Plant made anti-HIV microbicides- a field of opportunity

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Abstract

HIV remains a global burden and without an effective vaccine, it is crucial to develop microbicides to halt the initial transmission of the virus. Several microbicides have been researched with various levels of success. Amongst these microbicides, the broadly neutralising antibodies and peptide lectins are promising in that they can immediately act on the virus and have proven efficacious in *in vitro* and *in vivo* protection studies. For the purpose of development and access by the relevant

population groups, it is crucial that these microbicides be produced at low cost. For the promising protein and peptide candidate molecules, it appears that current production systems are overburdened and expensive to establish and maintain. With recent developments in vector systems for protein expression coupled with downstream protein purification technologies, plants are gaining credibility as alternative production systems. Here we evaluate the advances made in plant expression host and vector system development as well as the progress made in expressing HIV neutralising antibodies and peptide lectins using plant-based platforms.

Keywords: HIV, microbicides**,** antibodies, lectins, plant expression

1. Introduction

Recent reports show that the number of new HIV infections has declined by 21 % since the peak of the disease almost 15 years ago (UNAIDS, 2011). However, worldwide more than 34 million people are still living with the disease (UNAIDS, 2011). Furthermore, in sub-Saharan Africa, the most heavily HIV affected region, it is estimated that only 6.6 % of the population have been tested for HIV in 2009 (UNAIDS, 2010). Thus, globally there still exists a huge reservoir of HIV-infected people with the potential to infect millions more. Despite commendable research efforts over nearly 30 years, a protective HIV vaccine is still not available. Thus, it has become crucial to develop other strategies for disease prevention, such as microbicides that would effectively block the initial transmission of the virus. Women comprise 50% of the HIV infected population and are high risk candidates who are in many cases unable to protect themselves due to domestic violence, cultural and social habits, lack of education and financial security (UNAIDS, 2010). Due to these difficult socioeconomic conditions a successful microbicide should further lend itself to formulations that can be applied topically or orally in order for women to self-manage the use of it (Moscicki, 2008). The microbicide development field received a boost with the progress made in CAPRISA004 studies where it was demonstrated that a microbicide gel containing 1% tenofovir, a reverse transcriptase inhibitor, could prevent the risk of HIV infection by 38% (Karim et al., 2010).

Anti-HIV microbicide candidates comprise of surfactants, vaginal milieu protectors, viral entry inhibitors, reverse transcriptase inhibitors and other agents with an unknown mode of action (Cutler and Justman, 2008). Surfactants and vaginal milieu protectors were the first generation candidate microbicides (Cutler and Justman, 2008). These were broad acting and failed to produce effective HIV inhibition, even enhancing infection in some instances (Van Damme et al., 2002). Of these, N-9 was the first surfactant that was tested in a clinical trial (Garg et al., 2009). Although no adverse effects were reported for N-9 in preclinical and Phase I clinical trials, genital ulcers, irritation, inflammation and subsequent higher HIV risk were reported from Phase III trials (Garg et al., 2009; Mosciki, 2008). Further surfactant development as microbicides faded under the risk of vaginal damage and inconclusive clinical trials.

Vaginal milieu protectors stabilise the low mucosal pH. In this class of microbicides Acidform (Amphora, Instead Inc, Dallas, TX, USA) and BufferGel (Carbopol 974P, ReProtect, Baltimore, MD, USA) have been evaluated extensively, displayed good contraceptive properties and were shown to be well tolerated in human subjects (Mayer et al., 2001). Whilst *in vitro* anti-HIV activity has been reported for Acidiform, it has only been subjected to safety and acceptability pre-clinical studies (reviewed by Cutler and Justman, 2008; http://www.insteadsciences.com/amphora.htm#results). BufferGel failed to show reduction of HIV infectivity when compared to the placebo gel in a study that evaluated its effectiveness in reduction of HIV incidence in a high risk study group (Karim et al., 2011). It is thus likely that these vaginal milieu protectors will not be effective in preventing HIV transmission in single formulations and will probably be used in combination with other antiviral entities. In fact, Carbopol 974P is being used as the polymer base to formulate gels for the application of reverse transcriptase inhibitors such as Tenofovir and UC781 (Garg et al., 2010). Other strategies to maintain a healthy mucosal environment include the restoration of the microflora population by products such as Lactin V and MucoCept from Osel, Santa Clara, CA, USA (Moscicki, 2008).

Entry inhibitors are a group of microbicides that interact either with viral or host cell structures to prevent attachment, fusion and entry. The first type of entry inhibitors was chemical molecules such as anionic polymers that establish an interaction with

the virus based on surface charges (reviewed by Cutler and Justman, 2008). Most of these compounds failed to show significant protection in clinical trials, were associated with unwanted side effects and in some instances associated with an enhanced HIV infection risk (Pironne et al., 2011).

Subsequent microbicide development focused on more potent specialised molecules such as reverse transcriptase inhibitors, CCR5 antagonists and viral entry inhibitors. Reverse transcriptase inhibitors target viral enzymes (Campiani et al., 2002; Cihlar, 2006), CCR5 antagonists compete with the virus for host cell co-receptors (Baba, 2006; Schols, 2006; Schols, 2011), whilst entry inhibitors bind to viral envelope components to prevent entry of the virus into the cell (Balzarini, 2006; Botos and Wlodaver, 2005). In the later group, antibody and peptide lectins represent a class of molecules that are in advanced stages of development as microbicides.

To avoid repeating past failures, newly researched microbicide candidate molecules are currently undergoing strict evaluation in several preclinical test studies using specialised models and formulations (Buckheit et al.; 2010, Doncel and Clark, 2010; McGowan, 2009). Rigour is necessary in preclinical testing because clinical trials are complex and expensive (Minces and McGowan, 2010) and the largest market segment for HIV prophylaxis resides in resource limited countries who can ill afford the development costs. Because of these cost hurdles, it is crucial that microbicides be produced with minimum upfront capital outlay so as to facilitate development, testing and ultimate availability of the final product.

Plants are emerging as cost friendly alternative production systems for a variety of pharmaceuticals. Numerous therapeutic proteins have been produced in plant systems (Giddings et al., 2000; Ma et al., 2003). Protein based microbicides,-namely, neutralising antibodies and peptide lectins- lend themselves to production in plants (De Muynck et al., 2010; Matoba et al., 2010; Sexton et al. 2006; O'Keefe et al., 2009). Although these microbicides have been extensively studied in terms of their structure and mode of action, their production in plant host expression systems has not been audited to date. In this study, we evaluate the progress made in the expression and development of peptide and antibody candidate microbicides.

2. Plants as alternative production systems for anti-HIV microbicides

Over the past two decades plants have been extensively investigated as alternative production systems for pharmaceutical proteins. Even with careful consideration of existing production systems, plants provide several attractive features that are equivalent or more beneficial (Mett et al., 2008; Twyman et al., 2003 and 2005). Like mammalian and yeast cells, plants possess the cellular machinery which enables them to perform the post translational modifications essential for maturation and sometimes function of proteins. Unlike mammalian fermentation systems, plants are not at risk of being contaminated with human pathogens. Furthermore compared to mammalian and yeast systems, plant production systems are more easily scaled up; plants can either be propagated in large numbers in designated land plots or in contained greenhouses. Maintenance of plants in soil, hydroponic or cell culture is simple and cheap compared to the complex growth media and requirements of yeast and mammalian cell systems (Knäblein, 2005). Furthermore, plants provide a huge biomass in the form of green leafy tissue or as the numerous seeds of crops. The latter provides a further advantage of stable storage over longer time periods and high protein content that can be exploited for recombinant protein production (Lau and Sun, 2009). Whilst costs of downstream processing remain as high as that required for purifying proteins made from conventional systems; the burden can be alleviated by maximising production yields and utilising innovative purification strategies (Paul and Ma, 2011) to improve product recovery. Thus, for plant production systems the upfront investment required for infrastructure is lower, which potentially lowers the barriers to entry by more players or players in developing countries.

3. Challenges and developments in plant made pharmaceuticals (PMPs)

Considering the time and effort invested over twenty-years, relatively few plant made pharmaceuticals (PMPs) are currently marketed (Faye and Gomord, 2010). The main reason for this is that the production levels in plants were often too low to be commercially viable. Proteins were initially expressed in transgenic plants through stable nuclear transformation using *Agrobacterium* mediated delivery of binary

vectors or alternative methods such as biolistic introduction of DNA into plant cells (Banta and Montenegro, 2008). These are lengthy, labour intensive processes which mostly generated progeny with variable and, for the most part, low target protein accumulation levels. Expression of multiple component proteins such as antibodies required numerous crossings and screening of plants in breeding programmes. Transient expression with binary vectors was mainly used as a rapid screen to validate the expression potential of a gene and did not result in high protein accumulation (Gleba et al., 2005). Whilst viral vectors were useful to produce proteins in plants, they were limited by the insert size or the fidelity of the transcript (Pogue et al., 2002). Furthermore, systemic spread of the virus sometimes resulted in loss of the foreign gene insert and raised concerns of containment. However developments in this arena have resulted in some of the state of the art technologies in plant based protein expression.

Another perceived limitation for PMPs in clinical applications is the variation in plant glycan structure compared to that of humans (Gomord et al., 2005). This shortcoming is also typical of other systems such as yeast and insect cells (Mett et al., 2008). Thus far there has been no clinically significant evidence that plant specific glycans are immunogenic in humans (Van Der Veen et al., 1997). Data from the development of plant made glucocerebrosidase made in carrot which is now at very advanced stages of clinical development, has done a lot to allay such fears, with the plant made product proving to be not only well tolerate but perhaps even more efficacious and stable, a biobetter (Aviezer et al., 2009). Still the pressure remains on plant production systems to deliver therapeutic proteins with a humanised glycan profile. Several advances in expression vector systems and plant hosts have addressed some of these limitations to a large extent:

3.1. Development of expression vectors

To increase protein yield, second generation *Agrobacterium* binary vectors have incorporated various elements to enhance transcription and translation (Veluthambi et al., 2003). These improved vectors, used in conjunction with transient infiltration, have improved protein expression levels in plants. For instance, transient expression

of a human optimised HPV-16 L1 capsid protein gene using a specialised binary pTRA vector resulted in a yield of more than 0.5 g/kg of fresh leaf weight (17% total soluble protein, Maclean et al., 2007). Significantly lower levels were obtained when the same protein was transgenically produced in tobacco and potato resulting in the L1 protein accumulating to 0.5 and 0.2% of total soluble protein respectively (Bemielt et al., 2003).

Further development in vector systems has seen the merging of viral and binary vector technology to increase yields and address insert size restrictions, retention of target genes and containment issues. Icon Genetics (GmbH, Halle) developed a deconstructed viral vector system in which target genes and different viral vector components are carried on several pro-module vectors (Marillonet et al., 2004). In this system the viral coat protein has been removed to eliminate systemic spread. Instead agroinfiltration provides the delivery of the modules to the plant cell and limits replication to the infiltrated area. In the cell, high level expression is facilitated by a RNA dependant RNA polymerase. A site specific recombinase facilitates the assembly of the modules into a DNA molecule which is transcribed and spliced into a functional transcript. The transcript moves to the cytosol where it is translated into the specified protein. Alternatively target signals can be incorporated to direct proteins to specified subcellular compartments (Marillonnet et al., 2004). This system has been used to accumulate various proteins at levels over 4 g/kg plant material (Bendandi et al., 2010).

One shortcoming of the initial Icon system vectors was the inability to co-express more than one protein in the same spatial location (Giritch et al., 2006). This was problematic with the production of multi-component proteins such as immunoglobulins. A solution to this came by co-expression from two non-competing monopartite viral genomes such as TMV and PVX (Giritch et al., 2006). Alternatively, viral vectors derived from bi- or tri-partite viral genomes do not seem to be competing and are able to co-function in the same area.

The cowpea mosaic virus (CPMV) is a bipartite viral RNA genome from which two types of vector systems were developed. In the full-length system (wild type, wt) the coding sequence for the protein of interest is fused to the C-terminus of the RNA-2

polypeptide which is co-translationally released via 2A-peptide mediated cleavage (Sainsbury and Lomonossoff, 2008). Replication is facilitated by the co-expression of RNA-1. The full length version allows for local co-expression of two different proteins; however segregation of the co-expressed proteins occurs with systemic movement (Sainsbury et al., 2008). A deleted CPMV version of RNA-1 (hypertranslatable, HT) was developed which lacked the ability of systemic spread and was thus able to co-express more than one protein without the occurrence of segregation. Moreover the deleted CPMV system obtained higher expression levels than the full length version (Sainsbury et al., 2008 and 2010). Using this system, protein expression levels exceeded 0.3 g/kg protein (Sainsbury and Lomonossoff, 2008).

