Targeted expression of the anti-HIV microbicide lectin griffithsin in maize and tobacco

By

Hester Catharina (Therese) Stark

Thesis Presented for the Degree of

Doctor of Philosophy

In the department of Molecular and Cell Biology

University of Cape Town

Cape Town

July 2013

Supervisors: Prof E.P Rybicki

Dr. R.K. Chikwamba

Declaration

I, the undersigned hereby declare that the thesis submitted herewith for the degree *Philosophiae Doctor* to the University of Cape Town, contains my own independent work and hitherto has not been submitted for any degree at any university or faculty.

HCT Stark

July 2013-07-15

AKNOWLEDGEMENTS

This research forms part of a PhD study that is funded by the Council for Scientific and Industrial Research (CSIR), Biosciences, Pretoria, South Africa.

Thank you to both my supervisors, Dr. Rachel Chikwamba and Prof. Ed Rybicki for their guidance, enthusiasm and patience.

Dr. Barry O’Keefe (Molecular Targets Laboratory, Center for Cancer Research, NCI-Frederick, MD, USA) for providing the pET GRFT vector, *E.coli* purified GRFT and rabbit produced anti-GRFT immunoglobulins.

Dr. Kabamba Alexandre and Prof. Lynn Morris (National Institute for Communicable Diseases, Johannesburg, South Africa) for executing the neutralisation assays.

Prof. Robin Shattock and Naomi Armanasco (Imperial College, United Kingdom) for their assistance in executing the neutralisation assays.

Allan Hall and especially Chris van der Merwe (Electron microscopy facility at the University of Pretoria, Pretoria, South Africa) with their assistance in preparation of plant materialand imaging with the confocal and transmission electron microscopy.

Prof. Ed Rybicki (Department of molecular and cell biology, University of Cape Town) for providing the LBA4404 strain containing pBON-NSs (originally provided by Marcel Prins, Laboratory of Virology, Wageningen, the Netherlands).

To my colleagues for their support and practical guidance.

Then to my husband and son for their support and love that kept me motivated during my studies.

PUBLICATION AND CONFERENCE PRESENTATIONS OF WORK DONE WITHIN THIS DISSERTATION

1. Poster: (BIO-08,2008), Producing Anti-HIV Microbicides in Transgenic Maize T. Lotter and RChikwamba.

2. Poster: (Cape Bioforum), Production of an Anti-HIV Microbicidal Griffithsin Peptide in Maize. Therese Stark, MM O'Kennedy, Ed Rybicki and Rachel Chikwamba.

3. Poster: (CSIR's 3rd Biennial Conference, 2010). The effect of subcellular targeting on the expression and accumulation of Griffithsin in *N. benthamiana*. Therese Stark, Ed Rybicki and Rachel Chikwamba The poster won the "Best poster award" in the health section of the conference.

4. Presentation: (Pharmaplanta Consortium meeting, Heidelberg, Germany, 2006). Expression of Rabies Chimeric MAb heavy chain (E559VH-4E10CH) and MAb light chain (E559VL-4E10CL) in Zea Mays. (T. Lotter1, K. Ramessar, N. Dube and R. Chikwamba).

5. Presentation: (EMPRO Consortium meeting, Camogli Italy, 2007). Producing anti-HIV Microbicides in Transgenic Maize.

6. Presentation: (CSIR's 3rd Biennial Conference, Young Emerging Researcher Symposium, 2010). The effect of subcellular targeting on the expression and accumulation of Griffithsin in *N. benthamiana*.

JOURNAL ARTICLES

Lotter-Stark, H.C.T., Rybicki, E.P., and Chikwamba, R.K. (2012) Plant made anti-HIV microbicides-A field of opportunity. Epub 2012 Jun 28

ABSTRACT

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. 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). One of these lectins, namely Griffithsin (GRFT) was isolated from the blue green algae *Griffithsia* and is being developed as a leading anti-HIV microbicide peptide (Mori *et al*., 2005). As literature indicates, the optimal production of any protein is an empirical experimentation with different host systems, vector systems, codon optimisations and subcellular targeting. (Maclean *et al.*, 2007; Yang *et al.*, 2005). The latter sometimes results in unexpected locations which might reflect on an inherent property of the protein itself or specifically be associated with the plant organ involved (Chikwamba *et al.*, 2003). This again can influence protein yield- and activity, and impact downstream purification. In this study we aimed to compare expression levels using both *Zea mays* (maize) and *Nicotiana benthamiana* (tobacco) with relevant vector technologies. We expressed GRFT in maize using an endosperm specific maize expression vector with and without a signal peptide. In tobacco we utilised both the pTRA binary vector and magnICON deconstructed viral vector system to express GRFT with different subcellular targeting signals. Protein extractions were prepared from transgenic maize seed and infiltrated tobacco Expression of GRFT was confirmed using immunoblotting and accumulation levels were measured using ELISA. We concluded that tobacco was the optimal expression host in which subcellular targeting resulted in different expression levels, while in maize expression was significantly lower with subcellular targeting not resulting in major differences in expression levels. Tobacco produced GRFT was tested for efficacy against the HIV virus and proved to be as efficacious as purified *E. coli* produced GRFT.

ABBREVIATIONS

AA	galactosylated
ADCC	antibody dependent cellular cytotoxcity
AGnX	galactosyl-N-acetylglucosamine-Xylose
AH	actinohivin
BeYDV	bean yellow dwarf virus
bp	base pair(s)
BSA	bovine serum albumin
Bsp	bacterial signal peptide
CAPRISA	Centtre for the AIDS programme of research in South Africa
CaMV	cauliflower mosaic virus
CCR5	c-c chemokine receptor type 5
CHO	chinese hamster ovary cells
cm^2	square centimetre
CPMV	cowpea mosaic virus
CSIR	Council for Scientific and Industrial Research
CV-N	cyanovirin
CXCR4	c-x-c chemokine receptor type 4
°C	degrees celcius
dH_2O	distilled water
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DIG	digoxigenin

DNA	deoxyribonucleic acid
DMEM	Dulbecco's modified eagle medium
ECL	enhanced chemiluminescence
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
ELP	elastin-like polypeptide
ER	endoplasmic reticulum
FBS	fetal bovine serum
FDA	food and drug administration
Fig.	figure
GFP	green fluorescent protein
GNA	*Galathus nivalus* agglutinin
GnGn	N-acetylglucosamine
GlcNAc	N-acetylglucosamine
GnMX	N-acetylglucosamine-mannose-xylose
GnMXF	N-acetylglucosamine–mannose-xylose-fucose
GP1	glycoprotein
GRFT	Griffithsin
g	gram
g/l	gram per litre
H_2O	water
HC	heavy chain

He	helium
His	histidine
HIV	human immunodeficiency virus
HT	hyper translatable
HPV	human papillomavirus
HRP	horse radish peroxidase
IC_{50}	half maximal inhibitory concentration
IDA	information dependent acquisition
kDa	kilo dalton
LB	Luria-Bertani broth
LC	light chain
LIR	long intergenic region
LT-B	heat labile toxin
M	molar
mM	millimolar
MES	2-(N-morpholino)ethanesulfonic acid
mg	milligram
ml	millilitre
mg/ml	milligram per millilitre
MAb	monoclonal antibody
MS	Murashige and Skoog
Mbar	miilibar
MTT	tetrazolium-based colorimetric

MW	molecular weight
Ni-IDA	nickle iminodiacetic acid
Nm	nano-meters
NOS	nopaline synthetase
NSs	rNA silencing suppressor
kg	kilogram
OD_{450}	optical density at 450 nm
OD_{600}	optical density at 600 nm
OMT	oligo-mannose type
pAB	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pmi	phosphomannose isomerase
PMFS	peptide mass fingerprint sequencing
PMPs	plant made pharmaceuticals
PVX	potato virus x
PVDF	polyvinylidene difluoride
RLU	relative light unit
RNA	ribonucleic acid
rpm	revolutions per minute

RPMI	Roswell Park Memorial Institute medium
scFv	single-chain variable fragment
SDS	sodium dodecyl sulphate
SE	standard error
SIR	short intergenic region
SHIV	simian immunodeficiency virus
SSC	saline-sodium citrate
SUMO	small ubiquitin-like modifier
SVN	Scytovirin
$TCID_{50}$	median tissue culture infective dose
TEV	tobacco etch virus
TMB	3, 3’, 5, 5’–tetramethylbenzidine dihydrochloride
TMV	tobacco mosaic virus
TSP	total soluble protein
Tvsp	soybean vegetative storage protein terminator
VSV	vascular stomatitis virus
Wt	wild type
UNAIDS	United Nations Programme on HIV/AIDS
UK	United Kingdom
U.S.A.	United States of America
v/v	volume per volume

α	alpha
β	beta
μ	micro
γ	gamma

TABLE OF CONTENTS

CHAPTER ONE

Literature review

CHAPTER TWO

Expression of the anti-HIV lectin griffithsin in maize seed

CHAPTER THREE

Targeted transient expression of functional griffithsin (GRFT) in *Nicotiana benthamiana*

CHAPTER FOUR

CHAPTER 1.

Plant made anti-HIV microbicides - a field of opportunity

1.1 Introduction

Recent reports show that the number of new HIV infections annually has declined by 21 % since the peak of the disease almost 15 years ago. 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 had been tested for HIV by 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). While these were supposed to be broad acting, they 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 development of surfactants 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 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 were 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. The efficacy and application of these microbicides face a huge challenge in curbing the infection and spread of each virion.

It was clear since the development of the first generation of microbicides that their success was not only dependant on the molecule itself but also on formulation, acceptance and coherence of the users. The majority of candidate microbicides requires intravaginal application. The first generation of microbicides that were tested were formulated as gel or oral applications (http://www.microbicide.org, Microbicide pipeline update, 2009). Recent microbicides are formulated in long acting, slow release intravaginal rings (IVRs), rapidly integrating vaginal films and/or monthly injectable antiretrovirals (ARVs) (Karim et al., 2013). These release microbicides over extended time periods and might well be more acceptable to use that intravaginal gels or oral capsules that have to be applied or used daily. Furthermore the use of IVR indicated a higher dose of the released microbicide than would have been obtained by daily dosing (Johnson, 2012). Like the gels, newer formulations still allow woman initiated use without the leakage complaint that was associated with gel formulations. A limitation to the use of IVRs is the solubility of the microbicide in the IVR material (Friend and Kiser, 2013). This can be addressed by developments in IVR materials such as water swelling polyurethanes (Johnson et al., 2012).

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 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; 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, I evaluate the progress made in the expression and development of peptide and antibody candidate microbicides.

1.2 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 that 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.

1.3 Challenges and developments in plant made pharmaceuticals (PMPs)

Considering the time and effort invested over more than 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 (Bosch and Schots, 2010). Data from the development of the FDA approved glucocerebrosidase, produced in carrot cells, has done a lot to allay such fears, with the plant made product proving to be not only well tolerated but perhaps even more efficacious and stable, making it 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.

1.3.1 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 (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 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, such as chloroplasts (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 magnICON 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 cotranslationally released via picornavirus 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 version of CPMV 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 allowed 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 the bean yellow dwarf virus (BeYDV) based geminivirus vectors (Chen *et al*., 2011). Huang *et al*. (2010) and Regnard *et al*. (2010) developed viral vector systems based on the DNA genome of the bean yellow dwarf mastrevirus (BeYDV). The BeYDV system requires only two viral components: these are the long intergenic region (LIR) and the short intergenic region (SIR) control sequences, and the 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 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.

1.3.2 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 that 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 glucocerebrosidase, 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-acetylglucosaminyl transferase 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.

1.4 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-binding 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 fermenter 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 1.1 summarises the expression of these four antibodies in plant systems.

1.4.1 2G12

It has been reported that 2G12 neutralises A and B clade HIV-1 virus entry by recognition of a Man$\alpha 1 \rightarrow 2$Man 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 is 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 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 (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.

1.4.2 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-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 into *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.

1.4.3 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 latter 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.

1.4.4 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.

1.5 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; Hoorelbeke *et al.* 2013; 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 were 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 that 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 fungi display 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 would 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, classic 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 1.2 highlights the major findings.

1.5.1 CV-N

Cyanovirin (CV-N) is an 11 kDa protein that was isolated from the blue-green alga *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 dependent strains (Dey *et al*., 2000; Mori and Boyd, 2001). Anti-viral activity of CV-N against Hepatitis C (Helle *et al*., 2006) and Ebola viruses (Barrientos *et al*., 2003; Smee *et al*., 2008), SHIV (Boyd *et al*., 1997), Measles virus and Herpesvirus 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 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 challenges with HIV (Tsai *et al*., 2003). CV-N has been recombinantly produced in commensal 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* at 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 chimaeric 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 the protein 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 in hydroponic cultures. The CV-N gene was transformed into *N. tabacum* and expressed under the 35S CaMV constitutive promoter with an 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 comparably to 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. Elghabi *et al.* (2011) expressed GFP-CV-N fusions in tobacco chloroplast. Constructs containing GFP-CV-N and CV-N embedded in the GFP sequences resulted in expression levels of 0.3%.

1.5.2 GRFT

Griffithsin (GRFT) was isolated from the red prokaryotic 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 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). The homodimeric GRFT molecule contains six almost identical carbohydrate binding sites (Zoilkowska *et al*., 2006; 2007). These carbohydrate binding sites contains sequences that are highly conserved. Co-crystallisation of GRFT with glucose and N-acetylglucosamine showed that all six of the carbohydrate binding sites were occupied. This multivalent interaction of GRFT with carbohydrates forms the base of its high potency. Other reported lectins with a similar monosaccharide binding mode displays antiviral activity at concentrations of more than a 1000 fold higher than that of GRFT (Zoilkowska *et al*., 2007).

GRFT displayed broad activity against SARS coronavirus, Hepatitis C virus and HIV in very low concentrations (Meuleman *et al*., 2011; Mori *et al.* 2005; O'Keefe *et al*., 2010). GRFT has shown antiviral activity against HIV clades A, B and C, which 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 tropic strains. The lectin binds to and cross-links high mannose glycans on the HIV-1 viral envelope (Mori *et al.* 2005; Moulaei *et al*., 2010). It has also been reported that binding of GRFT to HIV-1 gp120 exposes the CD4 binding site making it more accessible for interaction with neutralising antibodies (Alexandre, *et al*., 2013). Banerjee and co-workers has showed that in the presence of GRFT, immune responses in mice are elevated against gp120 (Banerjee *et al.*, 2011). Recently Hoorelbeke *et al.*(2013) has shown that GRFT is effective in preventing DC-SIGN mediated transfer of HIV-1.

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 in order 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 in order to limit the risk of viral resistance developing. It is thus important that candidate microbicides should be compatible with other microbicides without compromising the efficacy and safety of the component molecules. GRFT was combined with other microbicides and evaluated for its possible synergistic inhibition of HIV (Férir *et al*., 2011; 2012). GRFT was tested in combination with tenofovir (nucleotide reverse transcriptase inhibitor), maraviroc (CCR5 HIV co-receptor inhibitor) and enfuvirtide (a gp41 fusion inhibitor). GRFT worked synergistically with these microbicides to inhibit HIV viral isolates of clade B and C (Férir *et al*., 2011). Furthermore the synergy of GRFT with “ microbicides of the same class” was investigated (Férir *et al*., 2012). GRFT was combined with carbohydrate-binding monoclonal antibody (MAb) 2G12, microvirin (MVN), banana lectin (BanLec), *Galanthus nivalis* lectin (GNA) and *Hippeastrum* hybrid agglutinin (HHA). In all except one (HHA) GRFT displayed a synergetic effect against HIV (Férir *et al*., 2012). When GRFT was combined with other microbicides, the potency of the combination was higher than that of GRFT alone.

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 which 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.

1.5.3 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 of 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 magnICON 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.

1.5.4 SVN

SVN was isolated from cyanobacterium *Scytonema Varium* (Bokesh et al., 2003). It is one of the smallest lectins described up to know. It has an approximate molecular mass of 9.7 kDa. Its 95 amino acid sequence displays significant internal sequence duplication and it has 10 cysteines forming five intrachain disulfide bonds. Furthermore the molecule appears to have two domains, the first domain (SD1) is formed by amino acid 1-48, while the second (SD2) is formed by 49-95 (Xiong *et al.*, 2006b). Although the primary sequence of SVN resembled the chitin binding motif of the *Volvox carteri* lectin, it does not bind to chitin (Bokesh *et al.*, 2003). SVN displayed activity against laboratory and primary HIV isolates in nano-molar concentrations (Bokesh *et al.*, 2003). SVN binds to glycosylated gp120, gp160 and gp 41. Biological active SVN was produced with an N-terminus thioredoxin in *E. coli* at levels of 5-10 mg/L purified SVN (Xiong *et al.*, 2006a). Interestingly, when the SD1 peptide was produced using the thioredoxin system, it displayed the same activity as the full SVN (Xiong *et al.*, 2006b). McFeeters *et al.* (2013) showed that by modifying proteins in the SD2 domain, the HIV inhibition potential of SVN could be improved.

1.6 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. I 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 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. While ER retention of 2G12 in *Arabidopsis* seed resulted in 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 led to 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 will add human type glycans to secreted proteins. Both 2G12 and 4E10 MAbs 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 cell-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 greatest 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 yet others up to fourfold more active. Impurities in the recovered products and glycan structures might play a role in decreasing activity, whilst aggregation and human-like glycan structures appear to improve activity, as was the case for 2G12 MAb 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 organisms. In general, these lectins do not accumulate 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 latter 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. 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 use of a TMV based viral vector, the lectin accumulated in tobacco leaves to more than 1g/kg protein. The AH lectin also accumulated to a level of 120 mg/kg by means of the magnICON 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 in order to evaluate the optimal expression conditions for that lectin.

Apart from expressing solitary candidate microbicides, plants were also able to produce fusion proteins 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 different modes of action, in order 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.

1.7 Concluding remarks

After more than twenty years of research, plants are on the brink of entering the playing field as protein production platforms for human therapeutics. Their progress from potential to actual production platforms 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 assumed to be 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.

Table 1.1 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 (Hypertranslatable, HT) CPMV 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 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	Sainsbury and Lomonossoff, 2008; Sainsbury *et al*., 2010

			Transient expression				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 2F5	Floss *et al*., 2008
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	Sexton *et al*., 2009

							assay the b12-CV-N fusion displayed higher potency than b12 alone. No comparison made with CHO produced b12.	
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

Table1.2 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
		CV-N GFP and 'N GFP-CV-N C'GFP	Strong tobacco plastid rRNA operon promoter (Prrn) Transgenic transformation	*Nicotiana tabacum* (*cv. Petit Havana*)	Chloroplast	0.3% TSP	Not evaluated	Elghabi *et al*. 2011
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 (magnICON) 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

CHAPTER 2.

Expression of the anti-HIV lectin griffithsin in maize seed

2.1 INTRODUCTION

Seeds are considered by some as the flagship for plant production of therapeutic molecules, with maize seed at the forefront (Lau and Sun, 2009). The first two commercial plant-made proteins, avidin and β-glucuronidase, were produced in maize seed (Hood *et al*., 1997; Witcher *et al*., 1998). Since then, numerous other pharmaceutical proteins have been produced in this crop of which some are already commercialised - like trypsin by Sigma (Lau and Sun, 2009) while others are in the pipeline for development as potential products (reviewed by Paul and Ma, 2011).

