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Abstract

Although the plant-made vaccine field started three decades ago with the promise of developing low-cost vaccines to prevent infectious disease outbreaks and epidemics around the globe, this goal has not yet been achieved. Plants offer several major advantages in vaccine generation, including low-cost production by eliminating expensive fermentation and purification systems, sterile delivery and cold storage/transportation. Most importantly, oral vaccination using plant-made antigens confers both mucosal (IgA) and systemic (IgG) immunity. Studies in the past 5 years have made significant progress in expressing vaccine antigens in edible leaves (especially lettuce), processing leaves or seeds through lyophilization and achieving antigen stability and efficacy after prolonged storage at ambient temperatures. Bioencapsulation of antigens in plant cells protects them from the digestive system; the fusion of antigens to transmucosal carriers enhances efficiency of their delivery to the immune system and facilitates successful development of plant vaccines as oral boosters. However, the lack of oral priming approaches diminishes these advantages because purified antigens, cold storage/transportation and limited shelf life are still major challenges for priming with adjuvants and for antigen delivery by injection. Yet another challenge is the risk of inducing tolerance without priming the host immune system. Therefore, mechanistic aspects of these two opposing processes (antibody production or suppression) are discussed in this review. In addition, we summarize recent progress made in oral delivery of vaccine antigens expressed in plant cells via the chloroplast or nuclear genomes and potential challenges in achieving immunity against infectious diseases using cold-chain-free vaccine delivery approaches.

Keywords

human infectious diseases, molecular farming, plant transformation, bioencapsulation, oral delivery, mucosal immune response

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Plant-made oral vaccines against human infectious diseases—Are we there yet?

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Summary

Although the plant-made vaccine field started three decades ago with the promise of developing low-cost vaccines to prevent infectious disease outbreaks and epidemics around the globe, this goal has not yet been achieved. Plants offer several major advantages in vaccine generation, including low-cost production by eliminating expensive fermentation and purification systems, sterile delivery and cold storage/transportation. Most importantly, oral vaccination using plant-made antigens confers both mucosal (IgA) and systemic (IgG) immunity. Studies in the past 5 years have made significant progress in expressing vaccine antigens in edible leaves (especially lettuce), processing leaves or seeds through lyophilization and achieving antigen stability and efficacy after prolonged storage at ambient temperatures. Bioencapsulation of antigens in plant cells protects them from the digestive system; the fusion of antigens to transmucosal carriers enhances efficiency of their delivery to the immune system and facilitates successful development of plant vaccines as oral boosters. However, the lack of oral priming approaches diminishes these advantages because purified antigens, cold storage/transportation and limited shelf life are still major challenges for priming with adjuvants and for antigen delivery by injection. Yet another challenge is the risk of inducing tolerance without priming the host immune system. Therefore, mechanistic aspects of these two opposing processes (antibody production or suppression) are discussed in this review. In addition, we summarize recent progress made in oral delivery of vaccine antigens expressed in plant cells via the chloroplast or nuclear genomes and potential challenges in achieving immunity against infectious diseases using cold-chain-free vaccine delivery approaches.

Keywords

human infectious diseases; molecular farming; plant transformation; bioencapsulation; oral delivery; mucosal immune response

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Conflict of interest

Henry Daniell is an inventor in several US and international patents on chloroplast transformation technology to produce biopharmaceuticals, in particular induction of oral tolerance.

Introduction

Traditional vaccines consist of inactivated or attenuated pathogens and are not entirely safe. Vaccine-derived poliovirus has been reported in a number of countries (Wang *et al.*, 2014). Live attenuated and killed viruses carry the risk of reverting to virulence, as well as other drawbacks in terms of antigenic variability between species, low levels of immunogenicity and possible gene transfer to wild-type strains (Adeniji and Faleye, 2015; Burns *et al.*, 2014). An outbreak of type 2 vaccine-derived polio in Nigeria was first detected in 2006, became endemic in Africa and persists today (Famulare and Hu, 2015). This large poliomyelitis outbreak, caused by type 2 circulating vaccine-derived poliovirus (cVDPV2), began in 2005 in northern Nigeria. According to the phylogenetic analysis of the P1/capsid region sequences of isolates from the 403 cases reported between 2005 and 2011, at least 7 of 23 independent type 2 vaccine-derived poliovirus (VDPV2) emergences established circulating lineage groups (Burns *et al.*, 2013). Therefore, new approaches are needed to improve current vaccines.

Plant-made subunit vaccines are heat stable, lack animal pathogen contamination and can be engineered to contain multiple antigens, such as those that are combined with subunits of cholera toxin (CT), for the protection of humans and animals against multiple infectious diseases (Davoodi-Semiromi *et al.*, 2010; Hefferon, 2013; Kwon *et al.*, 2013b; Scotti *et al.*, 2010). It is possible to harvest and process plant material on a large scale. When plants expressing a recombinant antigen are used as feed, they eliminate the purification requirement (Peters and Stoger, 2011). Plants offer general advantages for large-scale economic production, product safety and ease of storage and distribution (Holásková *et al.*, 2015). Plant-based oral vaccines could revolutionize the vaccine industry by reducing the cost of complex production systems, such as fermentation, purification, cold storage and transportation (Kwon *et al.*, 2013b). In addition, the use of plants to express pathogen subunit vaccine proteins allows the rapid production of diverse antigens that contain disulphide bonds, are glycosylated or require other post-translational modifications to achieve their desired biological function (Kwon *et al.*, 2013b; Yusibov *et al.*, 2011). The use of transgenic plants to produce subunit vaccine proteins has been developed as an alternative platform for the large-scale production and delivery of vaccines to induce protective immune responses via the mucosal immune system (Daniell *et al.*, 2009). Vegetable and fruit crops are ideal host systems for oral vaccine production. Potential plant species used for pharmaceutical protein production include alfalfa, carrot, lettuce, tomato, potato, maize, soya bean, rice and banana (Ahmad *et al.*, 2012; Azegami *et al.*, 2014).

Oral vaccines can effectively elicit humoral and cellular immunity of both the mucosal and systemic immune systems and eliminate undesired pain and discomfort from injections and needle-associated risks (Kwon *et al.*, 2013b). Plant-derived antigens can induce antigen-specific mucosal IgA and serum IgG synthesis when delivered orally to mice and humans (Sack *et al.*, 2015). Plant-based antigens can be fed directly to animals or humans without purification or processing. Transgenic plants are ideal for producing oral vaccines because the antigenic proteins are protected from the acidic environment in the stomach by the plant cell wall, enabling antigens to reach the gut-associated lymphoid tissue (GALT) (Limaye *et al.*, 2006; Ruhlman *et al.*, 2007; Verma *et al.*, 2010). Several recent studies have

unequivocally shown that bioencapsulation of antigens within the plant cell wall protects them from the acids and enzymes in the stomach (Boyhan and Daniell, 2011; Kwon *et al.*, 2013a; Shenoy *et al.*, 2014; Shil *et al.*, 2014). Although human digestive enzymes do not hydrolyse glycosidic bonds of plant cell wall carbohydrates, commensal microbes are able to digest and release protein drugs into the gut lumen. Bacteria inhabiting the human gut have evolved to hydrolyse almost all plant glycans (Martens *et al.*, 2011). Bacteria colonizing the large intestine are greater in number and digest plant cells that escape the upper gut (Flint *et al.*, 2008). Orally delivered insulin or exendin bioencapsulated in plant cells lowers blood sugar levels within 30 min (Boyhan and Daniell, 2011; Kwon *et al.*, 2013a). Histological studies provide ample evidence for the delivery of protein drugs across the gut epithelium throughout the ileum in the villi and Peyer's patches (Sherman *et al.*, 2014; Verma *et al.*, 2010; Wang *et al.*, 2015). Therefore, there is new evidence for the role of microbes in the small intestine in the release of protein drugs from plant cells in the gut lumen.

