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REVIEW

Plant-based strategies aimed at expressing HIV antigens and neutralizing antibodies at high levels. Nef as a case study

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Abstract The first evidence that plants represent a valid, safe and cost-effective alternative to traditional expression systems for large-scale production of antigens and antibodies was described more than 10 years ago. Since then, considerable improvements have been made to increase the yield of plant-produced proteins. These include the use of signal sequences to target proteins to different cellular compartments, plastid transformation to achieve high transgene dosage, codon usage optimization to boost gene expression, and protein fusions to improve recombinant protein stability and accumulation. Thus, several HIV/SIV antigens and neutralizing anti-HIV antibodies have recently been successfully expressed in plants by stable nuclear or plastid transformation, and by transient expression systems based on plant virus vectors or *Agrobacterium*-mediated infection. The current article gives an

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overview of plant expressed HIV antigens and antibodies and provides an account of the use of different strategies aimed at increasing the expression of the accessory multifunctional HIV-1 Nef protein in transgenic plants.

Keywords HIV · Nef · Multi-component vaccine · Plant made pharmaceuticals · Recombinant proteins · Heterologous expression systems

Introduction

As stated by the latest UNAIDS AIDS epidemic update (UNAIDS 2008 AIDS Epidemic update, http://www.unaids.org), a total of 33.2 million people were living with HIV in 2007. Twenty-five years since the discovery of HIV, an effective vaccine is not yet available. Current efforts in vaccine design have been focused on subunit vaccines. The viral envelope (Env) was initially considered the best candidate as an inducer of neutralizing antibodies, but viral variability represents a barrier to obtain a broad immune response (Walker and Burton 2008; Fauci et al. 2008). This problem could be overcome by a polyvalent HIV vaccine, identifying constant domains on the viral envelope necessary for virus–cell interaction (Zolla-Pazner 2004) and through structural studies of HIV envelope spikes created by gp41 and gp120 proteins (Walker and Burton 2008).

A vaccine able to induce sterilizing immunity still remains a major challenge and modern vaccinology is directed to inducing broad humoral and cellular immune responses. Therefore, novel vaccines are becoming increasingly complex, with several being constituted by a combination of different recombinant antigens. Both structural (Env, Gag) and non-structural HIV-1 gene products (Rev, Tat, Nef) are regarded as promising targets for multi-antigen vaccine development, able to induce durable antiviral immunity (Titti et al. 2007). Along with the development of subunit vaccines, antibodies with broad neutralizing activity against HIV-1 have been proposed for both prophylactic and therapeutic use (Trkola et al. 2005, 2008), although a major limitation to this treatment is represented by the necessity of a large-scale production system at low cost.

To achieve this aim the use of plants of agronomic relevance as bioreactors for large-scale production of biopharmaceuticals and vaccine components represents a safe and economical alternative to traditional production systems (Ma et al. 2003, 2005; Schillberg et al. 2005). A number of studies have already shown that structural and regulatory proteins of HIV or related viruses could be successfully expressed in plants by stable transformation or transient expression strategies (Table 1). Moreover, very recently different studies have been published on the over expression and characterization of plant-derived anti-HIV neutralizing antibodies (Table 2). In particular two mAbs, the anti-gp120 2G12 (Trkola et al. 1996) and the anti-gp41 2F5 (Conley et al. 1994), endowed with a broad HIV neutralising activity, have been extensively characterised. In the case of the 2G12 antibody, directing the expression to the seed endosperm of transgenic maize gave very promising results with production levels of 40–100 mg/kg dry seed weight (Ramessar et al. 2008; Rademacher et al. 2008). Moreover, both studies showed that the neutralising activity of the plant purified antibody was equivalent to or even better than that of the CHO (Chinese hamster ovary cells) derived 2G12. The same antibody was also transiently expressed in *N. benthamiana* by leaf agroinfiltration giving yields of 50 mg/kg of fresh biomass (Strasser et al. 2008). In the case of the mAb 2F5 expressed in transgenic tobacco plants it was shown that fusion of the heavy and light chains to an elastin-like peptide (ELP) repeat raised production yields mainly by enhancing protein stability, furthermore this strategy could simplify protein recovery and purification (Floss et al. 2008). The same antibody has been also expressed in transgenic tobacco cell suspension cultures yielding 6.4 mg/kg wet cell weight (Sack et al. 2007). In this case, although the plant derived 2F5 showed similar antigen binding activity compared to its CHO derived counterpart, HIV-1 neutralisation assays revealed a decreased efficiency. Taken together these results demonstrate the feasibility of expressing functional HIV-1 neutralising antibodies in plants at yields which could meet the requirements for a large-scale economical production.