Maize seed presents several alluring attributes for recombinant protein production, such as relatively good biomass yields, high protein content, low water content and protease activity, as well as a stable storage structure (Stoger *et al*., 2000; Rammessar *et al*., 2008; Lau and Sun, 2009). Furthermore, the seed composition is fairly homogeneous, which is advantageous for processing and purification. Also, molecular protocols for maize are well established and processing and purification can be aligned with current existing processing facilities.

Anti-HIV microbicides such as monoclonal antibodies and peptide lectins have been expressed successfully in plants, some of which expression occurred in seeds (recently reviewed by Lotter-Stark *et al*., 2012). Griffithsin (GRFT) is the most potent anti-HIV lectin reported thus far (Mori *et al.*, 2005). It displayed an excellent safety profile in animal models and human explant tissue and cells (Kouokam *et al.*, 2011; O’ Keefe *et al.*, 2009) and fared well in combination with other microbicide agents (Férir *et al.*, 2011).

Given the success of maize seed expression, I attempted the expression of the algal lectin, griffithsin (GRFT), in maize using an endosperm specific promoter. One of the considerations for production of a recombinant protein in any plant platform is the targeting of that protein to a specific compartment (Kusnadi *et al.*, 1997, Twyman *et al.*, 2005). The subcellular location of a recombinant protein can hugely influence the yield, functionality and subsequent purification of the recombinant protein. In the seed environment it appears that the inherent nature of the protein as well as tissue specific factors might also contribute to the ultimate location and accumulation levels of the protein (Chikwamba *et al.*, 2003; Hood, 2004; Drakakaki *et al.*, 2006). Thus, GRFT was produced as a secreted and non-secreted form in the endosperm of the seed of transgenic maize. I evaluated the yield, ease of purification and efficacy of the maize produced anti-HIV lectin.

2.2 MATERIALS AND METHODS

2.2.1 Construction of maize expression vectors and minimal transgene cassettes

Vector pET28 (+) containing the native griffithsin gene sequence (Mori *et al.*, 2005), was used as a template to amplify GRFT with forward primers bspGRNcoF (5’-CATGCCATGGTGAAGGTGAAGTGCTATGTGCTCTTCACTGCTCTCCTCAGC TCTCTTTGTGCTTATGGAAGCCTGACCCATCGCAAG-3’) or GRNcoF (5’-ATGCCATGGGCAGCCTGACCCATCGCAAG-3’) and reverse primer GRthrHisSacR (5’-ATGAGCTCTTAGTGATGATGATGATGATGGTGCCGCGCG GCACCAGGCCGCTGC TGTACTGTTCATAG-3’). GRFT amplification was carried out using the Long PCR Enzyme Mix (Fermentas, Thermo Scientific, GMBH). Amplification was done according to the following cycle: one cycle of 5 min at 95 °C; five cycles at 94 °C for 30 seconds, 30 °C for 30 seconds, 72 °C for 1 minute; five cycles at 94 °C for 30 seconds, 35 °C for 30 seconds, 72 °C for 1 minute; and thirty cycles at 94 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 1 minute; and 72 °C for 7 minutes. Amplified products were ligated into the pGEM-T-easy vector (Promega, Wisconsin, U.S.A.) and sequenced. Sequencing was done at the University of Cape Town. Genes were excised from the sequencing vector using *Nco*I and *Sac*I and ligated to *Nco*I and *Sac*I digested maize expression vector pRC4 (Chikwamba *et al.*, 2002a and b). All DNA fragments were gel purified using the DNA purification kit (Zymo Research, Irvine U.S.A.). All digestion enzymes were supplied by Fermentas. DNA fragments were ligated using either a Fast Link (Epicentre, Madison, U.S.A.) or T4 ligation kit (Fermentas).

2.2.2 Preparation of DNA for maize callus bombardment

Minimal transgene cassettes (Christou and Kohli, 2005) were generated for bombardment into maize Hi-II callus. The pRC4 vectors were digested with *Hind*III-*Nsb*I. Simultaneously the *pmi* (phosphomannose isomerase) selectable marker gene cassette was isolated from pNOV3604 (O'Kennedy *et al.*, 2004) using an *Asp*7181 and *Hind*III digestion. Microbicide gene cassettes were co-bombarded with the selectable marker gene cassette. Purified cassettes were coated onto 0.6 micron gold particles according to the manufacturer's recommendations (Biorad, Munich, Germany), except that extensive vortexing was used exclusively to resuspend the gold particles before coating; subsequent mixing was done by finger-tipping or short vortexing. Particle bombardment was performed with the PDS 1000/He gun (Biorad, Munich, Germany) at 650 psi.

2.2.3 Preparation of maize callus

Maize callus was prepared as follows: greenhouse-grown cobs of the Hi-II line (parent A×B offspring) were collected 10-13 days post pollination. Cobs were soaked in 70 % (v/v) ethanol for 1 minute, sterilised for 15 minutes in 2.5 % (v/v) sodium hypochlorite solution containing 0.1 % (v/v) of the surfactant Tween 20 (Merck, Darmstadt, Germany) and thoroughly rinsed with sterile distilled water. Immature zygotic embryos were aseptically excised from the florets using a dissecting microscope and placed with the scutellar side of the embryo in contact with the N6E callus induction medium (the composition of all tissue culture medium is summarised in Appendix A). Embroygenic callus was initiated for bombardment on NSE osmotic medium (NSE osm) for approximately 4 hours, where after it was bombarded with the gene cassettes. Bombarded calli were transferred to N6E medium 18 hours post bombardment to recover for approximately 4 days. Putative transgenic callus tissue was selected on N6S 12/6 media (selection media containing 12g/l mannose and 6g/l sucrose) for two weeks, then on N6S 13/4 (selection media containing 13g/l mannose and 4g/l sucrose) for another 4 weeks. Callus was matured on Reg I 26/8 media (maturation media containing 26g/l mannose and 4g/l sucrose) and regenerated on Reg II 13/4 media (regeneration media containing 13g/l mannose and 4g/l sucrose). Plantlets were rooted on ½ MS media.

2.2.4 PCR screening of putative transgenic maize

Regenerating plants were screened *via* PCR using vector and gene specific primers. DNA extraction from plants was based on the method of Dellaporta *et al.*, 1983. Briefly, leaf material was crushed in liquid nitrogen to a fine powder filling a third of the volume of a 2 ml Eppendorf tube to which 800 ul of extraction buffer (1 % Laurylsarkosyl, 100 mM Tris-HCl, 100 mM NaCl and 10 mM EDTA) was added and mixed. An equal volume of phenol (pH 7.8)/chloroform/isoamylalcohol (25:24:1) was added. Samples were vortexed and centrifuge at 5 000 rpm for 10 minutes. Supernatant was transferred to a new tube and mixed well with an equal volume of chloroform/isoamylalcohol (24:1). Samples were centrifuged as described and the supernatant transferred to a clean tube. DNA was precipitated with 1/10 volume 3 M NaAc pH 4.8 and 1 volume of isopropanol. Precipitated DNA was scooped out or centrifuged at 13 000 rpm for 30 minutes and transferred to 70 % ethanol. Samples were centrifuged at 5000 rpm for 5 minutes. The collected pellet was left until dry and dissolved in 30-100 ul TE supplemented with 2 µl of 10 mg/ml RNase. PCR screening was performed with 100 ng DNA and gene specific primers. DNA was screened for the presence of the GRFT gene using GRNcoF (described above) or GRscrF (5'-CTGACCCATCGCAAGTTCGG-3') and TvspR primers (5'-GCTCCTATTTGAATAGTGC-3'). To screen for the presence of the *pmi* gene, I used primers PMI-F (5'-CGTTGACTGAACTTTATGGTATGG-3') and PMI-R (5'-CACTCTGCTGGCTAATGGTG-3'). PCR was carried out using a 95 ºC 5 minutes denaturing step, a 30 cycle repeat consisting of 95 ºC 30 seconds, 57 ºC 45 seconds and 72 ºC 1 minute steps and final elongation for 7 minutes at 72ºC.

2.2.5 Southern blot

Southern blots were performed essentially according to the method of Southern (1975). GRFT and *pmi* probes were prepared by using a PCR dig labelling kit (Roche Biochemicals, Basil, Switzerland). DIG-labeled PCR products were cleaned using the DNA purification kit (Zymo research). Probe concentrations were established with a DIG -labeled positive control (Roche Biochemicals) in a spot assay. Approximately 12 ug of genomic DNA was digested with *Sac*I (40 u/ul, Roche Biochemicals) or *Hind*III (10 u/ul, Fermentas). Digested DNA was electrophoresed on 0.8 % TAE gel together with a DIG -labeled marker III (Roche Biochemicals). The gel was denatured by 2 × 15 minutes soak in (1.5 M NaCl; 0.5 M NaOH) at room temperature and then neutralised by 2 × 15 minutes soak in 1 M Tris-Cl pH 7.5/ 1.5 M NaCl at room temperature. Blotting was done using 20×SSC and positively charged nylon membrane (Roche Biochemicals). The membrane was prehybridised 3 to 4 hours at 42 ºC in DIG easy hybridisation solution (Roche Biochemicals; 15 ml/100 cm^2). Hybridisation was performed at 42 ºC overnight with the GRFT or *pmi* probes at 7.5 ng/ml hybridisation solution. The membrane was washed twice for 5 minutes at room temperature in 2 X wash (2 X SSC, 0.1 % SDS) and 3 × 15 minutes at 65 °C in 0.5 × wash (0.5 × SSC, 0.1 % SDS). The membrane was rinsed in wash buffer [Maleic acid buffer; 0.3% (v/v) Tween 20)] and incubated with blocking solution for 45 minutes at room temperature where after it was incubate for 30 minutes at room temperature with anti- DIG antibody (Roche) diluted 1: 50 000 in blocking solution. The membrane was washed four times for 15 min at room temperature in wash buffer (0.3 % Tween 20 in Maleic acid buffer). The membrane was equilibrated in 100 mM Tris HCl pH 9.5, 100 mM NaCl, covered with CDP-star Ready to use (Roche Biochemicals) for 5 minutes, and exposed to Amersham Hyperfilm (GE Healthcare Limited, UK) and developed after 3 hours.

2.2.6 ELISA

Kernels from Southern blot-positive events were selected for protein analysis. Endosperm was collected by hand-drilling using a Bosch hand drill. 1 X PBS buffer was added to the endosperm powder in a ratio of 1 mg: 10 ul, mixed thoroughly and incubated at 4 °C overnight. Supernatants, collected after centrifugation at 12000 rpm, were subjected to ELISA according to the method of Boyd *et al*. (1997). Briefly, wells of a 96 well ELISA plate (Nunc, Maxisorp) were coated with 100 ng gp120 (E clade gp120 provided by the National Institute for Communicable Diseases, NICD, South Africa) in PBS. Each well was blocked using 5 % fat free milk powder in PBS. Positive controls and protein extracts were incubated as 50 ul volumes in PBS. Rabbit polyclonal anti-GRFT (provided by Dr. Barry O'Keefe of the Molecular Targets Laboratory, Center for Cancer Research, NCI-Frederick, MD, USA), diluted 1:1000 in 1 X PBS, was added to the wells followed by rabbit horse radish peroxidase (HRP) conjugate (Sigma-Aldrich, A0545, Saint Louis, Missouri, U.S.A.) diluted 1:5000 in PBS. TMB (3, 3', 5, 5'–tetramethylbenzidine Dihydrochloride, Sigma-aldrich) was used as substrate for HRP, the colour reaction was stopped using 1 M H_2SO_4 and absorbance was measured at OD_{450}. Wells were washed (twice after coating and blocking steps and four times after sample and antibody incubations) with PBS-Tween 20 (0.1 % v/v, Merck). Incubation periods were carried out for 2 hours at 37 °C or overnight at 4 °C. ELISA data obtained was transformed by using the equation generated by the standard curve to calculate a ng/ul value. Knowing the amount of endosperm powder weighed, and the amount of buffer added a ng/mg value was calculated. This was then converted to mg/kg for maize.

2.2.7 Immunoblot

Protein extracts (35 ul) were electrophoresed on a 12 -15% poly-acrylamide gels. Gel to Sequi-Blot PVDF membrane (162-0184; Biorad, Hercules) transfer was performed with the semi-dry blotting system (Biorad) for approximately 1h at 15 V. Membranes were blocked in PBS supplemented with 5 % milk powder followed by incubation with anti-GRFT (1:1000) in blocking solution overnight at room temperature, followed by incubation with rabbit conjugate (1:5000) in PBS-Tween 20 (0.1 % v/v) for 2 hours. Washing steps between incubations were done 3×10 minutes with PBS-Tween 20 (0.1 % v/v). The ECL Plus Western blotting kit and ECL Hyperfilm (RPN 2124 and 28-9068-36 respectively, Amersham Life Science, Buckinghamshire, UK) were used for detection.

2.2.8 Partial purification of maize produced GRFT

Seeds were degermed and ground in a coffee grinder to a fine meal. Ground seed powder was mixed thoroughly with 1× PBS buffer (pH 7) or PBS-Tween 20 (PBS buffer containing 0.05% v/v Tween 20) in a 1:5 ratio. The suspension was incubated at 4°C overnight where after it was passed through a 100 µm mesh to retain the ground material. The milky liquid fraction was filtered through a 0.45 µm filter (Merck Millipore, Darmstadt, Germany) or centrifuged for 10 minutes at 10 000 rpm. The filtrate was concentrated using a 10 kDa membrane cut-off Vivaspin columns (Sartorius Stedim Biotech, GmbH, Goettingen, Germany). Gp120 binding ELISA (described previously) was used to determine the GRFT content in the different sample fractions.

2.2.9 Column purification of maize produced GRFT

Partially-purified maize extracts were applied onto a weak anion exchange column (PALL, Washington, U.S.A.), a C18-SPE column (Bonna Agela, Wilmington, U.S.A.), and His affinity columns (Zymo Research) for further purification and concentration. Samples were applied according to the manufacturer's guidelines. Flow through, washed and eluted fractions were evaluated by SDS-PAGE and immunoblot analysis as described previously.

2.2.10 Efficacy analysis of maize produced GRFT

Crude and partially-purified GRFT-containing maize extracts were tested for efficacy in a single-cycle neutralization assay (TZM-bl assay). The TZM-bl assay was set up with the extracts using the method previously described by Alexandre *et al.* (2010). Briefly, three-fold dilution series of GRFT in 100 µL of Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS (fetal bovine serum, growth medium) was prepared in a 96-well plate in duplicate. Two hundred $TCID_{50}$ of pseudovirus in 50 µL of growth medium was added and the mixture was incubated for 1 h at 37ºC. Then 100 µL of TZM-bl cells at a concentration of $1x10^5$ cells/mL containing 37.5 µg/mL of DEAE dextran was added to each well and cultured at 37°C for 48 hours. Infection was evaluated by measuring the activity of the firefly luciferase. Titres were calculated as the inhibitory concentration that causes 50% reduction (IC_{50}) of relative light unit (RLU) compared to the virus control (wells with no inhibitor) after the subtraction of the background (wells without both the virus and the inhibitor). As a control VSV-G (vascular stomatitis virus) was included in the assay.

Partially-purified GRFT maize extract was further evaluated for HIV inhibition against HIV-1 BaL infection of the C8166-R5 cell line. Briefly, C8166-R5 cells (0.4 x 105 cells/well) were exposed to a dilution series of the maize extracts for 1 hour prior to exposure to HIV-1BaL then cultured for a further 7 days in the presence of the maize extracts. Infection was measured by P24 ELISA. The extract was evaluated for any toxic effects using C8166-R5 cells and the MTT dye reduction assay. Briefly, C8166-R5 cells (0.4 x 105 cells/well) were exposed to a dilution series of compound for 7 days in the presence of compound. Cells were then washed with PBS and treated with MTT for 2 hours. Supernatant containing MTT was then removed and cells lysed to release the blue formazan product using 98% isopropanol with 2% 2N HCl.

2.3 RESULTS

2.3.1 Expression vector construction and minimal cassette generation

GRFT was amplified with and without the bacterial signal peptide (bsp) of the LT-B toxin (Mason *et al.*, 1998) at the 5' face and with a His-tag and threonine cleavage site at the 3'end. After sequence verifications the genes were cloned into vector pRC4 using the *Nco*I and *Sac*I sites to generate pRC-GRFT and pRC-bspGRFT maize expression vectors (Fig. 2.1 a and b).

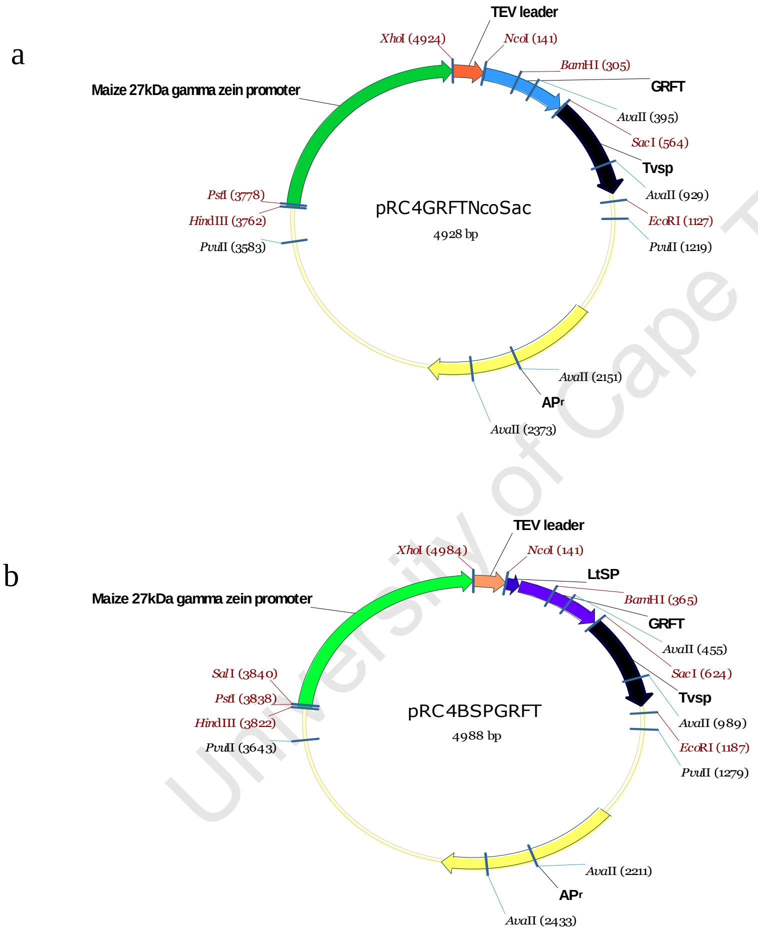

Fig. 2.1 Schematic representation of GRFT maize expression vectors pRC4-GRFT (a) and pRC4-bspGRFT (b). PCR amplified GRFT and bspGRFT (blue arrows) were cloned into the *Nco*I and *Sac*I sited of the vector. The vector contains the seed specific 27kDa γ-Zein maize promoter (Green arrow), the tobacco etch viral 5'leader sequence (TEV, orange arrow), and the soybean vegetative storage protein terminator (Tvsp terminator, black arrow).