The cholera toxin B subunit (CTB) from *Vibrio cholerae* and the heat-labile (LT) enterotoxin B subunit (LTB) of *Escherichia coli* are well-characterized bacterial proteins that have strong potential as mucosal carrier proteins (Chia *et al.*, 2011; Lakshmi *et al.*, 2013). Recombinant CTB was approved for human use a decade ago and is used by hundreds of millions of people worldwide (Hill *et al.*, 2006). When expressed in plants, CTB assembles into pentamers and acts as an effective carrier of fused foreign proteins to elicit immune responses in mice (Daniell *et al.*, 2001; Davoodi-Semiromi *et al.*, 2010). CTB and LTB are mucosal immunogens and induce both mucosal and systemic responses after administration through the mucosal surface or systemic delivery. The biological functions of CTB and LTB are dependent on their forming pentamers, which then bind to GM1-ganglioside receptors on the surface of the intestinal epithelial cells (Daniell *et al.*, 2001). Hence, both pentameric CTB and LTB function as carrier proteins for genetically fused antigens, and they can deliver these antigens across the mucosal epithelium to the underlying mucosa-associated lymphoid tissue (MALT) (Chia *et al.*, 2011; Czerkinsky and Holmgren, 2010; Davoodi-Semiromi *et al.*, 2010). CTB-fused autoantigens are ideal for the induction of oral tolerance, when delivered without priming (Ruhlman *et al.*, 2007; Sherman *et al.*, 2014; Verma *et al.*, 2010; Wang *et al.*, 2015).

Mechanism of the mucosal immune response

The mucosal immune system in the gut is regulated by mechanisms that initiate protective immune responses against pathogens while preventing responses to harmless intestinal antigens from food or commensals (Wang *et al.*, 2013). Lymphocytes in the mucosa are organized into structures known as MALT, which can be subdivided into the sites in which they are found, such as the bronchus-associated lymphoid tissue and the GALT (Czerkinsky and Holmgren, 2010). The GALT is the largest human mucosa and immunologic organ in the body. Within the intestinal GALT, Peyer's patches are the most recognized lymphoid organs, and they can be observed as lymphoid aggregates clustered in the ileum of the small intestine (Figure 1; Thanavala and Lugade, 2010). Microfold (M) cells residing in the follicle-associated epithelium (FAE) overlying the Peyer's patches are specialized epithelial cells that take up antigens or microorganisms from the intestinal lumen by endo-, phago-, or pinocytosis and transcytosis and transport them to the underlying immune system of the

mucosae (Azizi *et al.*, 2010). Most studies have identified both the mesenteric lymph nodes and the Peyer's patches as the major sites for antigen presentation and T-cell activation in response to orally delivered antigens in mouse models (Thanavala and Lugade, 2010). Antigen uptake could also occur by other mechanisms, for instance via the intestinal dendritic cells (DCs). It has been proposed that antigens taken up by M cells and transported into the Peyer's patches induce intestinal IgA antibodies, whereas those taken up by the DC induce systemic IgG antibodies (Hernández *et al.*, 2014). The immune response to intestinal antigens primarily produces IgA from B cells and generates T cells that secrete the Th2-associated cytokines interleukin 4 [IL-4, a cytokine involved in the differentiation of naive helper T cells (Th0 cells) to Th2 cells], interleukin 10 [IL-10, an anti-inflammatory cytokine also known as human cytokine synthesis inhibitory factor (CSIF)] and transforming growth factor-β (TGF-β, an immune suppressive cytokine involved in the induction of tolerance) rather than the Th1-associated interferon-γ (IFN-γ, a cytokine involved in the regulation of immune and inflammatory responses) (Lamichhane *et al.*, 2014). The ability of M cells in the Peyer's patches to take up and transcytose diverse numbers of microorganisms and antigens to antigen-presenting cells (APCs) has made M cells a prime target to enhance oral vaccine efficacy (Azizi *et al.*, 2010; Yuki and Kiyono, 2009).

Antigen uptake by the GALT is inefficient partly because of the proteolytic and acidic stomach environment. Bioencapsulated antigens fused with CTB are protected from stomach acids and enzymes but are released into the immune system in the gut (Davoodi-Semiromi *et al.*, 2010; Limaye *et al.*, 2006). Orally administered antigens form the pentameric structure required for binding to GM1 gangliosides on the intestinal epithelial cells, thereby allowing for endocytosis (Limaye *et al.*, 2006). Antigen–CTB-GM1 complexes then traffic through the trans-Golgi network (TGN) and into the lumen of the endoplasmic reticulum (ER), releasing the fused antigen via proteolytic cleavage of the furin cleavage site between CTB and the antigen in the TGN. Then, the antigen is exocytosed and released into the extracellular fluid, whereas CTB is retained intracellularly (Verma *et al.*, 2010). Antigens are primarily sampled and processed in the intestine by mononuclear phagocytes, including macrophages and DCs, which are critical for the differentiation, expansion and maintenance of Tregs (regulatory T cells expressing immune suppressive cytokines). Systemically delivered protein antigens in the gut have been found to be taken up by F4/80+ and CD11c+ cells in the ileum (Wang *et al.*, 2015).

Evidence for oral delivery with bioencapsulated vaccine antigens in mice

To understand the oral delivery route of plant-based recombinant proteins that circulate in the body after administration, mice were fed leaves expressing CTB fused with green-fluorescent protein (CTB–GFP) with a furin cleavage site between CTB and GFP. GFP fluorescence was observed in the intestinal mucosa, liver and spleen, indicating that CTB–GFP had been taken up by the enterocytes and the GALT through GM1 receptor binding of pentameric CTB–GFP (Limaye *et al.*, 2006). Haemophilia A and B patients are deficient in producing blood clotting factors VIII (FVIII) and IX (FIX), respectively, and their treatment involves intravenous infusion of these factors. However, some patients develop antibodies that are toxic and cause anaphylaxis. Therefore, FIX fused with CTB expressed in chloroplasts and bioencapsulated in plant cells has been orally delivered to induce tolerance.

The furin cleavage site is commonly used because furin is ubiquitous protease and is present in all cell types (Kwon *et al.*, 2013a). Oral delivery of protein drugs fused with furin was efficiently cleaved, releasing functional protein into the circulatory system (Kohli *et al.*, 2014; Kwon *et al.*, 2013a; Shenoy *et al.*, 2014; Shil *et al.*, 2014; Verma *et al.*, 2010). Consistent with previous findings, CTB was translocated across the epithelial cell barrier and did not co-localize with FIX due to efficient furin cleavage between FIX and CTB (Verma *et al.*, 2010; Wang *et al.*, 2015). Stains of the spleen showed positive labelling for FIX but not CTB, which was expected because CTB is retained within the cell, while cleaved FIX is in part systemically delivered (Verma *et al.*, 2010). Most importantly, the furin cleavage site (NH2-R-R-K-R-COOH) is cut without leaving any extra amino acid on the fused protein.

Immunohistochemical analysis of tissue from mice fed with CTB-FFIX (with furin cleavage site)-expressing plant cells twice per day for 2 days showed that the FIX antigen was delivered to the epithelial M cells and the $CD11c^{+}$ dendritic cells in the Peyer's patches that form the interface between the GALT and the luminal microenvironment. Moreover, the FIX antigen was also observed in the plasma and liver within 2–5 h of feeding, demonstrating systemic delivery of the FIX antigen into circulation (Kwon *et al.*, 2013b; Verma *et al.*, 2010). FVIII antigen that was orally delivered to the GALT was shown by immunostaining to be in the epithelial cells and delivered to the dendritic cells in the lamina propria and Peyer's patches of the small intestine. The furin cleavage site between the CTB and FVIII sequences facilitated the systemic delivery of the FVIII antigen after uptake in the gut. Further, heavy-chain (HC) antigen was observed in plasma samples and liver protein extracts from mice with haemophilia A 5 h after the last gavage (Sherman *et al.*, 2014). There are examples in which CTB fusion proteins induced tolerance (Ruhlman *et al.*, 2007; Sherman *et al.*, 2014; Verma *et al.*, 2010; Wang *et al.*, 2015). Unfortunately, CTB-fused vaccine antigens have not yet been investigated using histopathological studies. CTB-fused vaccine antigens stimulated antigen-specific IgG and IgA after priming and oral boosters, conferred protection against toxin/pathogen challenge, increased IL-10 but not FoxP3+ regulatory T cells [regulatory T cells expressing the transcription factor forkhead box P3 (FoxP3), which is involved in immunological self-tolerance], suppressed IFN-γ, interleukin-17 (IL-17, a cytokine involved in proinflammatory responses) and conferred immunity via the Th2 immune response (Davoodi-Semiromi *et al.*, 2010).

Stable transformation platforms used for plantderived vaccine production

Plants are proving to be attractive bioreactors to produce biopharmaceutical proteins, including vaccines, antibodies and immunomodulatory molecules such as cytokines. The main challenges for plant-derived recombinant antigens include low yields, the long process required to generate transgenic plant lines, and the associated scale-up costs (Hernández *et al.*, 2014). Thus, different strategies to improve protein yield have been investigated recently. Table 1 summarizes recent examples of plant-derived oral vaccines against human infectious diseases.