The first examples reported in the literature of expression in plants of HIV components relate to sequences encoding epitopes of the viral Env (see Table 1 for references). In both *env* products, gp120

Table 1 Plant expressed HIV-1 and SIV antigens

Protein/peptide	Plant	Plant expression system	Reference
Tat	Spinach	TMV plant viral vector	Karasev et al. (2005)
	Potato tuber	Nuclear transformation	Kim et al. (2004a)
	Tomato	Nuclear transformation	Ramírez et al. (2007)
	Tobacco	Nuclear transformation	Webster et al. (2005)
Nef	Tobacco	Nuclear transformation	Marusic et al. (2007), Barbante et al. (2008), de Virgilio et al. (2008)
	Tobacco	Plastid transformation	Zhou et al. (2008), McCabe et al. (2008)
	Tomato	Plastid transformation	Zhou et al. (2008)
p24	Tobacco	Nuclear transformation	Obregon et al. (2006)
	N. benthamiana	TBSV plant viral vector	Zhang et al. (2000)
	Tobacco	Nuclear transformation	Zhang et al. (2002)
	N. benthamiana	TMV plant viral vector	Meyers et al. (2008)
	N. benthamiana	Agroinfiltration	Meyers et al. (2008)
	Tobacco	Nuclear transformation	Meyers et al. (2008)
	Tobacco	Plastid transformation	Meyers et al. (2008)
	Tobacco	Plastid transformation	Zhou et al. (2008), McCabe et al. (2008)
	Tomato	Plastid transformation	Zhou et al. (2008)
p17/p24	*N. benthamiana*	Agroinfiltration	Meyers et al. (2008)
	Tobacco	Nuclear transformation	Meyers et al. (2008)
Pr55Gag	*N. benthamiana*	TMV plant viral vector	Meyers et al. (2008)
	N. benthamiana	Agroinfiltration	Meyers et al. (2008)
	Tobacco	Nuclear transformation	Meyers et al. (2008)
SIV_{mac} p27	Potato	Nuclear transformation	Kim et al. (2004b, c)
Env/gp120 V3 loop	*N. benthamiana*	TMV plant viral vector	Yusibov et al. (1997)
	Tobacco	TMV plant viral vector	Sugiyama et al. (1995), Beachy et al. (1996)
	Potato	Nuclear transformation	Kim et al. (2004d)
	N. benthamiana	TBSV plant viral vector	Joelson et al. 1997)
Env/gp 130 SIV_{mac}	Corn kernel	Nuclear transformation	Horn et al. (2003)
Env/gp41 ELDKWA epitope (662-667)	*N. benthamiana*	PVX plant viral vector	Marusic et al. (2001)
P1 peptide (649–684)	*N. benthamiana*	Agroinfiltration	Matoba et al. (2004)
Peptide (731–752)	Cowpea	CPMV plant viral vector	Porta et al. (1994), McLain et al. (1995, 1996), Durrani et al. (1998), McInerney et al. (1999)

TMV tobacco mosaic virus, *TBSV* tomato bushy stunt virus, *PVX* potato virus X, *CPMV* cowpea mosaic virus

and gp41 glycoproteins constant regions (epitopes) recognized by neutralizing antibodies have been identified (Zolla-Pazner 2004). Some of these protective epitopes (2F5, V3 loop, etc.) have been expressed, mainly fused to different plant virus capsid proteins, able to self-assemble and generate chimeric virus particles or chimeric virus like particles (VLP) (Table 1). Plant viruses and VLP were easily purified and their immunogenicity has been evaluated in animal models. Data have been reported about the capability of recombinant plant viruses to induce the production of antibodies able to neutralize

Table 2 Plant expressed anti-HIV1 neutralizing antibodies

Antibody	Species	Promoter	Compartment	Plant expression system	Yield	Reference
2G12	Maize	Endosperm specific	Secretory	Nuclear transformation	75 mg/kg seed dry weight	Ramessar et al. (2008)
	Maize	Endosperm specific	ER retention	Nuclear transformation	40 mg/kg seed dry weight	Rademacher et al. (2008)
	N. benthamiana	CaMV 35S	Secretory	Leaf Agroinfiltration	50 mg/kg fresh leaf weight	Strasser et al. (2008)
2F5	*N. tabacum*	CaMV 35S	ER retention	Nuclear transformation	ND	Floss et al. (2008)
	N. tabacum cell suspensions	CaMV 35S	ER retention	Nuclear transformation	6.4 mg/kg wet cell weight	Sack et al. (2007)