Digestion of pRC4 maize expression vectors using *Hind*III-*NsbI* generated DNA cassettes constituting of the seed specific 27 kDa gamma zein (γ-zein) maize endosperm specific promoter (Marks *et al.*, 1985), the tobacco etch viral 5'leader sequence (TEV, Gallie *et al.*, 1995), the bspGRFT and GRFT gene followed by the soybean vegetative storage protein terminator (Tvsp terminator, Rhee and Staswick, 1992) (Fig. 2.2 a and b). A selectable marker gene cassette was generated by *Asp*7181 and *Hind*III digestion of pNOV3604. The latter cassette contained an Ubiquitin promoter, the *pmi* gene and NOS (Nopaline Synthetase) terminator (Fig. 2.2c).

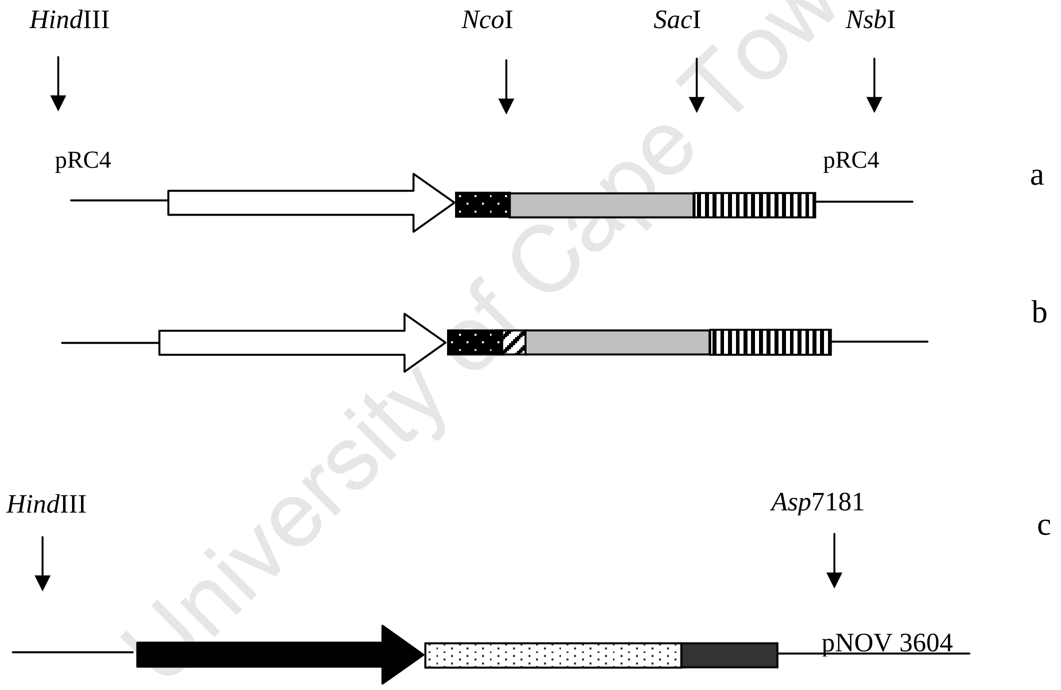

Fig. 2.2 Schematic representation of minimal cassettes generated for bombardment. Cassettes were generated containing the microbicide genes GRFT (a) and bspGRFT (b). The microbicides cassettes furthermore contained the 27 kDa γ Zein promoter (arrow); a Tobacco etch virus translational enhancer (TEV, dotted black box) and a soybean vegetative storage protein terminator (Tvsp; horizontal stripe box). GRFT (grey box) was cloned with and without the LT-B bacterial toxin signal peptide (bsp; diagonal striped boxed). The selectable marker cassette (c) contained an Ubiquitin promoter (black arrow), the *pmi* gene (white dotted box) and a NOS (Nopaline synthetase) terminator (black box).

2.3.2 PCR screening of maize transformants

Minimal cassettes containing a GRFT gene with or without a signal peptide as well as the *pmi* selectable marker gene were bombarded into maize Hi-II callus. Regenerated plantlets were screened *via* PCR using GRFT specific and *pmi* specific primers. The PCR results (Table 2.1) shows that there are no significant differences between stable integration of GRFT and bspGRFT using biolistic-mediated transformation of maize. The percentage co-integration of selectable marker gene and target gene achieved were similar for GRFT and bspGRFT, and the percentage of plants that contained either the selectable marker gene or a GRFT gene was similar between experiments. Furthermore the percentage of plants that generated seeds did not differ significantly between experiments. Thus, none of the inserts were recognised as potentially toxic and there did not seem to be a preference for seed production for cytosol expressed or secreted GRFT plants.

Table 2.1. PCR screening of regenerated plants for the presence of the GRFT and selectable marker *pmi* gene

Construct	*Plants screened*	*% Both genes*	*% Pmi only*	*% GRFT only*	*% Seed producers*
GRFT	38	84.2	2.6	2.6	71
bspGRFT	68	72.1	2.9	3.9	77.9

2.3.3 Southern blot analysis of maize GRFT transformants

Southern blot analysis was performed primarily on genomic DNA from the T1 generation of GRFT and bspGRFT transformed lines that were producing seed. For the GRFT T1 generation, genomic DNA was probed with griffithsin (GRFT) gene and *pmi* DNA probes (Fig. 2.3). In all events tested, a 100% co-integration of both genes into the genome was observed. Copy numbers varied from 3-7 for GRFT and 1-5 for *pmi*. For the bspGRFT T1 generation, genomic DNA was screened with a GRFT specific probe (Fig. 2.4). The copy number for the bspGRFT lines ranged between 1 and 6 copies. Thus the range of copy numbers for the GRFT, bspGRFT and *pmi* gene that were detected in the screened plants were similar.

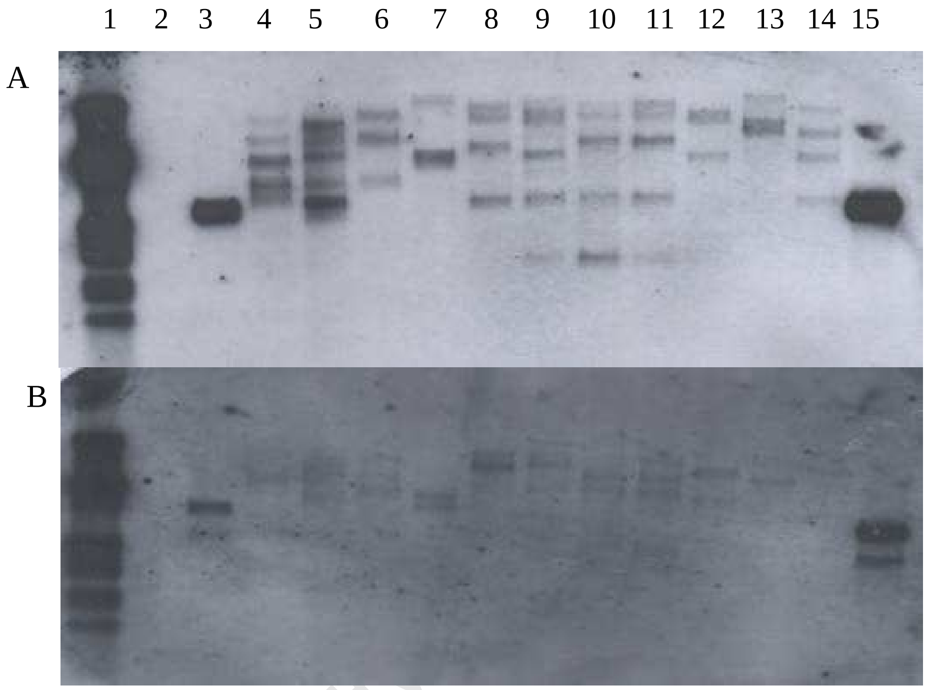

Fig. 2.3 Southern blot analysis of leaf extracted DNA from a maize T1 generation transformed with GRFT and *pmi* genes and probed with a GRFT (A) and pmi (B) specific probe. Lane 1 contains the marker, lane 2 contains untransformed Hi-II, lane 3 contains untransformed Hi-II spiked with two copies GRFT and *pmi*. Lane 15 contains untransformed Hi-II containing 20 copies GRFT and *pmi*. Subsequent lanes contain events A (lane 4), O (lane 5), B (lane 6), C (lane 7), D (lane 8), F (lane 9), H (lane 9), J (lane 10, 11), L (lane 12), N (lane 13) and I (lane 14).

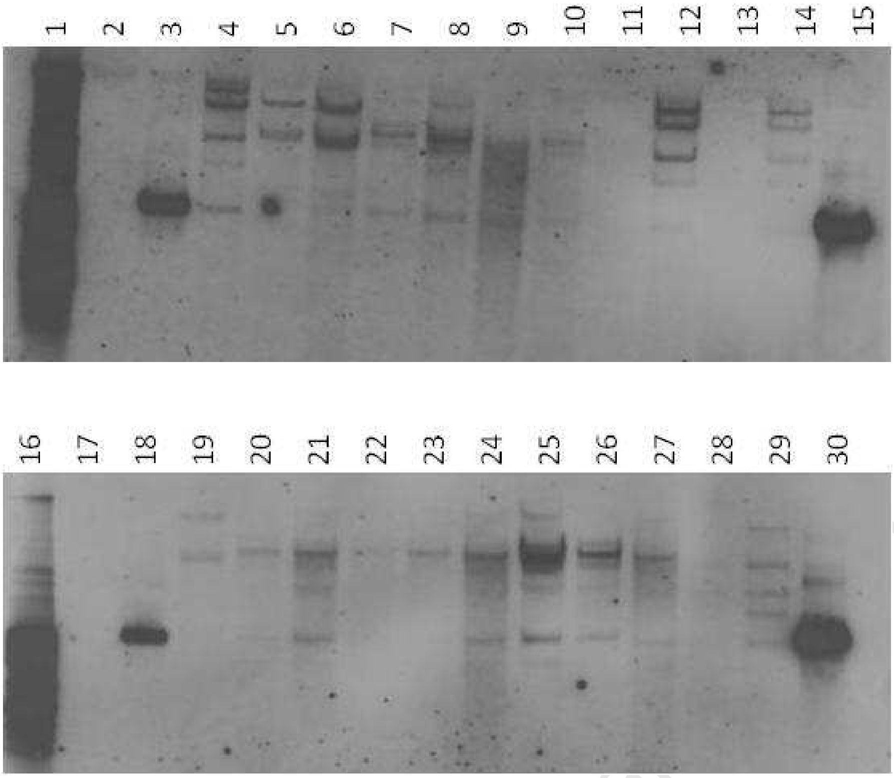

Fig. 2.4 Southern blot analysis of the T1 generation maize transformed with bspGRFT and *pmi* genes detected with a GRFT specific probe. Lane 1 and 16 contains the marker, lane 2 and 17 contains untransformed Hi-II, lane 3 and 18 contains untransformed Hi-II spiked with two copies GRFT and lane 15 and 30 contains untransformed Hi-II containing 20 copies GRFT. Subsequent lanes contain events bspGR_A (lane 4), bspGR_B (lane 5), bspGR_C (lane 6), bspGR_G (lane 7), bspGR_J (lane 8), bspGR_N (lane 9), bspGR_R (lane 10), bspGR_S (lane 11), bspGR_U (lane 12), bspGR_V (lane 13), bspGR_W (lane 14), bspGR_D (lane 19), bspGR_F (lane 20), bspGR_H (lane 21), bspGR_K (lane 22), bspGR_E (lane 23), bspGR_M (lane 24), bspGR_O (lane 25), bspGR_N (lane 26), bspGR_Q (lane 27), bspGR_Z (lane 28) and bspGR_AA (lane 29).

2.3.4 GRFT expression in maize endosperm

Endosperm was collected from seeds of mainly Southern blot-positive events. Total soluble protein (TSP) was extracted and microbicide expression levels were evaluated using ELISA. The average background ELISA value of untransformed seeds was approximately 0.37 mg/kg. In the T1 generation of plants that were transformed with the GRFT gene, expression levels of seeds above 37 mg/kg were plotted (Fig. 2.5). The majority of the transformed lines displayed expression levels between 0.37 and 2 mg/kg, while 12 lines had expression levels between 2 and 4 mg/kg and 12 lines displayed levels above 4 mg/kg. The highest expression observed was 13 mg/kg for line H1.5. The seed of this line was however not viable and did not generate a T2 generation. There was furthermore a variation of expression between lines of the same genetic event. On average, five seeds of each line were screened for GRFT expression. If the majority of these seeds displayed relatively good GRFT expression, additional seeds were also screened. These lines were selected to produce a T2 generation. Mainly seeds that showed expression levels above 1 mg/kg were planted.

Not all the drilled T1 seeds germinated and not all the T2 plants generated seeds. Thus a few T1 seeds were planted that was not drilled. These were mainly from line I since it expressed well in most of the seeds evaluated (like Ipl4). Five T1 lines were successfully advance to the T2 generation. As with T1, an average of five seeds was screened. If the majority of the five seeds in a line showed GRFT expression or if very good expression were detected for a seed, more seeds were screened from that line. Values that were above the M37W background value were plotted (Fig. 2.6). The bulk of the lines showed expression below 2 mg/kg. The highest expression level was obtained for I1.10.2 (9.4 mg/kg) and I1.10.3 (8.8 mg/kg). Their T1 parent, I1.10 expressed GRFT at 2.61 mg/kg. GRFT accumulation in G1.3.3 was also relatively high (6.2 mg/kg). The T1 parent of line G1.3 showed similar expression levels (6.4 mg/kg).

At this point some seeds of each line were selected and pooled for purification and efficacy analysis while the remaining seeds were selected to produce a T3 generation (Fig. 2.7). Like with T2 seed from good expressing lines were planted without ELISA screening. The bulk of the lines showed expression levels below 2 mg/kg. The highest expression levels were obtained for plants from line I. Since line I1 produced a lot of T3 seeds and showed fairly good expression levels it was a good candidate to produce subsequent generations. The T4 generation showed expression levels up to 4 mg/kg (Fig. 2.8).

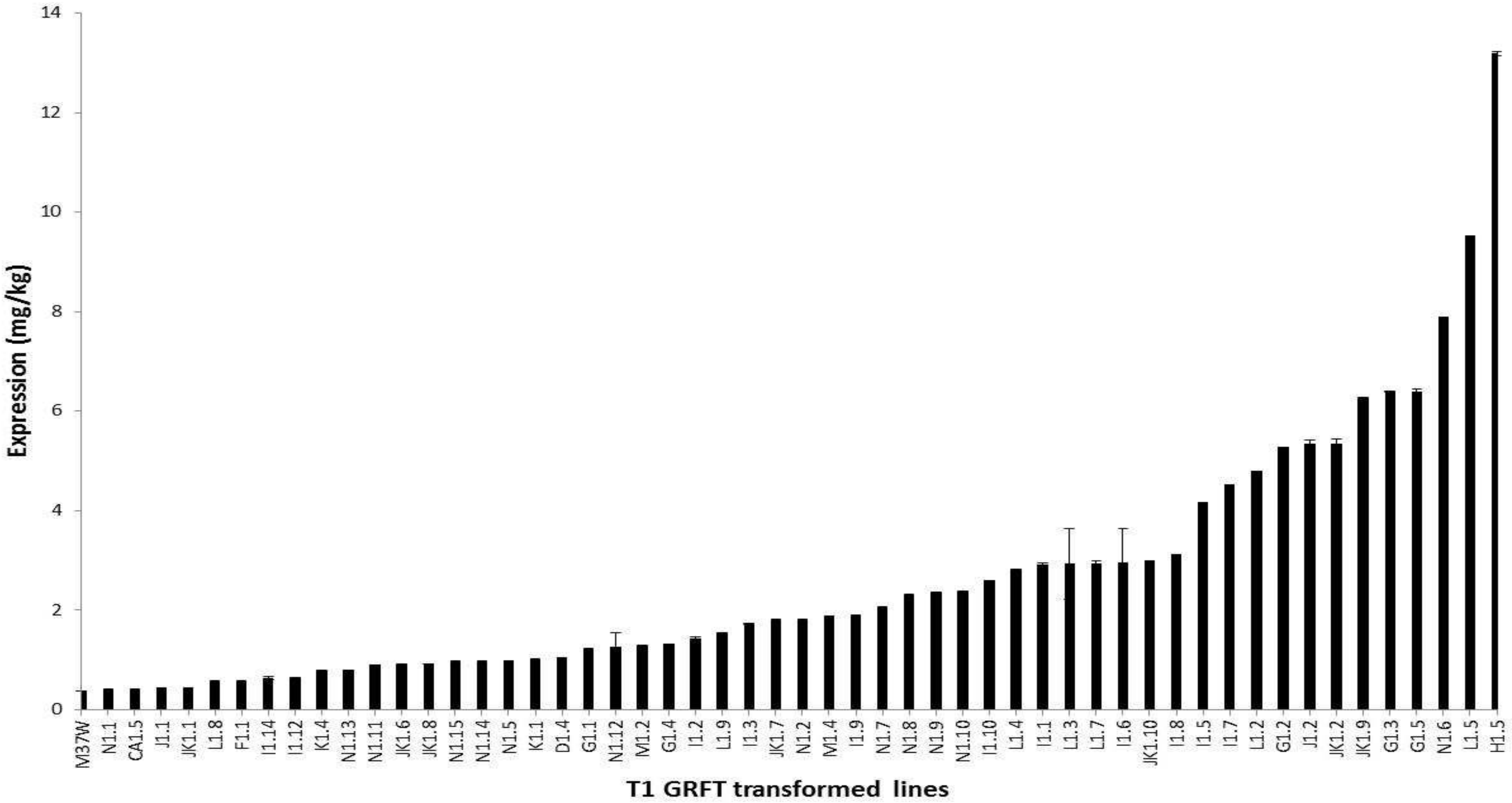

Fig. 2.5 Expression of GRFT (mg/kg) in maize T1 seed transformed with the native GRFT gene. Lines that expressed GRFT > M37W were plotted.