Several pharmaceutical molecules have been expressed in plants using Gemini viral bean yellow dwarf virus (BeYDV) based vectors (Chen et al., 2011). Huang et al. (2010) and Regnard et al. (2010) developed a viral vector system based on the DNA genome of the bean yellow dwarf Gemini virus (BeYDV). The BeYDV system requires only two viral components, the long intergenic region (LIR) and the short intergenic region (SIR) control sequences and Rep/RepA gene for co-expressing heteromeric proteins. In the system non-competing co-expression was achieved either from two replicons encoding different proteins or from a single replicon containing the different proteins. In *Nicotiana benthamina,* transient expression levels from the BeYDV vector were 3-to 7-fold more for EGFP and HIV-1 p24 compared to levels obtained using a binary pTRA *Agrobacterium tumefaciens* vector (Regnard et al., 2010). Furthermore, expression with BeYDV resulted in accumulation levels of 0.5 g/kg monoclonal antibody against Ebola virus GP1 (mAb 6D8) (Huang et al., 2010). It is anticipated that the system will be able to simultaneously produce as many as four different protein subunits.

3.2. Development of modified host systems

In eukaryotes the N-glycan biosynthesis pathway is conserved for the endoplasmic reticulum (ER) (Kukuruzinska and Lennon, 1998). Variations between species occur in modifications to glycan structures in processing steps after the protein exits from

the ER. In plants these variations depend on the protein itself, plant species and plant organ used for expression (Gomord et al., 2010). Unless the plant glycosylated form of the therapeutic protein is more attractive as is the case with the carrot cell produced glucocerebrosisdase (Shaaltiel et al., 2007), it is considered more ideal if plants are able to produce therapeutic proteins that have mammal-like glycans. Where glycan structure is not critical to protein function, recombinant proteins without glycan structures (Rodriguez et al., 2004) are desirable. Therapeutic proteins that are produced as aglycosylated forms are only feasible if the proteins need to stimulate an inflammatory response or in the case of a recombinantly produced antibody which does not require an effector function since glycan structures are often crucial for this biological function of the protein (Jefferis, 2009).

Another means to produce a protein that more closely resembles a humanised glycan profile is to restrict the recombinant protein to the ER by using KDEL, HDEL or SEKDEL ER retention signals (Ko et al., 2003; Triguero et al., 2005). For regulatory purposes it is generally regarded as “safer” to produce a native version of the protein (Fischer et al., 2012). However in the case of ER retention signals regulatory approval might be less stringent seeing that these sequences are also found in mammalian proteins. Of note is that the current carrot cell produced glucocerebrosisdase, which is on the verge of FDA approval, were produced with a storage vacuole targeting signal (Shaaltiel et al., 2007). Another argument for ER retention is that for the production of some proteins, ER retention is needed to increase the accumulation levels (Bortesi et al., 2009; Yang et al., 2005). On the other hand ER retention can result in the degradation of the protein or low stability and reduced half life of the therapeutic *in vivo* (Ko et al., 2003; Loos et al., 2010). Studies have also shown that ER retention of recombinantly produced proteins is not always successfully achieved, leading to some proteins leaking from the ER that are then further processed to contain complex immunogenic plant glycans (Floss et al., 2008; Loos et al., 2010; Rademacher et al., 2008).

Subsequently, improved plant hosts have been developed with the aim of humanising the glycan patterns of recombinant proteins. Plants such as *Arabidopsis*, tobacco and moss have been generated in which the plant specific glycosylation genes have been knocked out (Koprova et al., 2003; Schähs et al., 2007; Strasser et al., 2009). In these

mutants, plant specific α1,3-fucose and β1,2-xylose residues are replaced by complex N-acetylglucosamine (GnGn) structures. Further glycan improvements are made by co-expressing mammal like glycosylation and sialylation enzymes such as β1,4-galactosyltransferase (GalT), N-acetylglucosaminyltransferase III (GnTIII), core α1,6-fucosyltransferase, UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (GNE), N-acetylneuraminic acid phosphatase synthase (NANS), CMP-N-acetylneuraminic acid synthetase (CMAS), CMP-N-acetylneuraminic acid transporter (CMP-Neu5Ac) and α2,6-sialyltransferase (ST) in these plant glycosylation knock-out mutants (Casthilo et al., 2010; Castilho et al., 2011; Strasser et al., 2009). Resulting proteins not only lack plant specific glycans but also contain human glycan structures.

The current gel formulation of HIV neutralising antibodies (Morris and Lacey, 2010) and peptide lectins in gels (Tsai et al., 2003) suggests that these microbicides will most likely be applied topically. When these are exposed to the mucosal surfaces it will only have a limited interaction with the immune system and thus not cause inflammation. Thus for the production of these microbicides in plants the nature of the glycan structures on these microbicides might not be as important as the yield obtained.

4. Production of HIV neutralising antibodies in plants

Neutralising antibodies play a very important role in the development of vaccines for passive immunisation and as viral entry inhibitors (Reina et al., 2010). They are directed against the viral envelope protein and interfere with viral docking and fusion. They thus inhibit the infectivity of the virus and also potentially facilitate viral clearance *via* their Fc related effector functions (Hessell et al., 2007).

Several HIV-1 neutralising antibodies have been isolated from HIV infected individuals (Simek et al., 2009; Walker et al., 2009; Wu et al., 2010). Novel broad acting neutralising antibodies such as VRC01, VRC02, PG16 and PG9 were isolated which displayed a larger breadth and potency than some of the well-known neutralising antibodies (Wu et al., 2010). However the four well-known HIV-1

neutralising antibodies, namely, 2G12, 4E10, 2F5 and b12 have been well researched in terms of structure, interaction with the virus, protection in animal models and safety in clinical trials and were produced in various plant platforms. These antibodies have fared well in protecting macaques from systemic or vaginal Simian/Human Immunodeficiency Virus (SHIV) challenges and are well tolerated in human subjects (Armbruster et al., 2002 and 2004; Hessell et al., 2010; Mascola et al., 1999 and 2000; Parren et al., 2001). Furthermore, passive administration of neutralising monoclonal antibodies (MAbs) 4E10, 2F5 and 2G12 reduced viral rebound in established HIV infections (Trkola et al., 2005). Three of these neutralising monoclonal antibodies (MAbs) - 2G12, 4E10, 2F5 are being assessed as a gel formulated microbicide in a phase I clinical trial for their safety and pharmacokinetic effects (Morris et al., 2010; Morris and Lacey, 2010). So far vaccination attempts with these antibody epitopes have failed to produce equivalent neutralising antibodies in humans (Coëffier et al., 2001; Lenz et al., 2005; McGaugey et al., 2003). It is thus most likely therefore, that these antibodies will have to be administered passively and that a large production quantity will be required.

Mammalian cells are currently used for FDA approved therapeutic antibody production (Li et al., 2010). Given that the capacity of these traditional fermentor systems will not meet the demand, plants can be employed as alternative manufacturing platforms (Knäblein, 2005). Several antibodies have been successfully produced in plant platforms (De Muynck et al., 2010). Apart from yield, the glycan composition and efficacy will be important criteria for plant made manufacturing of these antibodies. Thus we evaluated the progress of plant production of these neutralising antibodies in light of these criteria. Table 1 summarises the expression of these four antibodies in plant systems.

4.1. 2G12

It has been reported that 2G12 neutralises A and B clade HIV-1 virus entry by recognition of a Manα1→2Man rich epitope on the exterior face of the gp120 protein (Binley et al., 2004; Scanlan et al., 2002). Monoclonal antibody (MAb) 2G12 can

activate the complement system and display antibody dependent cellular cytotoxcity (ADCC) against virus infected cells (Trkola et al., 1996). Passive infusion of 2G12 combined with other neutralising antibodies including 2F5, protected macaques from a vaginal and intravenous challenge with SHIV (Baba et al., 2000; Mascola et al., 2000). Passively infused 2G12 and 2F5 were well tolerated in human subjects in a phase-I clinical trial (Armbruster et al., 2002). Clinical trials with 2G12 produced in transgenic tobacco have commenced in 2009 (Paul and Ma, 2011).

MAb 2G12 is unique in its structure in that it naturally forms a single Fab region *via* domain swapping between the variable regions of the light chain (V_L) and heavy chain (V_H) and between the constant region of the light chain (C_L) and heavy chain (C_H1) respectively (Calarese et al., 2003; West et al., 2009). The 2G12 dimer has shown to be more than 50 times more efficient as the monomer in neutralising several HIV-1 strains in both *in vitro* (West et al., 2009) and *in vivo* assays (Luo et al., 2010).

The production of 2G12 has been actively pursued in maize, *Arabidopsis* and tobacco. MAb 2G12 was produced in the seed endosperm of two different maize lines under control of the rice glutelin (gt-1) promoter. The antibody was produced in Hi-II maize with endoplasmic reticulum (ER) retention signals (Rademacher et al., 2008) and in the elite maize line M37W as a secreted form (Ramessar et al., 2008). ER retained antibody accumulated to 30 ug/g in the T1 generation and 60 ug/g in the T3 generation whilst the secreted form reached 100 ug/g. Identification of the glycan structures showed that the majority of the ER retained antibodies contained oligo-mannose type glycans (OMT). However a few immunoglobulins contained glycans of the vacuolar type, indicating that ER retention was not completely successful. Different glycoforms were detected for the secreted 2G12 with the majority being single N-acetylglucosamine (GlcNAc) residues and the rest containing complex type fucose and xylose glycans with a small number also containing OMT type glycans.

The efficacy of the maize produced antibody was compared with the Chinese Hamster Ovaries cells (CHO) produced 2G12 derivative. Both the ER retained and secreted 2G12 had a similar antigen binding ability as the CHO produced 2G12. However in an HIV neutralisation assay, the ER retained and secreted forms were four and

threefold more effective than the CHO produced equivalent respectively. The increased potency was attributed to the dimerisation and aggregation of the antibody.

In *Arabidopsis*, 2G12 was expressed in both the leaves (Schähs et al., 2007) and seeds (Loos et al., 2010) of a knockout line (ΔXT/FT), that lacked the ability to generate immunogenic plant specific β 1,2-xylose and α1,3-fucose glycans (Strasser et al., 2004). In leaves, expression was driven by the 35S CaMV promoter without any ER retention signals. The antibody levels varied between 0.05 and 0.2% TSP in both the wildtype (wt) and ΔXT/FT line (Schähs et al., 2007). The glycans on the antibody that was produced in the ΔXT/FT line were mainly terminal N-acetylglucosamine (Gn) residues that lacked plant specific β 1, 2-xylose and α1,3-fucose residues. A small population of the antibody molecules produced contained OMT residues indicating that the processing of all antibodies was incomplete. Notably, the binding capacity of the ΔXT/FT produced 2G12 antibody was similar to the CHO produced 2G12.

For seed expression in *Arabidopsis* ΔXT/FT, expression of 2G12 was driven by the β-phaseolin promoter (Loos et al., 2010). The antibody was expressed with and without ER retention signals. There was no significant difference in accumulation levels between secreted and ER retained antibody. Expression levels peaked around 3.6 ug/mg. The N-glycan profile of the purified antibodies revealed that the secreted antibody of the wild type line contained complex N-glycans containing N-acetylglucosamine-Xylose-Fucose (GnGnXF) residues whilst the 2G12 produced in the mutant line contained a homogenous N-glycan structure consisting of N-acetylglucosamine (GnGn). The majority of the ER retrieved antibody of the wild type line contained oligo-mannosidic N-glycans, with a small amount of antibody carrying GnGnXF. The efficacy of the seed produced 2G12 in an HIV neutralisation assay was slightly inferior to the CHO derivative.