CaMV cauliflower mosaic virus, *ND* not determined

HIV in vitro (Yusibov et al. 1997; Marusic et al. 2001). Other studies demonstrated that Gag p17 and p24/p17 capsid proteins can be produced in plants using different expression strategies. A p24-immunoglobulin fusion molecule has been successfully expressed by nuclear transformation in tobacco plants and is able to elicit a T-cell and antibody immune response in mice (Obregon et al. 2006). Different results have been obtained with transiently expressed p17/p24 which proved non immunogenic in mice but was able to boost a humoral and T-cell response in mice primed with *gag* DNA vaccine (Meyers et al. 2008). Similarly, the HIV regulatory protein Tat produced in spinach did not induce a detectable antibody response in mice orally immunized with spinach leaves, but apparently mice were primed for the subsequent *tat* DNA immunization (Karasev et al. 2005). Nevertheless, Tat antigen expressed in tomato fruits was able to induce mucosal IgAs and serum IgGs with neutralizing activity after oral immunization in mice (Ramírez et al. 2007).

Nef protein as a plant-made HIV vaccine component

Among non-structural HIV-1 elements, the accessory protein Nef is considered a good candidate for the formulation of vaccines that combine both structural and functional viral components. Nef is expressed early during the viral life cycle and is necessary for both high viral load and disease progression. Moreover, *nef* genes are highly conserved in all primate lentiviruses (HIV-1, HIV-2, SIV). Experiments in a transgenic mouse model showed that Nef proteins of SIV and HIV are functionally interchangeable (Sinclair et al. 1997). In addition, data showed that patients classified as long-term non-progressors have alterations in the *nef* gene (Tobiume et al. 2002). Recently, promising studies on the generation of multi-component HIV vaccines, containing Nef, have been published, reporting the first clinical evaluation of a multi-component vaccine containing recombinant gp120 and Nef-Tat fusion proteins (Goepfert et al. 2007) and the effects of a multi-component genetic vaccine combining structural (Gag/Pol, Env) and regulatory (Rev, Tat, Nef) antigens in the SIV-*Macaca* animal model (Voss et al. 2003; Maggiorella et al. 2007).

It has been demonstrated that *nef* gene yields two main products: a full-length form of 27 kDa (p27) and a truncated form of 25 kDa (p25), translated from a second start codon and lacking the first 18 amino acids. The p27 protein is post-translationally modified by the addition of a myristoyl group to the N-terminus by which full-length Nef anchors to the cytosolic side of cellular membranes. The p25 was found in the cytosol, while the wild-type myristoylated p27 was mainly membrane associated (Yu and Felsted 1992). Nef contains several disordered regions, which cause difficulties in the resolution of its three-dimensional structure. However, the globular core domain and the N-terminal anchor domain have been characterised by X-ray crystallography, nuclear magnetic resonance (NMR) and NMR spectroscopy (Geyer et al. 1999; Geyer and Peterlin

2001). The combination of data obtained from both functional and structural studies allowed the identification of sequence motifs involved in protein modification, signalling and trafficking (Geyer et al. 2001b). In general Nef affects protein trafficking in the host cells, down modulates cell-surface molecules such as CD4 and MHC-I, while enhancing virus infectivity and accelerating disease progression in both humans and primate animal models (Roeth and Collina 2006).

Both p27 and p25 isoforms have been expressed in different biological systems such as *E. coli* (Azad et al. 1994), yeast (Macreadie et al. 1998; Sirén et al. 2006), insect cells (Kohleisen et al. 1996), mammalian cells (Cooke et al. 1997) and plant cells (Marusic et al. 2007; Barbante et al. 2008; de Virgilio et al. 2008; Zhou et al. 2008; McCabe et al. 2008).

In the following paragraphs we will focus on the different biotechnological approaches developed by several different laboratories aimed at obtaining high accumulation of Nef in stable transgenics.