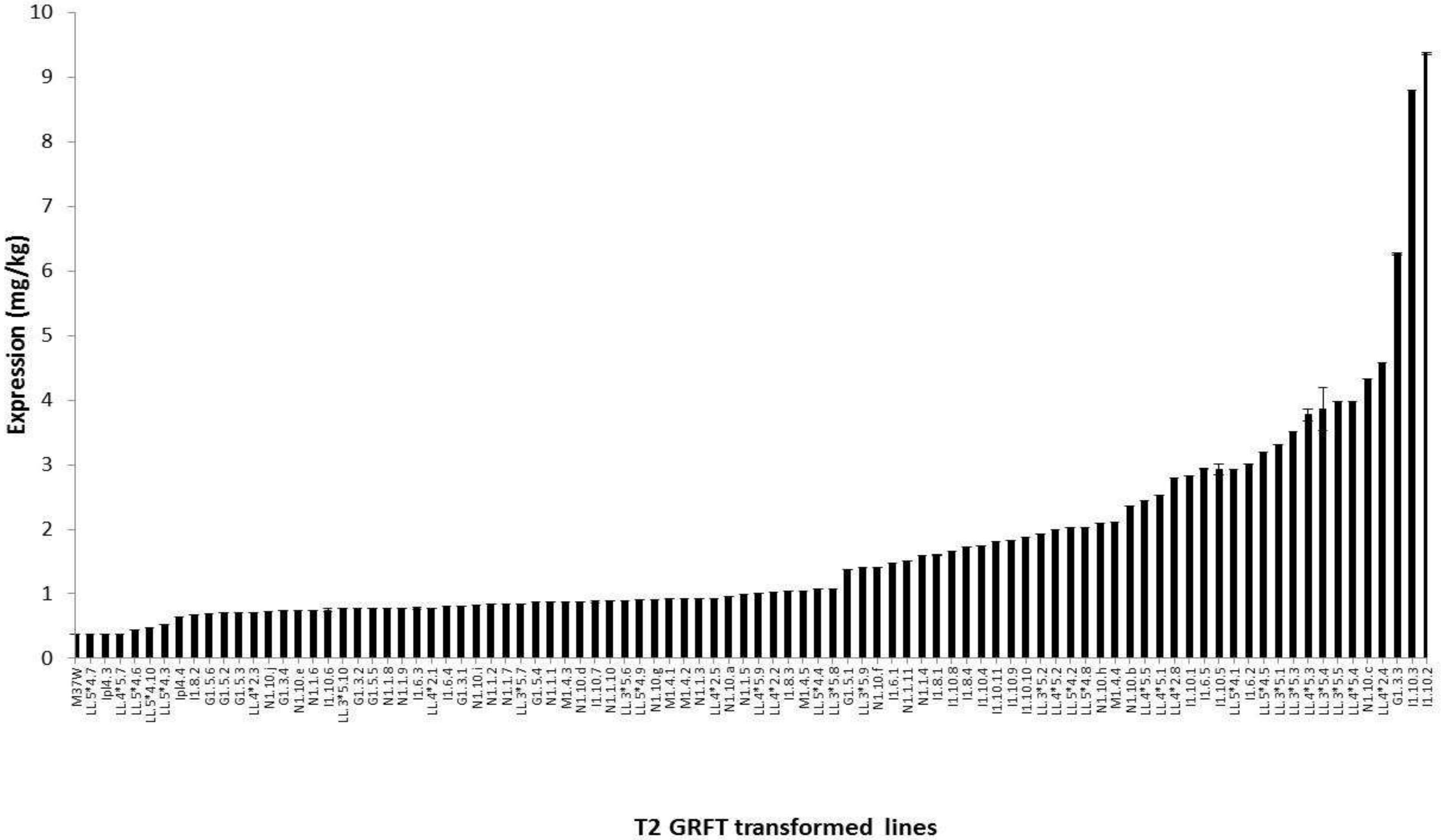

Fig. 2.6 GRFT expression (mg/kg) in seed of the T2 generation of maize lines transformed with the GRFT gene. Lines expressing GRFT above the levels of M37W are presented.

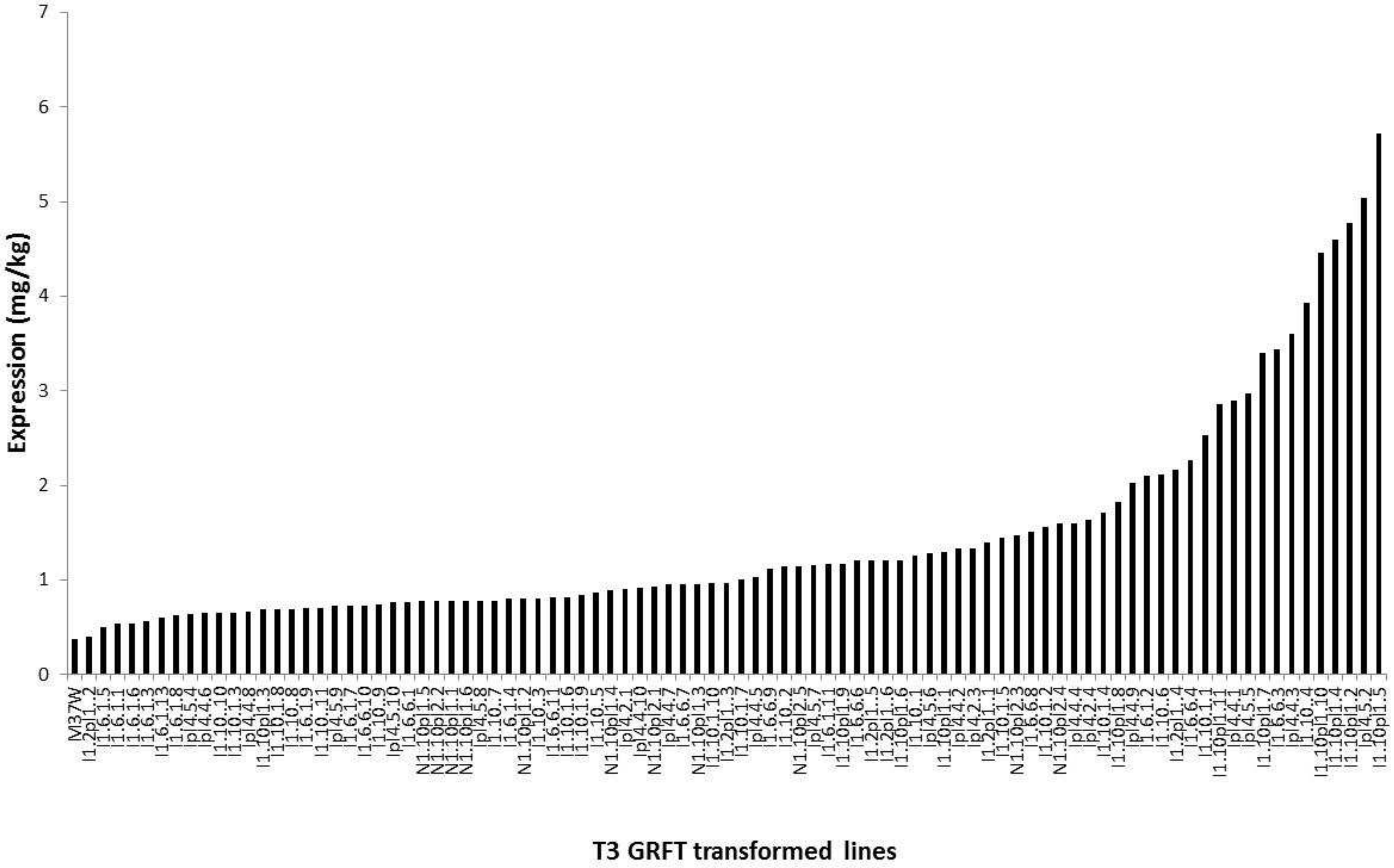

Fig. 2.7 GRFT expression (mg/kg) measured in seeds of the T3 generation of maize transformed with the GRFT gene. Lines that exhibited expression above M37W expression were plotted.

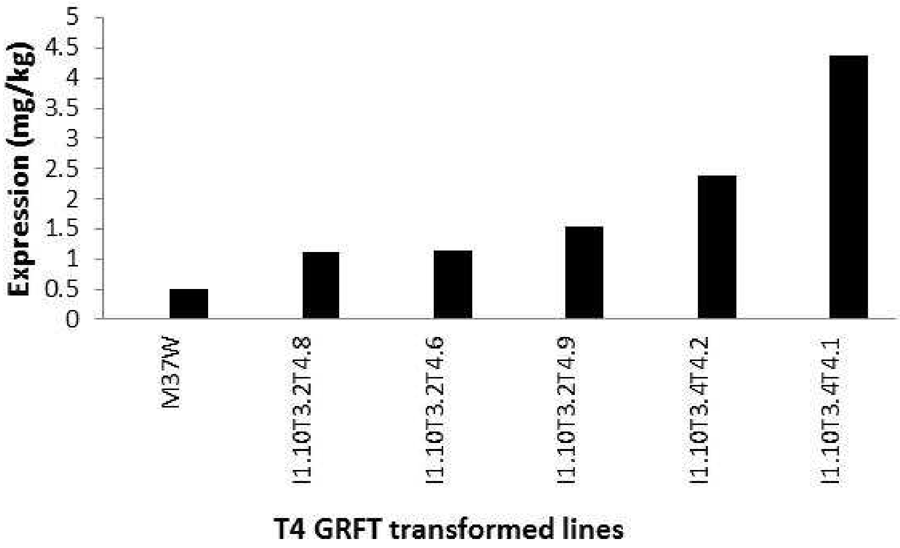

Fig. 2.8 GRFT expression (mg/kg) measured in seeds of the T4 generation of maize transformed with the GRFT gene. Lines that exhibited expression above that of M37W were plotted.

In the T1 generation of bspGRFT transformed maize, the bulk of the lines expressed between 0.37 (M37W) and 2 mg/kg (Fig. 2.9), similar to the T1 generation of GRFT transformed plants. The highest expression level detected was 3.7 mg/kg for line bspK (seed number 14). Again, expression varied between lines of the same genetic event. The same criteria as for T1 were used to generate T2 lines. Not all the drilled seed survived and those that survived did not all produce seed. In the T2 generation expression of GRFT was very low and only one line bspB.4.5 expressed GRFT above 1mg/kg. Since expression was low in the T2 generation, no further generations were produced.

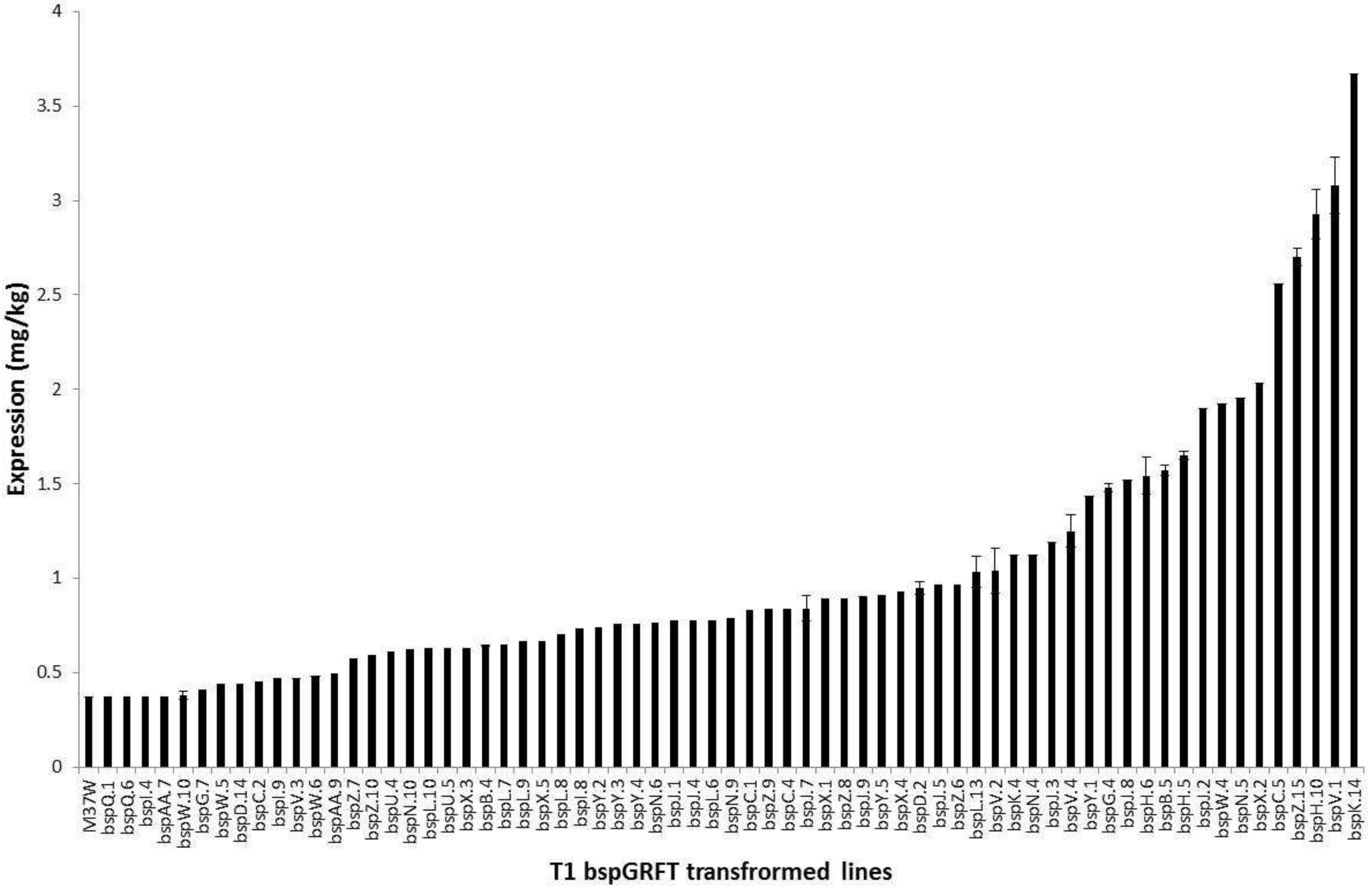

Fig. 2.9 Expression of GRFT (mg/kg) in maize seed of T1 lines transformed with the bspGRFT gene. Lines that expressed GRFT at levels greater than that of untransformed M37W are presented.

2.3.5 Immunoblot analysis of transgenic maize endosperm

Immunoblotting was performed on the endosperm protein extracts under denaturing conditions (Fig. 2.10 and 2.11). The purified *E. coli* produced GRFT was detected as a 15 kDa band with some residual dimer present as a 30 kDa band. In both GRFT transformed and untransformed maize endosperm protein extracts, bands were detected. For both the untransformed control and GRFT transformed lines a band that corresponds to 15 kDa was detected, although it seems more prominent in GRFT transformed maize extract. A band that could be the GRFT dimer (30 kDa) was observed for some GRFT samples (Fig. 2.10 lane 4 and 5; Fig. 2.11, lane 4, 5, 6 and 7). Larger bands ranging from 50 kDa to 100 kDa were also present in the GRFT extracts and untransformed maize control extracts. Apart from the 15 kDa band that was detected in the untransformed maize protein extract bands of approximately 27 kDa, 40 kDa, 50 kDa and 55 kDa were also detected.

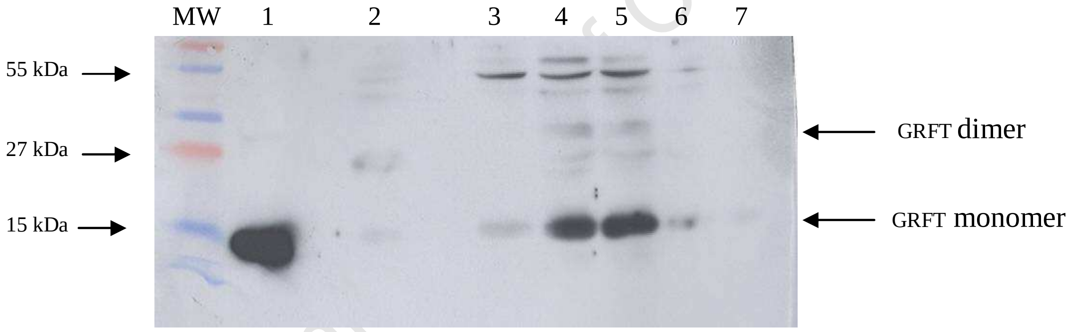

Fig. 2.10 Immunoblot analysis of endosperm extracts of T1 lines transformed with GRFT. MW is a DNA molecular weight marker (Fermentas, SM11811). Lane 1 contains *E. coli* produced recombinant GRFT (100ng). Lane 2 contains M37W untransformed endosperm protein extract. The rest of the lanes contains protein extracts from T1 GRFT transformed lines; lines K1.2 (lane 3), I1.1 (lane 4), I1.5 (lane 5), L1.2 (lane 6) and L1.5 (lane 7)

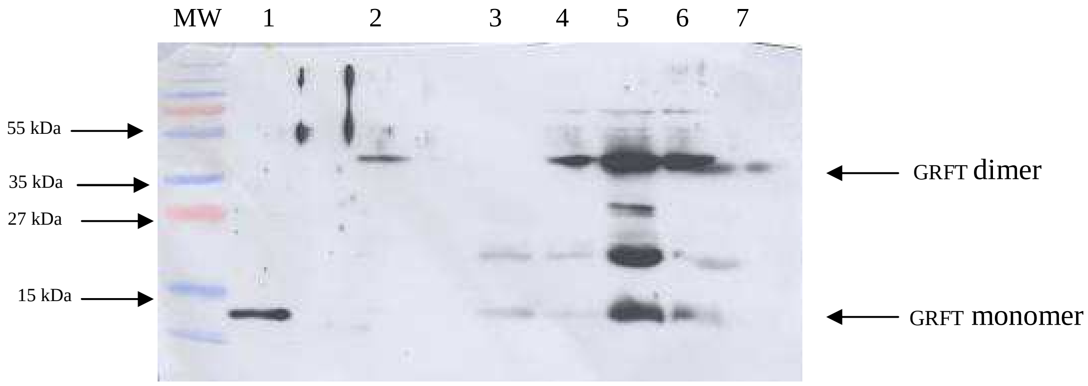

Fig. 2.11 Immunoblot analysis of endosperm extracts of T1 lines expressing GRFT. MW is a DNA molecular weight marker (Fermentas, SM11811). Lane 1 contains *E. coli* produced recombinant GRFT (100ng). Lane 2 contains Hi-II untransformed endosperm protein extract. Lane 3 to 7 contains protein extract from the endosperm of GRFT transformed lines I1.1; I1.3, I1.5; K1.1 and J1.2.

2.3.6 Partial purification of GRFT from maize endosperm extracts

GRFT maize endosperm extracts were subjected to partial purification processes prior to column purification. Protein extractions were prepared from ground maize meal using PBS buffer as well as PBS-Tween 20 (PBS-T) buffer. The GRFT content in the crude extraction was analysed by gp120 ELISA (Fig. 2.12). The OD readings for the untransformed maize were lower than the transformed maize extract (Fig. 2.12 A vs. B and C). Furthermore for the GRFT transformed maize, the presence of Tween in the extraction buffer resulted in the extraction of slightly less GRFT or it interfered marginally with the assay. Passing the crude extract through 100 µ mesh did not change the GRFT content significantly. However, filtering or centrifuging the crude extract results in a significant drop in the ELISA reading indicating a decrease in the GRFT content. Filtered maize GRFT extract was used in subsequent column purifications since using the crude extract blocked the flow through these columns.

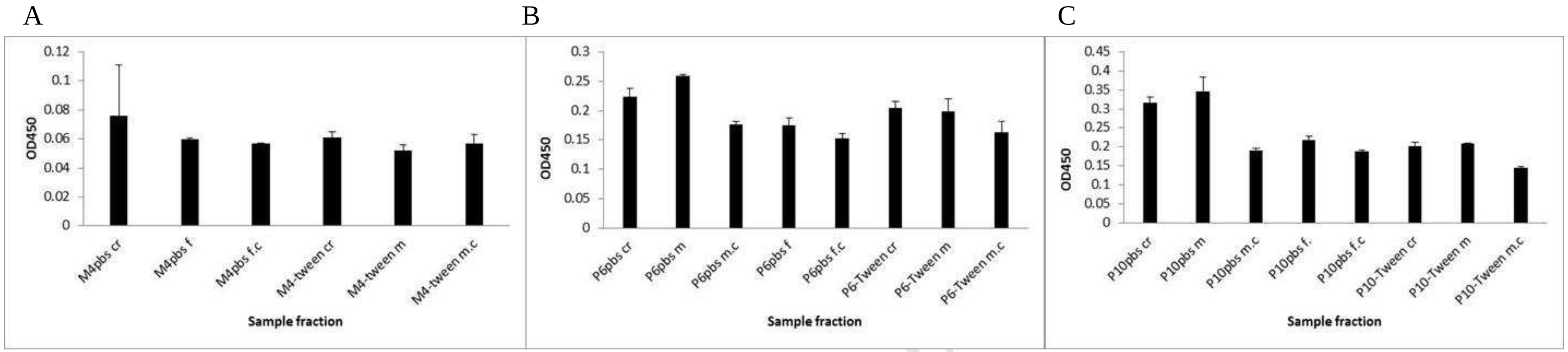

Fig. 2.12 Extraction and partial purification of GRFT maize protein extracts. Crude protein extracts (cr) were prepared from untransformed maize (A) and GRFT transformed maize (B and C) kernels. Extracts were prepared using PBS (pbs) or PBS supplemented with Tween-20 (tween) buffer. Extracts were flowed through a 100 µ mesch (m). Some of the fractions were further centrifuged (c) of filtered (f) to eliminate particles in the solution.

