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EXPERT REVIEWS

Plant-based anti-HIV-1 strategies: vaccine molecules and antiviral approaches

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Nunzia Scotti[†1], Luigi Buonaguro[2], Maria Lina Tornesello[2], Teodoro Cardi[1] and Franco Maria Buonaguro[2]

[1]*CNR-IGV, Institute of Plant Genetics, Via Università 133, 80055 Portici, Naples, Italy*
[2]*Istituto Nazionale Tumori 'Fond. G. Pascale' – IRCCS, Via Mariano Semmola 142, 80131 Napoli, Italy*
[†]*Author for correspondence:*
Tel.: +39 081 253 9482
Fax: +39 081 253 9481
nscotti@unina.it

The introduction of highly active antiretroviral therapy has drastically changed HIV infection from an acute, very deadly, to a chronic, long-lasting, mild disease. However, this requires continuous care management, which is difficult to implement worldwide, especially in developing countries. Sky-rocketing costs of HIV-positive subjects and the limited success of preventive recommendations mean that a vaccine is urgently needed, which could be the only effective strategy for the real control of the AIDS pandemic. To be effective, vaccination will need to be accessible, affordable and directed against multiple antigens. Plant-based vaccines, which are easy to produce and administer, and require no cold chain for their heat stability are, in principle, suited to such a strategy. More recently, it has been shown that even highly immunogenic, enveloped plant-based vaccines can be produced at a competitive and more efficient rate than conventional strategies. The high variability of HIV epitopes and the need to stimulate both humoral neutralizing antibodies and cellular immunity suggest the importance of using the plant system: it offers a wide range of possible strategies, from single-epitope to multicomponent vaccines, modulators of the immune response (adjuvants) and preventive molecules (microbicides), either alone or in association with plant-derived monoclonal antibodies, besides the potential use of the latter as therapeutic agents. Furthermore, plant-based anti-HIV strategies can be administered not only parenterally but also by the more convenient and safer oral route, which is a more suitable approach for possible mass vaccination.

KEYWORDS: HIV • plant-based pharmaceutical • protein accumulation • recombinant protein • transgenic plant • vaccine

The latest AIDS epidemic update in December 2009 reported that the number of people living with HIV worldwide continued to grow in 2008, reaching an estimated 33.4 million. Furthermore, the total number of HIV-infected individuals in 2008 was more than 20% higher than in the year 2000 [101].

In order to halt the spread of HIV, several strategies encompassing both treatment and prevention will need to be adopted. Hence, multidisciplinary approaches involving teams of scientists should be pursued to search for an HIV vaccine. As HIV is a highly variable virus, a breakthrough is not expected imminently. For a vaccine to be effective, the immune response should be broad and long-lasting, and the vaccine itself should be affordable to developing countries, and easy to transport, store and administer in ill-equipped health infrastructures.

Hundreds of vaccine approaches have been considered in the past 25 years since the discovery of HIV-1, for preventive as well as therapeutic immunotherapies. In this respect, vaccine models against structural (e.g., Env-gp120 membrane glycoprotein or Gag capsid protein) or regulatory proteins (especially Tat, Nef and Pol) have been developed [1,2]. The vast majority of the published molecules have shown some immunological efficacy, although not all of them have been tested for neutralizing and cross-neutralizing activity. Over 650 nonhuman primate trials [102] and more than 190 human clinical trials [103] have been performed with different immunogens, vaccine protocols and immunogenic end points. The overall achievements can be represented and summarized by some of the most recent trials: the two recombinant gp120-based, humoral-targeted vaccine trials conducted by VaxGen Inc. (CA, USA; AIDSVAX003 and AIDSVAX004) and the two Gag/Pol/Nef-based, T-cell-targeted adeno-5-based NIH-supported Vaccine Trials Network (HVTN502 and HVTN503) failed to demonstrate efficacy (reviewed in [2]). However, a recent trial based on four priming injections

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with an Env/Gag/Pro-based T-cell-targeted recombinant canarypox vector vaccine (ALVAC-HIV [vCP1521]) plus two booster injections of a recombinant gp120-based humoral-targeted subunit vaccine (AIDSVAX B/E) conducted in Thailand (RV144) showed, for the first time, reduced acquisition of HIV infection in vaccinated volunteers as compared with the placebo control group. The analysis displayed an overall reduction of 31.2% (p = 0.04) in the modified intention-to-treat (mITT) analysis, with a trend towards significance in the ITT and per-protocol (PP) populations [3]. Although not fully satisfactory, these results further supported the numerous earlier preclinical data, suggesting the need for a heterologous prime–boost strategy to elicit optimal humoral/cellular immune responses against HIV antigens, including protection against viral challenges (reviewed in [2,4,5]). Nevertheless, these large clinical studies (Table 1) have contributed to classify 'determinants' of HIV-induced disease progression, identify 'predictive' markers of HIV-related diseases and determine 'surrogate' end points in HIV vaccine trials [6]. These data will finally set the minimal end points required in any human clinical trial, and will strongly support a multicomponent approach, which should be easily accessible through a plant-based vaccine strategy.

In the meantime, several steps to express HIV epitopes have been taken by plant biotechnologists. Over the past decade, plants have been recognized as a promising production platform for recombinant pharmaceutical proteins [7]. They have been used to produce over 200 medically relevant proteins and their benefits make them a serious competitor to conventional systems for biopharmaceutical production [8]. The potential advantages of plants as factories include the ability to produce complex, correctly folded and post-translationally modified proteins, ease of scalability and reduced risk of contamination by human pathogens [9]. Furthermore, they allow the oral delivery of partially purified antigens, inducing both mucosal and humoral immunity, without the need for a cold chain [10]. However, low production yield is currently a major limitation observed in using plant systems, high-level expression being essential for economic and effective recombinant protein production [11].

