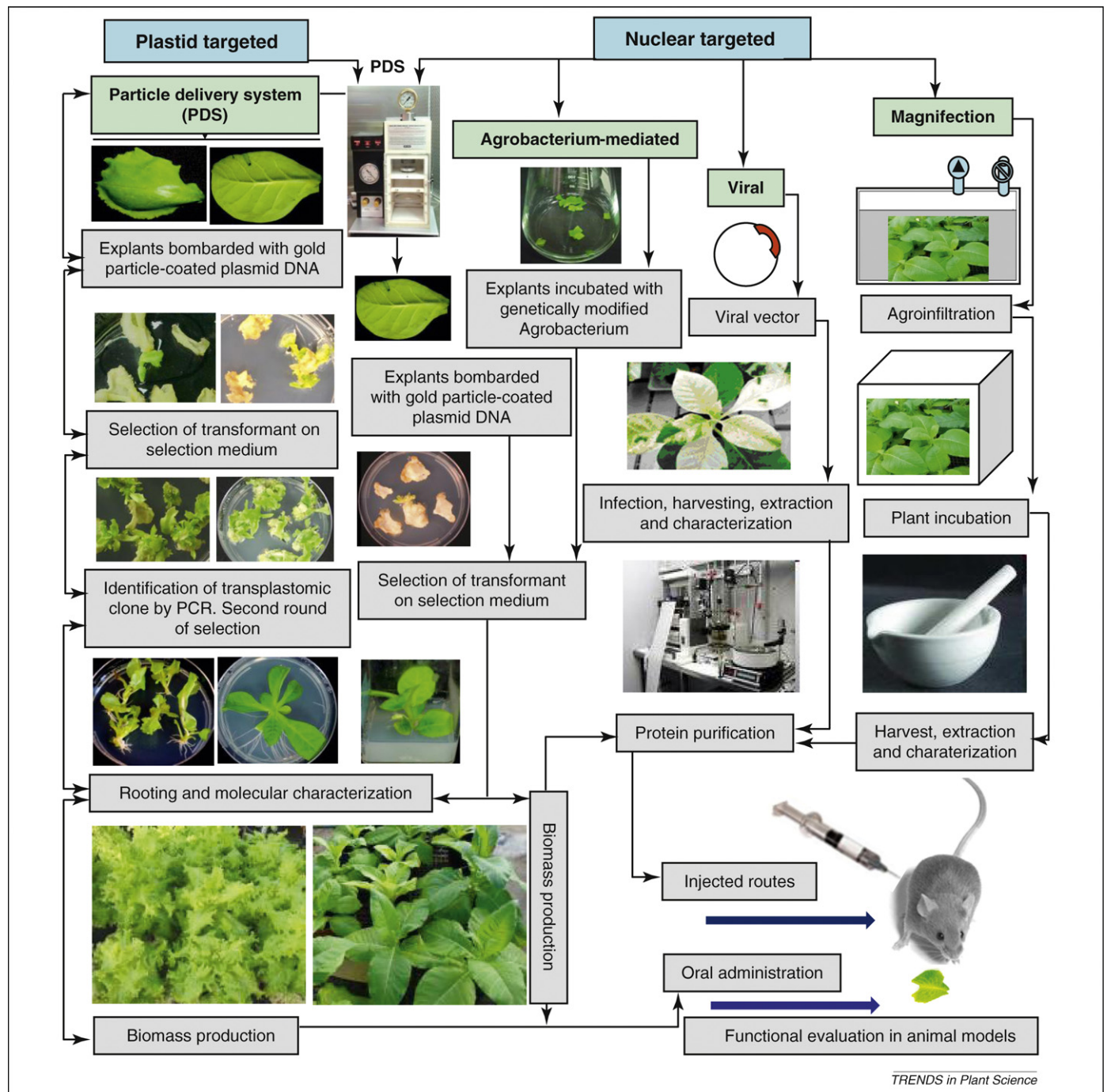


Figure 1. Schematic representation of vaccine antigens and biopharmaceuticals production in plants and their functional evaluation in animal models. Explants are tissues that have the potential for regeneration into mature plants. Here, we provide lettuce and tobacco as examples.

needed for transgene containment to facilitate field production of vaccines or biopharmaceuticals, when expressed via the nuclear genome. More generally, expression levels are target dependent and are a challenge for all recombinant systems.

Many functional assays (such as host cell protein) have not yet been developed for injectable products. Despite active research, there is limited published work on highly purified proteins and characterization of contaminants. It is well known that expression level varies depending on the developmental stage of leaves, the time of day and the regulatory sequences used, underscoring the importance of the developmental stage of the plants and the time of

harvest. Most importantly, achieving consistency of transgene expression in different batches is an important challenge. Therefore, harvested leaves should be homogenized and level of therapeutic protein must be determined for each harvested batch. There are limited studies on determination of stability of therapeutic proteins after harvest, processing and storage. Removal of Agrobacteria after vacuum infiltration of viral vectors is yet another challenge. All the above concerns need to be addressed with careful and reproducible investigations.