The filtered GRFT maize extract was applied to a weak anion exchange column. Fractions collected were evaluated on a SDS-PAGE and immunoblot (Fig. 2.13 A and B). The polyclonal GRFT antibody reacted with a protein of approximately 17 and 20 kDa in the untransformed maize GRFT extract. In the GRFT transformed extract and flow through bands that were larger than the GRFT monomer were detected, including a band that corresponds to the size of the GRFT dimer (approximately 30 kDa). A single band was present in the wash fraction but it was larger than that expected for the GRFT monomer. In the eluted fractions bands were detected that was of similar size of the *E. coli* produced GRFT monomer. The first eluted fractions also contained what seemed like a GRFT dimer. These were however only very faintly visible on the blot.

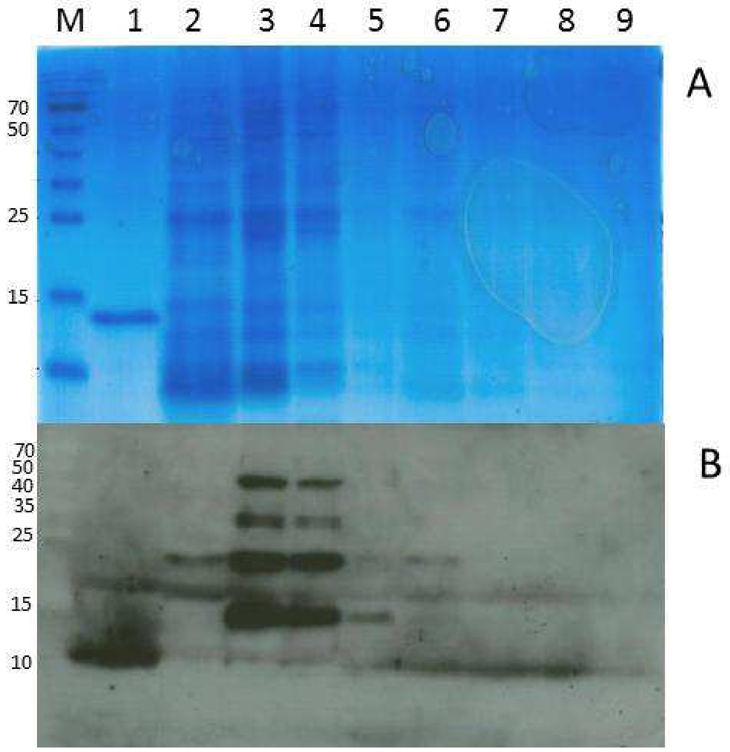

Fig. 2.13 GRFT maize sample was run through a weak anion exchange column. The fractions were separated on a 15 % SDS-PAGE gel and Coomassie stained (A) or detected with a GRFT specific antibody (B). Lanes represent the molecular marker (M), GRFT *E. coli* produced positive control (1), untransformed maize crude protein extract (2), GRFT transformed crude maize extract (3), fractions collected from the flow through (4), wash (5), elution with 0.5 M NaCl, 1 M NaCl, 1.5 M NaCl and 2 M NaCl (lanes 6, 7, 8 and 9 respectively).

The samples were re-dialysed and evaluated again using a Coomassie-stained gel and the corresponding immunoblot (Results not shown). Again, very faint bands were visible that corresponded to the *E. coli*-produced GRFT monomer at 15 kDa as well as bands of approximately 10 kDa. These bands were not visible on the PAGE gel.

Filtered GRFT maize extracts were also subjected to C18- SPE column purification. The fractions were subjected to SDS-PAGE and immunoblotting (Fig. 2.14 A and B). The crude and flow-through fractions contained bands that correspond to the GRFT monomer (15 kDa) as well as bands of 20 kDa, 28 kDa (which could be GRFT dimer that was not denatured to the monomer). In the dH_2O elution a band that corresponds to the GRFT monomer was detected, and a protein of approximately 20 kDa reacted with the antibody. In the dH_2O-methanol elution steps very faint bands were visible. The size of these also corresponded to that of the GRFT monomer. These bands were not visible on the PAGE gel.

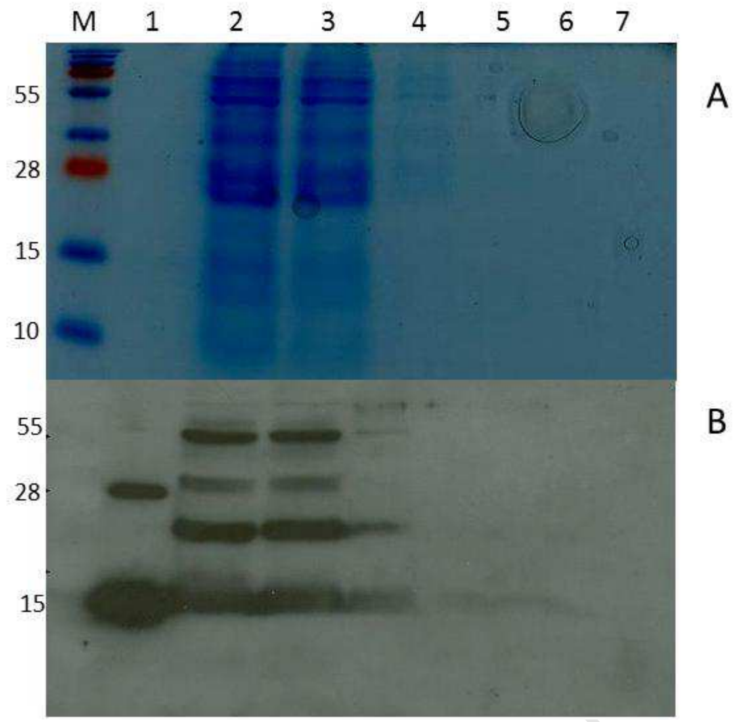

Fig. 2.14 GRFT maize protein extract was run through a C18-SPE column. The fractions were separated on a 15 % SDS-PAGE gel and Coomassie stained (A) and detected with a GRFT specific antibody (B). Lanes represent the molecular marker (M), GRFT *E .coli* produced positive control (1), GRFT maize crude extract (2), fractions collected from the flow through (3), wash (4), elution with dH_2O, 2:1 dH_2O/methanol, 2:1 methanol/ dH_2O (lanes 5 -7 respectively).

In another purification attempt, crude filtered GRFT maize protein extracts were concentrated using a 10 kDa membrane cut off spin column. Concentrated maize extract was applied to a nickel-His affinity columns and evaluated for GRFT content via SDS-PAGE and immunoblot analysis (Fig. 2.15). In the untransformed maize protein extract, bands of 20 and 35 kDa were detected in the wash fraction. In the GRFT transformed crude extract the same 35 kDa band was present. A band corresponding to the GRFT monomer was detected as well as a larger band of around 55 kDa. The latter as well as the 35 kDa band were detected in the washed fraction. No band that corresponds to GRFT monomer or dimer was detected in the eluted fraction.

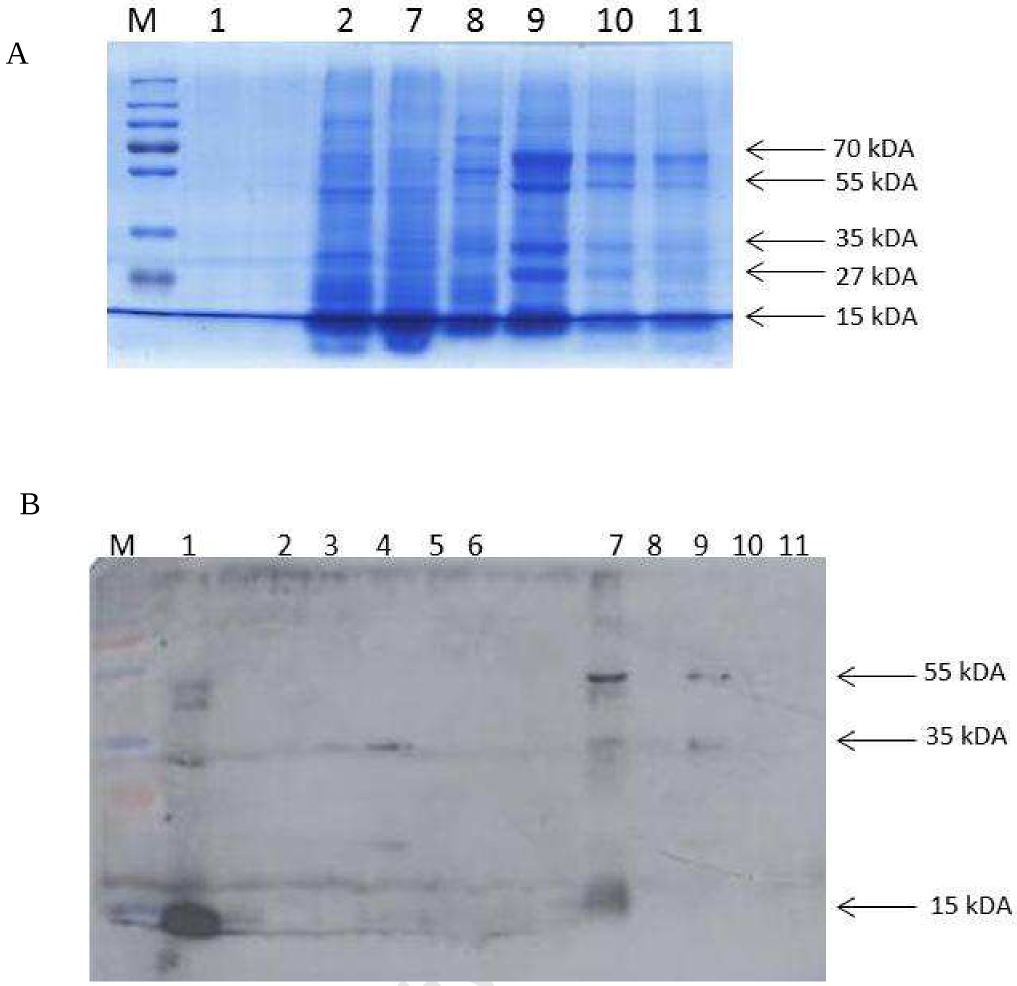

Fig. 2.15 Partial purification of GRFT maize extract using a His affinity column. Untransformed maize extract was used as a negative control. Fractions collected were analysed with SDS-PAGE analysis (A) and immunoblotting (B). Lanes are presented as follows; molecular weight marker (M). *E. coli* produced and purified GRFT (lane 1). M37W untransformed crude extract (lane 2), M37W flow through fraction (lane 3), M37W first and second wash fraction (lane 4 and lane 5 respectively), M37W eluted fraction (lane 6). GRFT transformed maize (Line O) crude extract (lane 7), GRFT transformed flow through fraction (lane 8), GRFT transformed first and second wash fraction (lane 9 and 10 respectively). GRFT transformed eluted fraction (lane 11).

The His-affinity purified fractions were evaluated for GRFT content using the gp120 binding ELISA (Fig. 2.16). In the untransformed line some activity were detected in the crude extract. A similar activity was detected in the flow through indicating that the protein did not bind the column. Although the immunoblot showed the presence of a band in the wash fraction, no activity was detected with the ELISA. For the GRFT transformed line, activity was detected in the flow through indicating that a large amount of protein did not bind. Activity was also detected in the crude extract, first wash and eluted fractions. Although the activity in the latter two fractions was very low compared to that observed for the starting crude material.

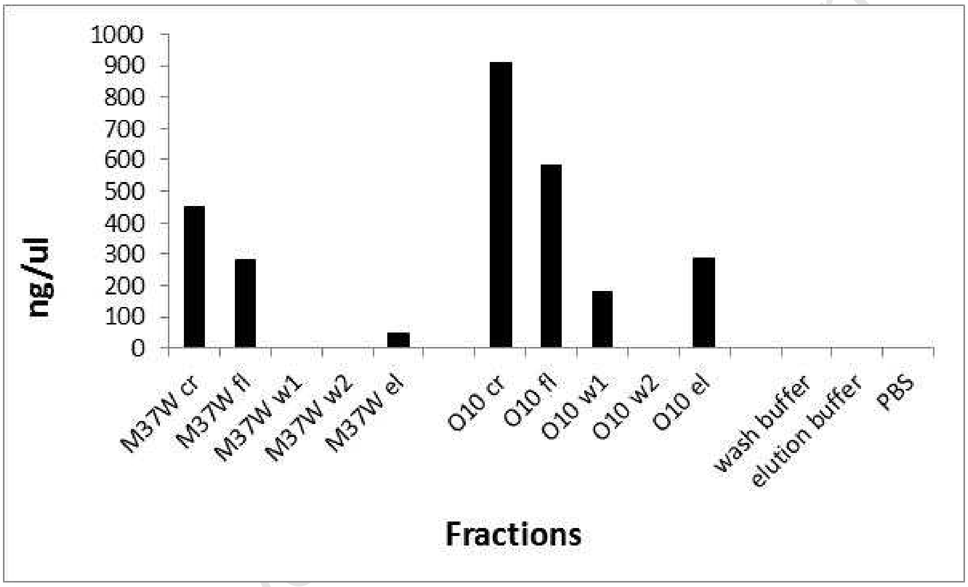

Fig. 2.16 Evaluation of GRFT content in different fractions from a His affinity purification. Untransformed M37W was used as a negative control. Protein extracts GRFT transformed line O was subjected to His purification. Fractions are presented as crude (cr), flow through (fl), first and second washed fractions (w1 and w2 respectively) and eluted fraction (el). The wash and eluted buffer as well as the PBS sample buffer were also evaluated.

2.3.7 GRFT efficacy analysis

A TZM-bl efficacy test was performed with crude untransformed (M37W) and GRFT transformed (GRFT-1 and GRFT-2) maize extracts (Table 2.2).

Table 2.2 Efficacy analysis of crude GRFT maize and untransformed maize extract against HIV pseudovirus QH0692.42 as measured in a TZM-bl assay. IC_{50} values are presented in ng/ml.

	GRFT-1	*GRFT-2*	*GRFT-3*	*M37W*	*E.coli GRFT*	*PBS*
QH0692.42	16.8	7.9	4.2	59	0.2	<20
VSV-G	<20	<20	<20	<20	No inhibition	<20
Gp120 binding as measured by ELISA	0.163 ng/µl	0.162 ng/ul	0.6 ng/ul	0	1mg/ml	0

Prior to efficacy analysis the concentration of the extracts was determined using gp120 binding ELISA. The M37W extract showed no gp120 binding activity while the GRFT transformed extract contained GRFT of 0.163 ng/ul and 0.162 ng/ul respectively. Both the GRFT and the untransformed maize extracts displayed a toxicity which caused the inhibition. Subsequent extracts were filtered and concentrated (GRFT-3). This extract showed a gp120 binding activity of 0.6 ng/ul. Both GRFT and untransformed maize extracts neutralised HIV pseudovirus QH0692.42; however, the control VSV-G virus was again inhibited by both GRFT and untransformed maize extracts. This is indicative that lack of HIV infection occurred due to cell toxicity rather than inhibition of the virus.

A previous cell line C8166-R5 did not seem to be sensitive for maize extracts (personal communication, Robin Shattock laboratory). Therefore, concentrated GRFT maize and untransformed maize extract were tested for HIV-BaL inhibition against C8166-R5 cells (Fig. 2.17.I and 2.17.II). Untransformed maize displayed a gp120 binding activity of 0.12 ng/ul while GRFT transformed maize extract displayed gp120 binding activity of 1.3 ng/ul. The concentration of the purified *E. coli* produced GRFT had a concentration of 21.5 ug/ul. Inhibition of infection was only observed at the highest concentration tested (50 ng/ml) for both purified *E. coli* produced GRFT and crude GRFT maize extract with the purified lectin being more efficient (Fig. 2.17.I). No inhibition was observed for untransformed maize protein extract (Fig. 2.17.I). However, when the effect of the extracts on cell viability was tested, it appeared that the GRFT maize extract reduced the cell viability significantly at the highest concentration tested (Fig. 2.17.II). An interesting observation was that the cell viability was also lower in the presence of pure GRFT and untransformed maize protein extract (Fig. 2.17.II). It is thus not conclusive if GRFT in the maize protein extract is able to inhibit the virus due to its lectin properties or due to its negative effect on the cell line.

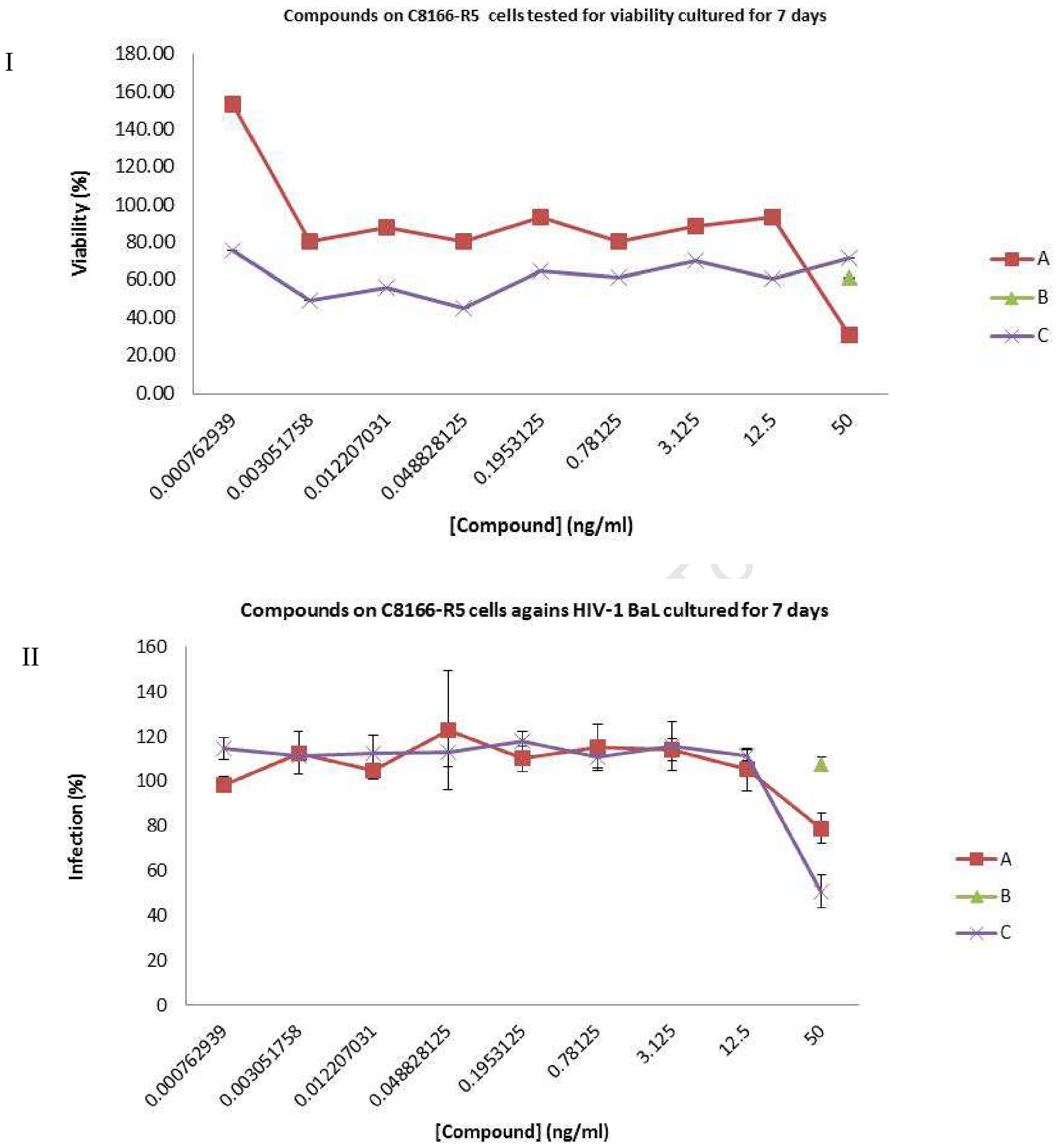

Fig. 2.17 Efficacy analysis of crude GRFT maize extract tested for HIV-BaL inhibition against C8166-R5 cells. Compounds were tested as crude GRFT maize protein extract (A), crude untransformed maize protein extract (B) and purified *E. coli* produced GRFT (C). Both the effect on cell viability (I) and inhibition of HIV infection (II) were measured. A single maximum measurement was performed for compound B.