In *N. benthamiana* leaves, 2G12 was transiently expressed using the full length and the deleted RNA-2 (HT) version of the CPMV vector (Sainsbury and Lomonossoff, 2008, Sainsbury et al., 2010). The antibody was expressed in both systems with and without ER retention signals. Overall higher antibody accumulation was obtained by

using the deleted CPMV vector and ER retention signals. Levels of 325 mg/kg were reported. The glycan analysis of the antibodies showed that ER retained forms consisted mainly of oligo-mannose type structures (OMT) with a few containing more complex glycans. Secreted antibodies contained complex GnGnXF, N-acetylglucosamine –Mannose-Xylose-Fucose (GnMXF) with a few OMT also present. *In vitro* evaluation of the binding ability and neutralisation efficacy of the *N. benthamiana* produced antibody showed that it was equal to the mammalian cell derived 2G12.

To further humanise the glycan structures on 2G12, Strasser et al. (2009) produced 2G12 in a *N. benthamiana* ΔXT/XT $GalT^{+}$ mutant line. This *N. benthamiana* line does not produce plant specific xylose and fucose glycans but produces partially humanised glycans *via* the activity of a highly active human derived β1, 4-galactosyltransferase. Although no mention was made of the accumulation levels, the 2G12 antibody produced in this system was fully galactosylated and was more effective in neutralising HIV-1 than the CHO produced version.

4.2. 2F5

MAb 2F5 displays a broader neutralisation activity than 2G12, inhibiting HIV isolates from clades A, B, D and E (Binley et al., 2004). It docks onto to the core epitope ELDKWA on the lipid embedded membrane proximal exterior region (MPER) of gp41 and potentially interferes with the fusion step of the virus (Binley et al., 2004; de Rosny et al., 2004; Franquelim et al., 2011; Muster et al., 1993). On its own and in combination with other antibodies including 2G12 and 4E10, 2F5 displayed the ability to protect against an intravenous, vaginal and oral challenge of SHIV in macaques (Baba et al., 2000; Hessell et al., 2010; Mascola et al., 1999 and 2000). Furthermore passive administration of this antibody did not seem to cause immune responses or other adverse effects in HIV infected human participants (Armbruster et al., 2004).

Production of 2F5 was explored in *Nicotiana* species. The heavy chain (HC) and light chain (LC) were expressed with SEKDEL retention sequences in tandem under control of the 35SCaMV promoter in *Nicotiana tabacum* L. cv bright yellow (BY-2) cell cultures (Sack et al., 2007). Accumulation of 2F5 reached a maximum of 2.9 mg/kg fresh weight and was further enriched by protein-A purification to 6.44 mg/kg wet cell weight. No degradation products were observed following purification, however minor impurities were detected. N-glycans were expected to be of the OMT, but this was not confirmed by analyses. Fc region binding between the BY-2 and CHO produced 2F5 was equivalent. However, the antigen binding capacity of CHO produced 2F5 (97%) was slightly superior to the plant derived 2F5 (89%). In HIV neutralisation studies the BY-2 produced antibody was threefold less efficient than the CHO produced counterpart. This lower potency was attributed either to the presence of impurities, the added SEKDEL motif or different glycan structures that could have interfered with the antibody access to the epitope.

To further enhance the accumulation of 2F5 in tobacco, the antibody was expressed as ER retained elastin-like polypeptide (ELP) fusions (Floss et al., 2008). The ELP peptide has been used to facilitate accumulation of proteins in green leaf tissue (Patel et al., 2007). Four transgenes; HC unfused, HC -ELP fused, LC unfused and LC - ELP fused were introduced in *N. tabacum* cv. Samsun NN. Plants were subsequently crossed resulting in combinations with neither gene carrying the fusion or both HC and LC carrying ELP fusions or either the HC or LC fused to ELP. Prior to crossing the transgenic lines, it was observed that the presence of ELP increased the accumulation of the chains with the LC accumulating to higher levels than the HC. In the crossed lines, the LC-ELP fusion facilitated a higher accumulation of the unfused HC as well. Accumulated total soluble protein (TSP) levels reached 0.3% for the LCELP-HC, 0.2% HCELP-LC, 0.6% for HCELP-LCELP and 0.1% for HCLC. The ELP fusion eased the purification process of the plant produced antibodies and did not interfere with the assembly of the antibody. The glycans of the plant produced 2F5 were mainly oligo-mannose type (OMT) with lesser amounts of complex glycans consisting of N-acetylglucosamine (GnGn), N-acetylglucosamine -Xylose (GnGnX), Galactosyl-N-acetylglucosamine-Xylose (AGnX), N-acetylglucosamine-Mannose-Xylose (GnMX) and N-acetylglucosamine –Xylose-Fucose (GnGnXF) moieties. 2F5 variants were all similar to the CHO produced 2F5 in their antigen binding capacity.

4.3. 4E10

MAb 4E10 is one of the most broadly neutralising antibodies that are active against several viral isolates of different clades including clade C, which is the most prevalent clade in the heavily affected sub-Saharan Africa region (Binley et al., 2004; Walker et al., 2009). Both 4E10 and VRC01 were able to neutralise over 90% of the key HIV subtypes (Walker et al., 2009; Wu et al., 2010). Although VRC01 is more potent, it uses a different mode of action with the virus than 4E10. VRC01 interacts with the envelope in a way that resembles the CD41-gp120 interaction (Li et al., 2011). The 4E10 epitope interaction is also somewhat complex; the antibody recognises a linear epitope adjacent to the 2F5 epitope on the membrane proximal exterior region (MPER) and interacts with the lipids on the cell membrane (Franquelem et al., 2011; Zwick et al., 2001). Whether lipid binding is involved in the broadly neutralising ability of 4E10 is still debatable (Scherer et al., 2010; Xu et al., 2010). Thus both antibodies can be used in combination against several HIV isolates.

In a phase I clinical trial, it was demonstrated that 4E10 can be safely administered to HIV infected participants alone or combination with 2F5 and 2G12 (Armbruster et al., 2004). When 4E10 was administered intravenously, rhesus macaques were protected from a mucosal challenge with SHIV (Hessell et al., 2010).

MAb 4E10 has been expressed *via* nuclear transformation in *N. benthamiana* (Strasser et al., 2009). It was produced in a wild type (wt), a glycoengineered ΔXT/FX mutant line and in a ΔXT/FX GalT+ line that produced an altered version of the human β1,4-galactosyltransferase. The glycans of the wt produced 4E10 contained N-acetylglucosamine –Xylose-Fucose (GnGnXF), N-acetylglucosamine (GnGn) for the XT/FX mutant and galactosylated (AA) glycans for the XT/FX GalT+ line. The later MAb form was more potent than the other plant made forms and more efficient than the CHO produced derivative in a neutralisation assay, possibly because of the galactosylated glycans that enhance the stability, half-life and functionality of the antibody.

4.4. b12

MAb b12 can effect HIV neutralisation across different clades from different geographic locations (Binley et al., 2004). Unlike other neutralising antibodies that are restricted to certain conformations of the virus, *in vitro* studies show that b12 can bind different conformations of the envelope (Eggink et al., 2007; Zhou et al., 2007). This antibody has been shown to protect macaques in a vaginal challenge with SHIV when administered systemically or topically (Parren et al., 2001; Veazey et al., 2003). MAb b12 was expressed in the milk of female mice and displayed the same HIV neutralisation ability as the CHO cell derived antibody (Yu et al., 2010). In combination with CD4-IgG2 (Pro542), b12 potently inhibited HIV infection of cervical tissue (Hu et al., 2004). More importantly, in this combination or administered alone, MAb b12 is able to stay associated with the virus that leaves the mucosal environment on migrating cells and prevents subsequent infection of target lymphocytes (Hu et al., 2004; Van Monfort et al., 2011). Other neutralising antibodies in the study did not display this property.

Sexton et al. (2009) produced b12 and a b12-CV-N (cyanovirin) fusion in *N. tabacum*. CV-N is a cyanobacterium lectin that displays potent anti-HIV activity (Boyd et al., 1997). Plants were generated that expressed b12 HC, LC or a fusion where CV-N was fused to the b12 HC. Subsequent crosses were performed to generate progeny that expressed both an unfused b12 (7.55ug/ml) as well as b12-CV-N fusion (2.45 ug/ml). The authors demonstrated that both modules of the fusion molecule were functional and the fusion molecule to be more potent than CV-N or b12 alone in an HIV neutralisation assay. The glycan profile of the plant made proteins was not presented.

5. Production of anti-HIV peptide lectins in plant systems

Lectins are proteins of non-immune origin that selectively bind to carbohydrate moieties (Goldstein and Hayes, 1978). These proteins have been isolated from all life

forms including bacteria, viruses, algae, mushrooms, nematodes and plants. Based on plant lectin information, 12 distinct families have already been described (Van Damme et al., 1998). Lectins have been useful for several applications including pest resistance in crop plants (Peumans and van Damme, 1995), therapeutic agents for cancer treatment (Liu et al., 2009 and 2010) and as anti-viral microbicide candidates (Francois and Balzarini, 2010).

The HIV envelope is heavily populated with mainly high mannose type glycans (Doores et al., 2010; Geyer et al., 1988). It comes as no surprise that the majority of these anti-HIV lectins show an affinity for mannose moieties (Botos and Wlodaver, 2005). By interacting with the glycan residues on the viral envelope they prevent attachment and fusion. Many of these lectins have a broad range of activity against different viral clades of various serotypes and co-receptor dependability. Furthermore, some have displayed the potential to inhibit viral capture and dissemination by DC-SIGN bearing host cells (Balzarini et al., 2007; Nabatov et al., 2008).

The anti-HIV peptide lectins fall into different families with different modes of interaction with mannose glycans. Furthermore variations occur in their quaternary structures, efficacy level towards HIV and immune stimulatory effect of human cells (Barre et al., 1996; Zoilkowska and Wlodaver, 2006). Anti-HIV lectins have been reviewed extensively with regards to structure and mode of binding (Balzarini, 2006; Francois and Balzarini, 2010; Zoilkowska and Wladover, 2006). The majority of these lectins are remarkably stable across broad pH ranges and high temperatures. This allows their manipulation in expression, purification, formulation and applications as microbicides. They represent a rich source of proteins that can be developed as anti-HIV microbicides.

The first group of anti-HIV lectins was originally isolated from plants (Van Damme et al., 1987). Thereafter numerous others have been isolated from other organisms including prokaryotic algae, bacteria, fungi and nematodes (Boyd et al., 1997; Bulgheresi et al., 2006; Chiba et al., 2004; Inokoshi et al., 2001; Mori et al., 2005; Zhao et al., 2010). Recombinant expression of lectins in plants has been applied to some extent to introduce pest resistance in valuable crops or promote rhizosphere

symbiotic associations (Rovenska and Zemek, 2006; Sreevidaya et al., 2005; Wang et al., 2005). Plant lectins which show anti-HIV activity have been isolated directly from their natural source such as the case of *Galathus nivalus* agglutinin (GNA) where the bulbs of snowdrop (*G. nivalus*) contain reasonably high levels of the lectin (Van Damme et al., 1987). On the other hand, HIV inhibiting lectins such as those from prokaryotes and some plants are produced in low quantities and it is thus not feasible for direct isolation from the source (Koshte et al., 1990; O' Keefe, 2001).

Lectins isolated from cyanobacteria, red algae and fungae displayed generally higher potency than most plant lectins and have been extensively researched as topical anti-HIV microbicides. Since the proteins seem to occur in low quantities in their native host, recombinant production in alternative systems, such as plants has been explored. As lectins occur naturally in plants, it seems that production of recombinant lectins from other sources will not be problematic. However, it has come to light that plants produce two types of lectins, classical and nucleocytoplasmic (Lannoo and Van Damme, 2010; Van Damme et al., 2004). Classical lectins reside in storage organelles whilst the nucleocytoplasmic lectins occur mainly in the cytoplasm. Generally classical lectins are synthesised with signal peptides, are produced in abundance and serve a defence and storage purpose for the plant. Nucleocytoplasmic lectins are produced without any signal peptides in small quantities and are thought to play a role in regulatory processes in the plant cell (Lannoo and Van Damme, 2010). It is thus evident that in a plant cell there is a clear distinction in signalling and abundance of different lectins with different roles. Thus, heterologous production of lectins in plants could have an effect on the viability of the plant cell.

The native roles of many of these HIV neutralising lectins have not been resolved and although the majority mainly bind to mannose residues on the viral envelope, one cannot rule out the possibility that other ligands may exist in the plant cell environment which may affect their expression, accumulation or recovery from the plant matrix. Thus subcellular targeting may play an important role in the resolution of the optimal compartment for high yield lectin accumulation that is not detrimental for plant cells during heterologous expression. Here the progress that has been made with heterologous expression of anti-HIV lectins in plants is briefly reviewed. Table 2 highlights the major findings.