The current focus for most groups is on purified antigens prepared from plants grown in contained conditions. This avoids environmental release and enables controlled plant

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Table 1. Recent vaccine antigens expressed via the plant nuclear genome that report immunogenicity or protection (for full length table see Appendix BTable S1 in the supplementary material online)

Antigen human vaccines (Stable expression)	Expression system	Expression level	Functional evaluation	Refs
Bacterial antigens				
Enterotoxigenic <i>E. coli</i> Heat-labile toxin B subunit (LTB)	Carrots	0.3% TSP	Immunogenic and protective against CT challenge	[15]
	Soybean	2.4% TSP	Immunogenic and partial protection against LT challenge in mice	[4]
Cholera toxin B subunit (CTB)	Tomato	0.081% TSP	Immunogenic by oral delivery to mice	[79]
	Rice	2.1% TSP	Immunogenic and protective against CT challenge to mice following oral administration	[16]
Viral antigens				
Hepatitis B virus surface antigen (HBsAg)	Potato	8.5 $\mu\text{g g}^{-1}$ FW	Immunogenic response in humans following oral administration	[19]
Hepatitis B virus surface antigen fused with preS1 epitope	Rice	31.5 ng g^{-1} DW	Immunogenic by intraperitoneal delivery to mice	[80]
Human group A rotavirus (VP6) protein	Alfalfa	0.06–0.28% TSP	Immunogenic in mice and offspring developed less severe diarrhea after challenge with simian rotavirus SA-11, indicating that antibodies generated in the dams provided passive heterotypic protection to the pups	[81]
Rotavirus (VP7)	Potato tubers	0.3–0.4% TSP	Immunogenic in mice following oral delivery. Neutralization activity against rotavirus	[82]
Norwalk virus capsid protein (NVCP)	Tomato fruit and potato	8% and 0.4% TSP	Elicit systemic and mucosal antibody responses in mice following oral administration	[21]
SARS-CoV S protein (S1)	Tomato and tobacco leaf	0.1% TSP	Immunogenic to mice following oral administration	[83]
Smallpox recombinant vaccine virus B5 antigenic domain (pB5)	Tobacco and collard leaf	Not reported	Antibody response in mice immunized parenterally and protects against lethal dose of vaccinia virus	[84]
Japanese encephalitis virus (JEV) envelope protein (E)	Rice	1.1–1.9 $\mu\text{g mg}^{-1}$ TSP	JEV specific neutralizing antibody detected in mice following intraperitoneal or oral administration	[85]
Protozoan antigens				
<i>Plasmodium yoelii</i> merozoite surface protein (PyMSP4/5)	Tobacco	0.02–0.04% TSP	Induces antigen-specific antibodies in mice following parenteral delivery	[86]
Human vaccines (viral/transient expression)				
Bacterial antigens				
<i>Yersinia pestis</i> F1 and LcrV antigens	Tobacco leaf tissue	380 and 120 $\mu\text{g g}^{-1}$ FW	Immunogenic and protective in monkeys against <i>Y. pestis</i> following subcutaneous injection	[57]
<i>Yersinia pestis</i> F1–V antigens	Tobacco leaf	1 and 2 mg g^{-1}	Immunogenic and protection in vaccinated guinea pigs against <i>Y. pestis</i> aerosol challenge	[26]
Viral antigens				
Smallpox recombinant vaccine virus B5 antigenic domain (pB5)	Tobacco leaf	Not reported	Antibody response in mice immunized parenterally and protective against lethal dose of vaccinia virus	[84]
Encoding domain III of the dengue 2 envelope protein (D2EIII)	Tobacco	0.28% TSP	Retains antigenicity and immunogenicity as well as inducing neutralizing antibodies in vaccinated animals	[87]
HIV entry inhibitors red algal protein griffithsin (GRFT)	Tobacco (TMV)	1 g kg^{-1} FW	Active against HIV at picomolar concentrations, directly virucidal via binding to HIV envelope glycoproteins and capable of blocking cell-to-cell HIV transmission	[75]
Pathogenic avian influenza virus (H5N1 subtype)	Tobacco	60 mg kg^{-1} FW	Immunogenic in mice and ferret and also protects ferrets against challenge infection with virus	[28]

growth but decreases the cost advantage versus field-grown plants because construction of greenhouses or growth rooms to meet regulatory approval is relatively expensive. In the case of oral delivery, moving forward on the most promising veterinary candidates should lay the foundation for human vaccines. Determining a regulatory pathway for oral products is a major hurdle, given

the limited resources of the prominent research groups in the field. Purified vaccines and therapeutic proteins might follow a similar regulatory path to recombinant proteins produced in other systems, with the focus on stability, potency and efficacy of the purified product. As with other purified protein subunit vaccines, adjuvants are needed and must be developed alongside target antigens.

Table 2. Biopharmaceutical proteins expressed via the plant nuclear genome (for full length table see Appendix BTable S2 in the supplementary material online)