2.4 DISCUSSION

The anti-HIV lectin GRFT was successfully expressed in transgenic maize seed. Expression was governed under an endosperm specific 27 kDa γ -zein promoter. The native griffithsin gene was expressed with and without the signal peptide from the LT-B toxin to evaluate the effect of subcellular targeting on mainly the yield obtained. In the T1 generation expression levels for cytosol targeted GRFT construct was higher than secreted GRFT construct. The highest expression level obtained for cytosol targeted GRFT was approximately 13 mg/kg, but these seeds were not viable and consequently did not generate a T2 generation for further analysis. These levels were significantly lower than that which has been reported for the maize-produced LT-B toxin (Chikwamba *et al.*, 2002b), 2G12 antibody (Ramessar *et al.*, 2007) and avidin (Hood *et al.*, 1997). The LT-B toxin was expressed using the 27 kDa γ -zein endosperm specific promoter at levels of 350 mg/kg; the 2G12 antibody was produced under the endosperm specific rice glutelin -1 promoter and resulted in 75 mg/kg extractable antibody, while avidin was produced up to 230 mg/kg under the constitutive ubiquitin promoter. All three molecules were expressed with a preceding signal sequence; the LT-B signal peptide preceding the LT-B toxin (which was also the signal peptide used in this study), an unspecified N terminal signal peptide preceding the 2G12 antibody and the barley α-amylase signal sequence at the N-terminus of avidin. The ELISA assays suffered from high background levels. Although I could have investigated methods to lower this, the low GRFT expression levels did not justify these attempts and would have also added costs to the process.

Several factors can affect the yield and functionality of a heterologously expressed protein. Two of these are the choice of promoter and subcellular targeting. Promoters can either drive constitutive or tissue specific expression. One might select a constitutive promoter to obtain a higher yield or a tissue specific promoter for the same purpose or for the purpose of containment. High expression levels have been obtained with both constitutive and seed specific promoters. Under the constitutive ubiquitin promoter, the level of expression for avidin reached 2.3% of total extracted seed protein (Hood *et al.*, 1997), while seed specific promoters delivered the same levels of expression; the 27 kDa γ -zein endosperm specific promoter was used to express the LT-B toxin at levels of 3.7% in maize seed (Chikwamba *et al.*, 2002b). From this study it is clear that a selected promoter will not always meet an expression expectation; where the 27 kDa γ -zein promoter was used to express the LT-B toxin, levels of 350 ug/kg were achieved in the third generation (Chikwamba *et al.*, 2002b). Expression levels with the same promoter were much lower than that obtained for GRFT.

Apart from promoter choice, other factors such as subcellular location can influence the final yield and functionality of a recombinant expressed protein. The endosperm environment seems to target proteins depending on their intrinsic properties combined with other tissue and species-specific factors (Chikwamba *et al.*, 2003; Hood, 2004; Drakakaki *et al.*, 2006). The cytosol is often not the location of choice for the expression of a recombinant protein since there is a high presence of proteolytic activity and little of the post translational activity that is often needed to obtain a functional protein. For the LT-B toxin, the cytosol was not the best location for production (Streatfield *et al.*, 2003). Low expression levels were also observed for scFv in the cytosol (Tavladoraki *et al*., 1993). Furthermore, high-level accumulation of the foreign protein can have a toxic influence on the plant cells (Hood *et al*., 1997). In this study, GRFT expression in the cytosol was higher than expression with the secretion peptide. In the majority of lines that expressed both cytosolic and secreted GRFT, no toxic effect on the seeds was observed: their seeds resembled untransformed control seeds in morphology. However the seed from the highest expressing line was not viable enough to generate a T2 generation. GRFT has been tested for cytotoxicity in several mammalian cell lines and did not show any significant adverse effects in the cells tested (Kouokam *et al*., 2011).

In ELISA tests the untransformed controls sometimes showed low residual gp120 binding activity. In immunoblot analysis the polyclonal antibody did react with untransformed maize extracts. Bands that were visible in these extracts were in the size range of the purified *E. coli* produced GRFT; also, bands of approximately 20 kDa, 30 kDa and 55 kDa were detected with the polyclonal antibody. In GRFT transformed samples bands were detected that corresponds to the GRFT monomer, dimer and also larger molecules were detected. The antibody could thus be detecting

GRFT, its dimer, GRFT aggregates, other seed lectins, and GRFT that might associate with other seed endosperm proteins. For GRFT expressed in *E. coli* larger proteins were also reported in the soluble cell lysate (Giomarelli *et al.*, 2006).

The detection of proteins in the extract from untransformed maize could be other seed lectins that reacted with the polyclonal antibody, since lectins from corn have been reported (Fouquaert *et al.*, 2009; Baker *et al.*, 2009; Jankovic *et al.*, 1999). The sizes of some of these were 11.5, 12.5 and 23 kDa (Jankovic *et al.*, 1999) - similar to bands detected in the untransformed maize extracts in the immunoblot analysis.

Purification of GRFT from maize seed was a challenge due to the low expression levels, and possibly also due to the association of GRFT with other seed components. GRFT content in the endosperm extract were influenced by centrifugation and filtration. Attempts were made to purify GRFT from the seed endosperm extract using different purification columns. Purification using weak anion exchange column and C18-SPE column seem to contain bands in the eluted fraction that were in the same size of GRFT but these were not visible on the PAGE gel to be excised and confirmed with peptide mass fingerprinting. Purification using a His affinity column did not result in any distinct GRFT bands in the eluted fractions. Also, GRFT content as measured by gp120 binding ELISA was very low. With the production of a fungal laccase in maize seed, the authors reported that the majority of laccase remained immobilised but active in the remaining solids of a seed extract (Baily *et al*., 2004). Jankovic *et al.* (1990) reported that extracted lectins from maize have the tendency to form aggregates with glycosylated proteins or self-aggregates, which leads to a decrease in lectin activity and complications in purification. This was also observed for mannose specific plant lectins of *Canavalia ensiformis*, lentil and or pea (Jankovic *et al.*, 1990).

Due to the low expression levels, efficacy analysis was attempted with crude protein extracts. The results indicated that the extract was toxic towards the cell line of the TZM-bl assay, which then prompted the use of partially purified concentrated maize protein extracts. These extracts were tested again against HIV pseudoviruses for efficacy in the TZM-bl assay. Once again, inhibition was attributed to toxicity rather than prevention of attachment. Previously a maize crude extract was tested against

C8166-R5 cell line without showing a negative effect on cell viability. Thus further efficacy analysis was pursued using a C8166-R5 cell line with HIV-BaL. Once more the maize extract had a negative effect on cell viability. There are reports of *in vitro* toxicity for lectins such as cyaonovirin-N (CV-N) (Huskens *et al.*, 2008), concanavalin A (Con-A) (Tsai *et al.*, 2003) and microvirin (MVN) (Tsai *et al.*, 2004). Unlike these lectins, *E. coli* produced GRFT has showed no significant effect on cell viability, this includes some of the cells used in this study (Alexandre *et al.*, 2010; Kouokam *et al*., 2011; Mori *et al*., 2005), even at high concentrations. Additionally, GRFT produced in *N. benthamiana* in a crude extract did not cause the toxicity effect observed from the untransformed maize and GRFT maize extracts (see Chapter 2 for GRFT tobacco efficacy tests). Lectins from maize have been reported to have agglutination activity and binds mainly mannose and N-acetyl-D-galactosamine (Baker *et al*., 2009; Jankovic *et al.*, 1999). Thus it is likely that a lectin that is present in the maize extract could have had the negative effect on the cells tested in this study.

Finally, I conclude that although maize has proven to be an excellent production platform for some pharmaceutical molecules, it was not suitable for GRFT in this case. In particular, expression levels were too low to be commercially viable from a yield and purification point of view.

CHAPTER 3.

Targeted transient expression of functional griffithsin (GRFT) in *Nicotiana benthamiana*

3.1 INTRODUCTION

Tobacco has been a major role player in the course of recombinant protein production in plants. It was one of the first plants to be genetically modified as well as being the first genetically modified plant to be subjected to field trial studies (Fraley *et al*., 1983; James and Krattiger, 1996). Furthermore, the first plant made pharmaceutical, the human growth hormone, was produced in tobacco in 1986 (Barta *et al*., 1986). Since then transgenic tobacco plants and cell suspension cultures have been used to produce many proteins of pharmaceutical interest. Thus far, the majority of plant derived antibodies have been produced in tobacco (Daniell *et al*., 2001; De Muynck *et al*., 2010). Tobacco also proved to be capable of producing other therapeutic molecules such as vaccines, microbicide peptides, cytokines and hormones (Daniell *et al*., 2001; Paul and Ma, 2011; Xu *et al*., 2011). Apart from having the advantage of large biomass and being a non-food crop, developments in transient expression technologies have enabled tobacco to produce some pharmaceutical molecules on a commercially viable scale (O'Keefe *et al*., 2009).

This expression platform has great potential to impact the treatment of pandemics such as HIV. Lectin microbicides have displayed the ability to inhibit HIV infection in *in vitro* and *in vivo* studies (Bewley *et al*., 2004; Boyd *et al*., 1997; Emau *et al*., 2007; Matoba *et al*., 2010; Sato *et al*., 2007; Tsai *et al*., 2003; Xiong *et al*., 2006). Peptide lectins with anti-HIV activity were isolated from natural sources which are difficult to propagate and often occur in low concentrations. This was also the case for griffithsin (GRFT), isolated from the red alga

Griffithsia (Mori *et al.*, 2005). Subsequently GRFT has been produced in tobacco spp. to very high levels using a TMV- based vector system (O' Keefe *et al.*, 2009).

As literature indicates, the optimal production of any protein is an empirical experimentation with different vector systems, codon optimisations, subcellular targeting. (Maclean *et al.*, 2007; Yang *et al.*, 2005). The latter sometimes results in unexpected locations which might reflect on an inherent property of the protein itself or specifically be associated with the plant organ involved (Chikwamba *et al.*, 2003). This again can influence protein yield and activity, and impact downstream purification. In this study we aimed to compare expression levels using different vector technologies, including an advanced Ti-plasmid based pTRA binary vector system (with the aid of silencing suppression), as well as a deconstructed viral vector system (magnICON). Both these systems were able to generate therapeutic proteins at high levels (Giritch *et al.*, 2006; Maclean *et al.*, 2007). With these vectors, the effect of subcellular location on plant cell viability and protein accumulation was investigated. Microscopic analysis was used to establish the location of the lectin under different targeting signals. Furthermore, the vitality of the plant produced microbicide was assessed in efficacy analysis in various assays.

3.2 MATERIALS AND METHODS

3.2.1 Vector construction

Vector pET28(c)+GRFT (Mori *et al.*, 2005) was used as template for all PCR amplifications. Two vector sets, the magnICON (Icon Genetics GmbH; Halle, Germany) and pTRA vector system (Fraunhofer Institute, Aachen, Germany) were used to construct the GRFT expression vectors. GRFT was amplified using the primers GRNcoF (5'-ATGCCA TGGGCAGCCTGACCCATCGCAAG-3') and GRthrHisSacR (5'-ATGAGCTCTTA GTGATGATGATGATGATGGTGCCGCGCGGCACCAGGCCGCTGCTGTACTGTTCAT

AG-3') for cloning into the *Nco*I and *Sac*I sites of the magnICON vector pICH11599 (Giritch *et al.*, 2006; Marillonnet *et al.*, 2004). To compile the pTRA vectors (Maclean *et al.*, 2007); the GRFT gene was amplified with GRNcoF and GRNotR (5'-TAGCGGCCGCGTACTGTTCATAGTAG-3') or GR_ctp_MluIF (5'-GTGGACGCG TTAGGTGCATGGGCAGCCTG-3') and GR_his_XbaIR (5'-GCTCTAGATTAG TGATGATGATGATGATGGC-3') for insertion into pTRAkc-ERH and pTRAkc-rbsc1-cTPrespectively. Likewise an *Nco*I - *Xba*I GRFThis amplicon was generated and inserted into the *Afl*III-*Xba*I and *Nco*I- *Xba*I sites of pTRAc and pTRAkc-AH respectively. Unless stated otherwise, plasmids were propagated in *E. coli* DH10B cells, all DNA preparation kits were from Zymo Research Corporation (California, USA) and restriction and ligase enzymes were provided by Fermentas GmbH (St Leon-Rot, Germany). All GRFT constructs generated contained a His tag at the C terminal end.

For immunolocalization GRFT-GFP fusions were constructed as follows: vector pICH7410 (Gils *et al*., 2005; Marillonnet *et al.*, 2004) was used as template for green fluorescent protein (GFP) amplification. A GRFT fragment was amplified with primers GRNcoF and GRBglIIR (5'TAAGATCTGTACTGTTCATAGTAGATGTCCAG 3'). A bsp GRFT fragment was amplified with bspGRNcoF (5'CATGCCATGGTGGAAGGTGAAGTGCTA TGTGCTCTTCACTGCTCTCCTCAGCTCTCTTTGTGCTTATGGAAGCCTGACCCATC GCAAG 3') and GRBglIIR primers. The bspGRFTNcoF primer incorporated the *Escherichia coli* (*E. coli*) heat labile enterotoxin signal peptide (Mason *et al.*, 1998) at the 5' end of the fusion. The GFP gene was amplified with primers GFPBglIIGly6F (5' GAAGATCTGGGGGAGGCGGTGGGGGAGTGAGCAAGGGCGAG 3') and GFPSacR (5'TAGAGCTCTTACTTGTACAGCTCGTC 3'). A GFP-SEKDEL fragment was amplified with GFPBglIIGly6Fand GFPSekdelSacR (5' TAGAGCTCTT AAAGTTCATCTTTCTCAGACTTGTACAGCTCGTC 3') primers. This amplification added the SEKDEL ER retention sequence at the 3' end of the fusion. Each fragment was cloned into pGEM-T-easy (Promega, Fitchburg, Wisconsinsin, USA) and sequenced. A three-way ligation was set up with the relevant fragments and *Nco*I *Sac*I digested vector pICH11599.GRFT was amplified with GR_ctp_MluIF (5' GTGGACGCGTTAGGT GCATGGGCAGCCTG-3') and GRBglIIR. A subsequent ligation was set up with the *Mlu*I GRFT *Bgl*II, *Bgl*II GFP *Xba*I and *Mlu*I, *Bgl*II digested pTRAkc-rbsc1-cTP.

3.2.2 Preparation of electrocompetent Agrobacterium cells

Agrobacterium tumefaciens GV3101::pMP90 (RK) was prepared for electroporation according to a method published by the University of Uppsala (http://lcpu.se/?page_id=280). Briefly, *Agrobacterium* was grown at 28°C on a fresh LB (Luria Bertani) plate containing kanamycin (25 µg/ml) and rifampicin (50 µg/ml). An overnight 20 ml liquid culture was prepared from the plate and used to inoculate a 200 ml LB culture. The latter was grown to an OD_{600} of 0.5. The culture was incubated on ice for 30 minutes prior to centrifugation at 4000 × g at 4 °C for 15 minutes. The cell pellet was resuspended in 100 ml ice cold HEPES (1mM). Cells were pelleted as described above and resuspended in 4 ml HEPES, centrifuged and resuspended in 500 µl ice cold glycerol (10%). Aliquots of 45 µl were frozen at – 80°C. For electroporation 50-100 ng of plasmid DNA were introduced *via* 1 mm gapped cuvettes (Sigma-Aldrich, St. Louis, USA) into the thawed cells at 1.44 kV, 200Ω, and 25uF (BIORAD Gene Pulser, BIORAD).

3.2.3 Agroinfiltration of GRFT pTRA and magnICON constructs into N. benthamiana

Recombinant pTRA *A. tumefaciens* colonies were prepared for agroinfiltration based on the method of Maclean *et al.* (2007) and Meyers *et al*. (2008). Colonies were selected on plates incubated at 28°C containing kanamycin (25 µg/ml), rifampicin (50 µg/ml) and carbenicillin (50 µg/ml). *A. tumefaciens* pTRA cultures and *A. tumefaciens* LBA4404 containing vector pBIN-NSs with the NSs silencing suppressor gene from tomato spotted wilt virus (TSWV, Takeda *et al*., 2002) was propagated overnight in induction medium (LB with 10 mM morpholineethanesulfonic acid, MES, pH5.6 supplemented with 20 µM acetosyringone, kanamycin (25 µg/ml), rifampicin (50 µg/ml) and 2 mM $MgSO_4$) at 28°C with agitation.

For infiltration, cells were pelleted at 8000 × g for 3 minutes. The pellet was resuspended in infiltration medium containing 10 mM MES and 10 mM $MgCl_2$ (pH 5.6) supplemented with 200 µM acetosyringone and further diluted in infiltration medium to an OD_{600} of 0.3. pTRA griffithsin cultures were combined in equal volumes with the NSs inhibitor or syringe injected without inhibitor into whole, uprooted *N. benthamiana* plants (6 to 7 weeks old).

