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Research review paper

## Plant made anti-HIV microbicides—A field of opportunity

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### ABSTRACT

HIV remains a significant 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, 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 rapidly gaining credibility as alternative production systems. Here we evaluate the advances made in host and vector system development for plant expression as well as the progress made in expressing HIV neutralising antibodies and peptide lectins using plant-based platforms.

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## 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 include 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; Moscicki, 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 Acidform, 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, 2011), whilst entry inhibitors bind to viral envelope components to prevent entry of the virus into the cell (Balzarini, 2006; Botos and Wlodawer, 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 (Mincos and McGowan, 2010) and the largest market segment for HIV prophylaxis resides in resource limited countries that 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; O'Keefe et al., 2009; Sexton et al., 2006). 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 in plants.

## 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, 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 certain 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 (Cunha et al., 2011a, b; 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 to improve product recovery (Paul and Ma, 2011). Thus, for plant production systems the upfront investment required for infrastructure is lower, which potentially lowers the barriers to entry by more players or especially 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 reasons for this are that the production levels in plants were often too low to be commercially viable, and regulatory approval of PMPs was perceived as being difficult to obtain. Recombinant 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; Vianna et al., 2011). 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 generally result in high protein accumulation (Gleba et al., 2005). Whilst viral vectors were useful to produce proteins in plants, these 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 significantly improved state-of-the-art technologies for 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 inappropriately immunogenic in humans (Van Der Veen et al., 1997). Another development that has done a lot to allay such fears is the recent FDA approval of a plant made therapeutic for Gaucher disease; glucocerebrosidase (also known as taliglucerase alfa or Eleyso), which is produced in carrot cells, has proven to be not only well tolerated but perhaps even more efficacious and stable—in other words, to be a “biobetter” (Aviezer et al., 2009). However, 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). Significant lower levels were obtained when the same protein was transiently produced in tobacco and potato resulting in the L1 protein accumulating to 0.5 and 0.2% of total soluble protein respectively (Biemelt 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, Germany) developed a deconstructed viral vector system initially based on Tobacco mosaic virus (TMV) in which target genes and different viral vector components are carried on several pro-module vectors (Marillonnet 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 dependent 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 RNA 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 Lomonosoff, 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, 2010). Using this system, protein expression levels exceeded 0.3 g/kg protein (Sainsbury and Lomonosoff, 2008).

Several pharmaceutically important molecules have been expressed in plants using vectors based on the ssDNA geminivirus bean yellow dwarf virus (BeYDV) (Chen et al., 2011; Huang et al. (2010) and Regnard et al. (2010) have independently developed vector systems based on the DNA genome of the virus. The BeYDV system requires only two viral components for co-expressing heteromeric proteins: these are the long intergenic region (LIR) and the short intergenic region (SIR) control sequences, and the single alternatively spliced gene for the replication-associated proteins Rep/RepA. In the system non-competing co-expression can be achieved either from two replicons encoding different proteins, or from a single replicon containing the different proteins. In *Nicotiana benthamiana*, 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. Several other geminiviruses and also the multicomponent ssDNA nanoviruses have been investigated for their potential as expression vectors; their further development could greatly expand the range of plant species that can be used for transient production of recombinant proteins (Rybicki and Martin, 2011).

#### 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 glucocerebrosidase (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 are desirable (Rodriguez et al., 2004). 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 that does not require an effector function since glycan structures are often crucial for this biological function of the protein (Jefferis, 2009). The current gel formulation of HIV neutralising antibodies (Morris and Lacey, 2010) and peptide lectins (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.

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 Protalix's carrot cell produced glucocerebrosidase, which has just received FDA approval, was 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 (Koprivova et al., 2003; Schähls et al., 2007; Strasser et al., 2009). In these mutants, plant specific  $\alpha$ 1,3-fucose and  $\beta$ 1,2-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  $\beta$ 1,4-galactosyltransferase (GalT), N-acetylglucosaminyltransferase III (GnTIII), core  $\alpha$ 1,6-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  $\alpha$ 2,6-sialyltransferase (ST) in these plant glycosylation knock-out mutants (Castilho et al., 2010, 2011; Strasser et al., 2009). Resulting proteins not only lack plant-specific glycans but also contain human glycan structures.