Pharmaceutical protein	Expression system	Expression level	Functional evaluation	Refs
Human growth hormone (hGH)	Rice suspension cells	57 mg l ⁻¹	The biological activity of shGH accumulated in the transgenic rice cell suspension culture was similar to that of the <i>E. coli</i> -derived recombinant hGH, as shown by proliferation of Nb2 node lymphoma cells	[88]
Mouse interleukin-12 (IL-12)	Tomato leaf and fruit	2.7–7.3 and 1–3.4 µg g ⁻¹ FW	Biologically active <i>in vitro</i> . The plant-produced mIL-12 induced the secretion of IFN-γ by T cells	[89]
Human epidermal growth factor (hEGF)	Tobacco	0.09–0.3% TSP	Plant-produced hEGF significantly stimulated Vero E6 cell expansion and proliferation similar to commercial hEGF products	[90]
Human basic fibroblast growth factor (bFGF)	Soybean	2.3% TSP	Mitogenic assay demonstrated that bFGF stimulated Balb/c 3T3 cells to proliferate in a dose-dependent manner, indicating similar biological activity as native bFGF	[91]
Human acid β-glucosidase (GCase)	Tobacco seeds	Not reported	Plant-derived GCase was taken up by fibroblasts of a Gaucher type II patient and lacks potentially immunogenic glycans	[33]
Human glucocerebrosidase enzyme (GCD)	Carrot cells	Not reported	Recombinant GCD in transgenic plant cells is biologically active. Phase I clinical trial have shown that no clinical or laboratory evidence of any significant innate or humoral immune reactions. A Phase III clinical trial is currently ongoing	[8]
Human cytokine granulocyte macrophage colony stimulating factor (GM-CSF)	Sugarcane	Undetectable–0.02% TSP	Human bone marrow cells (TF-1), which require GM-CSF for cell division, proliferated when growth media was supplemented with transgenic sugarcane extracts and had identical activity levels	[92]
Human granulocyte-macrophage colony stimulating factor (hGM-CSF)	Rice	1.2–1.3% TSP	Rice seed-derived hGM-CSF induces proliferation of TF-1 cells similar to <i>E. coli</i> -derived hGM-CSF	[93]
Macrophage colony-stimulating factor (M-CSF) 1	Tobacco	0.02–1.92% TSP	Plant-derived M-CSFsR inhibits colony formation of J6-1 cells	[94]
Human granulocyte-colony stimulating factor (hG-CSF)	Rice suspension cells	0.7% TSP	Plant-derived hG-CSF supports proliferation of the AML-193 cells similar to commercial <i>E. coli</i> -derived hG-CSF	[95]
Bone morphogenetic protein 2 (BMP2)	Tobacco	0.02% TSP	Application of hBMP2 to mouse C2C12 cell line significantly increased cell ALP activity but is lower than commercial rhBMP2	[96]
Human α-1-antitrypsin (AAT)	Tomato	0.44–1.55% TSP	Biologically active, showing high specific activity and efficient inhibition of elastase activity	[97]
Human IA-2 (IA-2ic), a diabetes-associated autoantigen	Tobacco (Transient)	0.5% TSP	Plant-derived IA-2ic protein is specifically recognized by human IA-2ic autoantibodies	[35]
Human fibroblast growth factor 8 isoform b (FGF8b)	Tobacco (transient)	90–150 µg g ⁻¹ FW	Plant-expressed FGF8b effectively increased the rate of cell proliferation of NIH3T3 as bacterially expressed mouse FGF8b	[36]

Over the past two decades, vaccine antigens expressed via the plant nuclear genome have elicited appropriate immunoglobulin responses and have conferred protection upon oral delivery [6,7] but no transgenic plant-based vaccine has yet moved beyond a Phase I clinical trial [7]. However, at least two therapeutics developed by Biolex (<http://www.biolex.com>) and Protalix (<http://www.protalix.com>) are moving through Phase II and Phase III [8–10].

Stably integrated nuclear transgenes

The earliest research using plants for the recombinant expression of vaccine antigens was performed using stable transformation of the nuclear genome of tobacco (*Nicotiana tabacum*) [11]. The ease of nuclear transgene methodology, started by *Agrobacterium*-mediated delivery of DNA, facilitated this approach, which was the dominant strategy for several years. Advantages include the ability to scale up

production of large amounts of vaccine antigens from transgenic seed stocks and the possibility of expression in fruits or other edible plant organs, enabling oral delivery of minimally processed materials. However, stably integrated nuclear transgenes typically yield relatively low levels of expression (<1% total soluble protein, TSP; Appendix BTable S1 in the supplementary material online), which can vary plant to plant and generation to generation, probably owing to gene silencing or position effect [12].

Many different vaccine antigens have been produced in various plant species, including tobacco and edible plants (e.g. *Solanum tuberosum*, *Solanum lycopersicum*, *Zea mays*, *Daucus carota* and *Glycine max*; Table 1, Appendix BTable S1 in the supplementary material online). Oral delivery of several plant-derived antigens has elicited antigen-specific antibodies in mice and, in a few clinical

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Table 3. Functional vaccine antigens and biopharmaceutical proteins expressed via the chloroplast genome (for full length table see Appendix BTable S3 in the supplementary material online)

