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Applications of Plant-derived Vaccines for Developing Countries

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Abstract

Plants offer tremendous advantages as cost-effective, safe and efficacious platforms for the large-scale production of vaccines and other therapeutic proteins. Plant-derived vaccines provide a way by which to enhance vaccine coverage for children in developing countries, and have the potential via oral administration to elicit a mucosal immune response. Plants have the added advantage of simultaneously acting as an antigen delivery vehicle to the mucosal immune system while preventing the antigen from degradation as it passes through the gastrointestinal tract. Transgenic plants, transplastomic plants and plant virus expression vectors have been designed to express vaccine epitopes as well as full therapeutic proteins in plant tissue. This review describes the use of different strategies to produce vaccines in plants against three deadly infectious diseases of the developing world today; human papillomavirus, human immunodeficiency virus and Ebola virus.

Keywords: Plant derived vaccines; Immune system; Infectious diseases

Introduction

In the third world, infectious diseases are the leading cause of infant mortality. A primary reason for this high prevalence continues to be the lack of cost-effective and easily accessible vaccines and other biopharmaceutical proteins, including monoclonal antibodies, which are readily available in industrialized countries. These discrepancies were addressed in 1992 at the Children's Vaccine Initiative, a platform by which globally accessible oral vaccines are generated through an assembly of philanthropic groups in conjunction with the World Health Organization. The initiative set out to develop novel vaccines that are inexpensive, efficacious, safe, easy to transport to remote areas, and temperature stable [1,2]. The employment of plants as potential production and delivery platforms for the expression of vaccines to infectious diseases is one promising approach that emerged from this Initiative. Plant-derived vaccines and therapeutic proteins retain similar biological activities as their mammalian-derived counterparts, unlike bacterial expression systems. Vaccines produced from plants have the dual advantage of acting as the vaccine delivery vehicle as well as protect the vaccine protein from degradation by the harsh environment of the gastrointestinal tract, so that it can reach the mucosal immune system more effectively [3]. Vaccines and therapeutic proteins can be either expressed stably as transgenic plants, or in a transient fashion using agro infiltration, or by infection with recombinant virus expression vectors [4-6].

Many orally delivered antigens are difficult to be recognized by the gut as foreign and as a result, cannot serve as immunogens. The use of adjuvants, which alter the immunogenic context through which an antigen can be encountered, can circumvent this obstacle. A frequently used mucosal imunogen used in the delivery of plant-derived vaccines is the cholera toxin subunit B (CT-B). CT-B is nontoxic to the cell, yet can alter the cellular environment to elicit an immune response to a given antigen; it can also act as an efficient transmucosal carrier molecule for plant-derived subunit vaccines [7]. Proteins which act as weak immunogens can thus be coupled to CT-B and expressed in plant tissue to elicit a stronger immune response [8].

Certainly a key driving force for the development of plant-derived biopharmaceuticals has been its expected potential to provide relief to Third World countries. At present, one fifth of the world's infants lack access to vaccines for commonly preventable infectious diseases [9]. As the West is less familiar with infectious diseases such as dengue fever, hookworm and rabies and treatments are poorly financed, the development of plant made vaccines could provide an opportunity to treat these 'orphan' diseases [9,10]. Moreover, vaccine proteins can be purified with fewer steps and at lower cost from plants than from mammalian cell culture, or depending on the intended usage, may require only partial purification.

Besides providing vaccines and therapeutic proteins to those who reside in developing countries, plant production platforms present other opportunities as well. Production platforms based upon plant viruses have been used to produce vaccines against global pandemics, such as Influenza virus, possible biological warfare agents, and even personalized medicine. Applications such as these offer compelling reasons toward further developing plant production platforms as a technology to produce biopharmaceuticals and other proteins.

One distinct attribute of the use of plants is that while a large number of mammalian therapeutic proteins are glycosylated, plant proteins also undergo post-translational modifications; that are similar, although not always identical to their mammalian counterparts. One shortcoming stemming from these subtle differences in glycosylation motifs between plant and mammalian derived therapeutic proteins could be an increase in allergic and other undesirable immune responses for patients [11]. Plants have been developed which produce 'humanized' therapeutic proteins through the result of altering a variety of plant glycosylation pathways [12,13]. For example, transgenic 'knockout' plants have been generated which lack plant-specific glycosylases, while other plants have been produced that express glycoproteins which are sialylated and O-glycosylated for correctly humanized protein expression [14].

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Additional modifications, including organelle specific transit signals, can also be constructed in plants [15].