Nuclear transformation system

Stable nuclear transformation involves transgene integration into the plant nuclear genome, leading to the expression of therapeutic proteins and Mendelian inheritance of the introduced trait (Figure 2). Stable integration into the nuclear genome allows for continual production of recombinant proteins, simultaneously reducing costs and simplifying production (Tremblay *et al.*, 2010). Nuclear-expressed recombinant proteins undergo typical eukaryotic post-translational modifications and can be stored in subcellular organelles or secreted, depending on the fused signalling peptides (Egelkrout *et al.*, 2012; Tremblay *et al.*, 2010). However, the potential for outcrossing with native species or food crops and the long production cycle of certain crops limit public acceptance of this method (Obembe *et al.*, 2011). The relatively low-level accumulation of recombinant proteins is a major limitation for generating a protective immune response. Modulation of gene expression in plants to enhance accumulation of target proteins could be achieved by using efficient promoters, adding specific signal sequences and optimizing several molecular factors like GC content, codon bias, incorporation of 5′ and 3′ regulatory sequences and elimination of cryptic splicing sites, putative polyadenylation signals, and mRNA-destabilizing sequences (Egelkrout *et al.*, 2012; Peters and Stoger, 2011).

The first plant-based oral vaccine, which used tobacco and potato to produce recombinant LTB from *E. coli*, induced low levels of both serum IgG and secretory IgA (sIgA) antibodies in mice after oral administration (Haq *et al.*, 1995). Various plants have been used to develop edible plant vaccines, including leafy crops, cereals, legumes, fruits and vegetables (Ahmad *et al.*, 2012). Although the first nuclear genome engineering was accomplished in 1995, two decades of research and development have not yet resulted in a single approved vaccine worldwide.

Chloroplast transformation system

Chloroplast transformation has been developed into a highly efficient expression system for recombinant protein production. In the chloroplast technology, site-specific integration of foreign genes into the chloroplast genome occurs by homologous recombination, eliminating the variation in expression caused by gene silencing, positional effects and pleiotropic effects among independent transgenic lines (Daniell *et al.*, 2009; Ruhlman *et al.*, 2010). Moreover, the prokaryotic nature of the chloroplast makes multigene engineering via chloroplast transformation possible (De Cosa *et al.*, 2001; Kumar *et al.*, 2012). Foreign gene products regulated by the endogenous *psbA* promoter and 5′-untranslated region (UTR) and the *psbA* 3′-UTR express up to 72% of the total soluble protein (TSP) of transplastomic plants (Ruhlman *et al.*, 2010). The species specificity of the regulatory sequences dramatically affects transgene expression levels (Ruhlman *et al.*, 2010). Use of the transcriptionally active spacer region between the *trnI* and *trnA* genes within the ribosomal operon and two copies of the transgene, which integrates into the inverted repeat regions of the chloroplast genome, resulted in the highest levels of transgene expression (Clarke and Daniell, 2011; Ruhlman *et al.*, 2010).

Field trials have been conducted a decade ago using transplastomic plants expressing biopharmaceuticals or vaccine antigens (Arlen *et al.*, 2007). Chloroplast genomes are

maternally inherited, offering transgene containment via pollen (Daniell, 2002, 2007). Antigen expression in leaves offers the opportunity to harvest them prior to the appearance of any reproductive structures, facilitating complete transgene containment via both pollen and seeds. Most importantly, a USDA-APHIS certification (dated 1/30/2013 to Dr. Daniell) stated that transplastomic lines do not fit the definition of a regulated article under USDA-APHIS regulations 7 CFR part 340 because there are no plant pest components. Therefore, >300 transplastomic lines expressing transgenes from different kingdoms were moved across state lines (from Florida to Pennsylvania) based on this USDA-APHIS certification. These recent developments should facilitate the commercial development of transplastomic lines expressing vaccines or biopharmaceuticals. However, the major limitation of recombinant proteins expressed in tobacco is that they are not suitable for oral delivery. Thus, optimized expression of antigen proteins in the chloroplasts of edible crops is necessary (Davoodi-Semiromi *et al.*, 2010; Ruhlman *et al.*, 2010).

Although chloroplast transformation protocols have been developed for a few edible crops like potato, carrot and tomato, the expression level of the foreign gene in the edible parts of these plants is not adequate for using such systems to produce vaccines or biopharmaceuticals. Compared with chloroplasts in photosynthetically active tissues, non-green plastids like chromoplasts generally have much lower gene expression activity due to the suppression of plastid gene expression through the interplay between transcriptional and translational control in non-green tissues (Caroca *et al.*, 2013). Therefore, edible leafy vegetables are ideal for biopharmaceutical applications. The lettuce chloroplast system has been successfully used to express a number of vaccines and biopharmaceuticals (Boyhan and Daniell, 2011; Davoodi-Semiromi *et al.*, 2010; Kanagaraj *et al.*, 2011; Maldaner *et al.*, 2013). Chloroplasts permit high gene expression levels and facilitate several post-translational modifications that are required for bioactivity such as pentamer assembly, disulphide bond formation, cyclization, protein lipidation and N-terminal methionine excision, but glycosylation does not occur in chloroplasts (Boyhan and Daniell, 2011; Davoodi-Semiromi *et al.*, 2010; Kohli *et al.*, 2014; Kwon *et al.*, 2013a; Scotti *et al.*, 2012; Sherman *et al.*, 2014; Shil *et al.*, 2014; Verma *et al.*, 2010). The expression of a thioredoxin in chloroplasts enhanced protein solubility, proper folding and disulphide bond formation (Sanz-Barrio *et al.*, 2011). The human papillomavirus (HPV) L1 protein was shown to self-assemble into virus-like particles (VLPs) within chloroplasts (Fernández-San Millán *et al.*, 2008; Waheed *et al.*, 2011a). Structures resembling VLPs were observed as aggregates of subviral particles in chloroplast extracts from transplastomic lettuce producing the dengue prM/E protein, suggesting that the prM/E proteins folded properly and were able to assemble into higher order structures (Kanagaraj *et al.*, 2011). Chloroplast-derived HPVL1 protein self-assembles into capsomeres, which play an important role in the induction of neutralizing antibodies and T-cell responses (Waheed *et al.*, 2011b). The L1 protein was also expressed, and it self-assembled into VLPs in tobacco chloroplasts, suggesting no processing requirement via ER for VLP formation in chloroplasts. Further, the HPV L1 protein showed high immunogenicity and neutralizing antibody production in mice (Fernández-San Millán *et al.*, 2008; Kanagaraj *et al.*, 2011).

Illustrative examples of plant-based vaccines against human infectious diseases

Respiratory infections

Influenza is a serious respiratory disease caused by influenza viruses. It has caused global pandemics, and its prevention is one of the world's greatest public health challenges because of mutations that cause antigenic variation in haemagglutinin (HA) (Shoji *et al.*, 2011). HA is a surface glycoprotein of the influenza virus and plays a key role in viral infectivity and pathogenesis. HA is also the main target for generating protective immunity against the influenza virus (Phan *et al.*, 2013). Recent outbreaks caused by the new H1N1 swine influenza virus infected a large number of humans and raised significant concerns as a global pandemic. The virus, A(H1N1) pdm09, is a triple reassortant with genes acquired from swine, avian and human influenza viruses and was first detected in humans in the United States in April 2009 (Cummings *et al.*, 2014). The highly pathogenic avian influenza A virus (H5N1) caused pandemics in poultry and carries a risk of global human infection due to wide circulation and rapid evolution of the virus (Lee *et al.*, 2015). The antigenicity of the HA protein depends on its proper folding and trimerization, and it also requires multiple post-translational modifications including disulphide bond formation and glycosylation (de Vries *et al.*, 2012). The expression of HA without its transmembrane domain from the A/Hong Kong/213/03 (H5N1) influenza virus strain fused with an ER-targeting signal at the 5′ end and the HDEL ER retention motif at the C-terminus resulted in its high-level accumulation in the ER (140 μg/g fresh weight, FW), *N*-glycosylation, protection from proteolytic degradation and long-term stability in *Arabidopsis*. Oral administration of freeze-dried leaf powder expressing this HA antigen and the adjuvant saponin together elicited not only high levels of HA-specific mucosal IgA and systemic IgG responses in mice, but also neutralizing antibodies and cellular immune responses, conferring protection against a lethal viral challenge. Although the trans-membrane domain is essential for the trimerization that is required for HA antigenicity, plant-based HA without the transmembrane domain still can induce strong HA-specific immune responses in mice (Lee *et al.*, 2015). The influenza virus nucleoprotein (NP) is a highly conserved multifunctional RNA-binding protein found in many different strains, making it a potential candidate for a universal vaccine. Oral immunization of maize-expressed H3N2 NP induced humoral immune responses in mice, showing the immunogenicity of this maize-based antigen and its potential as a universal flu vaccine candidate. The NP protein level in T1 transgenic maize seeds ranged from 8.0 to 35 μg/g of corn seed, and this level increased to up to 70 μg NP/g in T3 seeds. Cytokine analysis showed antigen-specific stimulation of IL-4 cytokines in splenocytes from mice orally administered with NP, further confirming a Th2 humoral immune response (Nahampun *et al.*, 2015).

