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The green vaccine: A global strategy to combat infectious and autoimmune diseases

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Abstract

Plant derived oral green vaccines eliminate expenses associated with fermenters, purification, cold storage/transportation and sterile delivery. Green vaccines are expressed via the plant nuclear or chloroplast genomes. Chloroplast expression has advantages of hyper-expression of therapeutic proteins (10,000 copies of trans-gene per cell), efficient oral delivery and transgene containment via maternal inheritance. To date, 23 vaccine antigens against 16 different bacterial, viral or protozoan pathogens have been expressed in chloroplasts. Mice subcutaneously immunized with the chloroplast derived anthrax protective antigen conferred 100% protection against lethal doses of the anthrax toxin. Oral immunization (ORV) of F1-V antigens without adjuvant conferred greater protection (88%) against 50-fold lethal dose of aerosolized plague (*Yersinia pestis*) than subcutaneous (SQV) immunization (33%). Oral immunization of malarial vaccine antigens fused to the cholera antigen (CTB-AMA1/CTB-Msp1) conferred prolonged immunity (50% life span), 100% protection against cholera toxin challenge and inhibited proliferation of the malarial parasite. Protection was correlated with antigen-specific titers of intestinal, serum IgA & IgG1 in ORV and only IgG1 in SQV mice, but no other immunoglobulin. High level expression in edible plant chloroplasts ideal for oral delivery and long-term immunity observed should facilitate development of low cost human vaccines for large populations, at times of outbreak.

Keywords

chloroplast genetic engineering; plant vaccine; infectious diseases; autoimmune; cholera; malaria; anthrax; plague; diabetes; HIV

Vaccination is considered to be the most efficient and cost-effective health intervention. However, the high cost of vaccination makes it unaffordable for most people living in developing countries, as the daily average income of nearly one billion people is less than US $1. Recent studies show that over 100 million people worldwide fall into poverty because of high cost of health care (WHO, 2008). Two decades ago, the full array of vaccines for children cost about US $84; currently these vaccines cost about US $1,200 or more, a 14-fold increase in cost.[1] Such high cost of current vaccines are due to their unnecessarily complex production and delivery methods, including the significant cost of fermentation systems to purification through the use of complex technologies and additional expenses associated with adjuvant, cold storage, transportation and sterile delivery. All current vaccines are produced via fermentation in various cell culture systems and no viable alternative to fermentation

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technology has yet emerged for mass-production of prohibitively expensive vaccines. This void can be addressed through the use of plant cells as bioreactors for the production and oral delivery of vaccine antigens. This novel approach uses freeze-dried plant cells for bio-encapsulation of vaccine antigens that protect them in the stomach from acids and enzymes but are released to the immune system in the gut when plant cell walls are digested by bacteria that colonize the gut.[2,3] Vaccine antigens expressed via the plant nuclear genome, in the past two decades, elicited appropriate immunoglobulin responses and conferred protection upon oral delivery.[4,5] However, no transgenic plant-based vaccine has moved beyond Phase I clinical trial, highlighting the need to explore new technologies. In this review, we discuss the potential of a new platform technology in which plant chloroplasts are used as bioreactors. This approach has several unique advantages over the nuclear genome, including hyper-expression of therapeutic proteins, efficient oral delivery and transgene containment.