Since the demand for biopharmaceuticals is expected to increase, different species (e.g., tobacco, tomato, spinach, carrot and maize) and expression strategies (i.e., transient and stable transgene expression) have been considered in order to improve recombinant protein yield in plants. In order to increase the use of plants as bioreactors, another important aspect to develop, previously overlooked, is downstream product processing, which accounts for approximately 80–90% of overall production costs. The new 'molecular farming' concept is based on the achievement of a processed product that has to be formulated reproducibly in order to ensure reproducible effects. In the recently funded EU Pharma-Planta project, the primary goal was to develop a complete production chain (from the expression platform to Phase I human trials) for candidate biopharmaceuticals for HIV/AIDS and three other key areas of health (TB, rabies and diabetes) [104].

In this article, we focus on the anti-HIV-1 strategies pursued in higher plants, aimed at expressing a single antigen (i.e., p24) as well as multiantigens (i.e., Nef–p24), including structural complexes such as virus-like particles (VLPs).

Production of HIV-1 antigens in plants

Many studies have demonstrated that plants can successfully express regulatory and structural HIV proteins (Table 2). Further information on the individual proteins is given in the following section.

Tat

Tat is a small regulatory protein, relatively well conserved across HIV clades, which is responsible for transactivation of virus transcription. It is synthesized early during HIV infection, and is essential for viral replication and cell-to-cell virus transmission [12–15]. Since a high anti-Tat antibody titer has been detected in asymptomatic HIV-infected individuals compared with progressed patients, it has been suggested that the presence of these antibodies has a protective role in disease progression [15]. For these reasons, it has been considered as a potential vaccine candidate.

To date, one study has presented data on production of SHIV 89.6p Tat and four on production of HIV-1 Tat protein in plants using both transient and stable expression systems [16–20]. The first plant-derived Tat protein was a fusion protein containing the cholera toxin B subunit (CTB) and the SHIV89.6p Tat protein produced in potato by *Agrobacterium tumefaciens* transformation methods [16]. Western and ganglioside M1-ELISA analyses detected the assembling of the fusion protein in pentameric structures and determined its yield (up to 0.007% of the total soluble

Table 1. HIV-1 vaccine tested in major Phase IIb/III clinical trials†.

Phase	Trial ID	Immunogen strategy	Candidate vaccines	Volunteers (n)	Start date
III	VAX 003	Protein	AIDSVAX gp120 B/E	2500	1999
III	VAX 004	Protein	AIDSVAX gp120 B/B	5400	1998
IIb	HVTN 502/Merck 023 (STEP study)	Viral vector – adeno	MRKAd5 HIV-1 Gag/Pol/Nef	3000	2005
IIb	HVTN 503 (Phambili)	Viral vector – adeno	MRKAd5 HIV-1 Gag/Pol/Nef	3000	2007
III	RV 144	Viral vector – pox/protein	ALVAC-HIV vCP1521 Env/Gag/Pro + AIDSVAX gp120 B/E	16,403	2003

†Information taken from database of AIDS vaccine candidates in clinical trials [103].

protein [TSP]), respectively. Karasev *et al.* used a tobacco mosaic virus (TMV)-based vector to express a synthetic plant codon-optimized *tat* gene in spinach leaves [17]. Despite the large Tat protein accumulation (up to 300 µg/g of fresh spinach leaves), oral immunization of mice was unable to induce a detectable HIV-specific immune response. Similar results were achieved by Webster *et al.* when two Tat constructs, containing the first exon or full-length *tat* gene, were stably expressed in the nuclear genome of tobacco plants [18]. In fact, they demonstrated that the plant-made Tat proteins were not immunogenic in Balb/C mice when administered intraperitoneally with or without Freund's adjuvant. By contrast, oral, intraperitoneal or intramuscular immunizations with protein extracts from transgenic tomato fruits expressing the *tat* gene induced a strong anti-Tat immune response comparable to those obtained with purified glutathione-S-transferase–Tat protein [19]. Interestingly, the fruit-specific expression of Tat protein seemed to be toxic to the plant, the fruits showing underdeveloped reproductive structures and no seeds [19]. Similar immunological results were achieved by Cueno *et al.*, expressing a plant-optimized *tat* and mutant (Cys30Ala/Lys41Ala) *tat* (*mtat*) genes fused with a β-glucuronidase (*gus*) gene in tomato [20]. Tomato extracts containing approximately 2–4 µg mTat/Tat–GUS fusion protein per milligram plant protein intradermally inoculated into Balb/C mice induced both humoral and cellular immune responses [20].

Nef

Nef is a small cytosolic protein expressed in the early stages of viral replication and is post-translationally modified by phosphorylation and attachment of myristic acid, which targets Nef to the cytosolic face of the plasma membrane [21]. Its ability has also been shown to influence progression to AIDS in HIV patients. This *in vivo* observation suggests that Nef represents a possible attractive target for the development of a therapeutic HIV vaccine [22]. Various biological systems have been used to produce the Nef protein. Recently, Marusic *et al.* summarized different biotechnological approaches to obtain high Nef accumulation in stable transgenic plants (Table 2) [23].

Table 2. Plant-derived HIV-1 vaccine antigens.