Vaccine antigens	Expression system	Expression level	Functional evaluation	Refs
Bacterial antigens				
Cholera toxin B (CtxB)	Tobacco Lettuce	4.1%, 8% and 12.3% TSP4.8% and 9.4% TSP	GM ₁ ganglioside-binding assay. Long-term protection (50% mouse lifespan) against CT challenge in both oral (100%) and subcutaneously (89%) immunized mice; protection correlated with CTB-specific IgA and IgG1 titers in oral and IgG1 in subcutaneously immunized mice; increasing numbers of IL-10+T cell but not Foxp3+ regulatory T cells, suppression of IFN- γ and absence of IL-17 were observed in protected mice	[3,66]
Tetanus toxin (TetC)	Tobacco	18–27% and 7–10% TSP	Mice developed systemic immune response and survived the tetanus toxin challenge	[54]
Anthrax protective antigen (Pag)	Tobacco	4.5–14.2% TSP	Macrophage lysis assay, systemic immune response, toxin neutralization assay, mice survived (100%) challenge with lethal doses of toxin	[55]
Lyme disease –OspA (OspA, OspA-T)	Tobacco	1% and 10% TSP	Systemic immune response in mice. Protected mice against <i>Borrelia burgdorferi</i>	[5]
Plague F1–V (CaF1–LcrV)	Tobacco	14.8% TSP	Immunogenic in mice (IgG1 titers). Oral delivery offered greater protection (88%) and immunity than subcutaneous (33%) injection when challenged with 50-fold lethal dose of aerosolized <i>Y. pestis</i>	[58]
<i>E. coli</i> enterotoxin B (LTB)	Tobacco	2.3% TSP	GM ₁ ganglioside-binding assay; oral immunization partially protected mice from CT challenge	[53]
Viral antigens				
Canine parvovirus (CTB–2L21, GFP–2L21)	Tobacco	31.1% and 22.6% TSP	Rabbit sera neutralized CPV in an <i>in vitro</i> assay	[59]
Hepatitis E virus (HEV E2)	Tobacco leaves Seeds	0.63–1.09 ng and 0.015–0.018 ng μg^{-1} TSP	Immune response in mice	[62]
Swine fever virus (CSFV E2)	Tobacco	1–2% TSP	Immune response in mice	[60]
Human Papillomavirus (L1)	Tobacco	20–26% TSP	Systemic immune response in mice after intraperitoneal injection and neutralizing antibodies were detected	[63]
Protozoan antigens				
Amoebiasis (LecA)	Tobacco	7% TSP	Systemic immune response in mice	[65]
Malaria (CTB– <i>ama1</i> and CTB– <i>msp1</i>)	Tobacco Lettuce	12.3% TSP9.4% TSP	Sera of immunized mice completely inhibited proliferation of the malaria parasite and crossreacted with the native parasite proteins/parasites in immunoblots and immunofluorescence studies, at the ring, trophozoite or schizont parasite stages	[3,66]
	Tobacco Lettuce	8% TSP4.8% TSP		
Autoantigens				
Diabetes – type 1 (CTB– <i>pins</i>)	Tobacco and lettuce	~16% TSP2.05–2.5% TSP	CTB–Pins treated mice showed significant decrease in inflammation (insulinitis) in non-obese diabetic mice; insulin-producing β -cells in the pancreatic islets of CTB–Pins-treated mice were highly protected, increase in insulin production with lower blood or urine glucose levels; increased expression of immunosuppressive cytokines (IL-4, IL-10)	[49]
Diabetes – type 1 (<i>hGAD65</i>)	Chlamydomonas	0.25–0.3% TSP	Immunoreactivity to diabetic sera	[67]
Biopharmaceutical proteins				
Interferon $\alpha 2b$ (IFN- $\alpha 2b$)	Tobacco LAMD Petit Havana	8.0–21.0% TSP2.0–14.0% TSP	Immunogenic in mice. Transgenic IFN- $\alpha 2b$ protected baby hamster kidney cells against cytopathic viral replication in vesicular stomatitis virus cytopathic effect assay, HeLa cells from HIV-1 entry and mice from a highly metastatic tumor line. Also, it increased the expression of major histocompatibility complex class I on splenocytes and the total number of natural killer cells	[41]
Insulin-like growth factor (IGF-1n, IGF-1s)	Tobacco	32.4% TSP32.7% TSP	Growth response in cultured HU-3 cells	[72]
Human alpha1-antitrypsin (A1A7)	Tobacco	2% TSP	Fully active and binds to porcine pancreatic elastase	[71]
Antimicrobial peptide (2 lysine-type protein)	Tobacco	~30% TSP	Bacteriolytic activity and kills <i>Streptococcus pneumoniae</i> , the causative agent of pneumonia	[43]

trials, in humans [13,14]. Vaccine antigens derived from enteric pathogens, such as Norwalk virus capsid protein (NVCP), rotavirus capsid protein and the bacterial ganglioside-binding protein cholera toxin B subunit (CTB) and *Escherichia coli* heat-labile toxin B subunit (LTB), have been extensively evaluated in nuclear transgenic plants (Appendix BTable S1 in the supplementary material online). For example, mice orally immunized with carrot-derived LTB [15] or rice-derived CTB [16] induce protection against cholera toxin (CT) challenge. Several other antigens from non-enteric pathogens such as F protein from respiratory syncytial virus [17] and surface antigen from hepatitis B [18,19] also stimulate antigen-specific antibodies after oral delivery. However, adjuvants might be required for such stimulation. For example, in the case of hepatitis B surface antigen (HBsAg) fed to mice in transgenic potato tubers [20], it was necessary to use CT, a potent mucosal adjuvant, to stimulate significant levels of anti-HBsAg antibodies, although the same HBsAg potatoes fed to humans without adjuvant provoked serum anti-HBsAg in most of the volunteers.