5.1 CV-N

Cyanovirin (CV-N) is an 11 kDa protein that was isolated from the blue-green algae *Nostoc ellipsosporum* with an EC_{50} value of 0.1 nM (Boyd et al., 1997). CV-N inhibited *in vitro* fusion of HIV-1 with target cells as well as subsequent viral spread between virus infected and uninfected cells. It displayed antiviral activity against primary and laboratory modified HIV strains of several clades including M, T and dual tropic viruses (Boyd et al., 1997). Furthermore CV-N inhibits gp120 binding to CCR5 or CXCR4 co-receptor dependant strains (Dey et al., 2000; Mori and Boyd, 2001). Anti-viral activity of CV-N against Hepatitis C (Helle et al., 2006), Ebola (Barrientos et al., 2003; Smee et al., 2008), SHIV (Boyd et al., 1997), measles and Herpes virus 6 (Dey et al., 2000) has also been reported.

CV-N has very low homology to other known protein sequences, but contains a sequence motif that is typical to the CV-N type lectin family (Boyd et al., 1997; Gustafson et al., 1997; Percudani et al., 2005; Van Damme et al., 1998). In solution, CV-N exists as a monomer or dimer depending on pH and temperature conditions (Barrientos et al., 2002 and 2004). CV-N interacts with terminal mannose residues of the oligomannose glycan structures of gp120 (Bewley and Otero-Quintero, 2001; Shenoy et al., 2001). The monomer contains two carbohydrate binding domains with different affinities to di-and trimannose respectively (Bewley and Otero-Quintero, 2001). Although anti-HIV activity has been reported for both monomeric and dimeric forms, it appears that the potency of CV-N depends more on the formation of multisite interactions with glycan residues rather than the affinity and presence of each binding domain (Barrientos et al., 2004; Fromme et al., 2007; Kelley et al., 2002).

In vitro test with host cells and CV-N displayed no or little loss of cell viability (Boyd et al., 1997) as a result of host cell exposure to CV-N. Furthermore, *in vivo* studies with gel-formulated CV-N caused no adverse effects in the test animals (Tsai et al., 2003). However, more extensive tests showed that CV-N induced the production of chemokines and cytokines and stimulated cell proliferation (Huskens et al., 2008).

This cytotoxicity was however not linked to its carbohydrate binding property. Thus with further development such as mutations or PEGylations (Zappe et al., 2008) that could potentially reduce the cytotoxicity of CV-N, the lectin might still be considered as a potential microbicide.

CV-N has been used in ground-breaking microbicide development work to pave the way for future development of lectins as viable microbicide molecules. It displayed the potential for lectins to be used as a gel formulated microbicide to protect against vaginal and rectal challenged with HIV (Tsai et al., 2003). CV-N has been recombinantly produced in commencial bacteria such as *Streptococcis gordonni* (Giomarelli et al., 2002; Pozzi et al., 2001) and *Lactobacillus jensinii* (Liu et al., 2006). CV-N displayed on the surface of *S. gordonni* was able to capture HIV virions, whilst if secreted from the bacteria it could bind to gp120. Recombinant *L. jensinii* were able to colonise the vagina in mice and secrete full length CV-N. *L. jensinii* produced CV-N was able to inhibit CCR5 HIV *in vitro* in nanomolar concentrations.

Several fusions of CV-N have been explored with different applications in mind: for example, to form high potency chimaeras CV-N has been fused to the broadly neutralising b12 antibody (Sexton et al., 2009) and to the linear 12pi peptide (McFadden et al., 2007). Both partners in the fusions were active and the new chimeras displayed similar stability and higher antiviral activity. CV-N was also fused to a *Pseudomonas* exotoxin A (Mori et al., 1997). The chimeric protein potently eliminated HIV infected cells that expressed gp120 on their surface.

The recombinant expression of CV-N in alternative systems has recently been reviewed by Xiong et al. (2010). In brief, initial production and purification in *Escherichia coli* was not optimal, resulting in low levels of CV-N accumulation. Further optimisations resulted in high accumulation levels but consisted of a heterogeneous CV-N population of intact, truncated and signal peptide containing CV-N forms. Chaperone fusions of CV-N resulted in homogenous CV-N that accumulated to 100 mg/L. Expression of CV-N was also pursued in yeast, which only resulted in low yields of non- functional protein.

Sexton et al. (2006) showed that it is feasible to produce CV-N in tobacco plants as well as hydroponic cultures. The CV-N gene was transformed into *N. tabacum* and expressed under the 35S CaMV constitutive promoter and ER targeting signal peptide. CV-N accumulated to 130 ng/mg fresh leaf weight (or 0.85 % TSP). Hydroponic cultures derived from the transgenic plants secreted CV-N at 0.4 ug/ml. Crude CV-N extracts from tobacco were able to inhibit HIV infection of TZM-bl cells comparable to that of purified *E. coli* derived CV-N. CV-N was also produced in tobacco as a fusion with the monoclonal antibody b12 (Sexton et al., 2009). The fusion accumulated at 2.45 ug/ml and was more active than CV-N or b12 alone.

5.2. GRFT

Griffithsin (GRFT) was isolated from the red algae *Griffithsia* (Mori et al., 2005). Its 121 amino acid sequence contains an unknown amino acid at position 31 and codes for a 12.7 kDa protein that is sequence unrelated to any known protein. In its folded state, the monomer displays the β-prism-I motif found in other lectins such as Jacalin, whilst the dimer is formed by a unique domain swopping between two GRFT molecules that are not typically found in this lectin family (Zoilkowska et al., 2006).

GRFT displayed broad activity against corona viruses and HIV (O'Keefe et al., 2010). GRFT has shown antiviral activity against HIV clades A, B and C which is prevalent in sub-Saharan Africa, India and the West. It is active against both clinical and laboratory adapted T and M tropic HIV-isolates and inhibits both CCR5 and CRCX4 orientated strains. Of all the prokaryotic anti-HIV lectins, GRFT is thus far one of the most potent and promising lectins for microbicide development with an EC_{50} value as low as 0.04 nM (Mori et al., 2005). The potency of this lectin is attributed to its remarkable dimeric structure that contains six mannose binding sites that are most likely spaced for optimal interaction and subsequent cross linking of the glycans of the gp120 coat protein (Moulaei et al., 2010; Zoilkowska et al., 2006).

Any successful topically applied microbicide must ultimately be able to function in the mucosal environment. Given that the infection rate of HIV is quite rapid, the microbicide should remain stable and efficacious to neutralise HIV immediately on

contact. Its presence furthermore should not compromise tissue viability or initiate an inflammatory response. In the light of these criteria, preclinical test shows that GRFT is a good microbicide candidate. GRFT is virucidal upon contact with the virus and remains stable over several hours prior to or after application (Emau et al., 2007). GRFT is stable and functional in cervical lavage fluid over a wide pH range. Furthermore GRFT was not cytotoxic to human and primate cell lines, does not initiate an inflammatory response and did not cause adverse effects in a rabbit vaginal irritation model (Emau et al., 2007; O Keefe et al., 2009). It is likely that an effective anti-HIV therapeutic will consist of more than one microbicides to limit the risk of viral resistance. It is thus important that candidate microbicides should be compatible with other microbicides without compromising efficacy and safety of the component molecules. GRFT was tested in combination with tenofovir (nucleotide reverse transcriptase inhibitor), maraviroc (CCR5 HIV co-receptor inhibitor) and enfuvirtide (a gp41 fusion inhibitor) against calde B and C virus isolates to evaluate possible synergestic effects of the lectin in combination with other microbicides (Férir et al., 2011). When GRFT was combined with other microbicides the potency of the combination was higher than that of the individual molecules.

Recombinant production of GRFT was initially pursued in *E. coli*. Although the lectin accumulated to 819 mg/L, 33% was irreversibly lost to inclusion bodies (Giomarelli et al., 2006). Whilst the expression of GRFT in *E. coli* illustrated the feasibility of an alternative production system of functional GRFT, it remains an expensive production platform with high optimisation and maintenance demands. O'Keefe et al. (2009) used a TMV based vector system for the transient production of GRFT in the cytosol of *N. benthamiana* leaves. GRFT accumulated to more than 1 g/kg fresh weight and allowed the purification of 60 g GRFT from 226.5 kg processed leaf material. The gp120 binding potential and efficacy of the plant made GRFT were similar to *E. coli* produced and native GRFT respectively, demonstrating the potential of plant expression approaches as viable alternatives for the production of the lectin for use as a candidate microbicide.

5.3. AH

Actinohivin (AH) is a lectin isolated from the actinomycete *Longispora abida* with a reported IC_{50} value of 2 nM (Chiba et al., 2004). AH harbours a lot of potential to be developed as an anti-HIV microbicide; the lectin inhibits both T and M tropic HIV strains and is particularly potent against C clade viruses (Chiba et al., 2004; Matoba et al., 2010). Furthermore AH exhibits an impressive safety profile; the lectin did not cause proliferation or mitogenic stimulation on host cells (Hoorelbeke et al., 2010). Unlike other prokaryotic lectins, AH binds to clustered high mannose type glycans instead of single moieties (Chiba et al., 2004; Tanaka et al., 2009). It is thus possible that the cluster binding of AH confers its specificity towards glycan types that are typical of the HIV envelope and is linked to its low mitogenic effects. Matoba et al (2010) investigated a plant based production system for AH. The native gene (Chiba et al., 2004) was expressed using the Icon system. Expression levels between 20 and 120 mg/kg were obtained. When the small ubiquitin-like modifier (SUMO) was fused to the N-terminus of AH, the protein levels accumulated to over 200 mg/kg in the apoplast (Davies, 2010). The plant produced AH was able to inhibit HIV mediated syncytium formation.

6. Summary

The burden of the HIV pandemic together with the lack of an effective vaccine, has spurred the development of several microbicidal candidate molecules to curb HIV transmission. Of these, neutralising antibodies and peptide lectins have shown encouraging potency and protection against the virus in *in vivo* and *in vitro* studies. Plants present a viable option for the cost effective production of protein based candidate microbicides. We reviewed here the progress made with the production of HIV neutralising antibodies and peptide lectins in plant systems.

So far the four HIV neutralising antibodies, 2G12, b12, 2F5 and 4E10 have been successfully made in plants. Expression levels in transgenic leaves, seeds and plant cell cultures were relatively low compared to that which is obtained with transient technologies. For instance, transient infiltration using new generation viral vector technologies such as the deleted CPMV vector resulted in 2G12 accumulating to 325 mg/kg.

Generally expression in various subcellular locations is used to optimise recombinant protein yield. In the case of antibodies, this aspect of expression also influences the glycan structure of the molecule. Also, depending on plant organ, subcellular targeting can influence the overall yield and efficacy of the plant made antibody. Where ER retention of 2G12 in *Arabidopsis* seed showed no significant difference in expression levels, it resulted in almost twofold lower levels of 2G12 in maize seed. In contrast to maize seed, ER retention of 2G12 in tobacco leaves improved yields. Furthermore ER retention does not seem to be entirely optimal, leaving a small fraction of immunoglobulins that will contain immunogenic plant glycans. Separating these during downstream processing will only add to production costs.

A promising solution lies in the use of a modified host system that is incapable of producing plant glycans but instead add human type glycans to secreted proteins. Both 2G12 and 4E10 were produced in such a modified *N. benthamiana* plant. The resulting antibodies contained only human like galactosylated glycans structures. Furthermore these galactosylated antibodies were more efficacious than the CHO produced equivalent. No mention was made of the accumulation levels of the antibodies in the mutant plant, but the ability to express MAb products with a human glycan profile represents a significant step towards the production in plants of fully functional proteins that are likely to be well tolerated.

It is difficult to say which factors have the most influence on the efficacy of plant made immunoglobulins. Whilst some plant made antibodies were as active as their CHO counterparts, others were less effective and others yet up to fourfold more active. Impurities in the recovered products and glycan structure might play a role in decreasing activity, whilst aggregation and human-like glycan structures appear to improve activity as was the case for MAb 2G12 antibody produced in maize and a modified tobacco. Furthermore, fusion with other anti-HIV agents also showed an increase in the potency of the antibody against the virus.