Antigen	Expression system	Plant species	Yield†	Ref.
CTB-Tat (SHIV 89.6p)	Nuclear transformation	Potato	0.007% TSP	[16]
Tat	TMV plant viral vector	Spinach	300 µg/g FW	[17]
	Nuclear transformation	Tobacco	0.01% TSP	[18]
	Nuclear transformation	Tomato	Not reported	[19]
Tat/mTat-GUS	Nuclear transformation	Tomato	4 µg/mg TP	[20]
Nef	Nuclear transformation	Tobacco	0.7% TSP	[24]
	Agroinfiltration	*Nicotiana benthamiana*	1.3% TSP	[28]
Nef-TA	Nuclear transformation	Tobacco	0.7% TSP	[25]
Zeolin-Nef	Nuclear transformation	Tobacco	1% TSP	[26]
Nef, Nef-p24, p24-Nef	Plastid transformation	Tobacco, tomato	40% TSP‡	[27]
Pr55^{gag}	TMV plant viral vector	*N. benthamiana*	2 µg/kg FW	[39]
	Agroinfiltration	*N. benthamiana*	44 µg/kg FW	[39]
	Nuclear transformation	Tobacco	48 µg/kg FW	[39]
	Plastid transformation	Tobacco	363 µg/g FW	[40]
p17–p24	Agroinfiltration	*N. benthamiana*	4.8 µg/g FW	[39]
	Nuclear transformation	Tobacco	230 µg/kg FW	[39]
p24	TBSV plant viral vector	*N. benthamiana*	5% TSP	[43]
	Nuclear transformation	Tobacco	0.35% TSP	[44]
	TMV plant viral vector	*N. benthamiana*	Not reported	[45]
	TMV plant viral vector	*N. benthamiana*	17.3 mg/kg FW	[39]
	Agroinfiltration	*N. benthamiana*	16.1 mg/kg FW	[39]
	Nuclear transformation	Tobacco	2.9 mg/kg FW	[39]
	Plastid transformation	Tobacco	450 mg/kg FW	[49]
	Nuclear transformation	*Arabidopsis*	366 µg/kg FW	[47]
	Nuclear transformation	Carrot	90 µg/kg FW	[47]
p24–IgA	Nuclear transformation	Tobacco	1.4% TSP	[48]
Chimeric peptides				
Env/gp120 (V3 loop)	TMV plant viral vector	*N. benthamiana*	Not reported	[50]
CTB–gp120 (V3 loop)	Nuclear transformation	Potato	0.004% TSP	[53]
Env/gp41	CPMV plant viral vector	Cowpea	Not reported	[51]
Env/gp41 (ectodomain)	PVX plant viral vector	*N. benthamiana*	Not reported	[52]
CTB–gp41 (ectodomain)	Nuclear transformation	*N. benthamiana*	0.2% TSP	[54]
HIV polyepitope–HBsAg	Nuclear transformation	Tomato	7 µg/g FW	[55]
	Nuclear transformation	Tobacco	0.02% TSP	[56]
	Nuclear transformation	*Arabidopsis*	0.026% TSP	[56]

†The yield was reported according to the original reference.
‡Yield was 40% TSP in leaves of tobacco and tomato, 2.5% TSP in green tomato fruits and not detectable in ripe fruits.
CPMV: Cowpea mosaic virus; CTB: B subunit of cholera toxin; Env: Envelope; FW: Fresh weight; GUS: β-glucuronidase; HBsAg: Hepatitis B surface antigen; mTat: Mutant Tat; PVX: Potato virus X; TA: Tail anchored; TBSV: Tomato bushy stunt virus; TMV: Tobacco mosaic virus; TP: Total protein; TSP: Total soluble protein.

Expression of the Nef protein alone by both nuclear (using different strategies) and plastidial transformations resulted in a much lower accumulation compared with fusion proteins [24–27].

Nef protein accumulation in the cytosol was variable in independent transgenic tobacco plants with an average value of 0.5% of the TSP [24]. Anchoring the Nef protein to the cytosolic face of the endoplasmic reticulum (ER), by fusing the Nef protein to the C-terminal portion of the mammalian ER isoform of cytochrome b5, produced a protein yield of approximately 0.7% of the TSP in transgenic tobacco [25]. In order to increase Nef protein accumulation, an alternative stable strategy, based on fusion of the entire chimeric zeolin sequence (composed by phaseolin fused to the N-terminal of γ-zein) to the *nef* gene, was pursued by de Virgilio *et al.* [26]; this fusion protein led to protein body formation and improved yield (up to 1.5% of the TSP). Recently, Lombardi *et al.* demonstrated that the plant expression level of Nef alone can be improved (up to 1.3% of the TSP) by using a transient expression system based on coagroinfiltration with two vectors containing the *nef* gene expression cassette and the coding sequence of the gene silencing viral suppressor P19 protein of the artichoke mottled crinkle virus, respectively [28].

To explore the potential of plastid transformation to produce components of an AIDS vaccine, Zhou *et al.* developed different expression constructs containing the HIV antigens p24 and Nef either alone or as a fusion protein [27]. All antigen combinations were expressed successfully, even if the highest Nef protein accumulation, up to 40% of the TSP, was achieved with a chimeric p24–Nef fusion protein in leaves of transplastomic tobacco and tomato plants [27]. Analyses of the p24–Nef expression levels during tomato fruit ripening detected protein accumulation (up to 2.5% of the TSP) only in green tomatoes, suggesting that the fusion protein may be less stable and/or synthesized at a lower rate in ripe fruits [27]. These results are due to the presence of chromoplasts (nonphotosynthetic plastids) in red fruits, which generally show downregulation of plastid gene expression [29].

A surprising finding was that, unlike the mild phenotype induced by overexpression of the p24–Nef fusion protein, the expression of Nef alone or as Nef–p24 fusion protein caused a dramatic pigment-deficient phenotype and partial protein degradation in older leaves, respectively [27]. These observations raise the possibility that the N-terminus of Nef could be directly responsible for the severe mutant phenotype. Indeed, the Nef N-terminus is known to be post-translationally modified by a lipid modification (myristoylation). It has recently been shown, for bacterial outer surface protein A (OspA) expressed in transplastomic tobacco plants, that when a lipid modification (palmitoylation) occurs inside the chloroplasts, it can produce the insertion of lipidated proteins into the thylakoid membrane, with consequent impaired photosynthesis and loss of photoautotrophic growth [30]. Furthermore, the absence of a similar phenotype in plants produced with the p24–Nef construct, and hence with a varying N-terminus, strengthens this hypothesis.

