Plant-made HIV vaccines and potential candidates

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Highlights

- HIV/AIDS is a partially treatable but not completely curable pandemic disease.
- Major advances have been made to treat patients living with HIV/AIDS.
- Developing HIV vaccines is an ongoing endeavor and moves at an accelerated pace
- Plant molecular pharming is a valuable tool in HIV/AIDS vaccine research.

Abstract

Millions of people around the world suffer from heavy social and health burdens related to HIV/AIDS and its associated opportunistic infections. To reduce these burdens, preventive and therapeutic vaccines are required. Effective HIV vaccines have been under investigation for several decades using different animal models. Potential plant-made HIV vaccine candidates have also gained attention in the past few years. In addition to this, broadly neutralizing antibodies produced in plants which can target conserved viral epitopes and neutralize mutating HIV strains have been identified. Numerous epitopes of glycoproteins and capsid proteins of HIV-1 are a part of HIV therapy. Here, we discuss some recent findings aiming to produce anti-HIV-1 recombinant proteins in engineered plants for AIDS prophylactics and therapeutic treatments.

Keywords: plant made pharmaceuticals; HIV vaccine; AIDS drug; plant molecular pharming/farming; multiepitopic HIV vaccine.

Introduction

Acquired Immunodeficiency Syndrome (AIDS) is one of the greatest challenges to global public health today. In 1981, AIDS was identified as a new disease characterized by the dramatic collapse of CD4+T cells and immune responses. This drastic depletion of the immune system can lead to an assortment of opportunistic pathologies in infected subjects. Two years after the discovery of AIDS, a novel retrovirus was identified as the causative agent of AIDS [1] and was formally renamed the Human immunodeficiency virus (HIV) in 1986. HIV belongs to lentiviruses (a subgroup of retrovirus family) and has a genome of two similar single-stranded RNA molecules and several viral proteins packaged within a capsid. Two types of HIV (HIV-1 and HIV-2) have been identified, based on their genetic particularities and various viral antigens. However, HIV-1, and its subtypes, is the major type of HIV responsible for the growing global AIDS pandemic. The HIV-1 genome encodes nine open reading frames. Three of these encode the Group-specific Antigen (Gag), DNA Polymerase (Pol), a reverse transcriptase, and envelope (Env) polyproteins, which are subsequently proteolyzed into individual proteins common to all retroviruses. Its viral envelope comprises trimers of non-covalently linked heterodimers, subunits gp120 and gp41. More than a dozen HIV-1 subtypes and hundreds of circulating HIV-1 recombinant forms (CRFs) have been identified so far [2]. The rapid evolution of HIV envelope (Env) protein (gp120) has serious consequences for immunity to HIV-1 [3].

Demand for HIV vaccines

By the end of 2018, the World Health Organization (WHO) estimated that 37.9 million people were living with HIV around the world, among them 23.3 million were receiving antiretroviral therapy (ART). Moreover, about 700,000 people contract HIV every year and thousands of others die from subsequent chronic AIDS. Africa remains the most severely affected region, accounting for approximately two thirds of people living with the virus. To complement the efforts of ART, a vaccine is essential to eradicate the HIV/AIDS pandemic and slow down its multifaceted damage. This is desperately needed, particularly in HIV endemic regions where people have little access to ART due to poor socioeconomic status and where molecular pharming can thrive [4] [5].

At present, there is no HIV vaccine commercially available. Putting in place a prophylactic HIV vaccine is an absolute necessity. The global HIV pandemic continues to grow (due to mortality, number of people living with the HIV-1 virus and newly infected people each

year), as demonstrated by WHO statistics. On the other hand, current ART medications are costly and require lifelong observance with many side effects.

As early as 1987, the United States carried out the first phase I trial of a candidate vaccine. Nowadays, more than 30 HIV vaccine candidates have been tried in Phase I/II clinical trials, conducted mainly in the US and Europe and involving thousands of healthy volunteers.