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB), is a leading bacterial infectious disease that is re-emerging due to drug-resistant strains worldwide (Lakshmi *et al.*, 2013). In 2013, there were 9.0 million cases of TB, with an estimated 480 000 multidrug-resistant TB cases and 1.1 million HIV-positive individuals. More than half (56%) of these cases appeared in South East Asia and the Western Pacific. Further, approximately 25% were in Africa, which suffered the highest rates of cases and deaths

relative to the population. China and India accounted for 11% and 24% of total cases, respectively (WHO, 2014a). The 6 kDa early secretory antigenic target (ESAT6) and culture filtrate protein 10 (CFP10) proteins are among the key cell virulence factors of MTB and have been expressed in transgenic carrot plants, where ESAT6 makes up <0.056% and CFP10 composes 0.002% of the total storage protein in carrot storage roots. Oral immunization of mice induced both cell-mediated and humoral immune responses (Uvarova *et al.*, 2013). Fusion of the ESAT6 antigen with other tuberculosis antigens, such as Ag85B or Mtb72F (a fusion polyprotein of two TB antigens, Mtb32 and Mtb39), and use of a transmucosal carrier such as CTB, LTB and LipY (a cell wall protein) to facilitate bioencapsulation/oral delivery, and further expression in various plant species (*Arabidopsis thaliana*, tobacco and lettuce), have been attempted (Floss *et al.*, 2010; Lakshmi *et al.*, 2013; Uvarova *et al.*, 2013). Compared with nuclear transgenic plants, the expression levels of CTB-ESAT6 and CTB-Mtb72F in transplastomic plants reached up to 7.5% and 1.2% of TSP, respectively, increasing antigen accumulation >100 fold (Lakshmi *et al.*, 2013). CTB-ESAT6 was expressed up to 0.75% of the total leaf protein in transplastomic plants. Western blot analysis of lyophilized lettuce leaves stored for up to 6 months at room temperature revealed the stability of the CTB-ESAT6 fusion protein, which retained proper folding, disulphide bond formation and assembly into pentamers for prolonged periods. ESAT6 is one of the secreted proteins in the ESX-1 system, which is involved in membrane pore formation during infection. A haemolysis assay indicated the ability of chloroplast-derived ESAT6 to lyse red blood cell membranes in a dose-dependent manner (Lakshmi *et al.*, 2013).

Gastroenteritis and hepatitis

Diarrhoeal infectious diseases (DID) are a major problem in developing countries, where poor sanitation prevails and food and water may become contaminated by faecal shedding (Böhles *et al.*, 2014). Traveller's diarrhoea and cholera, caused by enterotoxigenic strains of *Eschericia coli* (ETEC) and *Vibrio cholerae*, respectively, are two enteric diseases resulting in high mortality, especially in young children in developing countries (Karaman *et al.*, 2012). CTB was expressed in maize seeds driven by a γ-zein promoter and accumulated in the endosperm of transgenic maize kernels with an expression level of 0.0014% of the total aqueous soluble protein (TASP) in the T1 generation and significantly increased to 0.0197% of TASP in the T2 generation. Anti-CTB IgG and IgA were detected in the sera and in faecal samples from orally administered mice, and the mice were protected against CT holotoxin challenge (Karaman *et al.*, 2012). Inclusion of a heat-stable (ST) toxin into vaccine formulations is required, as most ETEC strains can produce both LT and ST enterotoxins. Transgenic tobacco plants carrying the LTB:ST gene accumulated up to 0.05% of TSP, and oral dosing with transgenic tobacco leaves elicited specific mucosal and systemic humoral responses in mice, although the authors did not provide antibody titres or any quantitative measurement of the response (Rosales-Mendoza *et al.*, 2011). In comparison, lettuce chloroplast-derived CTB-AMA1 and CTB-MSP1 expressed up to 7.3% and 6.1% of TSP, which is >100-fold higher expression than from the nuclear genome. CTB–proinsulin expressed up to 70% of TSP, suggesting that the fusion protein, not CTB, determines the expression level. CTB-specific antibody titres were incredibly high (up to 10

000 IgA, >800 000 IgG1) and also conferred protection against CT challenge in mice, providing long-term immunity (Davoodi-Semiromi *et al.*, 2010).

Hepatitis B virus attacks the liver and results in both acute and chronic disease, and it remains a major global health problem despite the availability of a safe and effective vaccine. Each year, hepatitis B infection causes approximately 780 000 deaths, 130 000 from acute hepatitis B and another 650 000 from liver cancer and cirrhosis due to chronic hepatitis B infection (Lozano *et al.*, 2012). The expression level of the major surface antigen of hepatitis B virus (P-HBsAg) reached 0.003–0.09% of TSP in transgenic potato. Mice produced specific faecal IgA and serum IgG antibodies against P-HBsAg after oral administration (Youm *et al.*, 2010). Herbicide-resistant lettuce was engineered to stably express the small surface antigen of hepatitis B virus (S-HBsAg) (Pniewski *et al.*, 2011). The progeny of these plants accumulated up to micrograms of antigen per gram of FW, and the S-HBsAG antigen was able to form VLPs (Pniewski *et al.*, 2011). Oral delivery of lyophilized lettuce containing low levels (100 ng) of VLP-assembled antigen with a long, 2-month interval between priming and boost administrations without adjuvant elicited both mucosal and systemic humoral anti-HB responses at the nominally protective level in mice. Lyophilized material, both as a powdered, semi-finished product or after conversion into tablets, preserved the S-HBsAg content for at least 1 year of room-temperature storage (Pniewski *et al.*, 2011). Bioencapsulated HBsAg expressed in maize reached between 0.08 and 0.46% of TSP and induced serum IgG and IgA in mice after oral administration (Hayden *et al.*, 2012b). High levels of HBsAg were obtained in maize grains, and supercritical fluid extraction (SFE)-treated maize material was used to form edible wafers. After feeding wafers containing approximately 300 μg/g HBsAg, mice showed robust serum IgG (20 000 mIU/mL) and IgA responses. Additionally, all mice administered the SFE wafers showed high sIgA and salivary IgA titres (142 mIU/mL) in faecal material, whereas Recombivax® Merck & Co., Inc., Whitehouse Station, NJ, USA (an injected commercial vaccine)-treated mice showed no detectable titre (Hayden *et al.*, 2014). Furthermore, mice boosted with orally administered HBsAg wafers displayed long-term memory mucosally and systemically, as evidenced by sustained faecal IgA and serum IgA, IgG and mIU/mL over 1 year (Hayden *et al.*, 2015). Freeze-drying of S-HBsAg expressed in lettuce leaf tissue without any purification step was shown to be an important factor affecting S-HBsAg preservation. This reproducible process provided a product with VLP content up to 200 μg/g dry weight. Long-term stability tests showed that the stored freeze-dried product was stable at 4 °C for 1 year but degraded at room temperature. Animal oral immunization trials induced systemic IgG in mice (293 mIU/mL), confirming the preservation of antigenicity and immunogenicity (Czyż *et al.*, 2014).

Human immunodeficiency virus

Human immunodeficiency virus (HIV), which causes acquired immune deficiency syndrome (AIDS), is one of the most severe infectious diseases worldwide. AIDS evolved as an alarming public health problem with very high costs for government agencies in most developing and African countries (Rosales-Mendoza *et al.*, 2012). The HIV-1 capsid protein p24 antigen expressed in both transgenic *Arabidopsis* and carrot showed a priming effect in mice and induced specific anti-p24 IgG in sera after an intramuscular p24 protein boost.