Gag polyprotein precursor (Pr55^{gag})

Pr55^{gag} is a polyprotein precursor cotranslationally myristoylated by cellular *N*-myristoyltransferase [31]. It represents the major structural protein complex, well conserved across diverse HIV-1 subtypes, that forms a submembrane shell in the immature virus particle [32]. The Gag precursor undergoes a process termed maturation in which the polyprotein is cleaved by a viral protease into four distinct domains: the N-terminal matrix domain (MA; p17), the central capsid domain (CA; p24), the nucleocapsid domain (NC; p7) and the C-terminal domain (p6). Two spacer peptides, p2 and p1, separate the p24/p7 and p7/p6 domains, respectively (Figure 1) [31]. Expression of the Gag protein in several recombinant systems showed its ability to assemble into noninfectious VLPs analogous to immature HIV virions [33–35]. Baculovirus-derived Pr55^{gag} VLPs presenting a clade A Ugandan gp120 produced an effective induction of cellular and humoral anti-HIV-1 systemic and mucosal immunity with cross-clade neutralizing activity [36–38].

Although VLP-based vaccines represent a promising HIV strategy, only two studies have demonstrated the expression of the Gag precursor in higher plants [39,40]. A very low Pr55^{gag} protein level (up to 48 µg/kg fresh weight [FW]) has been

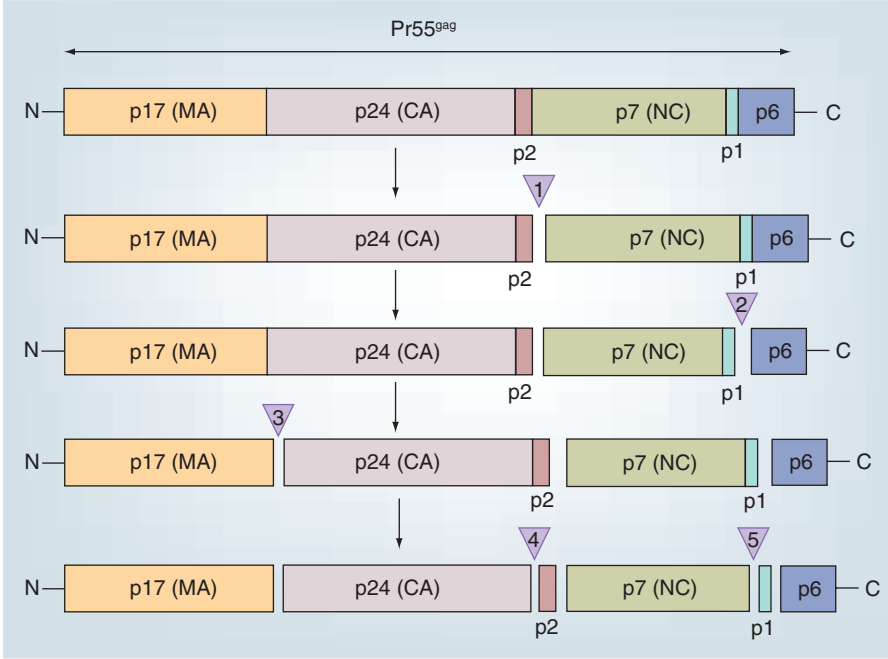

Figure 1. Sequential Gag precursor (Pr55^{gag}) processing. p17 (MA) subunit is involved in Gag targeting and subsequent binding to the plasma membrane; p24 (CA) forms the cone-shaped capsid shell of the mature virus; p7 (NC) is involved in packaging of the genomic RNA; p6 plays a role in release of the budding virus. p1 and p2 are spacer peptides. The numbers 1–5 in the triangles represent the sequentially ordered processing site of the Gag polyprotein precursor.
CA: Central capsid domain; MA: Matrix domain; NC: Nucleocapsid domain.
Modified from [31].

achieved in *Nicotiana* spp. by transient or stable nuclear transformation [39]. An increased protein yield (up to 4.8 µg/g FW) was obtained by the same authors with a chloroplast-targeted and transiently expressed p17–p24 truncated Gag protein. Although the p17–p24 protein was not immunogenic in mice when used in prime–boost vaccination, it was able to boost cellular and humoral responses in mice primed by a Gag DNA vaccine [39].

Various strategies (e.g., transient expression, stable nuclear and plastidial transformation) have been pursued for the expression of $Pr55^{gag}$ in order to investigate factors potentially affecting protein accumulation [40]. By means of transient transformation experiments, this study demonstrated, according to Meyers *et al.* [39], that the chloroplast is a suitable subcellular compartment for the expression of the full-length *gag* gene, being undetectable in other compartments (cytosol, apoplast, ER and mitochondrion) [40]. Hence, to examine the possible role of the different subunits on the lack of expression of the full $Pr55^{gag}$ precursor in other compartments, different vectors containing sequences coding for subunits p17, p24 and Δp17 (i.e., the entire precursor without the N-terminal p17) were produced and transiently expressed in both cytosol and chloroplast. This experiment revealed that only subunits p24 and Δp17 were expressed in the cytosol, while all other subunits accumulated in the chloroplasts [40]. These observations suggest a possible negative effect of the native N-terminus (p17) in the cytosol both for the initiation of translation and for protein stability. Based on the results of transient assays, stable transgenic plants targeting the full $Pr55^{gag}$ polyprotein to the chloroplast were produced. The protein expression level varied among independent transgenic lines between 0.01 and 0.1% of the TSP (equivalent to 28 mg/kg FW). Integration and expression of the *gag* transgene in the plastid genome, compared with the nuclear genome, revealed a significantly higher protein yield (up to 363 mg/kg FW). Furthermore, the highest Gag protein accumulation was obtained by fusing the $Pr55^{gag}$ polyprotein to the N-terminus of the plastid photosynthetic RbcL protein. This result underlines the importance of the transcription/translation control signals used in the construct [41]. The $Pr55^{gag}$ polyprotein was processed in a pattern similar to that achieved by the viral protease. Extended processing and reduction of all Gag polypeptides was observed in older leaves of mature plants. In addition, electron microscopy analysis showed that the Gag proteins were able to assemble into particles resembling VLPs produced in baculovirus/insect cells and *Escherichia coli* systems [40]. Owing to high Gag protein accumulation, these transplastomic plants showed a reduction in growth and a pigment-deficient phenotype in both heterotrophic shoot cultures *in vitro* and in autotrophic plants grown in soil (Figure 2) [40]. A toxic effect of the expressed recombinant protein was sometimes reported in other transplastomic plants and attributed to several factors [27,30,42]. Transmission microscopy analysis of these Gag transplastomic plants with a pigment-deficient phenotype showed plastids with irregular shape and size, bearing only rudimentary thylakoids dispersed in an electron-dense matrix compared with control plants [Scotti N, Unpublished Data]. The plant $Pr55^{gag}$-based VLPs showed considerable immunogenicity in mice following an immunization protocol with three intraperitoneal injections. Sera from the