Given that potatoes and most other plant organs (except seeds) have a limited shelf life in the fresh state, and vaccine dosage must be carefully monitored, it is unlikely that fresh, unprocessed plant materials will be used as vaccines. Thus, some form of processing, such as freeze-drying fruits or other plant organs, must be used to produce a product batch that can be validated for potency and stability. For NVCP, it was demonstrated that either freeze- or air-drying of transgenic tomato fruits yielded material that was at least as orally immunogenic as fresh transgenic tomato [21]. However, freeze-dried potato NVCP produced poor results compared with fresh potato NVCP [21], with oxidation of phenolic compounds in dried potato probably causing the problem. Thus, control of oxidation will be an important issue when tissues rich in phenolics and the enzyme polyphenol oxidase are used. The use of seed tissues is more promising, and such material has demonstrated extended stability for both human and animal vaccine candidates. Seed-based vaccines are particularly attractive for the development of veterinary products because of convenience in storage and delivery and have shown promising results in farmed animal studies [22].

Transient expression with viral vectors

Development of a plant viral vector system for transient expression has enabled rapid expression of recombinant proteins at levels higher than with stably integrated nuclear transgenes (Table 1, Appendix BTable S1 in the supplementary material online). Tobacco mosaic virus, although an RNA virus that replicates in the cytosol, can be delivered as a DNA construct that is transcribed in the nucleus to produce viral RNA. In one iteration, “magnIcon” technology has been used to express hepatitis B core antigen [23], HBsAg [24], NVCP [25] and plague antigens F1 and V [26] at levels of up to 1–2 mg g⁻¹ fresh leaf mass in *Nicotiana benthamiana*, and in a similar “Launch Vector” technology a range of candidates, including influenza targets, have been expressed at high levels in *N. benthamiana* [27,28]. Given that tobacco or

its close relatives, such as *N. benthamiana*, are the usual hosts for this system, and vectors are generally introduced by vacuum infiltration of *Agrobacteria*, the products must be purified for delivery as vaccines. This ensures removal of any noxious host plant compounds and removal of *Agrobacteria*. However, because purification is necessary, soluble antigens might lose oral immunogenicity owing to the removal of the encapsulation effect that might help protect them from the harsh stomach environment. Nonetheless, NVCP that was partially purified from leaves did induce serum IgG and mucosal IgA responses after oral delivery to mice [25]. One approach that could overcome this limitation is the expression of vaccine antigens as virus-like particles. This has been followed for a non-viral based transient expression system, with promising immunogenicity results following injection of purified influenza antigen [29]. This might be extendable to oral delivery.

A further DNA replicon system was developed using the geminivirus bean yellow dwarf virus (BeYDV) [30]. The construct is delivered by agro-infiltration, similar to the magnIcon and launch vector systems. Similar to the launch vector system [27], it can be used as a single vector, whereas the magnIcon system requires three separate vectors [25,26]. Expression of HBsAg and NVCP in the range of 0.5–0.8 mg g⁻¹ leaf mass was observed with the BeYDV replicon, which is similar to that obtained with the magnIcon system. The magnIcon system has also been adapted for IgG expression but requires the combination of a TMV (tobacco mosaic virus) replicon with a potato virus X replicon [31] and five different *Agrobacterium* vectors. The BeYDV replicon was also stably incorporated into the nuclear genome, with an inducible promoter driving the viral *Rep* gene to control gene amplification [32]. Tenfold increases in expression of NVCP in cultured tobacco cells were obtained after induction. This system might enable the use of stable transgenic lines with improved expression.

Nuclear-derived pharmaceutical proteins

Recombinant pharmaceutical proteins are useful for treatment of various conditions such as genetic diseases that result in the production of an insufficient quantity or quality of a particular protein. Given that many of these proteins would be administered parenterally, a high degree of purification under current Good Manufacturing Practice conditions is necessary for human use. Nuclear transgenic plant strategies have been used in many studies to demonstrate the production of many valuable human proteins, including epidermal growth factor, serum albumin, interferons, interleukins, lysozyme, lactoferrin and human acid β -glucosidase (Table 2, Appendix BTable S2 in the supplementary material online). However, as with vaccine antigens, expression levels are generally low. Specific assays demonstrated activity of the recombinant protein *in vitro* or in cell-based assays, but extensive animal studies have not yet been published. Although plant cells can synthesize and process mammalian proteins, in some cases, plant-specific glycosylation of ER- or cell surface-targeted proteins might produce undesired immunogenicity of the recombinant protein, which could diminish its half-life and utility. β -Glucosidase produced in seeds of tobacco lacks the plant-specific xylose

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and fucose residues that can be problematic [33]. If seed-based expression of other ER- or cell surface-targeted proteins yields similar results, then targeting of recombinant proteins to seed storage vacuoles will be an advantage. ER retention of proteins through the C-terminal inclusion of a KDEL or KDEL-like tag serves to limit glycosylation of recombinant proteins to core glycan structures, by comparison to cell surface-targeted proteins [4]. Also, recent advances in plant glycan engineering hold the promise of broadly applicable production of pharmaceutical glycoproteins in plants [34]. In addition, a few pharmaceutical proteins (human growth hormone, human fibroblast growth factor, human diabetes-associated autoantigen, etc.) have been produced in plants using the transient expression system [35–37], where subcellular targeting is again an option.