#### 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, 2004; Hessell et al., 2010; Mascola et al., 1999, 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 and Lacey, 2010; Morris et al., 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; McGaughey 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. Therefore 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 Man $\alpha$ 1 $\rightarrow$ 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 cytotoxicity (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  $\mu$ g/g

**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	<i>Zea Maize</i> Hi-II	Seed	Rice glutelin-1 (gt-1) endosperm promoter Transgenic transformation	ER retained	30 µg/g (T1), 33.2 µg/g (T2) and 60 µg/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-glycosylated 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	<i>Zea Maize</i> M37W	Seed	Rice glutelin-1 (gt-1) endosperm promoter Transgenic transformation	Secreted	100 µg/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	<i>Arabidopsis</i> xylose and fucose mutant ( <i>A. thaliana</i> 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	<i>Arabidopsis</i> wt and Xylose-fucose mutant ( <i>A. thaliana</i> ΔXT/FT)	Seed	Gateway vectors, β-phaseolin promoter Transgenic transformation	Secreted and ER retained	2.1 µg/mg for 2G12 secreted in XT/FT mutant, 3.6 µg/mg for 2G12 secreted in the wt and 3.0 µg/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	<i>N. benthamiana</i>	Leaf	Replicating CPMV full length vector and non-replicating deleted RNA-2 vector (Hypertranslatable, HT) CPMV vector Transient expression	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 GnGnXF, GnMXF and a few with OMT glycans.	In vitro binding assays as well as neutralisation assays show plant made 2G12 efficacy is comparable to CHO produced 2G12.	Sainsbury and Lomonosoff (2008), Sainsbury et al. (2010)
2G12	<i>N. benthamiana</i> wt; <i>N. benthamiana</i> β 1,2 xylose and α1,3-fucose mutant (ΔXT/FT) and <i>N. benthamiana</i> 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	<i>Nicotiana tabacum</i> Lcv 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	<i>Nicotiana tabacum</i> 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 2F5	Floss et al. (2008)
b12 and b12-CV-N fusion	<i>Nicotiana tabacum</i>	Leaf	CaMV 35S Transgenic transformation	Secreted	2.45 µg/ml (b12/CV-N) and 7.35 µg/ml (b12)	Not mentioned	Both b12 and b12-CV-N fusion were able to bind gp120. In a virus neutralisation assay the	Sexton et al. (2009)

Table 1 (continued)

Antibody	Plant host	Plant organ	Vector system and mode of transformation	Subcellular targeting	Yield	N-Glycan structures	Efficacy of plant made antibody	Reference
4E10	<i>N. benthamiana</i> wt; <i>N. benthamiana</i> $\beta$ 1,2 xylose and $\alpha$ 1,3-fucose mutant ( $\Delta$ XT/FT) and <i>N. benthamiana</i> $\beta$ 1,2 xylose and $\alpha$ 1,3-fucose mutant expressing $\beta$ 1,4-galactosyltransferase (GalT + $\Delta$ XT/FT)	Leaf	CaMV 35S promoter Transgenic transformation	Secreted	Not mentioned	GnGnXF (Wt); GnGn ( $\Delta$ XT/FT) and fully AA glycans forms (GalT + $\Delta$ XT/FT)	b12-CV-N fusion displayed higher potency than b12 alone. No comparison made with CHO produced b12. Plant derived GnGn and GnGnXF forms performed slightly better than CHO produced 4E10 in a syncytium assay, whilst the fully galactosylated AA form was more than three-fold 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.

in the T1 generation and 60  $\mu$ g/g in the T3 generation whilst the secreted form reached 100  $\mu$ g/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 Ovary 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 ( $\Delta$ XT/FT), that lacked the ability to generate immunogenic plant-specific  $\beta$  1,2-xylose and  $\alpha$ 1,3-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  $\Delta$ XT/FT line (Schähs et al., 2007). The glycans on the antibody that was produced in the  $\Delta$ XT/FT line were mainly terminal N-acetylglucosamine (Gn) residues that lacked plant-specific  $\beta$  1,2-xylose and  $\alpha$ 1,3-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  $\Delta$ XT/FT produced 2G12 antibody was similar to the CHO produced 2G12.