Mucosal Immunity and Plant-derived Vaccines

The mucosal membrane of the digestive, respiratory, and urogenital tracts are often sites of entry for infectious diseases. It is here where antigen uptake, processing, and presentation for eliciting mucosal responses take place. A major portion of the body's immune system is represented by the gut-associated lymphoid tissue (GALT). In the respiratory tract, antigens are taken up into alveolar spaces by antigen-presenting cells to regional lymph nodes [16]. Immune cells such as B cells can migrate via the lymphatic system to regional lymph nodes, where the primary immune response, including IgA and IgG antibody production, takes place [17].

Technology Platforms Used to Generate Biopharmaceuticals in Plants

Several approaches have been used to generate biopharmaceutical proteins in plants. Initial approaches involved the stable transformation of plant cells and their regeneratation into mature transgenic plants. This is routinely accomplished using either *Agrobacterium*-mediated transformation or biolistic delivery [18]. Chloroplast (transplastomic) transformation is another technique that is being developed; proteins that have been expressed from plastids are often expressed at much greater levels due to the high copy number of chloroplast genomes in each cell. Chloroplast transformants also have the added advantage of lacking transgene transmission through pollen. However, some caveats do exist. For example, proteins expressed in chloroplasts are more bacteria-like in terms of post-translational modifications; also, fewer plant species have been developed for chloroplast engineering at present.

As an alternative to the generation of transgenic plants from single cells, which can be a lengthy process, plant viruses have also been developed to express foreign proteins [19]. Plant viruses have small genomes and as a result are relatively easy to manipulate. Infection of plants with recombinant plant viruses expressing the protein of interest can be an extremely rapid process, and in most cases take only a few days to produce high yields [20]. Both plus-sense RNA viruses based on tobacco mosaic virus, potato virus X and cowpea mosaic virus, as well as a DNA virus known as a geminivirus have been designed to express foreign proteins such as vaccines in plants [21]. Specifically, geminiviruses such as bean yellow dwarf virus (BeYDV) possess small, single stranded DNA genomes that replicate in the nuclei of plant cells. Delivery of an expression vector based on geminivirus in plants results in very high levels of DNA replicons that in turn act as a template for transcription to generate high mRNA yields of the gene of interest. Thus, the transient expression of foreign genes based on plant viral vectors has become a reliable system for the production of vaccines against a variety of infectious diseases.

Case Studies for Plant-derived Vaccines in Developing Countries

To date, plant-made vaccines and monoclonal antibodies have been generated for several infectious diseases, including hepatitis B virus, influenza virus, rotavirus, Norwalk virus, enterotoxigenic *E. coli* and *Vibrio cholera*. Many of these have been described in detail in other reviews. The current paper focuses on research that has accumulated to date regarding three of the most difficult to control diseases found in developing countries today. Both human papillomavirus and human immunodeficiency virus remain a significant global burden. Although an effective vaccine against HPV does exist, it is too expensive to be considered for global use. To date, no vaccine exists for HIV, and the high cost of antivirals prevents their accessibility to the tens of millions in developing countries who are suffering from this disease. Finally, difficulties in controlling the spread of the newly emerging Ebola virus have attracted attention for its potential use as a biological warfare agent by terrorists. The following section describes the steps that have been taken in the design and generation of plant made vaccine and therapeutic agents to combat each of these pathogens.

Human Papilloma Virus (HPV)

Cervical cancer is the second most common of cancers in women, with over half a million new cases a year [22]. Of confirmed cases, more than 250,000 will die of the disease, and the vast majority of these deaths will take place in developing countries, particularly in Asia and sub-Saharan Africa. The number of new cases of cervical cancer has been steadily increasing every year. Cervical cancer is most frequently caused by Human papillomovirus (HPV), with HPV 16 and 18 being the most predominant types and responsible for HPV infection in more than 70% of the cases. HPV contains a double-stranded circular DNA genome, and is encapsidated as an unenveloped icosahedron by the (late) capsid proteins L1 and L2. Early protein E7 is believed to play a pivotal role in viral oncogenesis.

Human papillomavirus (HPV) is currently prevented by vaccines based upon virus-like particles (VLPs). However, these vaccines have limited availability in developing countries, as a result of elevated costs, particularly for resource-poor countries. As currently available vaccines to HPV are expensive and probably unaffordable for most women in these countries, the need to develop cost-effective vaccines for HPV is dire.