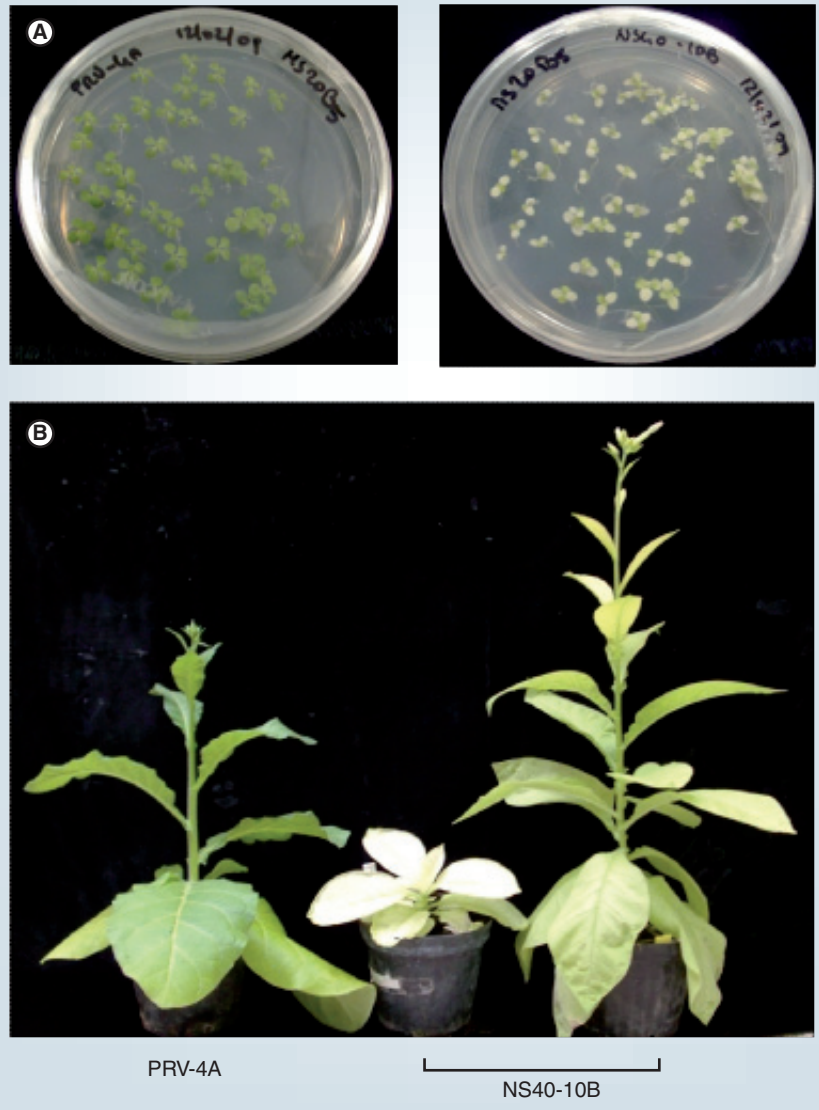

Figure 2. Phenotype of Gag (NS40-10B) and control (PRV-4A) transplastomic plants (A) *in vitro* or (B) in soil, from left to right 37, 60 and 114 days after transplantation.

immunized animals were positive in ELISA up to a dilution of 1:100,000 using VLPs as a target, and up to a dilution of 1:1000 against purified p24–Gag protein [Buonaguro L, Unpublished Data]. The observed immunogenic response was not significantly different from those reported using an equivalent quantity of VLPs prepared in a baculovirus system as immunogen [37,38].

Gag–p24

Unlike the $Pr55^{gag}$ polyprotein precursor, several studies demonstrated the production of the Gag–p24 subunit in plant cells using different strategies (Table 2). The first study concerning *in planta* expression of p24 protein (up to 5% of the TSP) was carried out using a tomato bushy stunt virus (TBSV)-based vector [43]. Subsequently, Zhang *et al.* expressed the full-length p24 coding sequence also in stable transgenic tobacco, but, in this case, a lower p24 protein yield (up to 0.35% of the TSP) was achieved [44].

Pérez-Filgueira *et al.* also pursued a transient expression strategy by cloning the *p24* gene into a new TMV-based vector containing a C-terminal histidine sequence [45]. The his-tagged p24 protein was purified from inoculated *Nicotiana benthamiana* leaves and used for immunogenicity tests in rabbits that developed a strong and specific humoral response to the p24 plant-derived protein. In addition, the immune sera was able to recognize the native p24 from a different clade expressed on the surface of a HIV-1 chronically infected HUT78/ARV T-cell line [45].

To increase the yield of plant-made p24 antigen, the relevant coding sequence was cloned in various vectors, containing signals to target the recombinant protein to the chloroplast and the ER, for transient and stable nuclear transformation in different plant species [39,46,47]. In transgenic *Arabidopsis*, it has been demonstrated that the presence of the ER retention signal increased the level of p24 protein by fivefold [47]. Immunological trials carried out on mice fed with fresh transgenic *Arabidopsis* plants showed a low anti-Gag IgG response that was significantly increased by a systemic boost with recombinant p37 Gag protein (containing p17 and p24 subunits) [46].