In the chloroplast technology, vaccine candidate genes are integrated into the chloroplast genome by homologous recombination,[6,7] eliminating variation of expression among independent transgenic lines. Similarly, gene silencing has not been reported in chloroplast transgenic (transplastomic) lines. High levels of expression of vaccine antigens, 100–1,000-fold higher than nuclear genomes is 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 crop to other related crops or weeds due to maternal inheritance of transgenes.[8] To date, 23 vaccine antigens against 16 different diseases have been expressed in chloroplasts (Table 1). The chloroplast expression system is quite versatile in synthesizing proteins as small as Magainin (20 amino acids)[9] or as large as protective antigen (83 kDa).[10] Plastids can also produce monomeric[11] or multimeric proteins.[12,13] Presence of chaperones or enzymes in the chloroplast creates a special niche for their assembly with suitable post-translational modifications (assembly of multimers, disulfide bonds, lipid modification, etc.). The chloroplast expression system has been used to produce a number of fully functional vaccine antigens against bacterial, viral or protozoan pathogens including cholera,[12,14] anthrax,[10,15] Plague,[2] Canine parvovirus,[16] CSFV,[17] EBV,[18] FMD,[19] HIV antigens p24,[20] HEV,[21] HPV,[11,22] Rotavirus,[23] amoebiasis[24] and Malaria.[14] Autoantigen expressed in chloroplasts, CTB-proinsulin[13] facilitated the delay of onset of diabetes and hGAD65,[25] has been expressed in algal chloroplasts.

In order to avoid purification and achieve oral delivery, expression of vaccine antigen in edible crops is necessary. Recently, we have stably integrated several therapeutic proteins and vaccine antigens against anthrax, cholera, malaria and autoantigen for diabetes in lettuce chloroplasts.[2,10,13,14] The level of expression in lettuce chloroplast is similar to tobacco, the most successful plastid transformation system. The carrot system was developed in 2004 to facilitate oral delivery;[26] however, its regeneration is extremely slow while lettuce can be transformed as rapidly as tobacco. Recently, HIV antigen p24 has been expressed in tomato leaf chloroplasts, but expression in fruits were very low.[27]

One of challenges associated with oral delivery of plant-derived vaccine antigens is delivery of adequate quantity of antigens to elicit the immune response. In this context, we have demonstrated that chloroplast-derived therapeutic proteins, upon oral delivery of plant cells, are protected in the stomach from acids/enzymes because of bio-encapsulation of the antigen by the plant cell wall. When the protected antigen is released in the gut, it is translocated from the gut lumen into the circulatory system via a receptor mediated oral delivery system upon binding to the epithelial receptor GM_1,[3] when the foreign protein is fused with CTB.

Mucosal associated lymphatic tissue (MALT) provides a unique architecture and the largest surface area for antigen entry into the body. For many diseases affecting the gastrointestinal,[28,29] respiratory and urogenital systems, the mucosal surface is the primary site for pathogen

entry into the body. Indeed total numbers of lymphocytes in the MALT are equal to total numbers of lymphocytes in the rest of the body combined, suggesting that the MALT plays a major role in health and in diseases.[30] Using chloroplast bioreactors and MALT, it is feasible to achieve immunity against infectious diseases such as cholera,[14,31,32] plague,[2] tetanus[33] or autoimmune diseases like type 1 diabetes.[13] Generation of local antigen-specific secretary IgA in oral delivery against vaccine antigen is crucial to confer immunity in oral immunization. Our studies show that orally immunized mice with plant-derived materials generated both local and systemic IgA. In contrast, subcutaneously immunized mice generated little or no IgA, suggesting production of antigen-specific IgA against gastrointestinal pathogen is mostly generated in the GALT and very little, in other part of the immune system.[14] Oral vaccination can radically alter the landscape of vaccination and offers a new tool to fight against infectious diseases in developing countries or against bioterrorism agents in developed countries. In this commentary we discuss some examples of vaccines or autoantigens that are expressed successfully using the chloroplast technology.

Anthrax Vaccine

Anthrax is an acute disease in animals and humans caused by the bacterium *Bacillus anthracis*, which is highly lethal in some forms. Anthrax does not spread directly from one infected animal or person to another, but spores can be transported by clothing, shoes, and even by mail as a biothreat weapon. The current licensed vaccine by BioPort Corporation is effective but is not toxin-free, which causes several side effects, and its limitations include batch-to-batch variability. The US Department of Health and Human Services (HHS) awarded VaxGen $877 million contract in 2004 to produce 75 million doses of anthrax vaccine using the conventional fermentation technology; however, this contract was withdrawn after failure in Phase I clinical trial. We have shown that tobacco chloroplast expressed PA to produce up to 360 million doses from one acre of tobacco; when subcutaneously immunized mice with chloroplast derived PA were challenged with lethal dose of toxin (LT) all (100%) of the immunized mice survived, while all control mice died after 50 hr of LT challenge.[10,15]