Targeting HIV-1 Nef to different plant cell compartments

Yield and stability of plant recombinant proteins are the main issues that must be addressed to develop competitive commercial products. Several approaches have been followed to express heterologous proteins at high levels and prevent protein degradation in plants (Benchabane et al. 2008). Subcellular targeting of recombinant proteins is one of the major research topics in the field of plant made pharmaceuticals. A number of strategies are now in place to direct proteins of interest to a specific cellular compartment (e.g., apoplast, plastid, mitochondrion, vacuole, endoplasmic reticulum) by the addition of specific targeting sequences (Streatfield 2007).

To achieve stable and high-level expression of Nef in transgenic plants a number of constructs were designed either for nuclear or plastid transformation, utilizing different signal or fusion peptides to direct the expression of the viral protein to either the cytosol or the secretory pathway. A schematic representation indicating the most successful Nef expression strategies and the relative yields are shown in Fig. 1 and will be reviewed in the following sections.

Nef is stable in the cytosol of plant cells

In the first study concerning plant recombinant Nef, a panel of constructs were designed to direct the expression of full-length N-terminal myristoylated (p27), non-myristoylated (p27 mut) and truncated form (p25) of Nef, to either the cytosol or the secretory pathway (Marusic et al. 2007). In this work, all constructs were tested initially by tobacco protoplasts transfection followed by pulse chase analysis to rapidly establish the most promising strategy for protein expression in stable transgenic plants. All cytosolic forms of Nef (p25, p27 and p27 mut) appeared stable while small amounts of myristoylated protein (p27) were associated to the plant cell membrane. Constructs encoding p25 and p27 mut were used to generate transgenic tobacco plants. Quantitative ELISA revealed variable expression levels in independently transformed plants ranging between 0.18 and 0.7% of total soluble protein (TSP) with an observed average value of 0.5% as reported in Fig. 1. A small-scale purification was successfully attempted by IMAC chromatography taking advantage of the $(His)_6$ tag fused to the C-terminus of p27 mut (Marusic et al. 2007). The use of recombinant Nef lacking myristoylation as part of a multicomponent vaccine is considered advantageous at present (Peng et al. 2006). Indeed, deletion or mutagenesis of the N-terminal myristoylation site has been shown to abrogate the capacity of Nef to down-regulate both MHC class I and CD4 cell-surface molecules (Peng and Robert-Guroff 2001), which normally prevent CTL-mediated lysis of HIV-1-primary infected cells (Collins et al. 1998).

Attaching Nef to the cytosolic face of membranes as a strategy to improve accumulation

A new, alternative strategy to improve p27 Nef accumulation in transgenic plants, recently developed by Barbante and colleagues (Barbante et al. 2008), involves anchoring the molecule to the cytosolic face of the endoplasmic reticulum (ER) membrane using the C-terminal portion of the mammalian ER isoform of cytochrome *b*5 (ER *b*5). ER *b*5 belongs to the tail-anchored (TA) protein family, which are bound to membranes by a C-terminally located hydrophobic sequence (Borgese et al. 2003). TA proteins do not