By contrast, Meyers *et al.* reported that the ER retention signal produced a high p24 yield (up to 16 mg/kg FW) only after transient expression, whereas in stable nuclear transgenic tobacco plants the highest p24 protein level (up to 3 mg/kg FW) was detected when the protein accumulated in the chloroplasts [39].

Different strategies for the expression of p24 antigen in transgenic tobacco plants were compared by Obregon *et al.*: expression of the unmodified *p24* gene product and its targeting to the plant endomembrane system, and the engineering of a p24 antigen fused to the constant regions of a human IgA heavy chain [48]. Both strategies resulted in the accumulation of correctly folded p24 protein in plants. The addition of the IgA α-chain sequence led to the formation of p24 dimers and to a much higher protein yield than p24 expressed alone (1.4 vs 0.1% of the TSP, respectively). Furthermore, immunological studies showed that the p24–IgA fusion protein was immunogenic in mice, priming T-cell responses and eliciting p24-specific antibodies [48].

In general, however, the best p24 accumulation level (up to 450 mg/kg FW, equivalent to ~4.5% of the TSP) was achieved by chloroplast transformation of the high-biomass tobacco variety Maryland Mammoth with a plastid-codon-optimized *p24* gene [49]. This study examined two transformation vectors containing different regulatory sequences, and the native and plastid-codon-optimized *p24* gene. Plants generated with the two vectors differed in terms of protein accumulation and stability, and phenotype. In particular, plants expressing the native p24 protein exhibited a normal green phenotype, but p24 protein accumulated only in the youngest leaves (up to 350 mg/kg FW, equivalent to 2.5% of the TSP). Similar results were obtained with *N. tabacum* cv. Petite Havana plants transformed with the same construct [27]. By contrast, some of the plants, produced with a different vector containing a plastid-codon-optimized *p24* gene, showed a yellow phenotype with the highest detectable p24 accumulation in all leaves, regardless of age. The yellow phenotype was associated, in these plants, with recombination between native and introduced direct repeat sequences of the *rbcL* 3′-untranslated region in the plastid genome [49].

Env epitopes in chimeric heterologous fusion proteins

The first HIV components expressed in plants reported in the literature are the epitopes of the Env (gp120 and gp41) protein (Table 2). These epitopes (V3 loop of gp120 and membrane-proximal part of the gp41 ectodomain) were expressed as fusion protein either to different plant virus capsid proteins (TMV, CPMV and PVX) or to the *CTB* gene [50–54]. Although in all cases a low protein yield was achieved, the recombinant epitopes produced by fusion with plant virus capsid proteins were easily purified and used for intraperitoneal, intranasal or oral immunization of mice [50–52]. In all cases, an HIV-specific immune response was detected. Moreover, Durrani *et al.* demonstrated that intranasal immunization with CPMV–gp41 fusion protein stimulates mucosal and systemic HIV-specific IgA and IgG better than oral immunization [51].

Recently, the hepatitis B virus surface antigen (HBsAg), able to assemble into VLPs, was used as a carrier of two different immunogenic chimeric HIV polyepitopes, including the Env–Gag and Pol–Env–Gag epitopes to produce tomato, and tobacco and *Arabidopsis* transgenic plants, respectively [55,56]. A very low chimeric protein yield was achieved for both HIV polyepitopes. Nevertheless, both transgenic tomato fruits and tobacco leaves were used in oral administration to Balb/C and humanized HSB mice by plant-prime–DNA-boost and DNA-prime–plant-boost immunization protocols, respectively [55,57]. The immunogenicity tests carried out with transgenic tomato fruits revealed a low induction level of specific HIV antibodies [55]; while those carried out with tobacco leaves led to low induction of $CD8^+$ T cells [57].

Plant-derived anti-HIV-1 preventive & therapeutic strategies

Since an effective HIV vaccine is still not in sight, other biotherapeutic approaches have gained considerable attention for the prevention of transmission and the possible treatment of infected individuals. In this respect, in recent years, research has

also focused on the production of other proteins such as microbicides and monoclonal antibodies for the prevention of HIV transmission. Despite the antiviral activity of these proteins, their practical application as topical microbicides is hampered by high production costs. For this reason, different proteins against HIV have been expressed in higher plants, as summarized in Table 3.

Microbicides

Microbicides (proteins or peptides) are products that can be applied to the genital apparatus to prevent sexual transmission of HIV. The first microbicide expressed in transgenic plants by stable nuclear transformation was cyanovirin-N (CV-N), a molecule able to inactivate a wide range of HIV strains by binding to gp120 [58]. Plant-derived CV-N accumulated as up to 0.85% of the TSP in transgenic tobacco leaves and its functional and anti-HIV-1 activity was demonstrated by specific binding to gp120 and protection of T cells from *in vitro* HIV infection [58]. Recently, Sexton *et al.* explored the possibility of expressing a fusion protein including the neutralizing monoclonal antibody (mAb) b12 and CV-N (b12–CV-N) in transgenic tobacco plants, which is predicted to have four binding sites for gp120 with two different specificities [59]. The b12–CV-N fusion protein was generated through cross-fertilization of two parent transgenic tobacco plants expressing different subunits (mAb b12 γ-chain/CV-N and mAb b12 κ-chain). Besides the correct assembling and functionality, the b12–CV-N fusion protein demonstrated an increased anti-HIV potency compared with b12 or CV-N alone [59].

A different strategy was pursued to express the red algal protein griffithsin (GRFT), a potent entry inhibitor able to inactivate HIV-1 in plants almost immediately on contact with the virus. Transient expression of GRFT in *N. benthamiana* via an infectious TMV-based vector produced more than 1 g of recombinant protein per kilogram of plant leaves [60]. The plant-derived GRFT has been evaluated for different chemical and functional properties: it proved stable in various physical conditions (e.g., low pH, organic solvents and lyophilization), active in a whole-cell anti-HIV assay at picomolar concentrations, and capable of blocking cell-to-cell HIV transmission. Assays carried out with the plant-derived GRFT and pseudoviruses derived from primary sexually transmitted isolates of HIV, representative of clades A, B and C, displayed a greater potency and a broader spectrum of activity than other agents that bind to similar oligosaccharide epitopes [60]. Furthermore, it has been demonstrated that the recombinant protein was nonirritating and noninflammatory in human cervical explants and *in vivo* in the rabbit vaginal irritation model [60].