Further, dose-dependent antigen analyses using transgenic *A. thaliana* revealed that low p24 antigen doses were superior to high doses, indicating the induction of tolerance (Lindh *et al.*, 2014). The accumulation of HIV-1 p24 and a fusion of p24 with the negative regulatory protein Nef (p24-Nef) reached 4% and 40% of TSP, respectively, in the leaves of transplastomic tobacco plants. Subcutaneous immunization with purified chloroplast-derived p24 induced a strong antigen-specific serum IgG response, with titres of up to 1 : 6400 for IgG1 and 1 : 3200 for IgG2a. Oral delivery of partially purified chloroplast-based p24-Nef fusion protein as a booster after subcutaneous injection with either Nef or p24 elicited strong antigen-specific serum IgG responses (in IgG titres of 3200–12 800). Additionally, after subcutaneous and oral immunization, both IgG1 and IgG2a subtypes, which correlate with cell-mediated Th1 and humoral Th2 responses, respectively, were detected in sera (Gonzalez-Rabade *et al.*, 2011). The synthetic C4V3 peptide, which includes the C4 domain and the V3 loop from HIV gp120, was introduced into tobacco chloroplasts and expressed at up to 25 μg/g FW in the leaves. Plant-derived C4V3 elicited both mucosal and systemic immune responses, but the antibody titres were not quantified; $CD4^+$ T-cell proliferation responses were observed (Rubio-Infante *et al.*, 2012). A lettuce-based C4(V3)6 multi-epitopic protein within the V3 loops, corresponding to five different HIV isolates including CC, MN, IIIB, RF and RU, elicited local and systemic immune responses when orally delivered to BALB/c mice. In addition, the induction of significant T-helper responses by the C4(V3)6 immunogen was shown in splenocyte proliferation assays (Govea-Alonso *et al.*, 2013). Multi-HIV, a multi-epitopic protein consisting of the C4, V1, V2, V3 domains and the ELDKWA epitope derived from the gp120 and gp41 envelope proteins of HIV, respectively, was expressed in tobacco chloroplasts. Mice orally immunized with the tobacco-derived Multi-HIV antigen indicated an immune response, but antibody titres were not provided. Furthermore, specific IFN-γ production was observed in both $CD4^+$ and $CD8^+$ T cells stimulated with HIV peptides, demonstrating that plant-derived Multi-HIV induces T helper-specific responses (Rubio-Infante *et al.*, 2015).

Human papillomaviruses

Cervical cancer caused by HPV infection is the fourth most common cancer among women worldwide and has become a global concern, particularly in developing countries, which bear approximately 80% of the burden (Ferlay *et al.*, 2015). Furthermore, HPV type 16 is by far the most prevalent type and is correlated with 54% of cervical cancer cases (Waheed *et al.*, 2012). Higher levels of specific IgG and IgA levels (<1 : 1000 for the L1/LT-B group and <1 : 500 for the L1 group) of HPV-16L1 (major capsid protein) were induced when mice were immunized with transgenic tobacco-derived HPV-16L1 combined with LTB by the oral route (Liu *et al.*, 2013). A novel HPV 16L1-based chimeric virus-like particle (cVLP) expressed in tomato plants contains a string of T-cell epitopes from HPV-16 E6 and E7 fusion at the C-terminus. Long-lasting specific IgG antibodies with neutralizing activity were detectable for 12 months after induction by immunization with cVLPs. Efficient long-term protection and tumour growth inhibition were elicited by TC-1 tumour cells expressing HPV-16 E6/E7 oncoproteins, whereas significant tumour reduction (57%) was observed in mice administered with these cVLPs (Monroy-García *et al.*, 2014).

Rabies

Rabies virus is an enveloped, negative-sense, single-stranded RNA virus of the genus *Lyssavirus* in the family *Rhabdoviridae*. This zoonotic disease causes acute, progressive, incurable viral encephalomyelitis and is usually transmitted through the bite of an infected animal, resulting in 40 000–100 000 human deaths annually worldwide (Hermann *et al.*, 2011). The expression level of the rabies virus glycoprotein protein (G protein) in transgenic maize kernels reached 25 μg/g FW. Neutralizing antibodies in sheep were induced after oral immunization with maize-derived G protein. Further, the degree of protection achieved with 2 mg of maize-based G protein was comparable to that of a commercial vaccine (Loza-Rubio *et al.*, 2012). Transgenic hairy roots of *Solanum lycopersicum* were engineered to express the rabies glycoprotein fused with ricin toxin B chain (rgp-rtxB) antigen driven by a constitutive CaMV35S promoter. The expression level of the RGP-RTB fusion protein in different tomato hairy root lines ranged from 1.4 to 8 μg/g of tissue. A partially purified RGP-RTB fusion protein was able to induce an immune response in BALB/c mice after intramucosal immunization, but the IgG titres were low (Singh *et al.*, 2015).

Malaria

Malaria is a mosquito-borne infectious disease caused by *Plasmodium* parasites (Jones *et al.*, 2013). According to the World, Malaria Report (2014), approximately 198 million clinical cases of malaria were reported worldwide in 2013, predominantly in developing countries in South East Asia and sub-Saharan Africa. Approximately 82% of malaria cases and 90% of malaria deaths occurred in the WHO African Region, mostly among children under the age of 5 years (WHO, 2014b). *Plasmodium falciparumis* is responsible for the majority of the over half a million malaria deaths per year, which are predominantly children under the age of five that live in indigent African nations (Gregory and Mayfield, 2014). A chloroplast-derived dual cholera and malaria vaccine expressing CTB fused with the malarial vaccine antigens apical membrane antigen 1 (AMA1) and merozoite surface protein 1 (MSP1) accumulated up to 13.17% and 10.11% of TSP in tobacco and up to 7.3% and 6.1% of TSP in lettuce, respectively. The AMA and MSP titres were lower than those of CTB, suggesting that the CTB antigen could saturate the immune system. Significant levels of antigen-specific antibody titres in orally immunized mice not only cross-reacted with the native parasite proteins in immunofluorescence studies and immunoblots, but also completely inhibited the proliferation of the malarial parasite (Davoodi-Semiromi *et al.*, 2010). Oral immunization of mice with the MSP1 and circumsporozoite protein (CSP) fusion protein (MLC) chimeric recombinant protein expressed in *B. napus* successfully elicited antigen-specific IgG1 production. Additionally, the Th1-related cytokines interleukin 12 (IL-12, a cytokine involved in the differentiation of naive T cells into Th1 cells), TNF (tumour necrosis factor, a cytokine involved in the inflammatory process and apoptosis) and IFN-γ were significantly increased in the spleens of immunized mice (Lee *et al.*, 2011).

Toxoplasma gondii—Infection by the intracellular parasitic protozoan *Toxoplasma gondii* can cause complications in pregnant women and in immunodeficient individuals such as patients with AIDS and organ transplant recipients (Guo *et al.*, 2015). Recent studies have shown the possible roles of chronic toxoplasmosis infection in the aetiology of certain mental disorders, such as schizophrenia (Parlog *et al.*, 2015). Expression of the *T. gondii*

dense granular protein 4 (GRA4) antigen via chloroplast transformation (chlGRA4) led to its accumulation to approximately 6 μg/g FW (0.2% of total protein) in tobacco plants. Oral immunization with chlGRA4 elicited both mucosal and systemic immunity (<1000 IgG titre) and also showed a 59% decrease in the brain cyst load of mice. Chloroplast-derived GRA4 induced a protective immune response against *Toxoplasma* infection by reducing parasite loads in mice, correlating with a mucosal and systemic balanced Th1/Th2 response (Del L. Yácono *et al.*, 2012). *Toxoplasma gondii* main surface antigen (SAG1) fused with the 90-kDa heat-shock protein from *Leishmania infantum* (LiHsp83) as a carrier was expressed in transplastomic tobacco plants. SAG1 protein expression levels reached up to 0.1–0.2 μg/g FW in tobacco and reacted with human seropositive samples in a functional analysis. Oral immunization with chLiHsp83-SAG1 induced a significant reduction in the cyst burden in mice, which correlated with an increase in specific anti-SAG1 antibodies (Albarracín *et al.*, 2015).