One of the first pioneers to take the challenge to produce a plant-made vaccine to task was Ed Rybicki's group in Cape Town, South Africa. The Rybicki lab started in the mid-1990's to generate a plant–made vaccine against HPV-16. However, this initial vaccine candidate failed to generate neutralizing antibodies. The group then made full length and truncated versions of coat protein L1 (with the C-terminal 22 aa missing, so that the resulting L1 lacked a nuclear localization signal) [23]. Although they were able to produce L1 as a single capsomere and in the form of virus like particles (VLPs) that could be neutralized with monoclonal antibodies (MAbs), the vaccines were too low in yield to utilize in an effective manner. When a plastid localization signal was included in the original constructs, yields not only increased by 2 orders of magnitude, but the vaccine elicited an immune response similar to both insect cell derived vaccines and commercially available vaccines. The overall yields increased again when a replicating geminivirus vector based expression system was employed and when the vaccine protein was expressed in transgenic tobacco (transplastomic) chloroplasts. The group currently focuses on using chimeric versions of L1/L2 epitopes as a vaccine as well as an E7 oncoprotein-derived vaccine against cervical cancer, both from HPV-16.

Other groups have employed different approaches to generate vaccines against cervical cancer in plants. For example, Waheed et al. [24] coupled the adjuvant-like Escherichia coli heat-labile enterotoxin subunit B (LTB) to HPV capsid protein L1 to increase its immunogenicity and reduce cost even further by removing the need for the co-administration of an adjuvant in the inoculation process, a feature that would be valuable in resource-poor regions [24]. By

expressing a modified version of HPV-16 L1 and LTB as a fusion protein in tobacco chloroplasts, Waheed et al. [24] were able to increase recombinant protein accumulation up to 2% of total soluble protein. A GM1-ganglioside binding assay and antigen capture ELISA confirmed that proper folding and display of conformational epitopes for both LTB and L1 had taken place. Unfortunately, plant transplastomic lines that expressed high levels of recombinant protein also exhibited chlorosis in the leaves, retarded growth and male sterility. Taken together, these results pave the way for the possible development of a low-cost adjuvant-coupled vaccine with potentially improved immunogenicity against cervical cancer.

In another example of a potential low cost vaccine made against HPV in plants, Cerovska et al. [25] utilized an epitope derived from HPV-16 L2 minor capsid protein and expressed it in a potato virus X (PVX)-based vector as part of a fusion protein along with the PVX coat protein (PVX CP) in transgenic Nicotiana benthamiana plants at high levels [25]. Immunogenicity of virus-like particles expressing the PVXCP: L2 epitope were tested after immunization of mice by subcutaneous injection, and antibodies against the PVX CP and the L2 epitope were identified.

It has long been thought that biopharmaceuticals based on the recombinant protein E7 of human papillomavirus (HPV) can serve as therapeutic vaccines and prevent the development of cancer in women infected with high-risk types of HPV such as HPV-16. Buyel et al. [26] have generated high yields (50% corresponding to 0.1 g of protein per 1 kg plant biomass) of a plant-produced HPV-16 E7GGG-lichenase fusion protein as a vaccine candidate in tobacco plants [26]. The target contains a modified HPV16 E7 protein internally fused to the surface loop of a truncated, hexa-His- and KDEL-tagged variant of a bacterial lichenase which has been previously shown to possess anti-cancer activity in an animal model.

Plant made vaccines against other human papillomaviruses have been attempted as well. Matić et al. [27] has endeavoured to generate a vaccine against type 8 of HPV in plants using a variety of constructs [27]. HPV-8 is often associated with epidermodysplasia verruciformis and nonmelanoma skin cancer in immuno-compromised individuals and at the moment, no vaccine against cHPVs is available. Using a binary vector with or without silencing suppressor constructs, a nonreplicating cowpea mosaic virus-derived expression vector and a replicating tobacco mosaic virus (TMV)-based vector, the authors found that the highest expression took place with the cowpea mosaic virus-based expression vector, and were able to show using electron microscopy that plant-made HPV-8 L1 proteins assembled into virus-like particles (VLPs) as well as paracrystalline arrays, thus suggesting that plants can be used to generate a promising vaccine candidate against HPV-8.

HIV

Although the human immunodeficiency virus (HIV) is one of the most devastating infectious diseases globally, attempts to develop an effective vaccine have been unsuccessful. HIV/AIDS has become an alarming public health problem and is very costly for the governments of most African and developing countries to contend with. It is critical that new antiviral agents and vaccines designed to combat HIV/AIDS be generated inexpensively so that they can be accessible by the segment of the population that need them most. As a result, the production of recombinant subunit vaccines which are capable of eliciting an effective and broad immune response and neutralize virus entry is a priority. To produce pharmaceutical proteins of interest in large-scale and at an affordable cost remains a challenge. In light of this, plants have gained credibility as attractive and affordable production systems for potential HIV vaccines.