Hurdles of HIV prophylactic vaccines

Despite a lot of human and financial resources that have been allocated to HIV research for decades, the development of an effective HIV vaccine is still elusive. Developing an effective preventive vaccine at low cost is a challenge for health professionals in the 21st century. The major hurdle behind this failure is the extraordinary genetic diversity of HIV-1 and, more specifically, the high level of variability within the viral envelope (Env) glycoprotein that is the target of neutralizing antibodies [6-8]. Due to high mutation rates (about 1–10 mutations per genome replication cycle), considerable conformational flexibility and extensive glycan coverage, the HIV-1 virus has developed a unique arsenal of mechanisms to evade neutralizing antibodies [9]. After infection, each host contains variants of HIV which are able to escape neutralizing antibodies (nAbs) that are produced. Although envelope structural modifications present a barrier to a broad-based immune response, this problem could be overcome by polyvalent HIV vaccines that recognize conserved domains on the viral envelope [10]. Accumulating evidence and multiple studies suggest that an effective HIV vaccine might be achievable in upcoming years.

Plant-based biofactories

Plant molecular pharming (PMP) is based on the ability of plants to express different genes for preventive and therapeutic purposes and it holds promise [11]. The production and commercialization of recombinant proteins and pharmaceutical compounds from plants has been optimized and relatively successful over the past few years [11-15]. The concept of PMP as a biofactory was demonstrated earlier on [16] and presents many advantages over conventional systems that rely on bacteria, yeast, insect and animal cell cultures for pharmaceutical products [13]. Engineered plants can properly assemble, fold, glycosylate and produce recombinant proteins at high scale and low cost without the risk of contamination by human pathogens [17]. Seeds harboring pharmaceutical proteins can also be stored at ambient

temperatures for months or years in certain instances without a loss in activity [18], thus removing substantial requirements of cold chain storage and transportation.

Examples of potential plant-derived vaccines for protection against different infectious diseases [19] and pathologies such as cancer [20], Influenza A virus subtype H1N1(H1N1), Hepatitis B and other pathogens responsible for global human pathologies have been reported [21,22]. Several plant secondary metabolites with antimicrobial properties have also been identified as inhibitors of the infection cycle of different pathogens. These metabolites are often part of a plant's defense strategy against biotic and/or abiotic stresses.

HIV vaccine candidates and antibodies expressed in plants

Several HIV antigens and neutralizing anti-HIV antibodies have been produced in various plant species since 1995 (Table 1) via stable *Agrobacterium*-mediated transformation or through transient expression systems using plant viral vectors (Figure 1). The ability to elicit multistage broadly neutralizing antibodies (bnAbs) is one of the current strategies used to develop HIV vaccines [23]. A novel anti-HIV-1 bispecific bNAb-lectin fusion protein has recently been expressed in *Nicotiana benthamiana* using a transient expression system [24]. The HIV-neutralizing human monoclonal antibody 2G12 has also been expressed in tobacco and is now approved for human phase I clinical trials [25]. The 2G12 antibody is one of the broadly neutralizing antibodies that specifically recognizes carbohydrate epitopes on the surface of HIV-1.

The identification of Epitope-focused Antigenic Domains (EADs) also offers a collection of possible targets for the rational design of HIV-1 vaccines [26]. Numerous epitopes of the Env glycoproteins and capsid proteins of HIV-1 are now found in the 'HIV Molecular Immunology Database'. These heterologous HIV-1 Env mixtures expose the immune response to a diversity of Env conformations [27]. An approach to the development of artificial proteins constructed using conservative T- and B-cell epitopes and their mimics is also promising for the development of vaccines against different viruses, including HIV [7,28]. The synthesis of vaccines against HIV-1 using virus-like nanoparticles is another area of research in HIV vaccines [29-32].

An example of a polyvalent vaccine is the C4(V3)6 chimeric multi-epitopic protein, modeled upon gp120 and generated to test its immunization capacity against HIV. This protein was expressed in bacteria, then expressed in tobacco and lettuce, and displayed potent antigenic HIV properties with no observed toxicity [33]. The characterization of the C4(V3)6 protein from lettuce showed that this multi-epitopic recombinant protein induced an immune

response when ingested by mice [34]. Additionally, C4(V3)6 derived from lettuce demonstrated enhanced immunogenic potential compared to *E. coli* produced epitopes. Further investigations of other multi-epitopic proteins were performed by fusing the multi-HIV epitope with glutathione-S-transferase and expressing the fusion protein in recombinant *E. coli* cells. The multi-HIV epitope construct was expressed in tobacco plants and elicited humoral responses in mice [35]. A multipotent HIV chimeric protein has also been synthesized in the moss plant *Physcomitrella patens* [36]. This protein was synthesized from gp41 and gp120 epitopes and used to produce the vaccine pol-HIV. Extracts of this recombinant protein were shown to induce specific antibodies in mice, signaling the potential of moss plants as an expression vehicle for the synthesis of HIV antigens and vaccines.