Chloroplast-derived vaccine antigens and therapeutics

In chloroplast technology, foreign genes are integrated into the chloroplast genome by homologous recombination [38], eliminating variation of expression among independent transgenic lines. Moreover, gene silencing has not yet been reported in chloroplast transgenic (transplastomic) lines. High levels of expression of vaccine antigens are facilitated by >10,000 copies of transgenes in each transformed plant cell. Chloroplast expression minimizes the risk of foreign gene transfer via pollen from genetically modified crops to other related crops or weeds owing to maternal inheritance of transgenes [39,40]. In addition, expression of therapeutic proteins in leaves facilitates their harvest before the development of any reproductive structures and offers additional opportunities for containment. Therefore, several chloroplast-derived vaccine antigens and biopharmaceutical proteins have been grown in field conditions and at least one such study has been published [41]. To date, 23 vaccine antigens against 16 different diseases and 11 biopharmaceutical proteins have been expressed in chloroplasts (Table 3, Appendix BTable S3 in the supplementary material online). The presence of chaperones and post-translational modification enzymes in the chloroplast creates a niche for target processing and assembly with suitable post-translational modifications (assembly of multimers, disulfide bonds, lipid modification, etc.), although glycosylation is not an option because N-glycosylation is not known to occur within chloroplasts. The chloroplast expression system has been used to produce several fully functional vaccine antigens against bacterial, viral and protozoan pathogens (Table 3, Appendix BTable S3 in the supplementary material online). Proteins that are difficult to express in other systems, such as antimicrobial proteins, have also been expressed in chloroplasts [42,43]. In addition to plants, *Chlamydomonas* chloroplasts have been used for expression of therapeutic proteins, although achieving higher levels of expression was a major challenge until recent improvements in this system [44]. Indeed, adequate levels of expression of therapeutic proteins in chloroplasts are not always possible and only a few proteins have been evaluated for functionality (Table 3, Appendix BTable S3 in the supplementary material online).

One major limitation in this field is that several vaccine antigens and therapeutic proteins have only been

expressed in tobacco. Tobacco is not edible and the addictiveness of nicotine also makes it unsuitable for oral delivery of therapeutic proteins. As an alternative, the carrot plastid transformation system was developed with adequate levels of gene expression in edible tissues to facilitate oral delivery [45]. However, carrot regeneration is slow and is not suitable for rapid production of therapeutic proteins. Although adequate expression of human immune deficiency virus (HIV) p24 antigen was observed in tomato leaves [46], there was a >90% reduction in green fruits and no expression was observed in ripe fruits. Lettuce (*Lactuca sativa* L.) chloroplast transformation has also been developed [47–49] and there has been success in expressing the first therapeutic protein (proinsulin) in lettuce chloroplasts [49]. Recently, this system has been optimized and several therapeutic proteins have been expressed [3]. The level of expression in lettuce chloroplasts is similar to that in tobacco chloroplasts and regeneration is as rapid as in tobacco, thus opening the door to practical oral delivery of chloroplast expressed proteins.

Another challenge is oral delivery of chloroplast-derived proteins in adequate doses to confer immunity or, in cases of autoantigens, to induce tolerance. In this context, it has been demonstrated that chloroplast-derived therapeutic proteins, delivered orally via plant cells, are protected from degradation in the stomach, presumably because of bioencapsulation of the antigen by the plant cell wall. To facilitate translocation of vaccine antigens or therapeutics from the gut lumen into the circulatory system, target proteins have been fused to the CTB transmucosal carrier protein, which can bind to the epithelial receptor GM₁ [50]. This approach has been widely applied to many orally delivered antigens, both for stable nuclear transgenics and for transplastomic approaches.

Chloroplast-derived bacterial antigens

Several vaccine antigens against bacterial diseases have been produced in chloroplasts (Table 3, Appendix BTable S3 in the supplementary material online). CTB subunit [51,52], the first vaccine expressed in chloroplasts, was shown to be functional by the GM₁ binding assay. Subsequently, a fusion of LTb and heat-stable toxin (ST) has been expressed in tobacco chloroplasts, and mice immunized with a chloroplast-derived LTb–ST fusion protein were partly protected against CT challenge [53]. Recently, two CTB fusion proteins were expressed in tobacco and lettuce chloroplasts [3]. Both subcutaneously and orally administered mice with chloroplast-derived CTB fusion proteins were protected against CT challenge. High levels of serum and intestinal CTB–IgA were detected in orally administered mice. Although none of the mice administered antigen subcutaneously had detectable CTB–IgA, high levels of IgG1 titer conferred protection (86%) against the CT challenge. Comparable patterns of IgG1 expression observed in orally administered mice in addition to intestinal and serum IgA conferred 100% protection and confirmed that oral vaccination results in both a mucosal and a systemic immune response [3]. Most importantly, long-term immunity and protection (up to 50% of mouse lifespan) was observed. This is a significant advancement because immunity is lost in the current cholera vaccine

in children within 3 years and it is not fully protective in adults. Vaccine antigen against tetanus also conferred complete protection against pathogen challenge [54]. Although chloroplast-derived outer surface lipoprotein A (OspA) generated a lower level of protective antibodies compared with lipidated *Escherichia coli*-derived OspA, the generated antibody level could protect against Lyme disease [5].