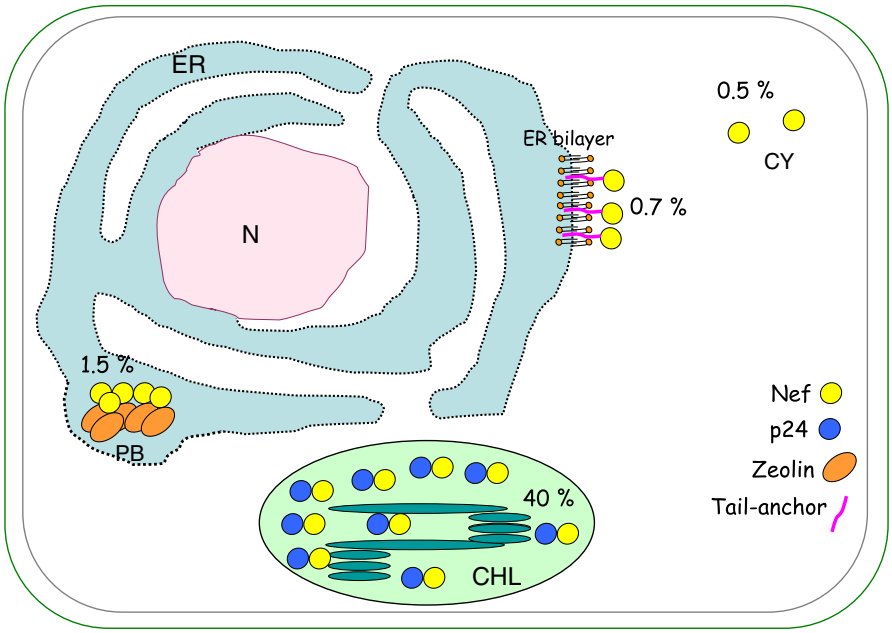

Fig. 1 Schematic representation of expression strategies used to accumulate HIV-1 Nef in plant cells. Nef fusion to the chimeric protein zeolin allows protein body formation (*PB*) within the endoplasmic reticulum (*ER*) lumen (de Virgilio et al. 2008); fusion to the C-terminal portion of the mammalian ER isoform of cytochrome *b*5 (*tail-anchor*) anchors recombinant protein to the cytosolic ER bilayer (Barbante et al. 2008); fusion to HIV-1 p24 and chloroplast (*CHL*) transformation (Zhou et al. 2008; McCabe et al. 2008); Nef cytosolic expression (*CY*) (Marusic et al. 2007). Nucleus (*N*). Protein expression levels are indicated and expressed as percentage of total soluble protein

have an N-terminal signal peptide and reach their destination post-translationally; their functional domains are exposed in the cytosol and only a short C-terminal segment is translocated (Borgese et al. 1993). Consistently, the resulting topology of the fusion molecule between Nef and the tail anchor of ER *b*5 (Nef-TA) maintains the entire Nef portion in its ideal cytosolic environment.

The Nef polypeptide anchored to the cytosolic face of the ER membrane accumulated to higher levels (more than threefold) compared to its soluble cytosolic counterpart in all transgenic plants analyzed (Barbante et al. 2008). The values were 0.7% of TSPs for Nef-TA with very little variability within each group. Analysis of mRNA levels and pulse-chase experiments with radioactive amino acids indicated that the higher accumulation of Nef-TA is due to increased protein stability. These results confirm that the ER membrane is a convenient target location for chimeric membrane proteins because of its intrinsic plasticity and remodelling ability (Borgese et al. 2006), which is attuned to the amount of protein that enters the secretory pathway. In addition, being anchored to the ER membrane, the Nef protein is likely to be less susceptible to cytosolic degradation processes mediated by the ubiquitin/proteasome pathway.

The successful production of recombinant vaccines in transgenic plants also depends on the ease and efficiency of purification. Nef can be removed in vitro from its TA taking advantage of an engineered thrombin cleavage site (Barbante et al. 2008). This opens the way to a purification procedure involving microsome preparation and then solubilization of the molecule in a pure or nearly pure form by thrombin cleavage. This new strategy could be generally used for production purposes, when it is necessary to maintain the heterologous protein in the reducing environment of the cytosol and at the same time limit molecular interactions with the host cell machinery.

Nef destiny in the secretory pathway: how to avoid quality control degradation

The secretory pathway has been exploited extensively for the production of recombinant pharmaceuticals in

plants. One of the reasons is that several classes of important pharmaceutical proteins are secretory proteins. The best known example is constituted by immunoglobulins, which fortunately are rather resistant to apoplastic proteases and therefore accumulate to high amounts (Hiatt et al. 1989; Ma et al. 1995). Further advantages reside in the finding that in all eukaryotic cells the ER, which is the port of entry to the secretory pathway, has low hydrolytic activity and that soluble proteins can easily be retained in this compartment by adding a signal peptide at the N-terminus and the ER localization signals HDEL or KDEL at the C-terminus (Vitale and Denecke 1999). Moreover, the ER of plant cells can tolerate high accumulation of protein without compromising cell functions (Wandelt et al. 1992). For proteins that naturally reside in compartments other than the ER but have a short half-life, targeting to the ER has often increased accumulation (Doran 2006), although there are exceptions (Patel et al. 2007; Yang et al. 2007).

Transient expression of Nef in the secretory pathway of tobacco protoplasts, by the addition of the signal peptide (sp) of the PR1 protein (Denecke et al. 1990) to the N-terminal of both p25 and p27 Nef variants, resulted in marked instability during 5 h chase suggesting misfolding and degradation by ER quality control (Marusic et al. 2007). The ER is a folding environment different from the cytosol, mainly because it leads to the formation of disulfide bonds and *N*-glycosylated proteins. These events have evolved to favour solubility, stability and in some cases biological activity of many secretory proteins, but when they occur on proteins that naturally reside in the cytosol and therefore are not disulfide-bonded, they obviously can change protein folding and have adverse effects. Experiments in which synthesis has been performed in the presence of the *N*-glycosylation inhibitor tunicamycin proved that indeed Nef is glycosylated when inserted into the ER, but also showed that the inhibitor does not improve Nef stability in the secretory pathway (Marusic et al. 2007). It therefore seems likely that disulfide bonds are responsible for Nef misfolding.