Antibodies

Monoclonal antibodies (anti-gp120 antibodies b12 and 2G12, and anti-gp41 antibodies 2F5 and 4E10) with broad HIV-neutralizing activity have been characterized and considered as alternative molecules to prevent HIV transmission [61–64]. However, only three of them (anti-gp41 2F5 and 4E10, and anti-gp120 2G12) have been produced in transgenic plants (Table 3). The antibodies have been expressed in higher plants both alone and as fusion protein with elastin-like peptides (ELPs) [65–71].

Expression of anti-gp41 2F5 in tobacco cell suspension cultures produced 1.8 mg of recombinant antibodies per liter of suspension culture [65]. Although the plant-derived 2F5 showed similar antigen-binding activity compared with its Chinese hamster ovary (CHO) counterpart, the HIV-1 neutralization assay revealed decreased efficiency. The same protein (antibody) has also been expressed in tobacco leaves with or without a C-terminal ELP fusion [66]. Characterization of the affinity-purified antibodies demonstrated that ELP fusions do not interfere with folding, assembly, trafficking in the secretory pathway or post-translational modification, but enhance stability and simplify recovery [66].

Expression of anti-gp120 2G12 antibody in the seed endosperm of transgenic maize gave promising results with production levels of 60–75 μg per gram of dry seed weight [67,68]. The ELP fusion to mAb 2G12 enhanced the accumulation of this antibody (up to 1% of the TSP) in tobacco leaves and seeds [70]. This increase in yield was greater than that reported for 2F5–ELP. Furthermore, in all studies, it has been demonstrated that the *in vitro* HIV-neutralization properties of the plant-derived 2G12 antibodies were equivalent to or better than those of the CHO counterpart [67,68,70].

Immunoglobulins need *N*-glycosylation to obtain full biological activity. In plants, however, complex-type *N*-glycans are generally smaller and contain β1,2-xylose and/or core α1,3-fucose, with high immunogenic activity. These differences have favored an argument against using plants as a production system for human *N*-glycosylated therapeutic proteins [72]. Specific antibodies anti-xylose and core α1,3-fucose are present in approximately 50% of human blood donors [73]. Recent studies have shown that the *N*-glycan composition can be modulated in *N. benthamiana* plants using RNAi technology to downregulate the expression of endogenous *fucT* and *xylT* genes [69]. In this study, the 2G12 antibody was transiently expressed, using the recently developed viral-based vectors [74], in both glycosylated stable mutants and wild-type plants. The mass spectrometry and immunoblot analyses of purified 2G12 antibody derived from a glycosylated *N. benthamiana* double mutant (ΔXT/ΔFT-2G12) showed a broadly homogeneous *N*-glycan profile with undetectable plant-specific xylose and α1,3-fucose residues. Furthermore, the ΔXT/ΔFT-2G12 glycoform was indistinguishable from its CHO counterpart in assembly, antigen-binding capacity and HIV neutralization activity [69].

The plant-derived 2G12 antibodies described in this article have been produced in different plant species. The Pharma-Planta consortium decided to choose, as its primary focus, the 2G12 antibody from tobacco leaf material to produce the first clinical batch under cGMP conditions and use it for clinical trials (Pharma-Planta Project Report 4) [104].

An innovative plant-based expression system was recently reported by Steinkellner's laboratory, who efficiently produced mAbs with a homogeneous β1,4-galactosylated *N*-glycosylation structure, the major *N*-glycan species present on serum IgG [71]. This result was achieved by the expression of a highly active

Table 3. Production of other proteins against HIV-1 in plants.

Protein	Expression system	Plant species	Yield†	Ref.
Microbicides				
CV-N	Nuclear transformation	Tobacco	0.85% TSP	[58]
b12–CV-N	Nuclear transformation	Tobacco	Not reported	[59]
Griffithsin	TMV plant viral vector	*Nicotiana benthamiana*	1 g/kg FW	[60]
Monoclonal antibodies				
2F5	Nuclear transformation	Tobacco cells	1.8 mg/l CSC	[65]
2F5-elastin-like peptides	Nuclear transformation	Tobacco	0.6% TSP	[66]
4E10	TMV plant viral vector	Glycoengineered *N. benthamiana*	Not reported	[71]
2G12	Nuclear transformation	Maize	60 µg/g DW	[67]
	Nuclear transformation	Maize	75 µg/g DW	[68]
	TMV plant viral vector	Glycoengineered *N. benthamiana*	110µg/g FW	[69]
	TMV plant viral vector	Glycoengineered *N. benthamiana*	Not reported	[71]
2G12-elastin-like peptides	Nuclear transformation	Tobacco	1% TSP	[70]

†The yield was reported according to the original reference.
CSC: Cell suspension culture; CV-N: Cyanovirin-N; DW: Dry weight; FW: Fresh weight; TMV: Tobacco mosaic virus; TSP: Total soluble protein.

modified version of the human β1,4-galactosyltransferase in glycoengineered plants lacking plant-specific glycosylation. In particular, anti-HIV mAbs (2G12 and 4E10) with fully β1,4-galactosylated *N*-glycans displayed an improved virus neutralization potency when compared with other glycoforms produced in plants and CHO cells. Production of mAbs containing such homogeneous *N*-glycan structures should be relevant to their application as preventive microbicides and possibly as therapeutic strategies [71].

Expert commentary

The plant-derived vaccine research field began in 1992 when Arntzen's group published a manuscript on production of HBsAg in transgenic tobacco, showing that the plant-made HBsAg formed particles antigenically and structurally similar to those derived from human serum and recombinant yeast [75]. Further, in subsequent years, the same group demonstrated the efficacy of plant-produced antigens for several pathogens (i.e., Norwalk virus capsid protein and *Vibrio cholerae* enterotoxin subunit [CTB]) in mice and in humans [76–79]. These observations prompted them to consider plants as an attractive production platform for biopharmaceuticals and vaccines. Since 1990, over 200 medically relevant proteins have been produced in transgenic plants, and various plant-based production systems have been developed for this purpose [8].