Dual Vaccine for Cholera and Malaria

Cholera is an epidemic disease caused by enterotoxin producing strains of the bacterium *Vibro cholerae*. Malaria is a vector-borne infectious disease caused by the protozoan parasite *Plasmodium falciparum*. Both these diseases cause high mortality in tropical and subtropical regions, including parts of the South America, Asia and Africa. The only available cholera vaccine is highly expensive; the immunity in children is lost in less than three years and adults are not fully protected.[34] No vaccine is yet available for malaria, with 500 million cases and one million deaths recorded every year. A native cholera toxin B subunit gene was expressed in the chloroplast, up to 4.1% of TSP, a 410-fold higher than the nuclear transformed plants[12] chloroplast-derived CTB was properly folded, disulfide bonded, assembled into functional oligomers and was fully functional by binding to GM_1, the intestinal epithelial receptor. Recently, CTB fused with *ama*-1and with *msp1* was expressed in tobacco and lettuce. The levels of expression was in the range of 8–12% of TSP in tobacco and 4–9% in lettuce. [14] Immunogenicity of fused CTB protein was observed in immunized BALB/c mice with cholera toxin (CT) challenge. Protection against CT challenge in both oral (100%) and subcutaneous (89%) mice correlated with CTB-specific titers of intestinal, serum CTB-IgA and -IgG1 in oral and only -IgG1 in subcutaneous treated mice.

The investigation of several malarial vaccine candidates led to the discovery of *ama1* and *msp1*, which are two of the leading asexual blood-stage malarial vaccine candidates. In this study, nine groups of mice (n = 10/group) were subcutaneously or orally immunized with purified or transplastomic leaf materials expressing cholera and malarial human vaccine

antigens, CTB-*ama1* and CTB-*msp1*. The levels of antigen-specific antibody titers of immunized mice was highly significant and resulted in inhibition of the proliferation of malarial parasite and cross-reacted with the native parasite proteins/parasites in immunoblots and immuno-fluorescence studies, at the ring, trophozoite or schizont stage of the malarial parasite. This is the first report of chloroplast derived dual vaccine.[14]

A plant optimized synthetic gene encoding for the LTB-ST (heat labile toxin B subunit) fusion protein has been expressed via the tobacco chloroplast genome. Orally immunized mice with LTB-ST transformed tobacco leaves induced both serum and mucosal LTB-ST specific antibodies. The challenged mice showed a decrease of intestinal fluid retention.

Plague Vaccine

Plague caused by *Yersinia pestis* has been classified by the Center of Disease Control (www.cdc.gov) as one of the six “biological agents” in “A” category.[35] We have recently reported[2] for the first time the effectiveness of chloroplast-derived oral vaccine that protected mice from live *Y. pestis* challenge. In this study, mice were immunized with plant material expressing two protective antigens F1 and V. When orally and subcutaneously immunized mice were challenged with 50-fold lethal dose of *Y. pestis* spores, only 33% of subcutaneously immunized mice were protected, while 88% of orally immunized mice survived. A comparison of splenic *Y. pestis* CFU counts showed that there were 10 billion spores found in the dead control mice whereas none in the orally immunized mice, demonstrating the power of mucosal immunity to combat several dreadful diseases. Protection was conferred by induction of Th2 immune response and generation of antigen-specific IgG1 antibody,[2] although there was no correlation with IgA.

HIV Vaccine

A number of relevant HIV-1 antigens have been expressed in plants nucleus.[5] HIV-1 p24, a 24 kDa capsid protein that encapsulates the genomic RNA of the HIV-1 virus was highly expressed in tobacco and tomato chloroplasts.[20,36] Interestingly, HIV-1 p24 is a non-glycosylated protein and is therefore suitable for chloroplast expression. However, immunogenetic and functional properties of this chloroplast derived vaccines remains to be evaluated. Although there is some progress expression of viral antigens, studies on functional evaluation is lagging behind.