Taenia solium—*Taenia solium* cysticercosis is an endemic parasitic disease that affects human health and the economy in developing countries. Cysticercosis cysts in the central nervous system produce neurocysticercosis (NCC) and are a common cause of acquired epilepsy (Garcia *et al.*, 2014). The S3Pvac vaccine components (KETc1, KETc12, KETc7 and GK1 [KETc7]) and the protective HP6/TSOL18 antigen were expressed using a Helios2A polyprotein system through the ‘ribosomal skip’ mechanism. The 2A sequence (LLNFDLLKLAGDVESNPG-P) derived from the foot-and-mouth disease virus induces self-cleavage events at the translational level, releasing the distinct antigens in a single transformation and expression event. Plant-derived Helios2A accumulated up to 1.3 μg/g FW in transgenic tobacco leaf tissue and was recognized by antibodies in the cerebrospinal fluid from patients with NCC in a functional assay. Further, orally immunized mice elicited an immune response, but antibody titres were not reported (Monreal-Escalante *et al.*, 2015).

Future perspectives

The plant-made vaccine field started two decades ago with the promise of developing low-cost vaccines to prevent infectious disease outbreaks and epidemics around the globe, but this goal has not yet been realized. A few clinical trials have been conducted a decade ago using lettuce leaves (Kapusta *et al.*, 1999), maize seeds (Tacket *et al.*, 2004) and potato tubers (Tacket *et al.*, 1998, 2000; Thanavala *et al.*, 2005) expressing different vaccines antigens. However, none of them advanced beyond phase I clinical trial or result in any licensed product. There are a number of major technical hurdles to achieve this goal, including inadequate expression levels in edible plant systems and the low success of oral priming to induce adequate immunity against pathogens (Egelkrout *et al.*, 2012; Pasetti *et al.*, 2011; Wahid *et al.*, 2011). The major advantage of the oral vaccination system is the stimulation of both mucosal and systemic immunity (Davoodi-Semiromi *et al.*, 2010). Compared with conventional vaccination by injection into the bloodstream with adjuvants, the mucosal immune system requires much higher antigen doses because the antigens do not cross the epithelial barrier and reach the immune system, but rather are degraded by acids and enzymes in the digestive system (Davitt and Lavelle, 2015). Although the latter concern is addressed by the bioencapsulation of vaccine antigens within plant cells, transmucosal carriers must be fused with the antigens for the delivery across epithelial barriers (with rare

exceptions in which an antigen such as CTB may have the ability to directly bind the GM1 receptor present in human gut epithelial cells). Foreign proteins without transmucosal carriers are not delivered to the immune system or into circulation (Kohli *et al.*, 2014; Limaye *et al.*, 2006). Very few vaccine candidates described above meet these requirements, and therefore, their efficacy has not been tested in suitable animal models or they were not effective enough to warrant further advancement to the clinic. Candidates that meet these criteria in successful studies and demonstrated the efficacy of oral vaccines in boosting the immune system and conferring greater/prolonged protection against pathogen challenge were not successful for priming. The requirement of priming by injection faces the same challenges of prohibitively expensive protein purification, cold storage/transportation and short shelf life or stability (Kwon *et al.*, 2013b). Therefore, the key advantages of low-cost and cold-chain-free plant-made vaccines have not yet been achieved using such technology. Furthermore, only a few vaccine antigens have been expressed in edible crops (lettuce), and those expressed in tobacco would face challenges in the FDA approval process because of concerns about nicotine in orally delivered drugs.

Plant-derived vaccine antigens can accumulate in and are protected by compartments such as plastids or seed storage organelles such as ER-derived protein bodies (PBs), as well as *de novo* formed protein storage vacuoles (PSVs). This bioencapsulation shields the antigens from chemical, thermal and enzymatic degradation (Kwon *et al.*, 2013b; Sack *et al.*, 2015). Freeze-dried leaf material expressing vaccine antigens can often be stored at ambient temperatures while maintaining antigen integrity (Kwon *et al.*, 2013b). Plant cells can be lyophilized and stored at room temperature without antigen degradation for several months or years (Kwon *et al.*, 2013a). Lyophilized leaves have various advantages over fresh materials such as long-term storage, increased antigen stability and content, and decreased microbial contamination (Kwon *et al.*, 2013b). After lyophilization, lettuce leaves showed a 22-fold increase in CTB-ESAT6 antigen content per gram compared with fresh leaves and could be stored at room temperature for up to 6 months (Lakshmi *et al.*, 2013). Freeze-dried and stored materials preserve the integrity and immunogenicity of the heterologous fusion protein, allowing efficient delivery to the GALT due to proper folding and assembly (Boyhan and Daniell, 2011; Kwon *et al.*, 2013b). However, HBsAg was not stable in lyophilized lettuce leaves stored at room temperature, suggesting that the compartment or level of an antigen's expression may play a role in its stability (Czyż *et al.*, 2014).

Seeds offer several alternative subcellular destinations for recombinant proteins, including PBs derived from the ER, PSVs, the surface of oil bodies and starch granules (Khan *et al.*, 2012). Seeds are also advantageous due to their ability to accumulate protein in a relatively small volume and the high stability of the recombinant protein in dry seeds, allowing batch processing and long-term storage (Peters and Stoger, 2011). Oral immunization with rice-based CTB stored at room temperature for more than 3 years still provided effective, long-term SIgA-mediated protection against CT- or LT-induced diarrhoea (Tokuhara *et al.*, 2010). Maize-derived HBsAg driven by the promoter of the cereal storage protein globulin1 showed high-level accumulation at a mean concentration of 0.51% TSP in T1 seeds. HBsAg expressed in maize seeds was heat stable; it could tolerate temperatures of up to 55 °C for 1 month without degradation. Optimal heat stability was realized after oil extraction of ground

maize material, either by SFE or hexane treatment (Hayden *et al.*, 2012a). High levels of HBsAg were obtained in maize grain, and SFE-treated maize material was formed into edible wafers. Mice fed with wafers containing approximately 300 μg/g HBsAg showed robust IgG and IgA responses in their sera (Hayden *et al.*, 2014).

Nonmammalian glycosylation and low recombinant protein yield are the two major challenges against the full utilization of plants as alternative bioreactors instead of mammalian cell culture. The challenge of choosing the most suitable host plant as an expression system for biopharmaceuticals, as well as downstream processing, has also received attention (Obembe *et al.*, 2011). Glycosylation is the covalent linkage of sugar molecules to proteins to improve their biological activity, folding, solubility and immunogenicity (Arcalis *et al.*, 2013). In plants, protein glycosylation occurs within the secretory pathway in the ER and the Golgi complex. Plants attach core α(1,3)-linked fucose and β(1,2)-linked xylose residues to the *N*-glycan of glycoproteins, whereas mammals add α(1,6)-linked fucose, β(1,4)-linked galactose and sialic acid residues to the *N*-glycan (Gomord *et al.*, 2010). Hence, to prevent allergic reactions and immunogenicity when plant-made therapeutic animal proteins are delivered to humans, it is necessary to engineer the host plant to perform authentic human *N*-glycosylation (Obembe *et al.*, 2011). Plant-derived recombinant human glycoproteins normally contain the carbohydrate groups α(1,3)fucose and β(1,2)xylose, which are not found in mammals, and they lack the terminal galactose and sialic acid residues that are required for the stability, activity and solubility of native human glycoproteins (Gomord *et al.*, 2010; Lico *et al.*, 2012). Despite these challenges, the first FDA approved plant-made biopharmaceutical is a glycoprotein, and so the limitations of plant glycosylation did not cause any problems; indeed, the terminal mannose residues facilitated the uptake of glucocerebrosidase by macrophages (Walsh, 2014).

Because the chloroplast is an *N*-glycosylation-free compartment, it offers unique advantages and disadvantages (Daniell *et al.*, 2009). Because plant glycosylation is different from human glycosylation, it requires careful modification of the glycosylation pathways to resemble human glycoproteins as discussed above to avoid any unintended immune responses. However, there are examples where glycosylation sites in human biopharmaceuticals like IGF-1 are inactivated to obtain fully functional proteins (Hede *et al.*, 2012; Philippou and Barton, 2014). Chloroplasts are ideal to express such proteins without glycosylation using the native sequence but are not suitable to express glycoproteins.