A few lectins with highly potent anti-HIV activity have been isolated from different biological organisms. In general, these lectins do not occur in large amounts in their natural sources, or these sources are difficult to propagate. Thus recombinant

expression of the lectins was pursued in other production systems such as bacteria and yeast. With the later systems, lectin production was not optimal resulting in heterogeneous products, non-functional products or products that formed insoluble inclusion bodies. Subsequently these lectins were produced in plants with generally improved outcomes with respect to these challenges.

Three potent anti-HIV lectins, CV-N, GRFT and AH have been expressed in tobacco. Stably transgenic expression resulted in significantly lower levels of accumulated product relative to transient production of lectins. The highest accumulation level reported thus far for a lectin produced in a plant is for GRFT. By using a TMV based viral vector the lectin accumulated in tobacco leaves to more than 1g/kg protein. The AH lectin also accumulated to a good level of 120 mg/kg using the Icon viral vector system. Furthermore all the plant produced lectins reviewed here seem to retain their native efficacy against the virus.

Subcellular targeting is an important aspect to consider when producing these lectins in plants since lectin expression may affect plant processes, yields and viability of the plant cell. The lectins reviewed here have either been produced as secreted or cytosolic proteins. It is not clear which compartment suits which lectin since high levels for both a cytosolic (GRFT) and a secreted lectin (AH) have been reported. Thus each lectin that is expressed in a plant system might have to be produced in different cell compartments to evaluate the optimal expression conditions for that lectin.

Apart from expressing solitary candidate microbicides, plants were also able to produce fusions of lectins and antibodies, either with each other (antibody –lectin fusions) or with entirely different molecules. Any administered HIV microbicide will most likely consist of several compounds with a different mode of action to ensure broad maximum activity without the risk of developing resistance. By producing fusion microbicides one combines the neutralisation potential of two molecules in a single production run, with positive implications for lowering cost and increasing efficacy. Additionally fusions can stabilise the target protein to ensure higher yields, as in the example of ELP fused to anti-HIV antibodies (Floss et al., 2008). Clearly,

production of microbicidal candidate molecules offers advantages beyond simple challenges of expression of efficacious molecules.

7. Concluding remarks

After twenty years of research, plants are on the brink of entering the playing field of protein production platforms for human therapeutics. Their progress from potential to actual production platform has been facilitated largely by technical developments in vector systems and plant hosts. For a disease such as HIV, where there is a desperate demand for an effective microbicide, these advances could potentially enable plants to meet the supply gap. Although several anti-HIV neutralising antibodies and peptide lectins have been produced in plants, only two have entered clinical trials (Twyman et al., 2012). MAPP66 is a cocktail of several antibodies produced by the magnICON system in *N. benthamiana* whilst plant made 2G12 entered clinical trials in 2011 (Twyman et al., 2012). Also plant made GRFT has passed pre-clinical studies and is safe to be evaluated in clinical trials. Advancements on plant made therapeutics in clinical development such as the use in advanced broad access trials of carrot cell glucocerebrosidase provide a new perspective on the potency and utility of PMPs. The hope is that as production and purification technology are more standardised in the field, and as more plant made candidates progress along the preclinical and clinical developmental pipeline, plants will become a source of routinely used, effective therapeutic and preventative biologics.

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References

Armbruster C, Stiegler GM, Vcelar BA, Jäger W, Michael NL, Vetter N, et al. A phase I trial with two human monoclonal antibodies (hMAb 2F5, 2G12) against HIV-1. AIDS 2002;16:227- 33.

Armbruster C, Stiegler GM, Vcelar BA, Jäger W, Köller U, Jilch R, et al. Passive immunization with the anti-HIV-1 human monoclonal antibody (hMAb) 4E10 and the hMAb combination 4E10/2F5/2G12. J Antimicrob Chemother 2004;54:915-20.

Aviezer D, Brill-Almon E, Shaaltiel Y, Hashmueli S, Bartfeld D, Mizrachi S, et al. A plant-derived recombinant human glucocerebrosidase enzyme - a preclinical and phase I investigation. *PloS One* 2009;4:e4792.

Baba M. Recent advances of CCR5 antagonists. Curr Opin HIV AIDS 2006;1:367-72.

Baba TW, Liska V, Hofmann-Lehmann R, Vlasak J, Xu W, Ayehunie S, et al. Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian–human immunodeficiency virus infection. Nat Med 2000;6:200-6.

Balzarini J. Large-molecular-weight carbohydrate-binding agents as HIV entry inhibitors targeting glycoprotein gp120. Curr Opin HIV AIDS 2006;1:355-60.

Balzarini J, Van Herrewege Y, Vermeire K, Vanham G, Schols D. Carbohydrate-binding agents efficiently prevent dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN)-directed HIV-1 transmission to T lymphocytes. Mol Pharmacol 2007;71:3-11.

Banta LM, Montenegro M. Agrobacterium and plant biotechnology. In: Tzfira T, Citovsky V, editors. Agrobacterium: From biology to biotechnology. Department of Biology, Williams College, Williamstown, MA 01267, USA 2008. p.73–147.

Barre A, Van Damme EJ, Peumans WJ, Rouge P. Structure-function relationship of monocot mannose-binding lectins. Plant Physiol 1996;112:1531-40.

Barrientos LG, Gronenborn AM. The domain-swapped dimer of cyanovirin-N contains two sets of oligosaccharide binding sites in solution. Biochem Biophys Res Commun 2002;298:598–602.

Barrientos LG, O'Keefe BR, Bray M, Sanchez A, Gronenborn AM, Boyd MR. Cyanovirin-N binds to the viral surface glycoprotein, GP1, 2 and inhibits infectivity of Ebola virus 1. Antiviral Res 2003;58:47–56.

Barrientos LG, Lasala F, Delgado R, Sanchez A, Gronenborn, AM. Flipping the switch from monomeric to dimeric CV-N has little effect on antiviral activity. Structure 2004;12:1799–807.

Bemielt S, Sonnewald U, Galmbacher P, Willmitzer L, Müller M. Production of human papillomavirus type 16 virus-like particles in transgenic plants. J Virol 2003;77: 9211–20.

Bendandi M, Marillonnet S, Kandzia R, Thieme F, Nickstadt A, Herz S, et al. Rapid, high-yield production in plants of individualized idiotype vaccines for non-Hodgkin's lymphoma. Ann Oncol 2010;21:2420-7.

Bewley CA, Otero-Quintero S. The potent anti-HIV protein cyanovirin-N contains two novel carbohydrate binding sites that selectively bind to Man8 D1D3 and Man9 with nanomolar affinity: implications for binding to the HIV envelope protein gp120. J Am Chem Soc 2001;123:3892-902.

Binley JM, Wrin T, Korber B, Zwick MB, Wang M, Chappey C, et al. Comprehensive cross-clade neutralization analysis of a panel of anti-human immunodeficiency virus type 1 monoclonal antibodies. J Virol 2004;78:13232-52.

Bortesi L, Rossato M, Schuster F, Raven N, Stadlmann J, Avesani L, et al. Viral and murine interleukin-10 are correctly processed and retain their biological activity when produced in tobacco. BMC Biotechnol 2009;9:22.

Botos I, Wlodawer A. Proteins that bind high-mannose sugars of the HIV envelope. Prog Biophys Mol Biol 2005;88:233–82.

Boyd MR, Gustafson KR, McMahon JB, Shoemaker RH, O'Keefe BR, Mori T, et al. Discovery of cyanovirin-N, a novel human immunodeficiency virus-inactivating protein that binds viral surface envelope glycoprotein gp120: potential applications to microbicide development. Antimicrob Agents Chemother 1997;41:1521-30.

Buckheit Jr RW, Watson KM, Morrow KM, Ham AS. Development of topical microbicides to prevent the sexual transmission of HIV. Antivir Res 2010;85:142–58.

Bulgheresi S, Schabussova I, Chen T, Mullin NP, Maizels RM, Ott JA. A new C-type lectin similar to the human immunoreceptor DC-SIGN mediates symbiont acquisition by a marine nematode. Appl Environ Microbiol 2006;72:2950-6.

Calarese DA, Scanlan CN, Zwick MB, Deechongkit S, Mimura Y, Kunert R, et al. Antibody domain exchange is an immunological solution to carbohydrate cluster recognition. Science 2003;300:2065-71.

Campiani G, Ramunno A, Maga G, Nacci V, Fattorusso C, Catalanotti B, et al. Non-Nucleoside HIV-1 reverse transcriptase (RT) inhibitors: past, present, and future perspectives. Curr Pharm Des 2002;8:615-57.

Castilho A, Strasser R, Stadlmann J, Grass J, Jez J, Gattinger P, et al. In planta protein sialylation through over-expression of the respective mammalian pathway. J Biol Chem 2010; 285:15923-15930.

Castilho A, Bohorova N, Grass J, Bohorov O, Zeitlin L, Whaley K, et al. Rapid high yield production of different glycoforms of Ebola virus monoclonal antibody. PLoS One. 2011; 6(10):e26040.

Chen Q, He J, Phoolcharoen W, Mason HS. Geminiviral vectors based on bean yellow dwarf virus for production of vaccine antigens and monoclonal antibodies in plants. Vaccines 2011;7:331-8.

Chiba H, Inokoshi J, Nakashima H, Omura S, Tanaka H. Actinohivin, a novel anti-human immunodeficiency virus protein from an actinomycete, inhibits viral entry to cells by binding high-mannose type sugar chains of gp120. Biochem Biophys Res Commun 2004;316:203–10.

Cihlar T. Nucleotide HIV reverse transcriptase inhibitors:tenofovir and beyond. Curr Opin HIV AIDS 2006;1:373-9.

Coëffier E, Clément J, Cussac V, Khodaei-Boorane N, Jehanno M, Rojas M, et al. Antigenicity and immunogenicity of the HIV-1 gp41 epitope ELDKWA inserted into permissive sites of the MalE protein. Vaccine 2001;19:684–93.

Cutler B, Justman J. Vaginal microbicides and the prevention of HIV transmission. The Lancet Infect Diseases 2008;8:685-97.

Davis KR. Development of novel vaccines and therapeutics using plant- based expression systems. Annual Report prepared for: U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland. 2010.

De Muynck B, Navarre C, Boutry M. Production of antibodies in plants: status after twenty years. Plant Biotechnol J 2010;8:529–63.

De Rosny E, Vassell R, Jiang S, Kunert R, Weiss CD. Binding of the 2F5 monoclonal antibody to native and fusion-intermediate forms of human immunodeficiency virus type 1 gp41: implications for fusion-inducing conformational changes. J Virol 2004;78:2627-31.

Dey B, Lerner DL, Lusso P, Boyd MR, Elder JH, Berger EA. Multiple antiviral activities of cyanovirin-N: blocking of human immunodeficiency virus type 1 gp120 interaction with CD4 and coreceptor and inhibition of diverse enveloped viruses. J Virol 2000;74:4562-9.

Doncel GF, Clark MR. Preclinical evaluation of anti-HIV microbicide products: new models and biomarkers. Antiviral Res 2010;88:S10–S18.

Doores KJ, Bonomelli C, Harvey DJ, Vasiljevic S, Dwek RA, Burton DR, et al. Envelope glycans of immunodeficiency virions are almost entirely oligomannose antigens. Proc Natl Acad Sci 2010;107:13800-5.

Eggink D, Melchers M, Sanders RW. Antibodies to HIV-1: aiming at the right target. Trends Microbiol 2007;15:291–4.

Emau P, Tian B, O'Keefe B, Mori T, McMahon J, Palmer K, et al. Griffithsin, a potent HIV entry inhibitor, is an excellent candidate for anti-HIV microbicide. J Med Primatol 2007;36:244-53.

Faye L, Gomord V. Success stories in molecular farming - a brief overview. Plant Biotechnol J 2010;8:525–8.

Férir G, Palmer KE, Schols D. Synergistic activity profile of griffithsin in combination with tenofovir, maraviroc and enfuvirtide against HIV-1 clade C. Virology (Epub ahead of print).

Fischer R, Schillberg S, Hellwig S, Twyman RM, Drossard J. GMP issues for recombinant plant-derived pharmaceutical proteins. Biotechnol Adv 2012;30:434-9.

Floss DM, Sack M, Stadlmann J, Rademacher T, Scheller J, Stöger E, et al. Biochemical and functional characterization of anti-HIV antibody–ELP fusion proteins from transgenic plants. Plant Biotechnol J 2008;6:379–91.