Amoebiasis Vaccine

Amoebiasis is another serious gastrointestinal infection caused by *Entamoeba histolytica*. It is estimated by the WHO that about 100,000 people die annually worldwide, but there is no approved vaccine available to fight against this pathogen. The infection begins with the adherence of amoeba to intestinal epithelial cell which is mediated by Gal/GalNAc lectin. Recombinant fragment of Gal/GalNAc lectin that is rich in cysteine known as *Lec* A, has potential to block the targeting amoeba. Recently, *Lec* A has been expressed in chloroplast, up to 6.3% of TSP or 2.3 mg Lec A/g leaf tissue.[24] Subcutaneous delivery of chloroplast-derived Lec A induced IgG1 titers up to 1:10,000, 10 times in mice higher than previous reports of subcutaneous delivery of full-length of native lectin antigen.[37] An effective vaccine for amoebiasis should induce both systemic and mucosal protection. Overexpression of *Lec* A in an edible crop would help oral delivery and confer immunity against amoebiasis.

Human Papillomavirus (HPV) Vaccine

Every year approximately 500,000 women develop cervical cancer caused by the human palillomavirus (HPV), 80% of them are from the third world countries. So far, 120 HPV strains

have been identified, but HPV-16 (51%) and HPV-18 (16%) are commonly found in the majority of patients (two-third). L1, a major structural protein of HPV capsid is highly conserved in all papilloma viruses. To date, only two subunits of vaccine is administered (a) bivalent that is used for HPV type 16 and 18 (b) tetravalent for HPV types 16, 18, 6 and 11; in both cases L1 antigen is used. HPV-16 L1 and HPV-11 L1 expressed in tobacco and potato via the nuclear genome[38,39] resulted in low levels of expression. In contrast, higher levels of L1 antigen (21% TSP)[22,40] was observed in chloroplasts and this induced systemic immune response in mice after intraperitoneal injection and neutralizing antibodies were detected.

Autoimmune Type 1 Diabetes (T1D)

Autoimmune disease is the malfunction of the immune system that results in destruction of autoantigens with devastating consequences. Therefore, it is of crucial importance to educate the immune system not to attack its own cells by induction of tolerance via expression of Foxp3 regulatory T-cells, ignorance or anergy or depletion. Successful oral administration of an autoantigen (e.g., insulin) and prevention of T1D has been shown earlier[41] and successful prevention/delay onset of T1D using plant-based vaccine technology has been shown by us and others.[13,42] In these studies proinsulin has been used as an autoantigen for restoration of broken tolerance in female NOD mice. We have recently shown that insulin producing β-cells were well protected in NOD mice orally gavaged with CTB-proinsulin whereas very few insulin-producing β-cells remained in control NOD mice with significant infiltration of pancreatic islets characteristic of lymphocytes (insulitis). Increased insulin production and lower urine and blood glucose levels were observed in NOD mice gavaged with CTB-proinsulin. Increased expression of immunosuppressive cytokines (IL4, IL10) was observed in pancreas of CTB-proinsulin treated mice, suggesting Th2 lymphocyte mediated oral tolerance as a likely mechanism for the prevention of insulitis and preservation of insulin producing β-cells.[13] Glutamic acid decarboxylase 65 (GAD65) has been shown to be an effective autoantigen for prevention/delay onset of T1D in NOD mice.[43] Recently, human GAD65 has been expressed in algal chloroplasts and its immunoreactivity with sera of diabetic NOD mice and its effect on proliferation of splenocytes of NOD mice has been reported.[25] Whether GAD65-derived algal can restore broken tolerance in diabetic prone NOD mice and prevent/delay onset of type 1 diabetes remains to be elucidated.