The chloroplast transformation system has been used successfully to develop vaccines against bioterrorism agents. The currently available anthrax vaccine obtained from the culture of *Bacillus anthracis* has undesirable side effects, such as local pain and edema, and relatively high rates of local and systemic reactions, including inflammation and flu-like symptoms. In an attempt to produce anthrax vaccine in large quantities that is free of extraneous bacterial contaminants, protective antigen (PA) was expressed in transgenic tobacco chloroplasts by inserting the *pagA* gene into the chloroplast genome [55,56]. High levels of IgG titers have been reported in mice immunized subcutaneously with partially purified chloroplast-derived PA and 100% of animals survived against lethal doses of toxin challenge. It is estimated that up to 360 million doses of fully functional anthrax vaccine could be produced from one acre of tobacco [55,56].

By contrast, there is no approved vaccine for bubonic or pneumonic plague, which is a potential agent for bioterrorism [57]. Recently, a plague F1-V fusion antigen has been expressed in chloroplasts and its functionality was investigated in mice [58]. Animals immunized either subcutaneously or orally with chloroplast-derived protective antigen F1-V showed significantly higher levels of serum F1-V specific IgG1 titers. Of the subcutaneously immunized mice, 33% survived. By contrast, 88% of the orally immunized mice were protected against a heavy dose of aerosolized *Yersinia pestis* plague challenge. A comparison of splenic *Y. pestis* CFU counts showed up to a 10-log reduction in the mean bacterial burden in survivors. This shows that oral delivery offers greater protection and immunity [58] and demonstrates that oral delivery of a chloroplast-derived vaccine can be an effective mode of delivery for plant-derived vaccine antigens.

Chloroplast-derived viral antigens

The first viral antigen capable of eliciting a protective immune response when expressed in chloroplasts was against canine parvovirus (CPV) [52,59]. Mice immunized intraperitoneally with chloroplast-derived 2L21 fused to CTB in leaf extract elicited anti-2L21 antibodies. Furthermore, the antibodies induced in rabbits by CTB-2L21-enriched plant extract efficiently neutralized CPV infection of CRFK cells [59]. The structural protein E2, a neutralizing antigen for classical swine fever virus (CSFV) was expressed in tobacco chloroplasts and subcutaneous immunization induced serum antibody reaction against CSFV; however, no immune response was detected by oral immunization [60], in contrast to studies described above with F1-V.

It has been shown that a chloroplast-derived subunit vaccine for hepatitis E virus ORF2-encoded pE2 peptide induce pE2-specific antibody in subcutaneously immunized mice [61,62]. Also, the major structural protein of

human papillomavirus, HPV-16 capsid, L1 was expressed from the tobacco chloroplast genome and the chloroplast-derived L1 protein displayed conformation-specific epitopes and assembled into virus-like particles visible by transmission electron microscopy [63,64]. Furthermore, chloroplast-derived L1 protein showed immunogenic and neutralizing antibodies in mice injected intraperitoneally [63].

Chloroplast-derived protozoan antigens

A potential anti-amoebic candidate, LecA, a surface antigen of *Entamoeba histolytica*, expressed in tobacco chloroplasts, resulted in high immunogenicity in mice following subcutaneous administration [65]. It has been reported that 29 million doses of vaccine antigen can be produced per acre of transplastomic plants [65]. Recently, malaria vaccine candidates AMA1 and MSP1 fused with CTB were expressed in tobacco and lettuce chloroplasts, with a significant level of antigen-specific titers in both oral and subcutaneously immunized mice [3,66]. Immunoblot and immunofluorescence studies have shown that the chloroplast-derived AMA1 and MSP1 inhibit the proliferation of the malaria parasite and crossreact with the native parasite proteins at the schizont, ring or trophozoite stage. *In vitro* parasite inhibition studies show that mice with higher anti-MSP1 antibody titer exhibit the highest inhibition [3,66].

Chloroplast-derived autoantigens

Type 1 diabetes (TD1) results from the autoimmune destruction of insulin-producing cells and current treatments are not only expensive but also provide only transient relief. Recently, human proinsulin fused with CTB was expressed in lettuce and tobacco chloroplasts [49]. It was shown that the pancreas of mice immunized with chloroplast-derived CTB-proinsulin had decreased infiltration of cells characteristic of lymphocytes (insulitis) and significantly preserved insulin-producing B-cells in the pancreatic islets with lower blood and urine glucose levels. Moreover, increased expression of immunosuppressive cytokines, such as interleukin-4 and interleukin-10 (IL-4 and IL-10), was observed in the pancreas of chloroplast-derived CTB-proinsulin treated mice, indicating T-helper 2 (Th2) lymphocyte-mediated oral tolerance [49]. Another autoantigen for the prevention and delayed onset of TD1, glutamic acid decarboxylase 65 (GAD65), has been expressed in *Chlamydomonas* chloroplasts and recombinant hGAD65 reacted with TD1 sera from non-obese diabetic mice and stimulated the proliferation of spleen lymphocytes from these mice [67].