Francois KO, Balzarini J. Potential of carbohydrate-binding agents as therapeutics against enveloped viruses. Med Res Rev 2010 Jun;23:1-39.

Franquelim HG, Chiantia S, Veiga AS, Santos NC, Schwille P, Castanho MA. Anti-HIV-1 antibodies 2F5 and 4E10 interact differently with lipids to bind their epitopes. AIDS 2011;25:419-28.

Fromme R, Katiliene Z, Giomarelli B, Bogani F, McMahon J, Mori T, et al. A monovalent mutant of cyanovirin-n provides insight into the role of multiple interactions with gp120 for antiviral activity. Biochem 2007;46:9199-207.

Garg AB, Nuttall J, Romano J. The future of HIV microbicides: challenges and opportunities. Antiviral Chem Chemother 2009;19:143-50.

Garg S, Goldman D, Krumme M, Rohan LC, Smoot S, Friend DR. Advances in development, scale-up and manufacturing of microbicide gels, films, and tablets. Antiviral Res 2010; S19-29.

Geyer H, Holschbach C, Hunsmann G, Schneider J. Carbohydrates of human immunodeficiency virus. Structures of oligosaccharides linked to the envelope glycoprotein 120. J Biol Chem 1988;263:11760-7.

Giddings G, Allison G, Brooks D, Carter A. Transgenic plants as factories for biopharmaceuticals. Nat Biotechnol 2000;18:1151–5.

Giomarelli B, Provvedi R, Meacci F, Maggi T, Medaglini D, Pozzi G, et al. The microbicide cyanovirin-N expressed on the surface of commensal bacterium Streptococcus gordonii captures HIV-1. AIDS 2002;16:1351-6.

Giomarelli B, Schumacher KM, Taylor TE, Sowder (II) RC, Hartley JL, McMahon JB, et al. Recombinant production of anti-HIV protein, griffithsin, by auto-induction in a fermentor culture. Protein Expression Purif 2006;47:194–202.

Giritch A, Marillonnet S, Engler C, Van Eldik G, Botterman J, Klimyuk V, et al. Rapid high-yield expression of full-size IgG antibodies in plants co-infected with noncompeting viral vectors. *Proc Natl Acad Sci USA* 2006;103:14701-6.

Gleba Y, Klimyuk V, Marillonnet S. Magnifection–a new platform for expressing recombinant vaccines in plants. Vaccine 2005;23:2042–8.

Goldstein IJ, Hayes CE. The lectins: Carbohydrate-binding proteins of plants and animals. Adv Carbohydr Chem Biochem 1978;35:127-340.

Gomord V, Chamberlain P, Jefferis R, Faye L. Biopharmaceutical production in plants: problems, solutions and opportunities. Trends Biotechnol 2005;23:559-65.

Gomord V, Fitchette A, Menu-Bouaouiche L, Saint-Jore-Dupas C, Plasson C, Michaud D, et al. Plant-specific glycosylation patterns in the context of therapeutic protein production. Plant Biotechnol J 2010;8:564–87.

Gustafson KR, Sowder (II) RC, Henderson LE, Cardellina (II) JH, McMahon JB, Rajamani U, et al. Isolation, primary sequence determination, and disulfide bond structure of cyanovirin-N, an anti-HIV (Human Immunodeficiency Virus) protein from the cyanobacterium Nostoc ellipsosporum. Biochem Biophys Res Commun 1997;238: 223–8.

Helle F, Wychowski C, Vu-Dac N, Gustafson KR, Voisset C, Dubuisson J. Cyanovirin-N inhibits hepatitis C virus entry by binding to envelope protein glycans. Journal Biol Chem 2006;281:25177-83.

Hessell AJ, Hangartner L, Hunter M, Havenith CEG, Beurskens FJ, Bakker JM, et al. Fc receptor but not complement binding is important in antibody protection against HIV. Nature 2007;449:101-4.

Hessell AJ, Rakasz EG, Tehrani DM, Huber M, Weisgrau KL, Landucci G, et al. Broadly neutralizing monoclonal antibodies 2F5 and 4E10 directed against the Human Immunodeficiency Virus type 1 gp41 Membrane-Proximal External Region protect against mucosal challenge by Simian-Human Immunodeficiency Virus $SHIV_{Ba-L}$. J Virol 2010;84:1302-13.

Hoorelbeke B, Huskens D, Ferir G, Francois KO, Takahashi A, Van Laethem K, et al. Actinohivin, a broadly neutralizing prokaryotic lectin, inhibits HIV-1 infection by specifically targeting high-mannose-type glycans on the gp120 envelope. Antimicrob Agents Chemother 2010;54:3287-301.

Hu Q, Frank I, Williams V, Santos JJ, Watts P, Griffin GE, et al. Blockade of attachment and fusion receptors inhibits HIV-1 infection of human cervical tissue. J Exp Med 2004;199:1065-75.

Huang Z, Phoolcharoen W, Lai H, Piensook K, Cardineau G, Zeitlin L, et al. High-level rapid production of full-size monoclonal antibodies in plants by a single-vector DNA replicon system. Biotechnol Bioeng 2010;106:9-17.

Huskens D, Vermeire K, Vandemeulebroucke E, Balzarini J, Schols D. Safety concerns for the potential use of cyanovirin-N as a microbicidal anti-HIV agent. Int J Biochem Cell Biol 2008;40:2802–14.

Inokoshi J, Chiba H, Asanuma S, Takahashi A, Omura S, Tanaka H. Molecular Cloning of actinohivin, a novel anti-HIV protein from an actinomycete, and its expression in Escherichia coli. Biochem Biophys Res Commun 2001;281:1261–5.

Jefferis R. Recombinant antibody therapeutics: the impact of glycosylation on mechanisms of action. Trends Pharmacol Sciences 2009;30:356–62.

Karim QA, Karim SSA, Frohlich JA, Grobler AC, Baxter C, Mansoor LE, et al. Effectiveness and safety of Tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. Science 2010;329:1168 – 74.

Karim SSA, Richardson BA, Ramjee G, Hoffman IF, Chirenje ZM, Taha T, et al. Safety and effectiveness of BufferGel and 0.5% PRO2000 gel for the prevention of HIV infection in women. AIDS 2011;25:957-66.

Kelley BS, Chang LC, Bewley CA. Engineering an Obligate Domain-Swapped Dimer of cyanovirin-N with enhanced anti-HIV activity. J Am Chem Soc 2002;124:3210-1.

Knäblein J. Plant-based expression of biopharmaceuticals. In: Meyers RA, editor. Encyclopedia of Molecular Cell Biology and Molecular Medicine, 2nd ed. Wiley and Sons; 2005;10:p.385-410.

Ko K, Tekoah Y, Rudd PM, Harvey DJ, Dwek RA, Spitsin S, et al. Function and glycosylation of plant-derived antiviral monoclonal antibody. Proc Natl Acad Sci USA 2003:100: 8013-8.

Koprivova A, Altman F, Gorr G, Kopriva S, Reski R, Decker EL. N-Glycosylation in the moss Physcomitrella patens is organized similarly to that in higher plants. Plant Biol 2003;5:582-91.

Koshte VL, Van Dijk W, Van Der Stelt ME, Aalberse RC. Isolation and characterization of BanLec-I, a mannoside-binding lectin from Musa paradisiac (banana). Biochem J 1990;272:721-6.

Kukuruzinska M, Lennon K. Protein N-Glycosylation: molecular genetics and functional significance. Crit Rev Oral Biol Med 1998;9:415-48.

Lannoo N, Van Damme EJ. Nucleocytoplasmic plant lectins. Biochim Biophys Acta 2010;1800:190–201.

Lau OS, Sun SS. Plant seeds as bioreactors for recombinant protein production. Biotechnol Adv 2009;27:1015–22.

Lenz O, Dittmar MT, Wagner A, Ferko B, Varauer-Uhl K, Stiegler G, et al. Trimeric membrane-anchored gp41 inhibits HIV membrane fusion. J Biol Chem 2005;280:4095-101.

Li F, Vijayasankaran N, Shen A (Y), Kiss R and Amanullah A. Cell culture processes for monoclonal antibody production. mAbs, 2010; 2: 466-77.

Li Y, O'Dell S, Walker LM, Wu X, Guenaga J, Feng Y, et al. Mechanism of neutralization by the broadly neutralizing HIV-1 monoclonal antibody VRC01. J Virol 2011;85: 8954-67.

Liu X, Lagenaur LA, Simpson DA, Essenmacher KP, Frazier-Parker CL, Liu Y, et al.. Engineered vaginal lactobacillus strain for mucosal delivery of the human

immunodeficiency virus inhibitor cyanovirin-N. Antimicrob Agents Chemother 2006;50:3250-9.

Liu B, Zhang B, Min M, Bian H, Chen L, Liu Q, et al. Induction of apoptosis by Polygonatum odoratum lectin and its molecular mechanisms in murine fibrosarcoma L929 cells. Biochim Biophys Acta 2009;1790:840–4.

Liu B, Bian H, Bao J. Plant lectins: potential antineoplastic drugs from bench to clinic. Cancer Lett (Shannon, Irel.) 2010;287:1–12.

Loos A, Van Droogenbroeck B, Hillmer S, Grass J, Kunert R, Cao J, et al. Production of monoclonal antibodies with a controlled N-glycosylation pattern in seeds of Arabidopsis thaliana. Plant Biotechnol J 2010;9:179–92.

Luo XM, Lei MY, Feidi RA, West (Jr) AP, Balazs AB, Bjorkman PJ, et al. Dimeric 2G12 as a potent protection against HIV-1. PLoS Pathog 2010;6:1661–71.

Ma JK, Drake PMW, Christou P. The production of recombinant pharmaceutical proteins in plants. Nature 2003;4:794-805.

Maclean J, Koekemoer M, Olivier AJ, Stewart D, Hitzeroth II, Rademacher T, et al. Optimization of human papillomavirus type 16 (HPV-16) L1 expression in plants: comparison of the suitability of different HPV-16 L1 gene variants and different cell-compartment localization. J GenVirol 2007;88:1460–9.

Marillonnet S, Giritch A, Gils M, Kandzia R, Klimyuk V, Gleba Y. In planta engineering of viral RNA replicons: efficient assembly by recombination of DNA modules delivered by Agrobacterium. Proc Natl Acad Sci USA 2004;101:6852-7.

Mascola JR, Stiegler G, VanCott TC, Katinger H, Carpenter CB, Hanson CE, et al. Protection of macaques against pathogenic simian/human immunodeficiency virus 89.6 PD by passive transfer of neutralizing antibodies. J Virol 1999;73: 4009-18.

Mascola JR, Stiegler G, VanCott TC, Katinger H, Carpenter CB, Hanson CE, et al. Protection of macaques against vaginal transmission of a pathogenic HIV- 1/SIV chimeric virus by passive infusion of neutralizing antibodies. Nat Med 2000;6:207-10.

Matoba N, Husk AS, Barnett BW, Pickel MM, Arntzen CJ, Montefiori DC, et al. HIV-1 neutralization profile and plant-based recombinant expression of actinohivin, an env glycan-specific lectin devoid of T-Cell mitogenic activity. PLoS One 2010;5:e11143.

Mayer KH, Peipert J, Fleming T, Fullem A, Moench T, Cu-Uvin S, et al. Safety and tolerability of BufferGel, a novel vaginal microbicide, in women in the United States. Clin Infect Dis HIV/AIDS 2001;32:476–82.

McFadden K, Cocklin S, Gopi H, Baxter S, Ajith S, Mahmood N, et al. A recombinant allosteric lectin antagonist of HIV-1 envelope gp120 interactions. Proteins: Struct Funct Bioinf 2007;67:617–29.

McGaughey GB, Citron M, Danzeisen RC, Freidinger RM, Garsky VM, Hurni WM, et al. HIV-1 vaccine development: constrained peptide immunogens show improved binding to the anti-HIV-1 gp41 MAb. Biochem 2003;42:3214-23.

McGowan I. Microbicide development. In: Celentano DD, Beyrer C, editors. Public Health Aspects of HIV/AIDS in Low and Middle Income Countries. Springer Science and Business Media; Part I; 2009.p.115-133.

Mett V, Farrance CE, Green BJ, Yusibov V. Plants as biofactories. Biologicals 2008;36:354–8.

Minces LR, McGowan I. Advances in the development of microbicides for the prevention of HIV Infection. Curr Infect Dis Rep 2010;12:56-62.