It has been shown that in the cytosol Nef folding is a slow process: at the end of 1 h pulse-labelling, Nef Cys residues were still available for unproductive in vitro disulfide bond formation, a feature that was abolished by folding only upon 2 h chase. It is conceivable that such a relatively long-term availability of Cys residues for interactions leads to in vivo formation of disulfide bonds in the ER oxidizing environment, leading to misfolding (Barbante et al. 2008). Addition of KDEL was unable to rescue a structurally defective protein from degradation by ER quality control (Frigerio et al. 2001). This was also true in the case of Nef, where addition of KDEL was incapable of rescuing the protein from rapid degradation in transient expression (Barbante et al. 2008). This further indicates that targeting Nef to the lumen of the ER is likely to trigger its rapid degradation, possibly by quality control.

As an alternative strategy, de Virgilio et al. (2008) tried to promote the formation of protein bodies (PB) containing Nef. PB are plant-specific, ER located, insoluble polymers naturally formed by a number of cereal seed storage proteins and are held together by inter-chain disulfide bonds (Shewry et al. 1995). A significant amount of information is available regarding the maize prolamin γ-zein, which forms PB and accumulates to high amounts also when expressed in vegetative tissues (Coleman et al. 1996; Geli et al. 1994). It has been previously showed that a fragment of γ-zein, corresponding roughly to the N-terminal half, is able to promote PB formation when fused to the vacuolar storage protein phaseolin in the chimeric protein zeolin (Mainieri et al. 2004). This leads to accumulation of zeolin up to 3.5% of tobacco leaf protein, roughly a tenfold improvement with respect to accumulation of phaseolin-KDEL (Mainieri et al. 2004). The first attempts to incorporate Nef into PB were however, disappointing: fusion of Nef to the same zein fragment used to construct zeolin, either in N-terminal or C-terminal position, resulted in very poor accumulation in transgenic tobacco leaves (M. de Virgilio et al., unpublished data). Pulse-chase in the presence of the vesicular traffic inhibitor brefeldin A and analysis of the processing of glycans indicated that the chimeric proteins are degraded by ER quality control. Conversely, fusion of the entire zeolin sequence to Nef (zeolin-Nef) lead to PB formation and markedly improved accumulation, to about 1.5% of leaf protein, consistently in twenty independent transgenic plants that were analyzed (de Virgilio et al. 2008). Pulse-chase experiments and comparison of transcript levels indicated that the increased accumulation was exclusively due to a marked improvement in protein stability. Electron

microscopy and subcellular fractionation showed that zeolin-Nef forms PB in the ER, even if these are smaller than those formed by zeolin.

The high instability of the fusions between Nef and the zein domain, and of Nef-KDEL, indicate that when a protein is recognized as structurally defective by the ER machinery, it is difficult to rescue it from quality control degradation. It is not known if the Nef-zein fusions are sorted for degradation before they have the possibility to form disulfide-linked polymers or after repeated unsuccessful attempts to polymerize, but these findings place the "approval" by the ER quality control machinery as a prerequisite for next steps in the synthesis of secretory proteins that lead either to polymerization and formation of PB or traffic along the secretory pathway to other destinations (de Virgilio et al. 2008; Vitale and Boston 2008). On the other hand, the successful accumulation of zeolin-Nef indicates that, when PB formation occurs, this rescues an heterologous unstable protein from degradation by quality control, markedly improving accumulation. Incorporation into insoluble PB can also provide an advantage in protein purification, but ultimately it will probably be necessary to remove the antigen from the appended sequence. In zeolin-Nef, a thrombin cleavage site was engineered between zeolin and the Nef sequence (de Virgilio et al. 2008); when the chimeric protein was solubilized with reducing agents, immunoprecipitated and treated in vitro with thrombin, Nef is released in a soluble form (M. de Virgilio and A. Vitale, unpublished data), indicating that, once released from zeolin, Nef is not grossly misfolded. However, further experiments are necessary to determine if the recombinant antigen can also be efficiently released from insoluble PB, thus making purification cost-effective.