A novel affinity ligand has also been recently described based on the red fluorescent protein DsRed (derived from *Discosoma* sp.) as a carrier of the epitope ELDKWA that can capture the HIV-neutralizing antibody 2F5 [37]. When the protein fusion DsRed-2F5-Epitope (DFE) was expressed in transgenic tobacco, a yield of 24 mg kg⁻¹ with up to 90% purity was obtained using heat treatment and immobilized metal-ion affinity chromatography for protein purification. Although DFE is not yet optimized, boosting its expression and increasing its capacity to some folds would make its use in the production and purification of idiotype-specific mAbs cost-effective [37].

In an attempt to assess the immunogenicity of a multivalent HIV vaccine including DNA vector alone or in combination with Env glycoprotein (gp120), it was found that the co-administration of gp120 Env protein components with DNA vaccine leads to early and potent induction of Env V1/V2 IgG binding antibody responses [38]. It was also recently reported that a short DNA vaccine vector (of 172 bp length) derived from the porcine circovirus PCV-1 capsid protein promoter, and expressing various HIV-1 subtypes, leads to high immunogenicity of the HIV-1 subtype C candidate DNA vaccines in rodents, at up to 10-fold lower doses than usually used [39].

A multi-epitopic HIV oral immunogenic protein derived from tobacco has been produced and characterized in orally immunized mice without adjuvant co-administration [40]. These multi-epitopic HIV constructs derived from tobacco chloroplasts were able to induce T-helper (i.e. CD4⁺ and CD8⁺ cells) specific responses such as IFN- γ -production after stimulation with HIV peptides. Together, these findings illustrate well the immunogenic potential of plant-made HIV multi-epitopic proteins in an oral immunization proof-of-concept [40].

Recently, evolutionarily conserved epitopes between Feline immunodeficiency virus (FIV) and HIV were explored as a means of developing an effective vaccine [41]. In this study, Sahay and colleagues identified overlapping peptides that elicited T cell immunity in HIV-infected people and FIV-vaccinated cats. Four of the conserved epitopes were then used to produce four multiple antigen peptides (MAPs) which were tested against FIV in cats. Increases in CD4⁺ and CD8⁺ T cell MAP responses were observed in nearly 80% of the cats, implying protection against FIV [41]. This criterion can be similarly applied in humans to select for protective HIV-1 peptides that can be used to formulate an effective vaccine against HIV-1. An *in silico* approach has recently been developed to predict anti-HIV activities and save time and money in screening for potential HIV/AIDS drugs [42].

Three proteins that can neutralize HIV were recently produced in rice endosperm [43]. These included the monoclonal antibody 2G12 as well as cyanovirin-N and griffithsin (GRFT). It was found that a cocktail of these components was able to neutralize HIV by binding to gp120. It was further discovered that the use of this cocktail in conjunction with rice endosperm crude extracts strengthened binding to gp120. When synthesized in transgenic plants, the extracts of these proteins also improved binding to gp120 while at the same time neutralizing the virus in a more potent manner than a conventionally produced cocktail [43]. Griffithsin (GRFT), an anti-HIV carbohydrate-binding protein (lectin) derived from algae and considered to be one of the most efficient viral entry inhibitors, has been synthesized in tobacco and lettuce plants at relatively high yield levels particularly when the open reading frame was fused to the Zera signal peptide [44]. Tobacco and lettuce may therefore be ideal host expression systems for the synthesis of GRFT as an HIV inhibitor.