One technique that has been pursued for the production of a vaccine against HIV has been the use of virus like particles based on the Gag protein. Gag VLPs are enveloped and similar in size as HIV virions; they are also are capable of eliciting strong humoral and cellular immune responses. Rybicki et al. [28] were one of the first research groups to attempt to generate an effective vaccine against HIV in plants [28]. Initial attempts to generate HIV Gag VLPs in plants using either the native gene or a plant codon usage-optimized version of the Gag gene proved to be unsuccessful. The authors improved their expression levels using a virus expression vector based on TMV, but the expression levels were still too low to be viable as a production platform. Eventually, Gag that was produced using a plant chloroplast expression strategy proved to be adequate in terms of expression levels and immunogenicity, suggesting that this may be the best prospect for a plant-made HIV vaccine.

Plants can be used as alternative expression systems to produce subunit vaccines against other HIV antigens, including early proteins such as Tat and Nef. For example, Rosales-Mendoza et al. expressed chimeric proteins comprising C4 and V3 domains from gp120, to elicit broadly neutralizing antibodies [29]. The research group generated a synthetic gene encoding C4V3, a recombinant protein which is known to induce both systemic and mucosal immune responses. C4V3 was expressed in tobacco chloroplasts at high levels. The protein was demonstrated to cross react with both an anti-C4V3 rabbit serum as well as with sera from HIV positive patients [30]. When orally administered using a four-weekly dose immunization scheme, the plant-derived C4V3 elicited both systemic and mucosal antibody responses in BALB/c mice, as well as $CD4^+$ T cell proliferation responses.

These findings support the viability of using plant chloroplasts as biofactories for HIV candidate vaccines, and could serve as important vehicles for the development of a plant-based candidate vaccine against HIV. The use of plant based microbicides to block transmission of HIV is also under development. Neutralising antibodies and peptide lectins can act on the virus at its initial stages of infection and are efficacious in *in vitro* and *in vivo* protection studies [31].

Ebola virus

Ebola virus is a member of the filoviruses and causes haemorrhagic fever in both humans and non-human primates. Ebola virus is considered to be a potential biological warfare threat that could be exploited by terrorists. Efforts have been made to generate a large stockpile of Ebola vaccine that would be inexpensive to produce. Recently, a plant geminivirus-based expression vector has been employed to cost-effectively produce an Ebola virus subunit vaccine. This was accomplished by fusing Ebola glycoprotein (GP1) to the C-terminus of the heavy chain of an IgG monoclonal antibody specific to GP1 and co-expressing the fusion protein in a bean yellow dwarf virus (BeYDV) replicon system [32]. Tobacco leaves expressing this vector produced assembled immunoglobulin, which could be purified by ammonium sulphate precipitation followed by protein G affinity chromatography. Mice injected with the plant-produced protein generated an antibody response that is comparable to one produced with a virus-like particle [33]. Since production in tobacco may not be optimal due to the presence of high levels of phenolics and toxic alkaloids, lettuce has also been explored as a potential production platform for expression of monoclonal antibodies against Ebola virus.

The use of lettuce is particularly attractive as it allows to unlimited quantities of inexpensive plant material to be grown for large-scale therapeutic protein production. Moreover, the work indicates that replicons of BeYDV can be stacked in tandem within the same vector molecule, so that more complex, multi-subunit proteins can be produced at high levels and their biological activities preserved.

Conclusions

It is largely hoped that plants will continue to become safe, efficacious and economical platforms for the large-scale production of vaccine and other biopharmaceutical proteins against a wide variety of infectious diseases. Recombinant proteins derived from plants are easy to produce in large quantities. For example, the ratio of 0.1 g of HPV protein produced per 1 kg plant biomass described previously remained essentially the same upon scale-up from 50 g to 1 kg of processed leaf biomass. The fact that plant-derived vaccines do not require refrigeration and are easily administered to people who reside in remote regions of the developing world makes them an attractive alternative to conventional vaccines, and can address gaps which exist at present in the infrastructure available to improve global public health. Plants provide a new way of thinking about how some of our worst diseases can be dealt with. The potential benefits for many of the world's poor are much too great to ignore.

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