For seed expression in *Arabidopsis*  $\Delta$ XT/FT, expression of 2G12 was driven by the  $\beta$ -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  $\mu$ g/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 Lomonosoff, 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*  $\Delta$ XT/XT GalT<sup>+</sup> 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  $\beta$ 1,4-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 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; Hessel et al., 2010; Mascola et al., 1999, 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 35S CaMV 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 LCELPEL–HC, 0.2% HCELPEL–LC, 0.6% for HCELPEL–LCELPEL 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 (Franquelim 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  $\Delta$ XT/FX mutant line and in a  $\Delta$ XT/FX GalT<sup>+</sup> line that produced an altered version of the human  $\beta$ 1,4-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<sup>+</sup> 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; Veazy 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 Montfort 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 (7.55  $\mu$ g/ml) as well as b12–CV–N fusion (2.45  $\mu$ g/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, 2010) and as antiviral 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 Wlodawer, 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 dependency. 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; Ziółkowska and Wlodawer, 2006). Anti-HIV lectins have been reviewed extensively with regards to structure and mode of binding (Balzarini, 2006; Francois and Balzarini, 2010; Ziółkowska and Wlodawer, 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 (Rovenská and Zemek, 2006; Sreevidya 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 (Koshite et al., 1990; O'Keefe, 2001).

Lectins isolated from cyanobacteria, red algae and fungi 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<sub>50</sub> 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 dependent strains (Dey et al., 2000; Mori and Boyd, 2001). Antiviral 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 and Gronenborn, 2002; Barrientos et al., 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 commercial bacteria such as *Streptococcus gordonii* (Giomarelli et al., 2002; Pozzi et al., 2001) and *Lactobacillus jensinii* (Liu et al., 2006). CV-N displayed on the surface of *S. gordonii* 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 chimaeras displayed similar stability and higher antiviral activity. CV-N was also fused to a *Pseudomonas* exotoxin A (Mori et al., 1997). The chimeric protein potentially 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 µg/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 µg/ml and was more active than CV-N or b12 alone.

**Table 2**  
Expression of anti-HIV peptide lectins in plants.

Origin	Organism	Lectin	Expression system	Expression host	Targeting	Levels	Efficacy	Reference
Cyanobacterium	<i>Nostoc ellipsosporum</i>	Cyanovirin (CV-N)	CaMV based vector Transgenic transformation	<i>N. tabacum</i>	Secreted	130 ng/mg (0.85% TSP leaf)	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	<i>Nicotiana tabacum</i> (var. <i>Xanthii</i> )	Not specified	Hydroponic 2.45 µg/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	<i>Griffithsia</i>	Griffithsin (GRFT)	TMV based vector Transient expression	<i>N. benthamiana</i>	Cytosol	> 1 g/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	<i>Longispora abida</i>	Actinohivin lectin (AH)	TMV based deconstructed viral vector (Icon) Transient expression	<i>N. benthamiana</i>	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.

## 5.2. GRFT

Griffithsin (GRFT) was isolated from the red alga *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  $\beta$ -prism-I motif found in other lectins such as Jacalin, whilst the dimer is formed by a unique domain swapping between two GRFT molecules that are not typically found in this lectin family (Ziółkowska 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 that are 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<sub>50</sub> 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; Ziółkowska 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 microbicide 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 synergistic effects of the lectin in combination with other microbicides (Féris 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<sub>50</sub> 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 vector 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 (Davis, 2010). The plant-produced AH was able to inhibit HIV mediated syncytium formation.

## 6. Summary

The burden of the continuing 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 four HIV neutralising antibodies—2G12, b12, 2F5 and 4E10—have been successfully made in plants. Expression levels in transgenic plant leaves and seeds and in plant cell cultures were relatively low compared to that obtained with transient expression 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.

Expression and targeting of product to various subcellular locations are generally 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 adds 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 glycan 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—namely, CV-N, GRFT and AH—have been expressed in tobacco. Stable 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 1 g/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). In addition, plant-produced GRFT has passed pre-clinical studies and is considered safe to be evaluated in clinical trials. Advancements in plant-made therapeutics in clinical development and regulatory approval 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 become 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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