Mass spectrometry analyses demonstrated that all the *N*-glycosylation sites of the extracellular domains of plant-based HA VLPs carry plant-specific complex or hybrid *N*-glycans having core α(1,3)-fucose and core β(1,2)-xylose epitopes and Lewis^a extensions (Le Mauff *et al.*, 2014). No hypersensibility or induction of IgG or IgE directed against these glycans of HA VLPs after immunization was observed in previous phase I and II clinical studies (Landry *et al.*, 2010). Furthermore, the identification of plant VLP raft markers confirmed that the VLP formation mechanism in *Nicotiana benthamiana* is similar to the natural process of influenza virus assembly in mammals (Le Mauff *et al.*, 2014). *Plasmodium falciparum* PfAMA1 consists of up to six recognition sites for *N*-linked glycosylation, which are absent in *P. falciparum*. Glycosylated and nonglycosylated

PfAMA1 accumulated to high levels in *Nicotiana benthamiana* after transient expression, and the glycosylated variant was confirmed to contain high-mannose-type *N*-glycans. Competition assays revealed that several epitopes were shielded from immune recognition by the *N*-glycans. Thus, *N*-linked glycosylation may improve efficacy by enhancing immunogenicity and/or focusing the response towards the corresponding epitopes by glycan masking (Boes *et al.*, 2015).

The ability to avoid inflammatory responses to dietary and microbial antigens in the gut mucosa is achieved by a mechanism termed oral tolerance (Oliveira *et al.*, 2015). Repeated oral immunization with large doses of the same antigen resulted in abrogated or decreased T cell-mediated responses in animal models (Azizi *et al.*, 2010). This phenomenon indicates the possibility of mucosal tolerance induced by orally delivered vaccines. The dominant target of oral tolerance for vaccine efficacy is the T-cell compartment, not the B cell compartment. A number of studies have shown that the frequency of antigen administration by the oral route is a critical factor for oral tolerance induction, indicating that multiple feedings by gavage were more effective than a single feeding of antigen to induce oral tolerance to inflammatory immune responses and autoimmune disease models (Guetard *et al.*, 2008; Oliveira *et al.*, 2015; Wang *et al.*, 2015). Continuous feeding correlates with the enhanced production of TGF-β and IL-10, which are important mediators of oral tolerance induction (Wang *et al.*, 2013). Several factors affect oral tolerance induction, including age, immunological status of the animal, dose and structure of the antigen, and the form of antigen feeding (Oliveira *et al.*, 2015). Indeed, oral delivery of several autoantigens expressed in plant cells induced high levels of tolerance (Ruhlman *et al.*, 2007; Sherman *et al.*, 2014; Verma *et al.*, 2010; Wang *et al.*, 2015).

Tolerance versus immunity is not determined by fusion tags, but by the process of priming with adjuvants. Although the molecular mechanism to distinguish participating subsets of dendritic cells is not fully understood, the protocol is clearly reproducible with adjuvants via injection conferring immunity (antibody production), whereas orally delivered antigens induce tolerance (suppresses antibodies). Subcutaneous injection of the purified antigen with a strong adjuvant assures presentation of the antigen in the context of a very strong activation signal during priming. This sets up an immune response that is further boosted orally. Therefore, the oral booster vaccine is effective when the systemic response has been set up by priming with an adjuvant (Davoodi-Semiromi *et al.*, 2010). The innate immune receptor NLRP10 plays an important role in activation of dendritic cells by adjuvants (Eisenbarth *et al.*, 2012). In the presence of inflammatory stimuli (adjuvants), local DCs become activated and present antigens for T-cell priming, locally and in the peripheral lymphoid tissues where DCs can migrate. Immature DCs induce Tregs that affect DC function and prevent stable DCs–effector T-cell contact, thereby priming the immune response (Guetard *et al.*, 2008). This is a very different scenario from the release of antigens into the gut immune system without priming, which is geared towards an anti-inflammatory response. The activation and maturation of DCs play a crucial role in the induction of tolerance or immunity upon antigen delivery. When antigens are presented to T cells by immature DCs in the absence of inflammation, they induce tolerance. Furthermore, by secreting cytokines or by cell-to-cell contact, Tregs interfere with DC maturation, shifting

DCs into tolerogenic function (Figure 1). Tregs begin suppressive effect by secretion of cytokines IL-10 and TGF-β, which induce apoptosis or cell-cycle arrest in effector T cells and block co-stimulation and maturation of dendritic cells. However, it has been demonstrated there is no difference in the behaviour of $CD4^+$ T cells during primary exposure to antigen in priming or tolerizing conditions. Immune priming is associated with the formation of large, stable clusters of $CD4^+$ T cells around DCs, whereas during tolerance induction, smaller, more short-lived clusters are formed. Subsequently, T cells return to rapid migration, but this may take longer under conditions of priming than initiation of tolerance (Shakhar *et al.*, 2005; Zinselmeyer *et al.*, 2005). These observations demonstrate that altering the interaction between T cells and DCs can have profound consequences for the induction of immunity. Therefore, the interaction between Tregs and DCs plays a major role in orally delivered vaccine antigens bioencapsulated in plant cells.

Thus, potential plant-based oral vaccines require suitable immunization protocols and antigen formulations to ensure antigen stability through the alimentary tract and a balance between immunity and oral tolerance, such as targeting pivotal APCs, co-administration with a mucosal adjuvant for oral vaccine formulation, the optimization of oral antigen delivery and its dosage and feeding frequency (Azegami *et al.*, 2014; Pniewski *et al.*, 2011; Wang *et al.*, 2013). Moreover, the temporal sequence of administration, with initial systemic priming and mucosal boosting combined with the usage of certain adjuvants, is likely to prevent mucosal tolerance induction (Azizi *et al.*, 2010). These challenges should be carefully addressed as this technology reaches clinical evaluation.

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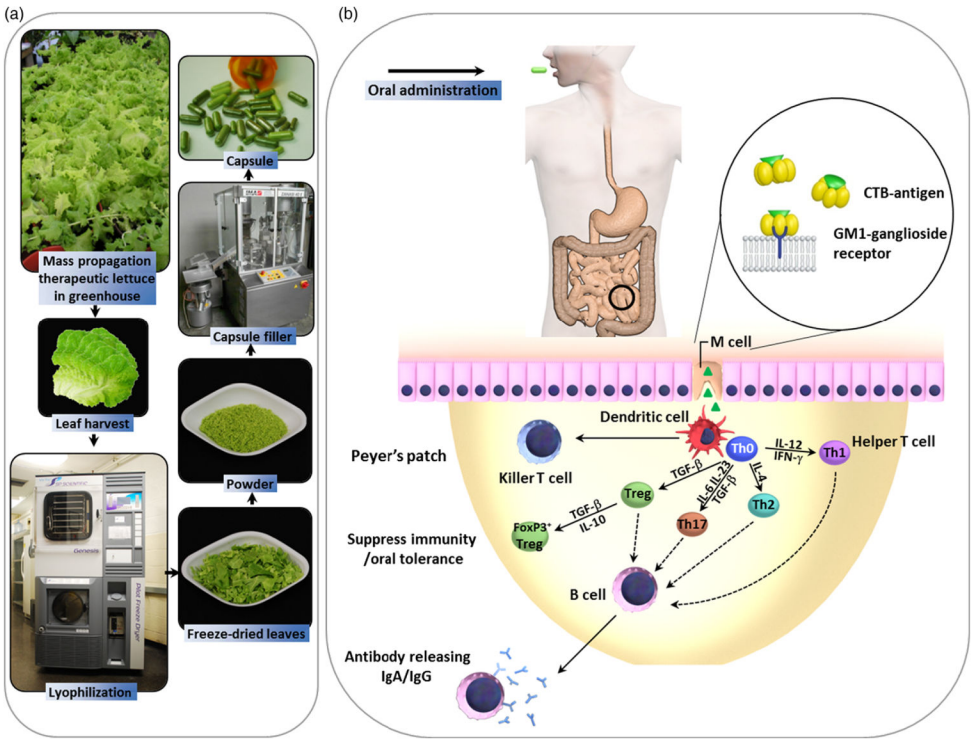

Figure 1.

An outline for the process of oral delivery of plant-derived vaccine antigens: (a) Foreign genes are introduced and expressed in lettuce chloroplasts via particle bombardment. After confirmation of stable integration of foreign genes into all of the chloroplast genomes in each plant cell (achieving homoplasmy) and characterization of dosage and functionality, transplastomic lines are transferred to the greenhouse to increase biomass. Harvested leaves are lyophilized in programmed machines to maintain sublimation temperature below 20 °C, powdered and stored in moisture-free environment. (b) Proposed mechanism of plant-derived oral vaccines. Orally administered CTB-fused antigens are taken up by M cells located in the FAE through the binding to GM1-ganglioside receptor. Antigens are then captured by antigen-presenting cells, such as DCs, inducing antigen-specific T and B cells. Th1-associated cytokines, such as IFN-γ, and Th2 cytokines, such as IL-4, IL-10, play important roles in cell-mediated immunity and humoral immunity. Mucosal DCs induce FoxP3+ Tregs via the production of TGF-β but that concomitant retinoic acid signalling boosted this process. TGF-β mediates immune tolerance via induction and maintenance of FoxP3+ Treg, which suppress Th1 and Th2 responses.