Mori T, Shoemaker RH, McMahon JB, Gulakowski RJ, Gustafson KR, Boyd MR. Construction and enhanced cytotoxicity of a cyanovirin-N-Pseudomonas exotoxin

conjugate against human immunodeficiency virus-infected cells. Biochem Biophys Res Commun 1997;239:884–8.

Mori T, Boyd MR. Cyanovirin-N, a potent human immunodeficiency virus-inactivating protein, blocks both CD4-dependent and CD4-independent binding of soluble gp120 (sgp120) to target cells, inhibits sCD4-induced binding of sgp120 to cell-associated CXCR4, and dissociates bound sgp120 from target cells. Antimicrob Agents Chemother 2001;45:664 -72.

Mori T, O'Keefe BR, Raymond C, Sowder (II) RC, Bringans S, Gardella R, et al. Isolation and characterization of griffithsin, a novel HIV-inactivating protein, from the red alga Griffithsia sp. J Biol Chem 2005;280:9345-53.

Morris G, Chindrove S, Woodhall S, Wiggins R, Vcelar B, Lacey C. A prospective randomized double blind placebo-controlled phase 1 pharmacokinetic and safety study of a vaginal microbicide gel containing three potent broadly neutralizing antibodies (2FS, 2G12, 4E10) (MabGel). Microbicides 2010 (Pittsburgh, PA), LB1, p. 229.

Morris GC and Lacey CJN. Microbicides and HIV prevention: lessons from the past, looking to the future. Curr Opin Infect Dis 2010; 23:57-63.

Moscicki A. Vaginal microbicides: where are we and where are we going? J Infect Chemother 2008;14:337-41.

Moulaei T, Shenoy SR, Giomarelli B, Thomas C, McMahon JB, Dauter Z, et al. Monomerization of viral entry inhibitor griffithsin elucidates the relationship between multivalent binding to carbohydrates and anti-HIV activity. Structure 2010;18:1104-15.

Muster T, Steindl F, Purtscher M, Trkola A, Klima A, Himmler G, et al. A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. J Virol 1993;67:6642-7.

Nabatov AA, de Jong MA, de Witte L, Bulgheresi S, Geijtenbeek TB. C-type lectin Mermaid inhibits dendritic cell mediated HIV-1 transmission to CD4+ T cells. Virology 2008;378:323–8.

O'Keefe BR. Biologically Active Proteins from Natural product Extracts. J Nat Prod 2001;64:1373-81.

O'Keefe BR, Vojdani F, Buffa V, Shattock RJ, Montefiori DC, Bakke J, et al. Scaleable manufacture of HIV-1 entry inhibitor griffithsin and validation of its safety and efficacy as a topical microbicide component. Proc Natl Acad Sci USA 2009;106:6099-104.

O'Keefe BR, Giomarelli B, Barnard DL, Shenoy SR, Chan PKS, McMahon JB, et al. Broad-spectrum in vitro activity and in vivo efficacy of the antiviral protein griffithsin against emerging viruses of the family Coronaviridae. J Virol 2010;84:2511-21.

Parren PW, Marx PA, Hessell AJ, Luckay A, Harouse J, Cheng-Mayer C, et al. Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro. J Virol 2001;75:8340-7.

Patel J, Zhu H, Menassa R, Gyenis L, Richman A, Brandle J. Elastin-like polypeptide fusions enhance the accumulation of recombinant proteins in tobacco leaves. Trans Res 2007;16:239-49.

Paul M, Ma JK-C. Plant-made pharmaceuticals: Leading products and production platforms. Biotechnol Appl Biochem 2011; 58:58-67.

Percudani R, Montanini B, Ottonello S. The Anti-HIV cyanovirin-N domain is evolutionarily conserved and occurs as a protein module in eukaryotes. Proteins: Struct Funct Bioinf 2005;60:670–8.

Peumans WJ, Van Damme EJ. Lectins as plant defense proteins. Plant Physiol 1995;109:347-52.

Pironne V, Wigdahl B, Krebs FC. The rise and fall of polyanionic inhibitors of the human immunodeficiency virus type 1. Antiviral Res 2011;90:168-82.

Pogue GP, Lindbo JA, Garger SJ, Fitzmaurice WP. Making an ally from an enemy: plant virology and the new agriculture. Annu Rev Phytopathol 2002;40:45–74.

Pozzi G, Giomarelli B, Maggi T, Medaglini D, Mori T, Boyd M. Mucosal delivery of microbicides by recombinant commensal bacteria:expression of the HIV-inactivating protein cyanovirin-N in gram-positive bacteria. AIDS 2001;15:S58.

Rademacher T, Sack M, Arcalis E, Stadlmann J, Balzer S, Altmann F, et al. Recombinant antibody 2G12 produced in maize endosperm efficiently neutralizes HIV-1 and contains predominantly single-GlcNAc N-glycans. Plant Biotechnol J 2008;6:189–201.

Ramessar K, Rademacher T, Sack M, Stadlmann J, Platis D, Stiegler G, et al. Cost-effective production of a vaginal protein microbicide to prevent HIV transmission. Proc Natl Acad Sci USA 2008;105:3727–32.

Regnard GL, Halley-Stott RP, Tanzer FL, Hitzeroth II, Rybicki EP. High level protein expression in plants through the use of a novel autonomously replicating geminivirus shuttle vector. Plant Biotechnol J 2010;8:38–46.

Reina JJ, Bernardi A, Clerici M, Rojo J. HIV microbicides: state-of-the-art and new perspectives on the development of entry inhibitors. Fut Med Chem 2010;2:1141-59.

Rodriguez M, Ramirez NI, Ayala M, Freyre F, Perez L, Triguero A, et al. Transient expression in tobacco leaves of an aglycosylated recombinant antibody against the epidermal growth factor receptor. Biotechnol Bioeng 2004;89:188-94.

Rovenská GZ, Zemek R. Host plant preference of aphids, thrips and spider mites on GNA-expressing and control potatoes. Phytopar 2006;34:139–48.

Sack M, Paetz A, Kunert R, Bomble M, Hesse F, Stiegler G, et al. Functional analysis of the broadly neutralizing human anti-HIV-1 antibody 2F5 produced in transgenic BY-2 suspension cultures. FASEB J 2007;21:1655-64.

Sainsbury F, Lavoie P, D'Aoust M, Vézina L, Lomonossoff GP. Expression of multiple proteins using full-length and deleted versions of cowpea mosaic virus RNA-2. Plant Biotechnol J 2008;6:82–92.

Sainsbury F, Lomonossoff GP. Extremely high-level and rapid transient protein production in plants without the use of viral replication. Plant Physiol 2008;148: 1212-8.

Sainsbury F, Sack M, Stadlmann J, Quendler H, Fischer R, Lomonossoff GP. Rapid transient production in plants by replicating and non-replicating vectors yields high quality functional anti-HIV antibody. PLoS One 2010;5:1-9.

Scanlan CN, Pantophlet R, Wormald MR, Saphire EO, Stanfield R, Wilson IA, et al. The broadly neutralizing anti-human immunodeficiency virus type 1 antibody 2G12 recognizes a cluster of 132 mannose residues on the outer face of gp120. J Virol 2002;76:7306–21.

Schähs M, Strasser R, Stadlmann J, Kunert R, Rademacher T, Steinkellner H. Production of a monoclonal antibody in plants with a humanized N-glycosylation pattern. Plant Biotechnol J 2007;5:657–63.

Scherer EM, Leaman DP, Zwick MB, McMichael AJ, Burton DR. Aromatic residues at the edge of the antibody combining site facilitate viral glycoprotein recognition through membrane interactions. Proc Natl Acad Sci USA 2010;107:1529-34.

Schols D. HIV coreceptor CXCR4 antagonists. Curr Opin HIV AIDS, 2006;1:361-6.

Schols D.HIV co-receptor inhibitors as novel class of anti-HIV drugs. Antiviral res 2011;71:216-26.

Sexton A, Drake PM, Mahmood N, Harman SJ, Shattock RJ, Ma JK. Transgenic plant production of cyanovirin-N, an HIV microbicide. FASEB J 2006;20:356-8.

Sexton A, Harman S, Shattock RJ, Ma JK. Design, expression, and characterization of a multivalent, combination HIV microbicide. FASEB J 2009;23:3590 -600.

Shaaltiel Y, Bartfeld D, Hashmueli S, Baum G, Brill-Almon E, Galili G, et al. Production of glucocerebrosidase with terminal mannose glycans for enzyme replacement therapy of Gaucher's disease using a plant cell system. Plant Biotechnol J 2007;5: 579–590.

Shenoy SR, O'Keefe BR, Bolmstedt AJ, Cartner LK, Boyd MR. Selective interactions of the human immunodeficiency virus-inactivating protein cyanovirin-N with high-mannose oligosaccharides on gp120 and other glycoproteins. J Pharmacol Exp Ther 2001;297:704-10.

Simek MD, Wasima R, Priddy FH, Pung P, Carrow E, Laufer DS, et al. Human immunodeficiency virus Type 1 elite neutralizers: individuals with broad and potent neutralizing activity identified by using a high-throughput neutralization assay together with an analytical selection algorithm. J Virol 2009;83:7337-48.

Smee DF, Bailey KW, Wong MH, O'Keefe BR, Gustafson KR, Mishin VP, et al. Treatment of influenza A (H1N1) virus infections in mice and ferrets with cyanovirin-N. Antiviral Res 2008;80:266–71.

Sreevidya VS, Hernandez-Oane RJ, So RB, Sullia SB, Stacey G, Ladha JK, et al. Expression of the legume symbiotic lectin genes psl and gs52 promotes rhizobial colonization of roots in rice. Plant Science 2005;169:726–36.

Strasser R, Altmann F, Mach L, Glössl J, Steinkellner H. Generation of Arabidopsis thaliana plants with complex N-glycans lacking β1,2-linked xylose and core α1,3-linked fucose. FEBS Lett 2004;561:132-6.

Strasser R, Castilho A, Stadlmann J, Kunert R, Quendler H, Gattinger P, et al. Improved virus neutralization by plant-produced anti-HIV antibodies with a homogeneous 1,4-galactosylated N-glycan profile. J Biol Chem 2009;284:20479-85.

Tanaka H, Chiba H, Inokoshi J, Kuno A, Sugai T, Takahashi A, et al. Mechanism by which the lectin actinohivin blocks HIV infection of target cells. Proc Natl Acad Sci USA 2009;106:15633-8.

Triguero A, Cabrera G, Cremata JA, Yuen C, Wheeler J, Ramírez NI. Plant-derived mouse IgG monoclonal antibody fused to KDEL endoplasmic reticulum-retention signal is N-glycosylated homogeneously throughout the plant with mostly high-mannose-type N-glycans. Plant Biotechnol J 2005;3:449–57.

Trkola A, Purtscher M, Muster T, Ballaun C, Buchacher A, Sullivan N, et al. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. J Virology 1996;70:1100-8.

Trkola A, Kuster H, Rusert P, Joos B, Fischer M, Leemann C, et al. Delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies. Nat Med 2005;11:615-622.

Tsai CC, Emau P, Jiang Y, Tian B, Morton WR, Gustafson KR. et al. Cyanovirin-N gel as a topical microbicide prevents rectal transmission of SHIV89. 6P in macaques. AIDS Res Human Retrovirus 2003;19:535–41.

Twyman RM, Stöger E, Schillberg S, Christou P, Fischer R. Molecular farming in plants: host systems and expression technology. Trends Biotechnol 2003;21:570-8.

Twyman RM, Schillberg S, Fischer R. Transgenic plants in the biopharmaceutical market. Expert Opin Emer Drugs 2005;10:185–218.

Twyman RM, Schillberg S, Fischer R. The production of vaccines and therapeutic antibodies in plants. In: Wang A, Ma S, editors. Molecular farming in plants:recent advances and future prospects. Springer, NY, p 145-159.

Van Damme EJM, Allen AK, Peumans WJ. Isolation and characterization of a lectin with exclusive specificity towards mannose from snowdrop (Galanthus nivalis) bulbs. *FEBS* Lett 1987;215:140-4.

Van Damme E, Peumans W, Barre A, Rouge P. Plant lectins: a composite of several distinct families of structurally and evolutionary related proteins with diverse biological roles. Crit Rev Plant Science 1998;17:575-692.