A plant virus vector system based on Tobacco mosaic virus has also been used to produce GRFT in *N. benthamiana*. GRFT accumulated to more than 1 g/kg fresh weight in fresh leaf material and exhibited gp120 binding potential and efficacy that was similar to that produced in *E. coli* and native GRFT respectively. Fuqua *et al.* (2015) were able to develop an inexpensive and industrial scale purification system for GRFT that could be viable in resource-poor settings [45]. The authors' technique could provide multi-kilogram quantities of GRFT at low cost. A further study demonstrated the feasibility of plant-produced GRFT using a Tobacco mosaic virus vector inoculated into *N. benthamiana* plants via a high-pressure spray [46]. According to the model set up by these authors, 20 kg of GRFT can be generated every year, with a yield of 0.37 grams of GRFT per kg of plant biomass and a projected cost estimated at \$USD 0.32 per dose (3 milligrams). This is in comparison to the current cost of the pre-exposure prophylaxis drugs emtricitabine and tenofovir disoproxil

fumarate (which ranges from \$1,300 to over \$1,700 per month). Using transient plant-based platforms for the production of antibodies can reduce cost and capital investment by about 50% compared to traditional biomanufacturing platforms that use mammalian cells [47].

Tobacco has also been used to synthesize the lectin, cyanovirin-N (CVN) [48]. Another plant lectin (BanLec) seems to effectively neutralize HIV-1 by targeting a subset of dense mannose glycans of the glycosylated HIV-1 envelope protein, gp120, via bidentate interactions spanning two carbohydrate binding sites present on each BanLec monomer [49]. BanLec (banana lectin) was initially isolated from bananas with anti-HIV replication activity [50].

B cell epitopes for the production of broadly neutralizing antibodies (bNAbs) against HIV also exist. bNAbs can induce either passive or innate cellular responses to HIV-1 [51]. HIV Env gp140 antigens have recently been produced in a transient expression system in *Nicotiana benthamiana* by using two virus isolates (CAP256 SU and Du151) [52]. The HIV antigen, P24, has been synthesized in the green alga *Chlamydomonas*, with the recombinant protein constituting 0.25% of the total cellular protein [53]. Constructs of P24 and Gag, a HIV-1 coat protein gene, have been inoculated into *N. glutinosa* and *N. benthamiana* plants where it was discovered that P24 replicated in the inoculated plants while Gag did not [54]. This demonstrated the presence of P24, and therefore the potential of high-throughput p24 production in plants. It has been previously shown that a transient expression system using the gene silencing suppressor protein P19 helped increase the expression of HIV-1 Nef in *N. benthamiana* plants [55].

Conclusion and Perspective

There is an urgent need to uproot the HIV/AIDS epidemic everywhere in the world in whatever way possible by targeting its conserved and/or variable structures. The facility and cost effectiveness of plant expression systems can help expedite preventive and therapeutic HIV vaccine development. Once an effective HIV vaccine is developed sooner or later in whatever biological approach, plant molecular pharming may turn out to be a solution of choice for producing such vaccine thanks to its numerous advantages over other bio-drug production platforms. Economical and highly efficacious plant-made biopharmaceuticals, including vaccines, broadly neutralizing monoclonal antibodies and antiretroviral microbicides could have a significant impact on HIV epidemiology, incidence and prevalence. Ultimately, plants that harbor therapeutic molecules can grow almost everywhere without the need for sophisticated or expensive infrastructures to produce potential HIV vaccine candidates so that poor people can have access to these vaccines easily. Kessans *et*

al. (2016) demonstrated the potential of combining a plant vaccine into a heterologous prime/boost vaccination regimen, in which plant made vaccines, would provide suitable booster vaccines due to the large amount of vaccine material required for repeated booster shots [56]. Others have also demonstrated in animal studies the value that PMP could have for effective vaccine prime/boost strategies [57,58].

The next steps will be to continue the push toward the development of facilities which enable plant-made vaccines and antiretrovirals to be produced in resource-poor settings [59]. HIV vaccines and antiretroviral proteins that could meet the world's demands could never be produced under the current prohibitively expensive restrictions of mammalian cell production and purification systems. Plant-based platforms, on the other hand, stand a reasonable chance of meeting this goal [60,61].

Conflict of interest: none.

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The authors showed that when recombinant *A. tumefaciens* strains encoding the soluble HIV-1 gp140 antigens made from CAP256 SU and Du151 Env genes were introduced into *N. benthamiana* plants, both proteins resulted in severe pathology in the plants. It was discovered that the pathology caused by Du151 gp140 was more severe, with necrosis observed after only 5 days. More plants were infiltrated with these antigens leading to the mean recovery of 4.9 mg/kg and 6.2 mg/kg for Du151 and CAP256 SU respectively.
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Table 1. Examples of plant-derived vaccine candidates produced in plants and tested in animals against HIV.