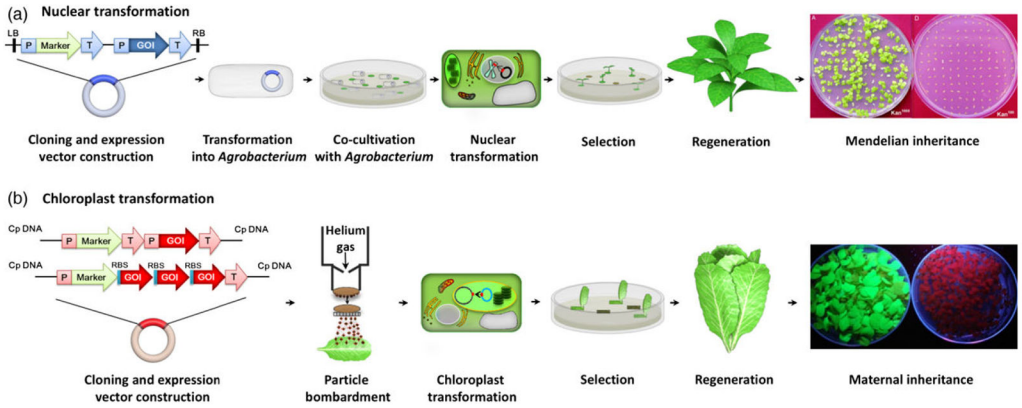

Figure 2.

Schematic representation of stable nuclear and chloroplast transformation processes. (a) Gene(s) of interest (GOI) are introduced into plant chromosomes via *Agrobacterium*-mediated transformation, followed by selection and regeneration. Phenotypic segregation of the progeny via Mendelian inheritance is observed. (b) Chloroplast transformation using particle gun bombardment of chloroplast vectors is followed by two to three rounds of antibiotic selection and subsequent regeneration of homoplasmic transformants. GOI can be engineered to express single or multiple genes. Transgene integration is mediated by site-specific recombination, and maternal inheritance is shown in T1 progeny. P, promoter; Marker, antibiotic selectable marker gene; T, terminator; LB, left border sequence; RB, right border sequence; Cp, chloroplast; and RBS, ribosome-binding site.

Table 1

Recent examples of plant-derived oral vaccines against human infectious diseases

Pathogen	Antigen	System/Plant	Expression level	Immunogenicity	Reference
Virus					
Hepatitis B virus	Surface antigen	Nuclear/Potato	0.003–0.09% of TSP	Serum IgG and mucosal IgA in mice	Youm *et al.* (2010)
	Surface antigen	Nuclear/Lettuce	5–10 μg/g FW	Serum IgG and secretory IgA in mice	Pniewski *et al*. (2011)
	Surface antigen	Nuclear/Maize seeds	0.08–0.46% of TSP	Serum IgG and IgA in mice	Hayden *et al*. (2012b)
	Surface antigen	Nuclear/Lettuce	200 μg/g DW	Systemic IgG in mice	Czyż *et al*. (2014)
	Surface antigen	Nuclear/Maize seeds	0.51% of TSP	Serum and mucosal immunities in mice	Hayden *et al*. (2014)
Human immunodeficiency virus (HIV-1)	p24-Nef	Chloroplast/Tobacco	40% of TSP	Cell-mediated and humoral immunities in mice	Gonzalez-Rabade *et al*. (2011)
	C4V3	Chloroplast/Tobacco	25 μg/g FW	Systemic and mucosal antibody, CD4+ T-cell proliferation in mice	Rubio-Infante *et al*. (2012)
	C4(V3)6 multi-epitopic protein	Nuclear/Lettuce	240 μg/g DW	Cell-mediated and humoral immunities in mice	Govea-Alonso *et al.* (2013)
	p24	Nuclear/*Arabidopsis* Nuclear/Carrot	17–366 ng/g FW 90 ng/g FW	Sera IgG in mice	Lindh *et al.* (2014)
	Multi-HIV	Chloroplast/Tobacco	100 μg/50 mg DW	Antibody and cellular responses in mice, specific IFN-γ production	Rubio-Infante *et al*. (2015)
Human papillomavirus (HPV)	HPV16-L1	Nuclear/Tobacco	0.22%–0.31% of TSP	Cell-mediated and humoral immunities in mice	Liu *et al.* (2013)
	HPVL1-E6/E7	Nuclear/Tomato	0.05% to 0.1% of TSP	Persistent IgG and neutralizing antibodies, tumour growth inhibition in mice	Monroy-García *et al*. 2014;
Influenza virus	H3N2 nucleoprotein	Nuclear/Maize seeds	8.0–35 μg/g of corn seed	Humoral immune responses in mice	Nahampun *et al.* (2015)
Rabies virus	G protein	Nuclear/Maize seeds	25 μg/g FW	Neutralizing antibodies in sheep	Loza-Rubio *et al*. (2012)
	G protein	Nuclear/Tomato hairy roots	0.9–1.1% of TSP	Serum IgG and Th2 lymphocyte response in mice	Singh *et al.* (2015)
Bacteria					
Enterotoxigenic *Escherichia coli*	LTB	Nuclear/Rice callus	0.12% of TSP	Humoral immunity in mice	Kim *et al*. (2010)
	LTB:ST fusion protein	Nuclear/Tobacco	0.05% of TSP	Mucosal and systemic humoral immunities in mice	Rosales-Mendoza *et al*. (2011)

Pathogen	Antigen	System/Plant	Expression level	Immunogenicity	Reference
Mycobacterium tuberculosis	ESAT6	Nuclear/Carrot	0.056% of TSP	Cell-mediated and humoral immunities in mice	Uvarova *et al.* (2013)
	CFP10	Nuclear/Carrot	0.024% of TSP		
Vibrio cholerae	CTB	Nuclear/Rice seeds	2.1% of TSP	Humoral immunity in mice, protection from CT and LT challenge	Tokuhara *et al.* (2010)
	CTB	Nuclear/Maize seeds	0.0014–0.0197% of TSP	Serum and mucosal immunities in mice, protection from CT and LT challenge	Karaman *et al*. (2012)
Protozoa					
Plasmodium falciparum	AMA1	Chloroplast/Tobacco Chloroplast/Lettuce	13.17% of TSP 7.3% of TSP	Long-term cellular, humoral immunities in mice and parasite inhibition	Davoodi-Semiromi *et al*. (2010)
	MSP1	Chloroplast/Tobacco Chloroplast/Lettuce	10.11% of TSP 6.1% of TSP		
Plasmodium vivax	MSP1 and CSP fusion protein	Nuclear/*Brassica napus*	ND	IgG1 production, increase in IL-12, TNF, and IFN-γ in mice	Lee *et al.* (2011)
Toxoplasma gondii	GRA4	Chloroplast/Tobacco	6 μg/g FW	Systemic and mucosal immunity, parasite reduction in mice	Del L. Yácono *et al*. (2012)
	SAG1	Chloroplast/Tobacco	0.1–0.2 μg/g FW	SAG1-specific antibodies, cyst burden reduction in mice	Albarracín *et al*. (2015)
Parasite					
Taenia solium	Helios2A multi-epitope polyprotein	Nuclear/Tobacco	1.3 μg/g FW	Specific antibodies in mice	Monreal-Escalante *et al*. (2015)

AMA1, malaria apical membrane antigen 1; C4V3, synthetic peptides comprising the V3 loop and the C4 domain from HIV gp120; CTB, cholera toxin B subunit; CFP10, culture filtrate protein 10; CSP, circumsporozoite protein; DW, dry weight; E6/E7, oncogenes of HPV; ESAT6, 6 kDa early secretory antigenic target; FW, fresh weight; G protein, rabies virus glycoprotein protein; GRA4, dense granular protein 4; L1, HPV major capsid protein; LTB, heat-labile enterotoxin B subunit; MSP1, merozoite surface protein 1; ND, not determined; Nef, HIV accessory protein negative factor; p24, HIV capsid protein; SAG1, major surface protein; ST, heat-stable enterotoxin; TSP, total soluble protein.