Regulation of the Immune System by the Green Vaccine

The GALT is highly organized in the Peyer's patches (PPs) and lamina propria as antigens encounter the antigen presenting cells (APCs) via the microfold cells (M cells) or captured by the APCs directly and then will be presented to T-cells. The PPs has a very unique architecture that does not exist in any other part of the immune system of the body.[44–46] Presence of regulatory cytokines such as TGFβ1, IL-10 and other determining molecules such as IL-4 and retinoic acid (RA) secreted from different T-cells and dendritic cells (DCs) makes it a unique educational microenvironment where conventional T-cells will be educated as regulatory T-cells (Tregs) expressing master regulatory transcription factor Foxp3 after receiving signals from tolerized antigen-experienced DCs. The de-novo generated Tregs and tolerogenic DCs will enter into the mesenteric lymph node and will travel into the pancreas where they guard insulin producing β-cells from destruction of the autoreactive T-cells.[47] The plant-based vaccine generates both cellular and humoral immune system. It appears that the green vaccine generates different immune responses such as Th1 (IgG2, IFNγ, IL-2), Th2 (IL-4, IgG1, IL-10, IgA), Tr1 (IL-10), Th3 (TGFβ1) and regulatory immune responses dependent upon the nature of the disease and route of vaccinations.[2] In many studies, cellular component of the immune response has not been investigated and only the humoral immune response has been characterized. A Th2 immune response to vaccine antigen is the most common form of the immune response against the vaccine antigen and secretion of IL-10, IL4 and local intestinal

IgA and IgG1 are signatures of this response, while IL-10 and TGFβ1 is a Tr1 and Th3 immune response, respectively. Increasing number of $Foxp3^+$ regulatory T-cells has also been shown to be associated with oral vaccination using plant-derived materials. This very crucial subpopulation of $CD4^+$ T-cells has been shown to express constitute level of IL-2Rα (CD25) and a very important transcription factor namely forkhead box P3 (Foxp3) possess suppression properties and will suppress proliferation of responding $Foxp3^-$ T-cells. Secretion of local soluble IgA in the GALT is a unique characteristic of oral vaccination and this is due to presence of a unique microenvironment in the gut mucosa to generate and secrets IgA.[48] The IgA plays an inimitable role in the mucosal immunity against infectious agents[49] and locally expressed in orally vaccinated mice mainly but little or not at all by any other means of vaccination.

Conclusions

Chloroplast transgenic technology offers an environmentally friendly and cost effective system for generation of vaccines and other therapeutic proteins. Published literature in this field demonstrates that chloroplast-derived vaccine antigens expressed in edible crops is indeed an efficient tool for oral delivery of vaccine antigens and can induce appropriate antigen-specific immune response and confer protection against dreadful pathogens. One of the most important issues in any clinical trial is “safety” and it appears plant-derived vaccines could offer a safer approach because it is needle free and requires no adjuvants or other chemicals. Bioencapsulation of vaccine antigens in plant cells provide an ideal low cost delivery system for large-scale distribution at times of crisis. Several products expressed via the plant nuclear genome have entered human clinical trials so far.[5] Most importantly, oral delivery provides both mucosal and systemic immunity, thereby conferring higher levels of protection than vaccines delivered using current technologies. Currently, other than the rotavirus, there is no other example of oral vaccines in the US and the mucosal immune system has not been utilized to confer immunity against invading pathogens. Oral polio vaccine was discontinued in the US because one in 2.4 million cases contracted polio from the live attenuated oral vaccine. However, such problems are not associated with subunit vaccines because only one or two antigens are used that are incapable of causing any disease. Therefore, it is important to understand and utilize the power of mucosal immune system for delivery of vaccines. High level protection observed against several pathogens with chloroplast-derived antigens, makes green vaccines yet another new platform for advancing towards human clinical studies.