For the magnICON vectors the *Agrobacterium* preparation and infiltration was done as described by Giritch *et al*. (2006) and Marillonnet *et al*. (2004). Briefly, overnight-grown *Agrobacterium* cultures containing pICH11599 GRFT and GRFT-GFP fusions, the pICH7410 GFP control, the pICH14011 integrase pro module, the pICH17620 apoplast and pICH17388 cytosol module (Marillonnet *et al*., 2004) were sedimented at 8 000 × g for 3 minutes and diluted to OD_{600}~ 0.4 in infiltration buffer (10 mM MES, pH 5.5 and 10 mM $MgSO_4$). Equal volumes were mixed to a final OD_{600}~ 0.4 and syringe injected into leaves of 6 to 7 week old *N. benthamiana* plants. Infiltrated plants were grown at 25°C under a 16 /8 hour light/dark cycle and subsequently harvested at 4, 6, 8, 10 and 12 days post infiltration (dpi).

For large scale infiltrations, *Agrobacterium* cultures were grown as described above, however dilutions were adapted to OD_{600}~0.06 for magnICON and pTRA cultures. Whole *N. benthamiana* plants of approximately 6 weeks old were submerged in the diluted *Agrobacterium* suspension. A vacuum of -600 mbar was applied for 30 seconds. Infiltrated plants were grown as described above. Leaf material of infiltrated plants was collected at days 4, 6 and 8 post infiltration.

Harvested material from infiltrated plants was crushed in liquid nitrogen and mixed with 1 × PBS (15 mM KH_2PO_4, 81 mM Na_2HPO_4, 27 mM KCl and 140 mM NaCl) buffer in a 1 to 2 ratio, where after the extract was centrifuged for 10 minutes at 14 000 rpm and the supernatant collected.

3.2.4 ELISA

ELISA was performed according to the method of Boyd et al. (1997). Each well of a 96 well maxisorp plate (NUNC, Nalgene, Thermo Scientific, Waltham, Massachusetts, USA) was coated with 100 ng gp120 in 1 × PBS (gp120, clade E was provided by the National Institute for Communicable Diseases, NICD, South Africa). Wells were blocked using 5% fat free milk powder in 1 × PBS. For the standard, purified E. coli produced GRFT was diluted two fold from 200 to 0.78 ng GRFT/well. Positive controls and tobacco extracted protein were incubated as 50 ul volumes. Rabbit polyclonal anti-GRFT (provided by Dr. Barry O'Keefe of the Molecular Targets Laboratory, Center for Cancer Research, NCI-Frederick, MD, USA), diluted 1:1000 in PBS was added to the wells followed by goat anti-rabbit HRP conjugated antibody (Sigma-Aldrich) 1:2500 in PBS. Detection was done with 3,3′,5,5′-Tetramethylbenzidine substrate (TMB, Sigma-Aldrich) and colour reaction measured at OD450. Wells were washed (twice after coating and blocking and four times after sample and antibody addition) with PBS supplemented with 0.1% Tween-20 (Merck, Darmstadt, Germany) and incubation periods were 2 hours at 37°C or overnight at 4°C. ELISA data was converted in by using the standard curve equation to obtain a ng/ul value. Since tobacco GRFT levels were high the samples in the ELISA was diluted. Thus the dilution factor was accounted for. This generated a ng/ ul value. The volume and the leaf mass of the sample were known and were worked back to mg/kg.

3.2.5 PAGE and Immunoblot analysis

The concentration of total soluble protein (TSP) was determined using the Bradford assay (Bradford, 1976). Protein extracts (30 ug TSP each) were mixed with 6 × loading buffer (100 mM Tris, pH 6.8, 2% SDS, 5% ß- mercaptoethanol, 15% glycerol and 0.2% bromophenol blue). For denaturing conditions samples were electrophoresed on a 15% (29:1

acrylamide:bisacrylamide ratio) or on 12% (19:1 acrylamide:bisacrylamide ratio) acrylamide gels according to the method of Laemmli (1970). For non-denaturing conditions 10 % acrylamide gels were used and SDS and ß- mercaptoethanol were omitted. Tris-tricine gels were prepared according to the method of Bonifacino (2002). Proteins were visualised by Coomassie staining or transferred using a semi-dry blot system (BIORAD) onto a PVDF membrane (Amersham Life Science, Buckinghamshire, UK), and then challenged with rabbit polyclonal anti-GRFT (1:1000) in PBS supplemented with 0.1% Tween-20 and 5% fat free milk powder followed by goat anti-rabbit HRP-conjugated antibody (Sigma) diluted 1:5000 in PBS with 0.1% Tween-20. Between adding the antibodies, immunoblots were washed 3 times for 10 minutes in PBS with 0.1% Tween-20. Chemiluminescence detection was performed using ECL detection kit according to the method described by the supplier (Amersham Life Science). Colour detection was performed using 3, 3′, 5, 5′-Tetramethylbenzidine substrate (Sigma).

3.2.6 Peptide mass fingerprinting using mass spectrometry

PAGE-separated bands were excised for in-gel digestion according to the method described by Shevchenko *et al*. (2007). Briefly, gel bands were destained using 50mM NH4HCO3/50% methanol followed by in-gel protein reduction (50mM dithiothreitol in 25 mM NH4HCO3) and alkylation (55 mM iodoacetamide in 25 mM NH4HCO3). Proteins were digested over night at 37°C using 5 – 50 µl, 10 ng/µl tryspin depending on the gel piece size. Peptides were extracted using 50% acetonitrile (ACN)/5% formic acid (FA) and vacuum dried. For MS analysis trypsin digested samples extracted from gel-plugs were re-suspended in 40 ul 2% ACN/0.2 % FA and centrifuged at 13000 rpm, 4 °C for 15 minutes. Samples were analysed on a Dionex Ulitomate 3000 RSLC system coupled to a QSTAR ELITE mass spectrometer. Samples were de-salted on an Acclaim PepMap C18 trap (75 µm x 2 cm) for 8 minutes at 5 µl/min using 2% ACN/0.2% FA. Peptides were separated on Acclain PepMap C18 RSLC column (75 µm x 15 cm, 2um particle size) connected to the trap column via 10-port switching valve. Peptide elution was achieved using a flow-rate of 500 nl/minute with a gradient: 4-60% B in 30 minutes (A: 0.1% FA; B: 80% ACN/0.1% FA).

Nano-spray was achieved using a MicroIonSpray head assembled with a New Objective, PicoTip emitter (o.d. 360 µm; i.d. 10 µm; tip i.d. 10 µm). An electrospray voltage of 2.0 - 2.8 kV was applied to the emitter. The QSTAR ELITE mass spectrometer was operated in Information Dependent Acquisition (IDA) using an Exit Factor of 2.0 and Maximum Accumulation Time of 2.0 seconds. MS scans were acquired from m/z 400 to m/z 1500 and the three most intense ions were automatically fragmented in Q2 collision cells using nitrogen as the collision gas. Collision energies were chosen automatically as function of m/z and charge.

3.2.7 Purification of GRFT

Total soluble protein was extracted as described above. The extract was applied to a Protino Ni- IDA His affinity column and the protocol followed as prescribed by the manufacturer. Fractions collected during the purification process were analysed *via* PAGE and immunoblot as described above.

3.2.8 Confocal microscopy

For confocal imaging infiltrated and control leaves were subjected to hand sectioning. Fine leaf sections were incubated in distilled H_2O for imaging. Images were captured using a Zeiss Universal microscope equipped with epifluorescence condenser III RS and a FITC filter set comprising exciter filter (BP 450-490),chromatic beam splitter (FT 510), and barrier filter (LP 520), and Kodak Ektachome Elite 400 colour film. The optimal exposure time was 30 seconds. The light source was provided by a HBO 50 W high-pressure mercury bulb.

3.2.9 Transmission electron microscopy (TEM)

Plant material was prepared by excising squares of 2mm from infiltrated and control un-infiltrated leaves. The material was fixed in 4% formaldehyde in 0.075 M phosphate buffer (pH7.4) for 1 hour at room temperature. Material was rinsed three times for 10 minutes each in 0.075M phosphate buffer. The plant material was subsequently dehydrated for 10 minutes each in an ethanol series of 30%, 50%, 70%, 90%, 100%, 100%, 100%. The material was infiltrated in 50% London Resin (LR white) in ethanol for 1hour then in pure LR white in ethanol for approximately 4 hours. The material was allowed to polymerise at 60°C for 24 hours. Microtome sections of 0.5µm were prepared from polymerised material using a Reigher Ultra Cut E microtome (Vienna, Austria). Plant microtome slices were placed on gold grids (G200ht gold grids, BIORAD).

3.2.10 Immunogold labelling

Tobacco leaf sections mounted on the grids were incubated in blocking buffer (PBS, pH 7.5 5% BSA) for 2 hours at room temperature. The grids were then incubated with rabbit anti-GRFT antibody, diluted to 1:75 in PBS, pH 7, supplemented with 0.5% BSA for 2 hours at room temperature. Grids were subsequently washed three times for 15 minutes in PBS buffer. Grids were incubated in goat produced anti-rabbit gold conjugated (10 nm) secondary antibody (Sigma-Aldrich) diluted1:25 in PBS with 0.5% BSA, pH 7, supplemented with 0.5% BSA buffer for 2 hours at room temperature. Grids were washed twice with PBS as described before and once with distilled water for 15 minutes. Grids containing control uninfiltrated wild type *N. benthamiana* were used as two controls; incubated in PBS buffer only and incubated with antibodies as the infiltrated leaf samples. Immunogold labelled grids were allowed to air dry before imaging under a Jeol 2100 F transmission electron microscope (Tokyo, Japan).

3.2.11 Efficacy analysis

A single-cycle neutralization assay (TZM-bl assay), a peripheral blood mononuclear cell (PBMC) cell assay and inhibition of DC-SIGN mediated transfer were used to evaluate the efficacy of *N. benthamiana* produced GRFT. The TZM-bl assay was set up as described previously (Alexandre *et al*., 2010; Montefiori, 2004). Briefly, a three-fold dilution series of GRFT in 100 μL of DMEM with 10% FBS (Fetal Bovine Serum, growth medium) was prepared in a 96-well plate in duplicate. Two hundred $TCID_{50}$ (tissue culture infective dose) of pseudovirus in 50 μL of growth medium was added and the mixture was incubated for 1 hour at 37ºC. Then 100 μL of TZM-bl cells at a concentration of 1x105 cells/mL containing 37.5 μg/mL of DEAE dextran was added to each well and cultured at 37º C for 48 hours. Infection was evaluated by measuring the activity of the firefly luciferase. Titres were calculated as the inhibitory concentration that causes 50% reduction (IC_{50}) of relative light unit (RLU) compared to the virus control (wells with no inhibitor) after the subtraction of the background (wells without both the virus and the inhibitor).

For the PBMC protocol a three-fold dilution series of GRFT in 40 μL of RPMI 1640 containing 20% FBS and interleukin-2 (growth medium) was prepared in triplicate in a U-bottom 96-well plate. Five hundred $TCID_{50}$ of HIV-1 primary isolate in 15 μL of growth medium was added to each well and the plate was incubated at 37ºC for 1 hour. Then 100 µL of 5 x 106 cells/mL phytohaemagglutin/interleukin-2 stimulated PBMC (PHA-PBMCs) was added to each well. The following day cells were washed 3 times with RPMI 1640 with 20% FBS and resuspended in 155 μL of fresh growth medium. The culture supernatant was collected twice daily and replaced with an equal amount of fresh growth medium. For each harvest the p24 antigen concentration in the virus control wells was measured by ELISA using the Vironostika HIV-1 Antigen Microelisa System (Biomerieux, Boseind, the Netherlands), according to the manufacturer’s instructions. The inhibitory activity of the lectins were measured at the time-point that corresponded to the early part of the linear growth period of the virus control (Zhou and Montefiori, 1997). The IC_{80} were calculated by plotting the lectin concentration vs. the percentage inhibition in a linear regression using GraphPad Prism 4.0.

To evaluate DC-SIGN mediated transfer inhibition 5 x 104 Raji/DC-SIGN cells/ well were incubated with HIV-1 pseudovirus in a U bottom 96 well plate at 37°C for 1 hour. The cells were then washed three times with plain RPMI 1640 by centrifuging at 2000 rpm for 5 minutes to remove unbound viruses. A fivefold dilution series of GRFT in 150 μL of DMEM (Dulbecco's modified Eagle medium) with 10% FBS was then added to the virus bound Raji/DC-SIGN cells. This was followed by an incubation at 37°C for 1 hour and transfer of 100 μL from each well to the corresponding well of a flat bottom 96 well plate. Subsequently, 3 x 104 TZM-bl cells / well were added to the plate that was then placed at 37°C for 48 hours. HIV-1 infection was evaluated by comparing the relative light unit (RLU) between wells treated with GRFT and untreated wells (control). The IC_{50} were calculated by plotting the lectin concentration vs. the percentage inhibition in a linear regression using GraphPad Prism 4.0

3.3 RESULTS

3.3.1 Vector construction

The native GRFT gene was inserted into the magnICON expression vector resulting in pICHgrft (Fig.3.1). pCIHgrft was combined with the pICH17388 cytosol or pICH1762 apoplastpromodules (Fig. 3.1) to target GRFT expression in the cytosol (pICHgrft-Cyto) and apoplast (pICHgrft-Apo) respectively. Recombination was facilitated by the pICH14011 integrase promodule (Fig. 3.1). The amplified native GRFT gene was furthermore inserted into the advance binary pTRA vectors to construct pTRA-Cgrft, pTRA-cTPgrft, pTRA-APOgrft,and pTRA-ERgrft(Fig. 3.2). These vectors contain signalling information for expression of foreign genes in the cytosol, chloroplast, apoplast and endoplasmic reticulum. Table 3.1 summarises the griffithsin vectors and associated subcellular locations.

For microscopic evaluation of subcellular location, GRFT was fused to the GFP reporter gene as encoded by the magnICON pICH7410 (Gils *et al*., 2005) vector. GRFT was linked to GFP via a six glycine linker. The fusion was cloned as is into the magnICON vector resulting in pICHgrftGFP (Fig. 3.3a) for targeting to the cytosol and apoplast using cytosol or apoplast magnICON targeting modules (Fig. 3.1). The fusion was also constructed with the LT-B signal peptide and SEKDEL ER retention sequence to construct pICHbsp-grftGFP-SEKDEL (Fig. 3.3b) to microscopically evaluate ER retention. Finally a pTRA-cTPgrftGFP fusion was constructed to determine GRFT targeting to the chloroplast (Fig. 3.3c). Table 3.2 summarises the GRFT vectors constructed for subcellular location studies.

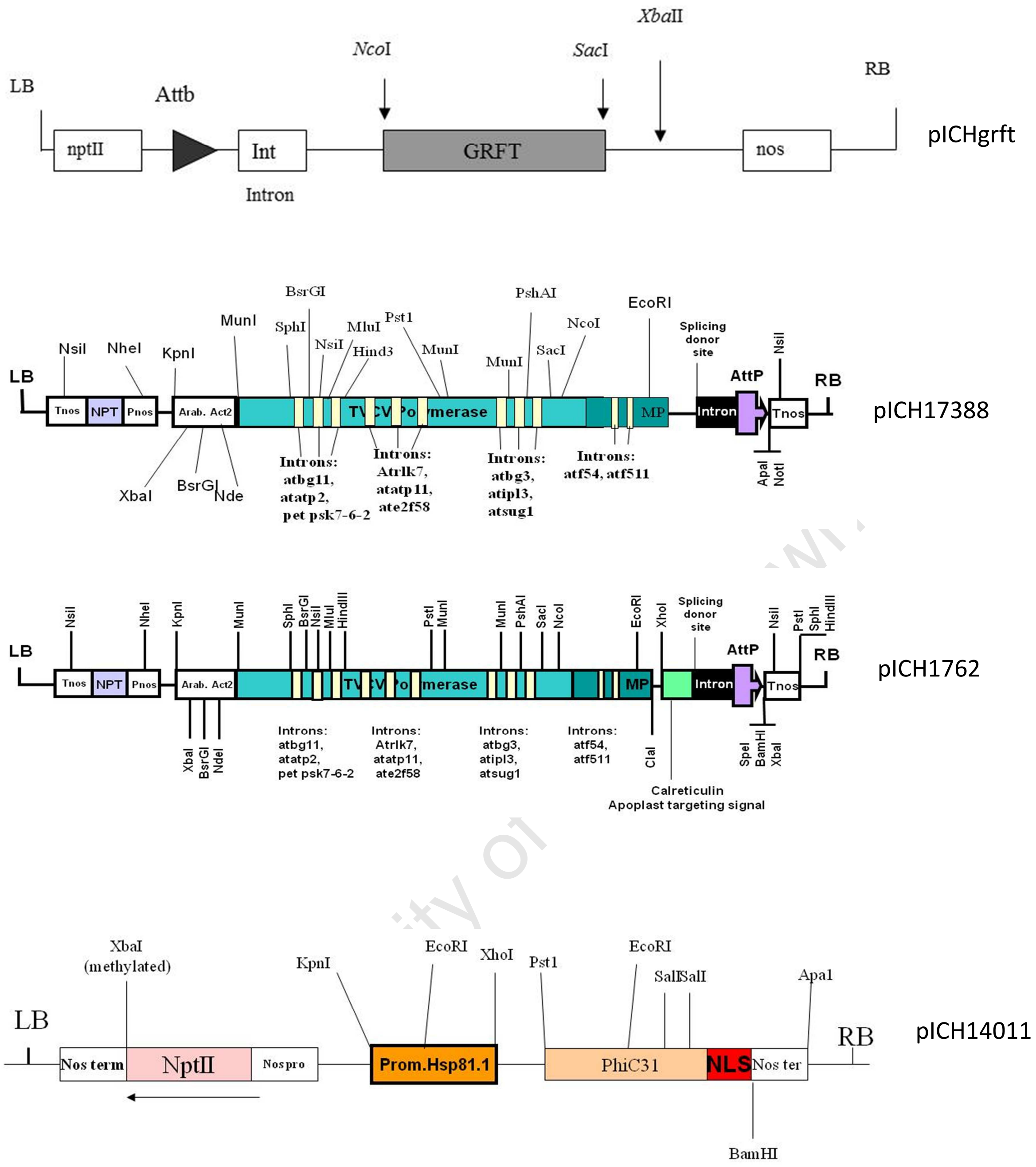

Fig. 3.1 The native GRFT gene was amplified with *Nco*I and *Sac*I sites for insertion into pICH11599 5' promodule resulting in pICHgrft. The GRFT promodule were combined with either a cytosol targeting (pICH17388) or apoplast targeting (pICH1762) 5' promodule. Recombinase between promodules was facilitated by a *Streptomyces* phage C31 integrase encoded by the PhiC31 gene carried on the pICH14011 promodule.