Van Damme L, Ramjee G, Alary M, Vuylsteke B, Chandeying V, Rees H, et al. Effectiveness of COL-1492, a nonoxynol-9 vaginal gel, on HIV-1 transmission in female sex workers: a randomised controlled trial. The Lancet 2002;360:971–7.

Van Damme EJ, Barre A, Rougé P, Peumans WJ. Cytoplasmic/nuclear plant lectins: a new story. Trends Plant Science 2004;9:484-9.

Van Der Veen MJ, van Ree R, Aalberse RC, Akkerdaas J, Koppelman SJ, Jansen HM, et al. Poor biologic activity of cross-reactive IgE directed to carbohydrate determinants of glycoproteins. J Allergy Clin Immunol. 1997;100:327-34.

Van Montfort T, Nabatov AA, Geijtenbeek TBH, Pollakis G, Paxton WA. Efficient capture of antibody neutralized HIV-1 by cells expressing DC-SIGN and transfer to CD4+ T lymphocytes. J Immun 2011;178:3177-85.

Veazy RS, Shattock RJ, Pope M, Kirijan JC, Jones J, Hu Q, et al. Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120. Nat Med 2003;9:343-6.

Veluthambi K, Gupta AK, Sharma A. The current status of plant transformation technologies. Curr Science 2003;84:368–80.

Walker LM, Phogat SK, Chan-Hui P, Wagner D, Phung P, Goss JL, et al. Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. Science 2009;326:285-8.

Wang Z, Zhang K, Sun X, Tang K, Zhang J. Enhancement of resistance to aphids by introducing the snowdrop lectin genegna into maize plants. J Biosci 2005;30:627–38.

West AP, Galimidi RP, Foglesong CP, Gnanapragasam PNP, Huey-Tubman KE, Klein JS, et al. Design and expression of a dimeric form of human immunodeficiency virus type 1 antibody 2G12 with increased neutralization potency. J Virol 2009;83:98-104.

Wu X, Yang Z, Li Y, Hogerkorp C, Schief WR, Seaman MS, et al. Rational design of envelope identifies broadly neutralizing human monoclonal antibodies to HIV-1. Science 2010;329:856–61.

Xiong S, Fan J, Kitazato K. The antiviral protein cyanovirin-N: the current state of its production and applications. Appl Microbiol Biotechnol 2010;86:805-12.

Xu H, Song L, Kim M, Holmes MA, Kraft Z, Sellhorn G, et al. Interactions between lipids and human anti-HIV antibody 4E10 can be reduced without ablating neutralizing activity. J Virol 2010;84:1076-88.

Yang J, Barr LA, Fahnestock SR, Liu Z-B. High yield recombinant silk-like protein production in transgenic plants through protein targeting. Transgenic Res 2005;14:313-24.

Yu X, Pollock D, Duval M, Lewis C, Meade H, Cavacini L, et al. Neutralization of HIV by milk expressed antibody. Poster presented at Fedation of Clinical Immunology Societes (2010) Boston.

Zappe H, Snell ME, Bossard MJ. PEGylation of cyanovirin-N, an entry inhibitor of HIV. Adv Drug Del Rev 2008;60:79-87.

Zhao S, Zhao Y, Li S, Zhao J, Zhang G, Wang H, et al. A novel lectin with highly potent antiproliferative and HIV-1 reverse transcriptase inhibitory activities from the edible wild mushroom Russula delica. Glycoconjugate J 2010;27:259-65.

Zhou T, Xu L, Dey B, Hessell AJ, Van Ryk D, Xiang SH, et al. Structural definition of a conserved neutralization epitope on HIV-1 gp120. Nature 2007;445:732–7.

Ziółkowska NE, Wlodawer A. Structural studies of algal lectins with anti-HIV activity. Acta Biochim Pol 2006;53:617–26.

Ziółkowska NE, O'Keefe BR, Mori T, Zhu C, Giomarelli B, Vojdani F, et al. Domain-swapped structure of the potent antiviral protein griffithsin and its mode of carbohydrate binding. Structure 2006;14:1127-35.

Zwick MB, Labrijn AF, Wang M, Spenlehauer C, Saphire EO, Binley JM, et al. Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. J Virol 2001;75:10892-905.

Table 1 Expression of anti-HIV neutralising antibodies in plants

Antibody	***Plant host***	***Plant organ***	***Vector system and mode of transformation***	***Subcellular targeting***	***Yield***	***N-glycan structures***	***Efficacy of plant made antibody***	**Reference**
2G12	*Zea Maize* Hi-II	Seed	Rice glutelin-1 (gt-1) endosperm promoter Transgenic transformation	ER retained	30 ug/g (T1), 33.2 ug/g (T2) and 60 ug/g (T3)	HC produced as different sized molecules. Larger glycoforms contained mainly OMT glycans, and a few had complex MMXF, MUXF and GnMXF residues. The smaller HC glycoform was a-glysosylated whilst others contained single GlcNAc residues.	*In vitro* binding activity similar than CHO derivative. Neutralisation fourfold higher than CHO equivalent.	Rademacher et al., 2008
2G12	*Zea Maize* M37W	Seed	Rice glutelin-1 (gt-1) endosperm promoter Transgenic transformation	Secreted	100 ug/g	Different glycoforms detected; more than half contained single GlcNAc, others contained complex glycans containing fucose and xylose, whilst a few contained OMT glycans.	*In vitro* binding activity was similar to CHO derivative. Neutralisation threefold higher than CHO equivalent.	Ramessar et al., 2008
2G12	*Arabidopsis* xylose and fucose mutant (*A. thaliana* ΔXT/FT)	Leaf	CaMV 35S promoter Transgenic transformation	Secreted	0.05-0.2 %TSP	Wt contained complex GnGnXF/ GnMXF/ MGnXF/MMXF type glycans. The ΔXT/FT mutant produced 2G12 contained mainly complex GnGn with a few containing OMT.	Efficacy was comparable to CHO produced 2G12 in *in vitro* ELISA binding assay.	Schähs et al., 2007,
2G12	*Arabidopsis* wt and Xylose -fucose mutant (*A. thaliana* ΔXT/FT)	Seed	Gateway vectors, β-phaseolin promoter Transgenic transformation	Secreted and ER retained	2.1 ug/mg for 2G12 secreted in XT/FT mutant, 3.6 ug/mg for 2G12 secreted in the wt and 3.0 ug/mg for 2G12 ER retained in the wt	Complex GnGnXF for secreted wt 2G12 whilst complex GnGn type glycans for 2G12 produced in ΔXT/FT. Majority of ER retained wt 2G12 carried OMT whilst a few contained GnGnXF.	Neutralisation efficacy was slightly weaker than CHO produced 2G12.	Loos et al., 2010
2G12	*N. benthamiana*	Leaf	Replicating CPMV full length vector and non-replicating deleted RNA-2 vector	Secreted and ER retained	325 mg/kg obtained with ER retention using the HT CPMV	ER retained forms contained mainly OMT with a few containing complex glycans. The secreted antibody contained complex	*In vitro* binding assays as well as neutralisation assays show plant	Sainsbury and Lomonossoff, 2008; Sainsbury et al., 2010

			(Hypertranslatable, HT) CPMV vector Transient expression			GnGnXF, GnMXF and a few with OMT glycans.	made 2G12 efficacy is comparable to CHO produced 2G12.	
2G12	*N. benthamiana* wt; *N. benthamiana* β 1,2 xylose and α1,3-fucose mutant (ΔXT/FT) and *N. benthamiana* β 1,2 xylose and α1,3-fucose mutant expressing β1,4-galactosyltransferase (GalT+ΔXT/FT)	Leaf	CaMV 35S promoter Transgenic transformation	Secreted	Not mentioned	GnGnXF (wt); GnGn (ΔXT/FT) and fully AA forms (GalT+ΔXT/FT)	Plant derived GnGn and GnGnXF forms slightly better than CHO produced 2G12 in a syncytium inhibition assay. The fully galactosylated AA was more than threefold more active than other plant glycoforms.	Strasser et al., 2009
2F5	*Nicotiana tabacum* L.cv bright yellow cell cultures (BY-2).	Cell culture	CaMV 35S Transgenic transformation	ER retained	Prior to purification accumulated to 2.9 mg/kg fresh cell weight. Post purification reached 6.44 mg/kg wet cell weight.	Not evaluated but predicted to be OMT.	BY-2 derived 2F5 similar Fc region binding but less antigen binding activity compared to CHO produced 2F5. In an HIV neutralisation assay, the BY-2 produced 2F5 was inferior to CHO produced counterpart.	Sack et al., 2007
2F5	*Nicotiana tabacum* cv. Samsun NN	Leaf	CaMV 35S Transgenic transformation	ER retained, ELP fusions	TSP of 0.1% HCLC, 0.2% HCELP-LC, 0.3% LCELP-HC and 0.6 % HCELP-LCELP	Majority OMT, a few carrying complex GnGnX and AGnX. Trace amounts of complex GnMX, GnGn and GnGnXF detected	Binding assay shows that all ELP variants and non fused plant made 2F5 antibodies had similar antigen binding ability compared to the CHO produced	Floss et al., 2008

							2F5	
b12 and b12-CV-N fusion	*Nicotiana tabacum*	Leaf	CaMV 35S Transgenic transformation	Secreted	2.45 ug/ml (b12/CV-N) and 7.35 ug/ml (b12)	Not mentioned	Both b12 and b12-CV-N fusion were able to bind gp120. In a virus neutralisation assay the b12-CV-N fusion displayed higher potency than b12 alone. No comparison made with CHO produced b12.	Sexton et al., 2009
4E10	*N. benthamiana* wt; *N. benthamiana* β 1,2 xylose and α1,3-fucose mutant (ΔXT/FT) and *N. benthamiana* β 1,2 xylose and α1,3-fucose mutant expressing β1,4-galactosyltransferase (GalT+ΔXT/FT)	Leaf	CaMV 35S promoter Transgenic transformation	Secreted	Not mentioned	GnGnXF (Wt); GnGn (ΔXT/FT) and fully AA glycans forms (GalT+ΔXT/FT)	Plant derived GnGn and GnGnXF forms performed slightly better than CHO produced 4E10 in a syncytium assay, while the fully galactosylated AA form was more than threefold as efficacious than other plant glycoforms.	Strasser et al., 2009

AA: Galactosylated residues; CaMV: Cauliflower Mosaic Virus; CHO: Chinese Hamster Ovaries (cells); CPMV: Cowpea Mosaic Virus; ER: Endoplasmic reticulum; F: Fucose residue; GlcNAc/Gn: N-acetylglucosamine residue; HC: Heavy chain; M: Mannose; OMT: Oligo-mannose type Wt: Wild type; X: Xylose residue

Table 2 Expression of anti-HIV peptide lectins in plants

Origin	***Organism***	***Lectin***	***Expression system***	***Expression host***	***Targeting***	***Levels***	***Efficacy***	***Reference***
Cyanobacterium	*Nostoc ellipsosporum*	Cyanovirin (CV-N)	CaMV based vector Transgenic transformation	*N. tabacum*	Secreted	130 ng/mg (0.85% TSP leaf) 0.4ug/ml Hydroponic	Plant made CV-N bound to gp120. Showed inhibition of syncytium formation.	Sexton et al., 2006
		CV-N b12 fusion	CaMV based vector Transgenic transformation	*Nicotiana tabacum* (var. *Xanthii*)	Not specified	2.45 ug/ml (b12/CV-N)	The b12-CV-N fusion was able to bind gp120 and in a virus neutralisation assay it displayed higher potency.	Sexton et al., 2009
Algal	*Griffithsia*	Griffitsin (GRFT)	TMV based vector Transient expression	*N. benthamiana*	Cytosol	> 1g/kg	Plant made GRFT efficacious against HIV strains of different clades and co-receptor type. Plant produced GRFT prevented HIV infection in cervical explants.	Emau et al., 2007; O'Keefe et al., 2009
Fungae	*Longispora abida*	Actinohivin lectin (AH)	TMV based deconstructed viral vector (Icon) Transient expression	*N. benthamiana*	Secreted	20-120 mg/kg	Tobacco produced AH bound to gp120 and inhibited HIV mediated syncytium formation.	Matoba et al., 2010

CaMV: Cauliflower Mosaic Virus; HIV: Human Immunodeficiency Virus; TMV: Tobacco Mosaic Virus; TSP: Total Soluble Protein