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Table 1

Vaccine and autoantigens produced in chloroplasts

Vaccines antigens	Gene	Plant species	% of TSP	Functional evaluation	Year	Literature cited
Bacterial antigens						
Cholera toxin B	*Ctx*B	Tobacco Lettuce	4.1, 8, 12.3 4.8, 9.4	GM1 ganglioside-binding assay. Long-term protection (50% mouse life span) 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 $IL10^{+}$ T-cell but not $Foxp3^{+}$ regulatory T-cells, suppression of IFNγ and absence of IL-17 were observed in protected mice.	2001, 2009	12, 14
E. coli enterotoxin B	*LTB*	Tobacco	2.5	GM1 ganglioside-binding assay.	2003	50
Tetanus toxin	*Tet*C	Tobacco	25, 10	Mice developed systemic immune response and survived the tetanus toxin challenge.	2003, 2005	33, 51
Mutant of *E. coli* toxin	*LTK*63	Tobacco	3.7	GM1 ganglioside-binding assay.	2004	52
Anthrax protective antigen	*Pag*	Tobacco	19	Macrophage lysis assay, systemic immune response, toxin neutralization assay, mice survived (100%) challenge with lethal doses of toxin.	2004 2005	10, 15
Lyme disease -OspA	OspA, OspA-T	Tobacco	1, 10	Systemic immune response in mice. Protected mice against *Borrelia burgdorferi.*	2006	53
Plague F1-V	*CaF*1-*Lcr*V	Tobacco	14.8%	Immunogenic in mice (IgG1 titers). Oral delivery offerred greater protection (88%) and immunity than subcutaneous (33%) injection when challenged with 50-fold lethal dose of aerosolized *Y. pestis.*	2008	2
E. coli enterotoxin B	*LTB*	Tobacco	2.3	GM1 ganglioside-binding assay; oral immunization protected mice from CT challenge	2009	31
Viral antigens						
Canine parvovirus	*CTB-2L21* *GFP-2L21*	Tobacco	31.1, 22.6	Rabbit sera neutralized CPV in an in vitro assay.	2004, 2005	16, 54
Hepatitis E virus	*HEV E2*	Tobacco		Immune response in mice.	2006	55
Swine fever virus	*CFSV E2*	Tobacco	1–2	Immune response in mice	2008	56
Human Papillomavirus	*L1*	Tobacco	24	Systemic immune response in mice after intraperitoneal injection, and neutralizing antibodies were detected.	2008	11
Foot-and-mouth	*CTB-VP1*	Chlamy	3	Not reported	2003	46
Rotavirus	*VP6*	Tobacco	3	Not reported	2004	23
Hepatitis C	*NS*3	Tobacco	2	Not reported	2005	57
Epstein-Barr virus		Tobacco	0.004%	Not reported	2006	18
Swine fever virus	*CFSV E2*	Chlamy	1.5–2	Not reported	2007	58
Human Papillomavirus	*L1*	Tobacco	1.5	Not reported	2008	22
HIV	*p24*	Tobacco	2.5	Not reported	2008	36
HIV	*P24-Nef*	Tobacco & Tomato	40♣	Not reported	2008	20
Protozoan antigens						
Ameobiasis	*Lec*A	Tobacco	7	Systemic immune response in mice.	2007	24
Malaria	*CTB-ama1 & CTB-msp1*	Tobacco	12.3	Sera of immunized mice completely inhibited proliferation of the malarial parasite and cross-reacted with the native parasite proteins/parasites in immunoblots and immuno-fluorescence studies, at the ring, trophozoite or schizont stage of the malarial parasite	2009	14
		Lettuce	9.4			
		Tobacco	8			
		Lettuce	4.8			
Autoantigens						
Diabetes—Type 1	*CTB-pins*	Tobacco & Lettuce	~16% ~2.5%	CTB-prins treated mice showed significant decrease in inflammation (insulitis) in non-obese diabetic mice; insulin-producing β-cells in the pancreatic islets of CTB-	2007	13

Vaccines antigens	Gene	Plant species	% of TSP	Functional evaluation	Year	Literature cited
Diabetes—Type 1	*hGAD*65	Chlamy	0.3	Pins-treated mice were highly protected, increase in insulin production with lower blood or urine glucose levels; Increased expression of immunosuppressive cytokines (IL4, IL10) Immunoreactivity to diabetic sera	2008	25

♣-40% in leaves of both tobacco and tomato, 2.5 % in green tomato and none in red- ripe tomato