Chloroplast-produced Nef

Plastid (chloroplast) transformation has emerged as an attractive tool for plant biotechnologists (Maliga 2004; Bock 2007; Koop et al. 2007). The major advantages of this technology are high-level foreign protein accumulation, ranging between two and more than 70% of TSP (Oey et al. 2008), and the absence of epigenetic effects (gene silencing and position effects). Moreover, the maternal plastid inheritance, which prevents pollen-mediated gene flow from the genetically modified plant to other plants, greatly increases transgene containment (Bock 2001; Ruf et al. 2007; Svab and Maliga 2007). Transgenic chloroplasts as bioreactors for the production of vaccines and biopharmaceuticals are very promising as indicated from a number of published studies (Daniell 2006).

Zhou and colleagues have explored the potential of the plastid transformation as an alternative tool to produce high levels of HIV-1 Nef and p24 antigens in plant cells (Zhou et al. 2008). For this purpose, a series of constructs were designed to express the p27 Nef protein either alone or as p24-Nef or Nef-p24 fusion proteins. As the analysis of the codon usage in the native *p24* and *nef* gene revealed significant differences from that in the (AT)-rich plastid genome, both genes were re-synthesized. All constructs were utilised to transform tobacco (cv. Petite Havana) plastids and the transplastomic (i.e., plastid-transformed) lines were purified to homoplasmy, the genetically stable state in which all wild-type plastid genomes are eliminated and a homogeneous population of transgenic plastid genomes is present (Bock 2001; Maliga 2004). Analysis of p24-Nef and Nef-p24 fusion proteins showed that both can be expressed to relatively high levels in chloroplasts. Some lines harbouring *nef* as single gene, displayed a mutant phenotype with green and yellow sectors. *nef* mRNA and protein expression analysis showed that high levels of *nef* transcripts accumulated only in the yellow tissue while low amounts of *nef* mRNA were found in the green sectors. Consistent with this, when expression was analyzed at the protein level, high accumulation levels of Nef were seen only in the yellow plants (Zhou et al. 2008). Sequencing of the promoter region in several independent green and yellow lines showed that the phenotype was due to an homologous recombination event between a short and a full-length version of the tobacco plastid ribosomal RNA operon promoter. These results indicate that, if expressed to high levels, the Nef protein may be deleterious to the chloroplast by interfering with organellar functions (Zhou et al. 2008).

As the best results in terms of protein expression levels were obtained with the p24-Nef fusion protein, the correspondent gene was cloned in a new expression vector constructed to avoid recombination events. This construct was introduced into the

tobacco and tomato plastid genomes. Transplastomic tobacco and tomato plants were analysed and protein accumulation was found to be close to 40% of the leaf's total protein and easily detectable by Coomassie staining. Transcript and protein accumulation were analysed in different ripening stage of tomato fruit and while green tomatoes accumulated the fusion protein to 2.5% of the total protein, the protein was not detectable in ripe fruits. However, the use of a green-fruited tomato variety, commercially available, seems the solution for the production of the antigens in an edible fruit (Zhou et al. 2008). It is also noteworthy that, despite the enormous expression levels obtained with the optimized *p24-nef* fusion gene, the transplastomic plants did not display the same strong phenotype as the *nef* plants, suggesting that fusion to p24 may alleviate the negative effects of Nef on chloroplast function.

As the tobacco cultivar generally used for plastid transformation is a relatively small variety (cv. "Petit Havana"), which produces only about 100–200 g of leaf material per plant, the yield of recombinant protein could be greatly increased if a high biomass cultivar was used. Philip Dix's group in Maynooth reports the production of high yields of HIV p27 Nef protein through plastid transformation of tobacco cv. Maryland Mammoth, a high biomass cultivar which produces about 1.5 kg leaf material per plant (M. S. McCabe et al., unpublished data).

All the transplastomic plants expressing Nef exhibited a yellow phenotype, as had previously been found in plants expressing the HIV p24 protein using an otherwise identical vector (McCabe et al. 2008). The cause of the pigment deficiency has not been explored further in these plants. It results in a reduction in growth rate but the plants still grow well, remain vigorous and reach flowering after about 120 days. Seedling tests revealed 100% yellow seedlings with no sign of segregation, supporting homoplasmy of these lines, which was confirmed by long-range PCR. Nef expression levels were estimated to be 300 μg/g fresh weight (equally ~6% of TSP). The purification of Nef from Maryland Mammoth chloroplasts by ammonium sulphate precipitation followed by citric acid/sodium citrate treatment and cation exchange chromatography, a procedure previously used with success with HIV p24 (McCabe et al. 2008), was unsuccessful and the protein appeared unstable under these extraction conditions. Refinements in the extraction procedure are under investigation (M. S. McCabe et al., unpublished data). The tobacco cultivar Maryland Mammoth is a strong candidate for the production of commercial quantities of recombinant HIV proteins (including Nef), via chloroplast transformation.