Chloroplast-derived human blood proteins

Several chloroplast-derived pharmaceutical proteins have been produced (Table 3, Appendix B Table S3 in the supplementary material online). For example, human somatotropin was expressed in tobacco chloroplasts and functionality was shown by using a rat lymphoma cell line, Nb2 proliferation assay [68]. Human serum albumin expressed in tobacco chloroplasts formed inclusion bodies [69]. Human interferon gamma (IFN- γ) expressed in chloroplasts protected a human lung carcinoma cell line against

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infection with the encephalomyocarditis virus [70]. Chloroplast-derived IFN- α 2b had similar activity to commercially produced PEG-IntronTM, protecting cells against cytopathic viral replication in the vesicular stomatitis virus cytopathic effect assay, inhibiting early-stage HIV infection, and increasing the expression of major histocompatibility complex class I on splenocytes and the total number of natural killer cells. IFN- α 2b purified from transplastomic lines protected mice from a highly metastatic tumor line following intraperitoneal injection [41]. Human alpha1-antitrypsin (A1AT) expressed in tobacco chloroplasts binds to porcine pancreatic elastase [71], although a detailed functional evaluation is needed. Insulin-like growth factor 1 (IGF-1) was expressed in tobacco chloroplast [72]. Mass spectrometry confirmed the identity of purified chloroplast-derived human IGF-1. The biological activity of the chloroplast-derived and the commercially synthesized human IGF-1 have similar levels of mitotic activity, as shown by a mammalian cell (human HU-3 cells) proliferation assay [72]. Proper functionality of several of these human blood proteins show that required disulfide bonds are formed in chloroplasts [41,68,70]. Therefore, proper post-translational modifications and functionally active chloroplast-derived therapeutic proteins have opened the door for the production of low-cost plant based therapeutic proteins.

Conclusions

When delivered orally, plant-derived vaccines and biopharmaceuticals have the advantage that there is no need for expensive fermentation and purification systems and other expenses associated with cold storage, transportation and sterile delivery. Although plant-made vaccines have been developed and advanced for two decades, none of them have advanced beyond Phase I human clinical trials. Crucial evaluation of various expression systems highlights the advantages and disadvantages of each system. Stable nuclear expression systems have been developed over three decades and are available in a large number of crops, with the ability to facilitate tissue-specific expression and inducible expression. However, as seen in the list of vaccine antigens and biopharmaceuticals expressed via the nuclear genome (Appendix B Tables S1 and S2 in the supplementary material online), expression levels are often inadequate for commercial development, although adequately expressed antigens have advanced into clinical trials in a few cases. Chloroplasts generally offer higher levels of expression of vaccine antigens against bacterial, viral and protozoan pathogens and biopharmaceuticals, and transgene containment via maternal inheritance. Chloroplast system for expression of therapeutic proteins has been developed in the past 8 years and recent development of an efficient lettuce chloroplast system for oral delivery is a significant advancement. However, glycoproteins cannot be expressed in chloroplasts in their glycosylated form. Although viral expression systems produce therapeutic proteins quickly, they are generally not suitable for oral delivery [73]. Therefore, costs associated with purification, cold storage, transportation and sterile delivery would be major challenges for the viral systems. The downstream processing cost is typically 80% of the pro-

duction cost and a significant challenge for the future is to reduce this [7]. However, research into the viral system has made significant progress recently, including the development of an antibody for passive immunity against non-Hodgkin's lymphoma in Phase I clinical trials [74], testing F1-V plague vaccine in monkeys [57] and the development of anti-HIV peptides [75]. The transient expression technology is well suited for pandemic responses, such as that faced with H1N1 influenza strains.

The ultimate success of plant-based production systems for subunit vaccine and protein therapeutic candidates will probably depend on lead commercially viable targets successfully transitioning through clinical trials over the next few years. Plant-based production systems for recombinant proteins have been researched over the past 20 years, with a proliferation of academic groups and biotechnology companies, together with groups from larger agrochemical companies, developing the expression technologies and lead product candidates. This effort needs to be repaid with positive results in later clinical studies for the field to continue to advance. Notable early clinical stage successes have been recorded, advancing several oral (or edible) vaccine candidates into Phase I trials [14,19,76–78]. However, none of these candidate products have progressed further, largely because they either represented proof-of-principle targets or impractical oral delivery vehicles. During the past 5 years, insufficient funding and lack of commercial interest in plant-based oral vaccines has led to this approach slowing, and no oral vaccine candidates have recently entered the clinic. The challenges of guiding novel plant-based oral vaccines through the regulatory process currently outweigh available resources. More recently, there has been an increased focus on using plant-based production systems to generate purified recombinant proteins for vaccines and therapeutics to be delivered by injection. Notable recent examples that have had clinical trial success include a personalized therapeutic vaccine for non-Hodgkin's lymphoma assessed in a Phase I clinical trial [74], IFN- α 2b to combat hepatitis C assessed in a Phase IIa clinical trial (<http://www.biolex.com>), and human glucocerebrosidase to combat Gaucher's disease, which has now progressed into a Phase III clinical trial [8]. These and similar purified protein products represent the current best options for making plant-based production systems for human vaccines and therapeutics a reality. In these cases, the emphasis is on demonstrating that the plant production systems can generate antigen or therapeutic protein of comparable safety potency and efficacy to other recombinant systems, without added concerns of an oral recombinant plant delivery vehicle. On a parallel track, a plant-produced vaccine candidate for Newcastle disease in chickens was approved for use in 2006. This again focused on a protein product purified from plant cells and emphasizes that plant-produced targets can progress along the regulatory path.

In summary, the availability of diverse plant-based production systems offers opportunities to produce a wide array of antigens and biopharmaceutical proteins against diverse diseases, and for different means of delivery to be explored. However, greater research emphasis is needed on large-scale production, purification, functional characterization,

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oral delivery and preclinical evaluation for this field to move forward.

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Supplementary data

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