The first HIV component expressed in plants was the V3 loop epitope of Env glycoprotein (gp120) by TMV plant viral vector [50]. As previously discussed, plants were subsequently used to pursue a variety of anti-HIV strategies. Both HIV and related proteins have been expressed in different plant species, using various gene transfer and expression strategies. Yield and stability of plant recombinant proteins are, in fact, the main issues that must be addressed to develop competitive commercial products. Only for few HIV proteins (Nef, $Pr55^{gag}$ and its p24 subunit) has the comparison of different plant production systems been possible [24–28,39,40,43–45,47–49]. These studies showed that the most promising strategy, considering protein accumulation and transgene containment, was plastid transformation [27,40,49]. Although disulfide-bond formation and protein lipid modification can occur inside the chloroplasts [30,80], a drawback of plastid transformation is the lack of glycosylation. Expression of Nef and $Pr55^{gag}$ precursor by plastid transformation produced transplastomic plants with a pigment-deficient phenotype, probably owing to the N-terminus of Nef and $Pr55^{gag}$ [27,40].

Although immunological studies are available for few plant-derived HIV proteins, results show an immunogenicity higher than, or comparable to, other systems. In particular, the Env-based vaccines expressed in *N. benthamiana* [50,52] have also shown the ability to induce IgG with neutralizing activity. The recent discovery that the human influenza virus H1N1 hemagglutinin (HA) is able to induce assembly of particles at the plant plasma membrane, with subsequent accumulation of several VLPs at budding sites [81], suggests that further studies should be performed to verify whether Env glycoproteins are also able to assemble into VLPs when fused to the HA transmembrane region. This approach would make an enveloped plant-derived Env-based VLP besides the chimeric nonenveloped VLP obtained following the fusion of specific Env epitopes to the self-assembling HBsAg available to immunological studies [55]. Moreover, it is important to develop multicomponent prime–boost vaccine strategies that can induce both humoral as well as cellular immune responses, with plant immunogens alone or in combination with different immunogens expressed in other systems (including priming DNA or live vectors). Furthermore, plant products can contribute to the containment of HIV infection with products able to back up our immune system (such as adjuvants for active immunotherapy, and mAbs for passive immunotherapy), as well as reduce/minimize viral exposure (i.e., microbicides for their local antiviral activities) [35].

Although it has been amply demonstrated that the diversity of plant-based production systems and their advantages are particularly attractive for vaccine development, several challenges still lie ahead. For this field to move forward, greater research emphasis is needed on large-scale production, purification, functional characterization, oral delivery, preclinical evaluation and public acceptance. In particular, both scientists and industry need to address several issues concerning readily available clinical trials (beyond Phase I), containment, product quality, biochemical characterization and toxicity testing of the final product.

Five-year view

Although HIV vaccine trials in humans have so far resulted in either no protection at all or in relatively unimpressive levels despite the measurable immunogenicity of administered HIV antigens, the development of an effective HIV vaccine is still an achievable goal [82]. However, fundamental knowledge is required concerning the nature, quality and quantity of immune responses to be induced, the antigens to include and how to overcome high sequence variability; it must also be decided whether preventive vaccine strategies should focus on protection from infection or from disease progression [82]. For this purpose, flexible, large-scale and long-term funding mechanisms involving multidisciplinary teams should be launched to obtain greater innovation and shorten the timeline to a safe and effective HIV vaccine [83]. In this context, as recently demonstrated, plants could contribute both as a source of novel proteins related to HIV [84,85] and as a production system, especially by using new high-yielding transient expression systems [60,81,86].

In *Hypericum perforatum* callus culture (St John's wort), a novel protein (p27SJ) able to suppress the transcription of HIV-1 genome in several human cell types (primary culture of microglia and astrocyte) has been identified, suggesting its potential in developing a therapeutic advance to control HIV-1 transcription and replication in cells associated with virus infection in the brain [84,85].

In the cyanobacterium *Scytonema varium*, an interesting anti-HIV protein (scytovirin) with a potent anticytophatic activity has been isolated [87]. This lectin (95 amino acids) inhibits HIV-1 infection by binding to glycans of viral gp120, gp160 and gp41 proteins, and is able to neutralize HIV-1 subtype C isolates from blood and cervicovaginal lavages [88].

Encouraging recent examples (antibody against non-Hodgkin's lymphoma, HIV microbicide and swine influenza) demonstrated that plant production systems can generate antigens or therapeutic proteins of comparable safety, potency and efficacy to other expression systems [60,81,86]. In particular, the study of non-Hodgkin's lymphoma documented a rapid production and recovery of idiotypic single-chain antibodies (scFv) derived from each patient's tumor, and immunization of patients with their own individual therapeutic antigen [86]. The plant-based transient swine influenza manufacturing platform produced, instead, large-scale good manufacturing practice-grade influenza VLPs with unprecedented speed (only 2 weeks were required to obtain the first proof of feasibility that the antigen was accumulating at high levels in agroinfiltrated plants), and demonstrated in preclinical studies that low doses of purified plant-derived VLPs induced a strong and broad immune response in mice and ferrets [81].

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Key issues

- An effective HIV vaccine should ensure a broad and long-lasting immune response, be easy to transport, store and administer.
- Plant-based production systems are potentially attractive for vaccine development.
- Many biotechnological hurdles related to the use of plants as biofactories have been overcome.
- Greater research emphasis is needed on large-scale production, functional characterization and clinical evaluation of plant-derived recombinant proteins.

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103 IAVIReport: AIDS vaccine trials database www.iavireport.org/trials-db

104 Pharma-Planta: resources www.pharma-planta.org/resources.htm