Conclusions

The last AIDS epidemic update indicates that a vaccine able to induce neutralizing immunity is a priority. The goal of the HIV-1 vaccination should be to obtain an antibody barrier able to neutralize the virus preventing viral entry and to induce a durable cellular immune response overcoming strain-specific immunity (Walker and Burton 2008). In this context the design of a multi-antigen protein vaccine should include different viral components conserved among several strains. Studies concerning the generation of multi-component HIV vaccines, containing Nef, have been published (Goepfert et al. 2007; Voss et al. 2003; Maggiorella et al. 2007) and the results are very promising.

As the vast majority of people who need HIV vaccine live in developing countries, low-cost production should be considered an important issue together with the effectiveness of the vaccine design. In this context plants represent a cost-effective alternative to the expression systems frequently used to produce vaccine components (Ma et al. 2005; Schillberg et al. 2005).

In the present review we gave an overview of the different expression strategies developed to over produce HIV neutralizing antibodies and HIV antigens in plants, specifically focusing the attention on Nef protein.

Results obtained with the expression of HIV neutralising antibodies in transgenic plants are very promising, and indicate that plant-produced antibodies are generally comparable in terms of antigen binding and HIV neutralization activity to their CHO-derived counterparts. Moreover, the high expression yields documented for the 2G12 antibody produced in maize seed endosperm make this plant expression platform particularly attractive and economically advantageous (Ramessar et al. 2008). Yet, a possible limitation to the applicability in therapy of plant-derived mAbs could be represented by the difference in N-glycan composition from their mammalian

counterpart which could lead to immunogenicity problems (Jin et al. 2008). To address this limitation different strategies have been successfully adopted to modify the glycosylation pattern of plant derived mAbs which include the modulation of plant glycosylation using RNA interference (Cox et al. 2006; Strasser et al. 2008), the co-expression of specific human glycosyltransferases (Bakker et al. 2006; Fujiyama et al. 2007), or the exploitation of specific knockout transgenic lines that synthesize complex *N*-glycans lacking immunogenic xylose and fucose epitopes (Schähs et al. 2007) and/or the retention of the immunoglobulin in the endoplasmic reticulum (ER) (Triguero et al. 2005).

In this work we particularly focused our attention on the different strategies attempted to express HIV-1 Nef in planta. The highest Nef accumulation has been obtained in chloroplasts of transplastomic vegetative tissue as fusion to p24 yielding up to 40% of TSP (Zhou et al. 2008), while expression of Nef alone in this organelle resulted in a much lower accumulation suggesting that protein degradation can be a limiting factor also in plastids. Attempts to express Nef alone in the cytosol demonstrated very low accumulation levels below 0.5% of TSP (Marusic et al. 2007), although it has been found that anchoring Nef to the cytosolic face of the ER by addition of a C-terminal tail-anchor domain can improve protein stability and accumulation (Barbante et al. 2008). The strategy of targeting Nef alone into the lumen of the ER proved deleterious for its stability, probably because of folding defects recognized by the quality control machinery. In this case Nef fusion to the vacuolar storage protein zeolin increases accumulation levels by preventing protein degradation in the secretory pathway (de Virgilio et al. 2008). The results are promising, however, they do not equal those obtained in leaf chloroplasts.

In conclusion, although absolute comparisons in terms of protein yield between the different expression strategies discussed cannot be made, general considerations can be drawn from the results obtained in this collaborative research. The expression of Nef protein alone in different cell compartments tended to result in low accumulation levels. Conversely, the fusion to stabilizing protein components appears to be at the moment the most promising approach to enhancing yields, although in this case efficient extraction and purification of the antigen still has to be demonstrated.

In the light of these results, as the immunogenicity of a number of HIV-1 antigens produced in plants has been already reported, future work will move on from optimisation of expression and aim at testing the immunological properties of plant expressed Nef to verify the possibility of including it with other HIV antigens, already expressed in plants, in a potential HIV vaccine.

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