HIV Antigen	Produced in (plant)	Tested in (animal)	Observed Immunogenicity	Reference
C4(V3)6	Lettuce	Mice	Induction of humoral immune response. Induction of cell-mediated immune response.	[34]
C4V3	Tobacco	Mice	Induction of systemic mucosal antibody response; induction of mucosal antibody response; induction of T cell proliferation.	[62]
CTB-MPR	Tobacco	Mice	Induction of serum antibodies; induction of mucosal antibodies.	[63]
CTB-P1	Tobacco	Mice	Serum IgG response; mucosal IGA response; induction of immunological memory.	[64]
Gp41	Tobacco	Mice	Serum antibody responses; CD4 and CD8 T cell responses.	[56]
Multi-HIV	Tobacco	Mice	Induction of antibody and cellular responses. Induction of T cell immune response.	[40]
Multi-HIV	Tobacco	Mice	Induction of humoral response.	[35]
p17/p24	Tobacco	Mice	Induction of humoral immune response. Induction of T cell immune response.	[65]
p24	Tobacco	Mice	Serum IgG response.	[66]
p24	Arabidopsis	Mice	Serum IgG response.	[67]
p24	Tobacco	Rabbit	Induction of specific humoral immune response.	[68]
P24	Arabidopsis	Mice	Serum IgG response.	[69]
Poly-HIV	<i>Physcomitrella patens</i>	Mice	Induction of specific antibody response.	[36]

Figure 1A-1B- Plant-made HIV vaccines

Figure 1A. Stable *Agrobacterium tumefaciens*-mediated transformation. HIV-1 gene (up-left) is introduced into plant tissues within an optimized expression cassette of Ti-plasmid (Ti: for tumor inducing) of *A. tumefaciens*. Wounded leaf explants are transformed by insertion of T-DNA (a transferable T-DNA segment from the Ti-plasmid) into plant genome (1-2). Transgenic cells harboring HIV transgene can grow on kanamycin-resistant medium (3). Organogenesis generates plantlets, then plants cultivated in greenhouse setting 6 months later (4-5). The insertion of HIV-1 gene in plant tissues is verified by PCR/RT-PCR analysis prior to purification of the corresponding recombinant protein (6-7).

Figure 1-B. Transient expression process in plants mediated by *Agrobacterium tumefaciens*. HIV-1 gene (up-left) is introduced into plant tissues within an optimized expression cassette of Ti-plasmid (Ti: for tumor inducing) of *A. tumefaciens* (1). *Agrobacterium* mixture is introduced into *N. benthamiana* leaf tissues by agroinfiltration. Either (a) by weak vacuum agroinfiltration from leaf shoots (0.8-0.9 bar for 20-30 seconds) or (b) by needleless syringe agroinfiltration at the level of the epidermis on the back side (2). Leaf explants are transformed by insertion of Ti- T-DNA (a transferable T-DNA segment from the Ti-plasmid) into plant genome (3). After infiltration with the *A. tumefaciens* mixture, the leaf tissues are incubated for 4-6 days (full expression of a HIV-1 gene in agroinfiltrated leaves takes place 3–4 days later (4). Then, downstream processes are performed: leaf homogenization, purification by dialysis (remove salts), HIV-1 protein concentration (5). The insertion of HIV-1 gene in plant tissues is verified by PCR/RT-PCR analysis, prior to purification of the recombinant proteins. 6).* Transient expression is a fast and efficient process for producing wide amounts of recombinant proteins, compared to the stable transformation process via *Agrobacterium tumefaciens*. Plant species, development stage and physiological status of plant play a crucial in the efficiency of the agroinfiltration process for producing recombinant proteins.

Abbreviations: HIV: Human immunodeficiency virus. (*)In disarmed Ti-T-DNA (Binary vector): an expression cassette used for the insertion and expression of the HIV-1 gene in plants. LB: left border; Tnos: terminator of the gene coding for nopaline synthase; HIV-1 gene coding for an antigen chimeric protein; p35s: 35s promoter of CaMV (cauliflower mosaic virus); nptII: gene encoding neomycin phosphotransferase, conferring resistance to kanamycin; pnos: promoter of the gene coding for nopaline synthase; RB: right border.