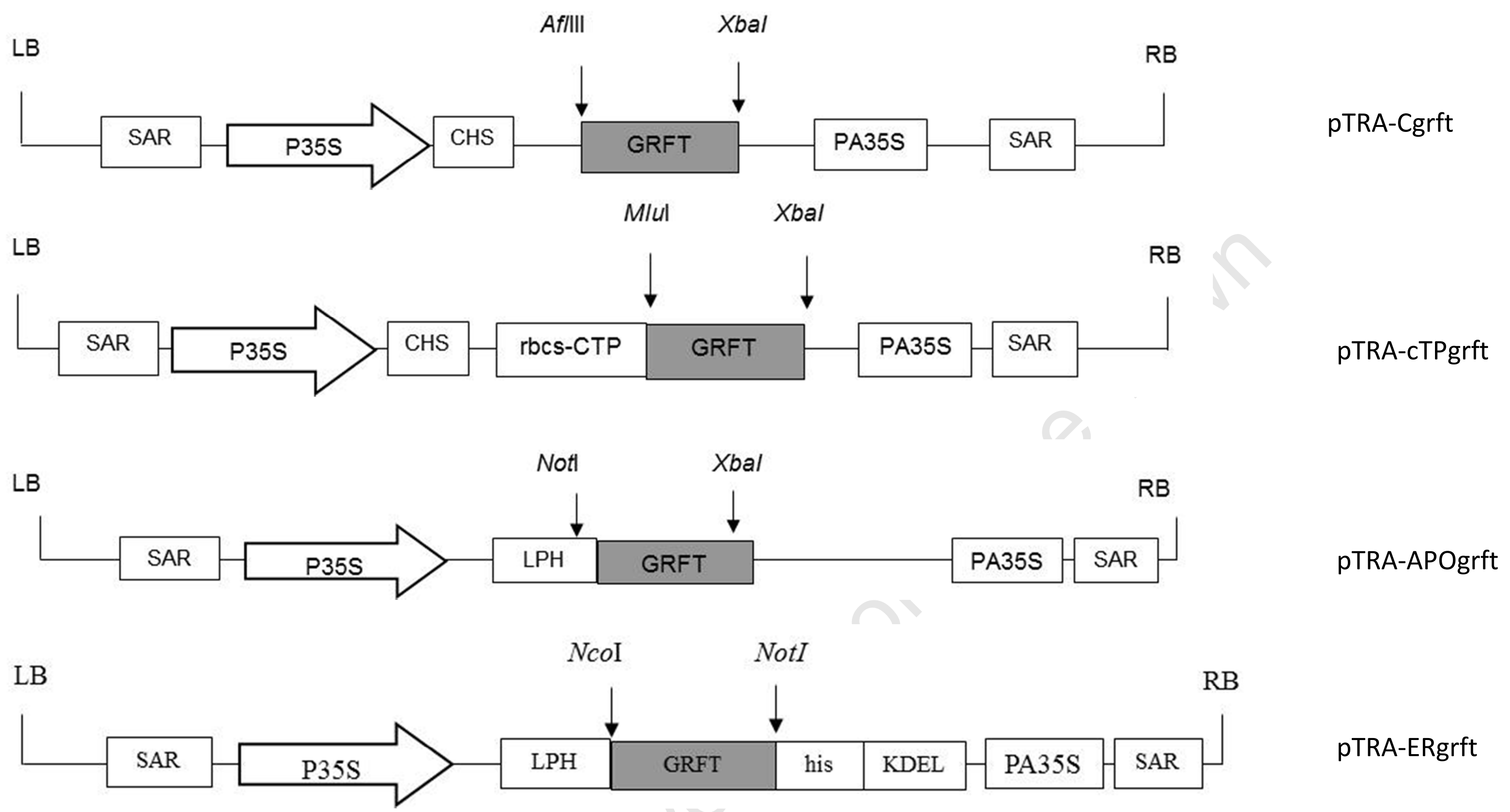

Fig. 3.2 Schematic illustration of pTRA vectors that were used for expression of the native GRFT gene. GRFT was cloned into the AflII-XbaI site of pTRAc (cytosol targeting) resulting in pRA-Cgrft. A MluI and XbaI GRFT pcr amplicon was inserted into rbsc1-cTP-PTRA (chloroplast targeting) to generate pTRA-cTPgrft. To generate pTRA-APOgrft aNcoI –XbaI GRFT pcr product was cloned into pTRAkc-AH (apoplast targeting). pTRA-ERgrft was generated by cloning an NcoI NotI GRFT amplicon into pTRAck-ERH(ER targeting

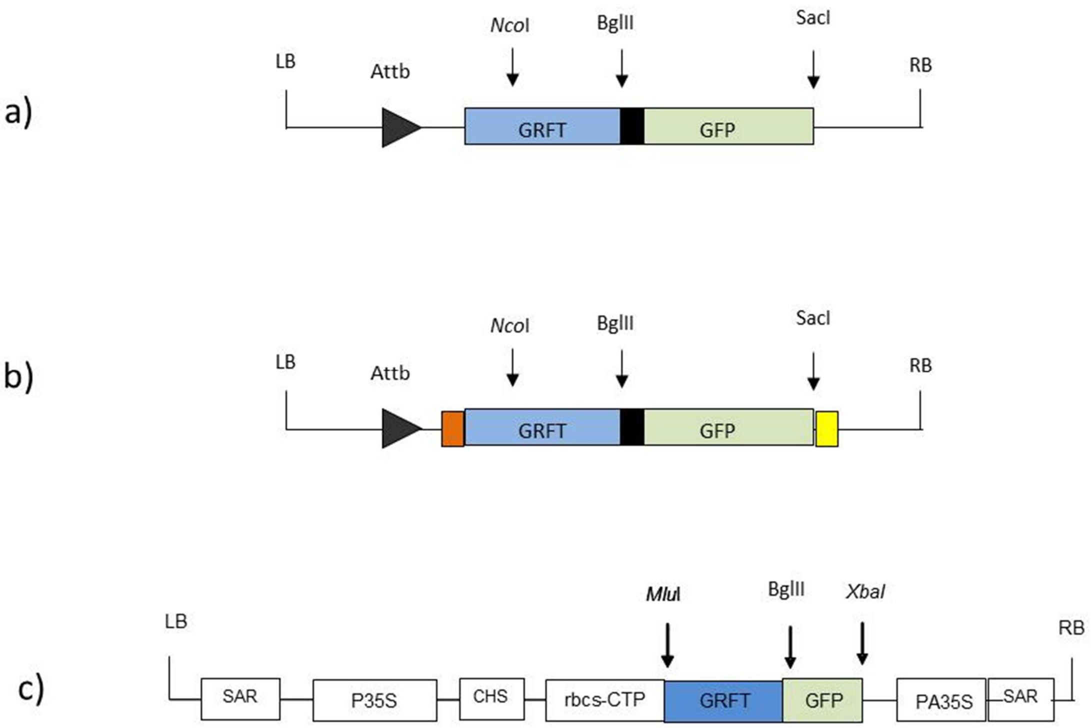

Fig. 3.3 GRFT-GFP fusions were constructed by cloning GRFT (blue block), GFP (green block), bspGRFT (bsp is represented by the orange block) and GFP-SEKDEL(SEKDEL is represented by the yellow block) combinations into pICH11599resulting in pICHgrftGFP (a) andpICHbsp-grftGFP-SEKDEL (b). pICHgrftGFP was infiltrated with cytosol and apoplast targeting promodules while pICH-bsp-grftGFP-SEKDEL was infiltrated with the cytosol targeting pro module. A MluF GRFT and BglIIGFP fragment were ligated pTRAcTP resulting in pTRA-cTPgrftGFP (c), the latter was infiltrated as with the NSs silencing suppressor

Table 3.1 Summary of the vector constructs used for *Agrobacterium*-mediated transient expression of griffithsin.

Vector module	Subcellular compartment
pTRA-based vectors	
pTRA-Cgrft	Cytosol
pTRA-cTPgrft	Chloroplast
pTRA-APOgrft	Apoplast
pTRA-ERgrft	Endoplasmic reticulum
MagnICON TMV-based Provectors	
pICHgrft-Cyto	Cytosol
pICHgrft-Apo	Apoplast

Table 3.2 Summary of the vector constructs used for microscopic evaluation of subcellular targeting of griffitsin in *N. benthamiana*.

Vector module	**Subcellular compartment**
GRFTgfp fusion vectors	
pICHgrftGFP-Cyto	Cytosol
pICHgrftGFP-Apo	Apoplast
pICHbsp-grftGFP-SEKDEL	Endoplasmic reticulum
pTRA-cTPgrftGFP	Chloroplast

3.3.2 Effects of GRFT expression using the pTRA vector system

pTRAgrft vectors were infiltrated into *N. benthamiana* both with and without the NSs silencing suppressor from the Tomato spotted wilt virus (TSWV) to assess the effect of silencing suppression on GRFT accumulation. GRFT accumulation was measured by gp120 binding ELISA (Fig. 3.4). With these vectors, the cytosol was the optimal compartment for GRFT expression (Fig. 3.4A). In the presence of NSs, GRFT accumulated to around 1 g/kg and in the absence of silencing suppression to around 300 mg/kg. Thus, NSs silencing inhibition contributed to a 3-fold increase in GRFT yield. In the presence of NSs expression was high from as early as 4 days post infiltration (dpi) and seemed to increase further on. In contrast, expression in the absence of NSs remained constant over the period analysed. With cytosolic expression the leaf tissue remained healthy over the period of evaluation (Fig.3.5A).

With chloroplast directed expression, the presence of NSs increased GRFT levels by 3 fold (Fig.3.4B). With NSs mediated silencing suppression GRFT accumulated over 350 mg/kg and in the absence of NSs to approximately 100 mg/kg. An interesting observation with GRFT expression in the chloroplast was a sudden drop in GRFT levels 8 dpi in the presence of NSs and 10 dpi in the absence of NSs. This phenomenon was observed in several biological repeat experiments. Expression of GRFT in the chloroplast had no negative effect on plant tissue viability (Fig.3.5B).

In the apoplast the presence of the silencing suppressor enhanced GRFT levels again by an overall average of 3 fold (Fig.3.4C). Under the influence of NSs silencing suppression GRFT accumulated to more than 200 mg/kg and seems to be significantly higher from 10 dpi. Without suppression, levels of 47 mg/kg were obtained and expression were fairly constant over the period analysed. Expression of GRFT in the apoplast had a detrimental effect on plant tissue with leaf tissue damage visible from 4 dpi (Fig.3.5C)

In the endoplasmic reticulum, the presence of NSs did not seem to have a significant enhancing effect on GRFT levels (Fig.3.4D). In the presence of NSs, GRFT accumulated to a maximum level of 150 mg/kg while in the absence of NSs it accumulated to 120 mg/kg. However in the presence on NSs GRFT seem to increase later after infiltration while in the absence of silencing suppression GRFT seem to slightly decrease. Similar to the apoplast, GRFT expression in the ER had a negative impact on the leaf tissue viability (Fig. 3.5D).

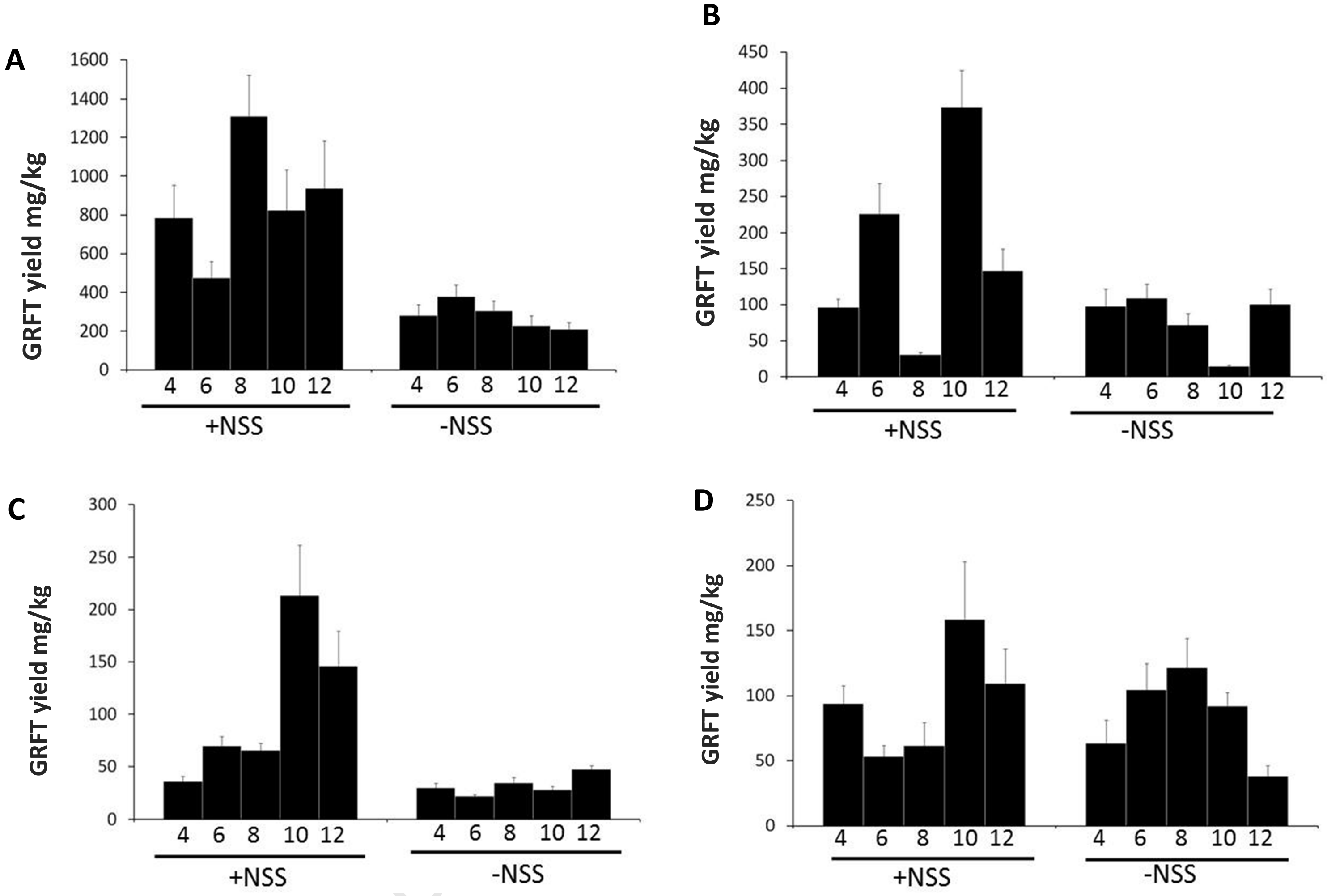

Fig. 3.4 pTRA GRFT expression levels as measured by gp120 binding ELISA on 4, 6, 8 and 12 days post infiltration (dpi). pTRA vectors pTRA-Cgrft (A), pTRA-cTPgrft (B), pTRA-APOgrft (C) and pTRA-ERgrft (D) were infiltrated with and without the NSs silencing inhibitor. Error bars indicate the difference in expression levels as calculated as standard error (SE) for three biological replicate experiments

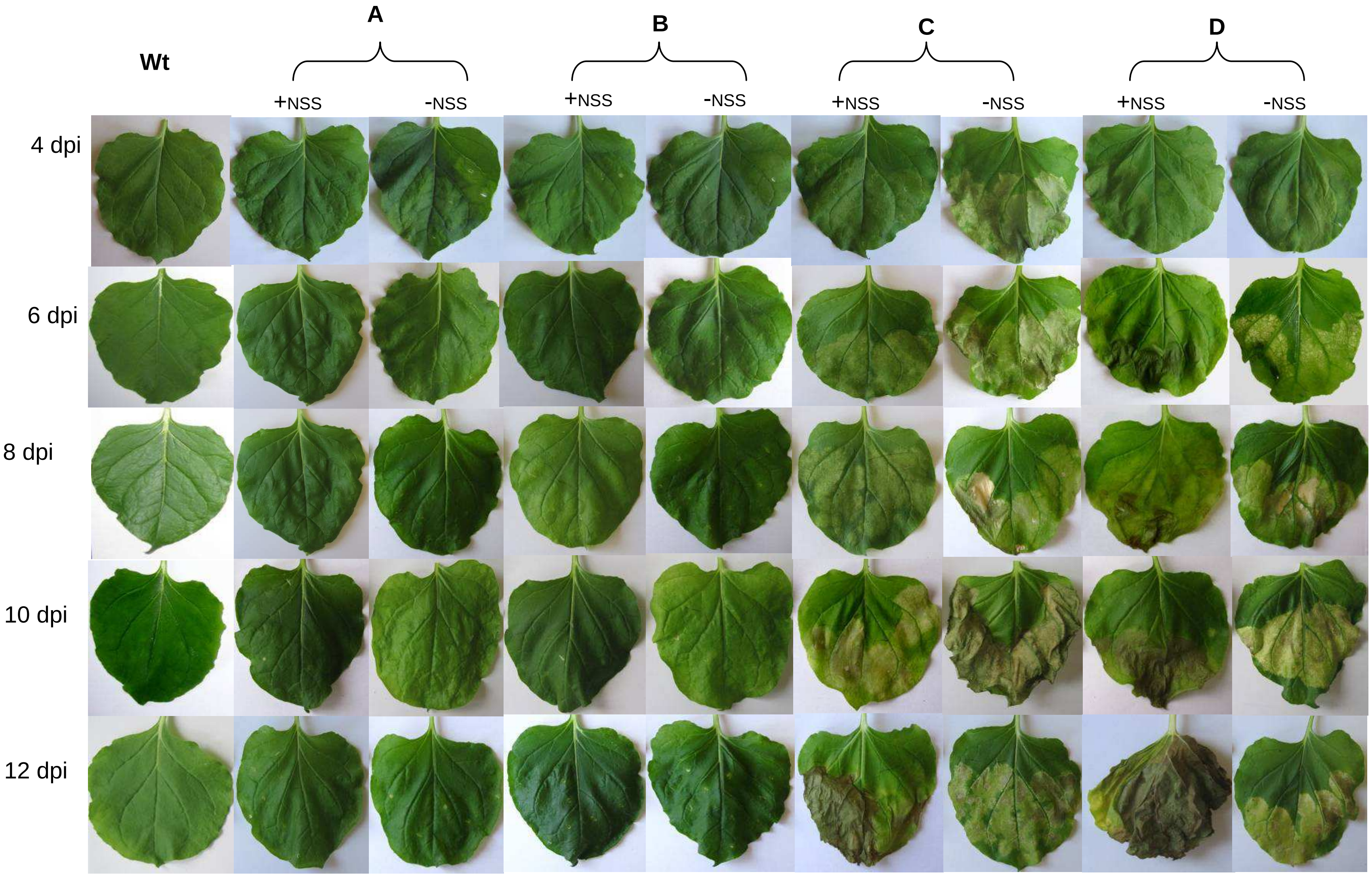

Fig. 3.5 The effect of intracellular localisation of griffithsin on *N. benthamiana* leaf phenotype over a time course. pTRA-based constructs pTRA-Cgrft (A), pTRA-cTPgrft (B), pTRA-APOgrft (C) and pTRA-ERgrft (D) were infiltrated with and without the NSs silencing inhibitor

3.3.3 Expression of GRFT with the magnICON vector system

GRFT was expressed in *N. benthamiana* leaves with the high output magnICON vector system. The latter consist of different vector modules that reassemble in the plant. In this study the modules consisted of a 3' module that contained the native GRFT gene and two 5' promodules which directed expression either to the cytosol or apoplast. An integrase module facilitated the recombination of the 5' and 3' promodules. With the magnICON system the cytosol was also the optimal location for GRFT accumulation; the lectin accumulated to over 1.5 g/kg (Fig. 3.6). These levels were observed from 4 days post infiltration (dpi) and remained fairly constant over the period analysed. Expression of GRFT in the cytosol with the magnICON system had no negative effect on leaf tissue (Fig. 3.7). Expression in the apoplast resulted in maximum GRFT levels of approximately 34 mg/kg (Fig. 3.6). Again GRFT levels observed remained relatively constant from 4 to 12 dpi. In contrast to cytosolic expression GRFT accumulation in the apoplast was detrimental to tissue (Fig. 3.7). The necrosis was visible as early as 4 dpi.

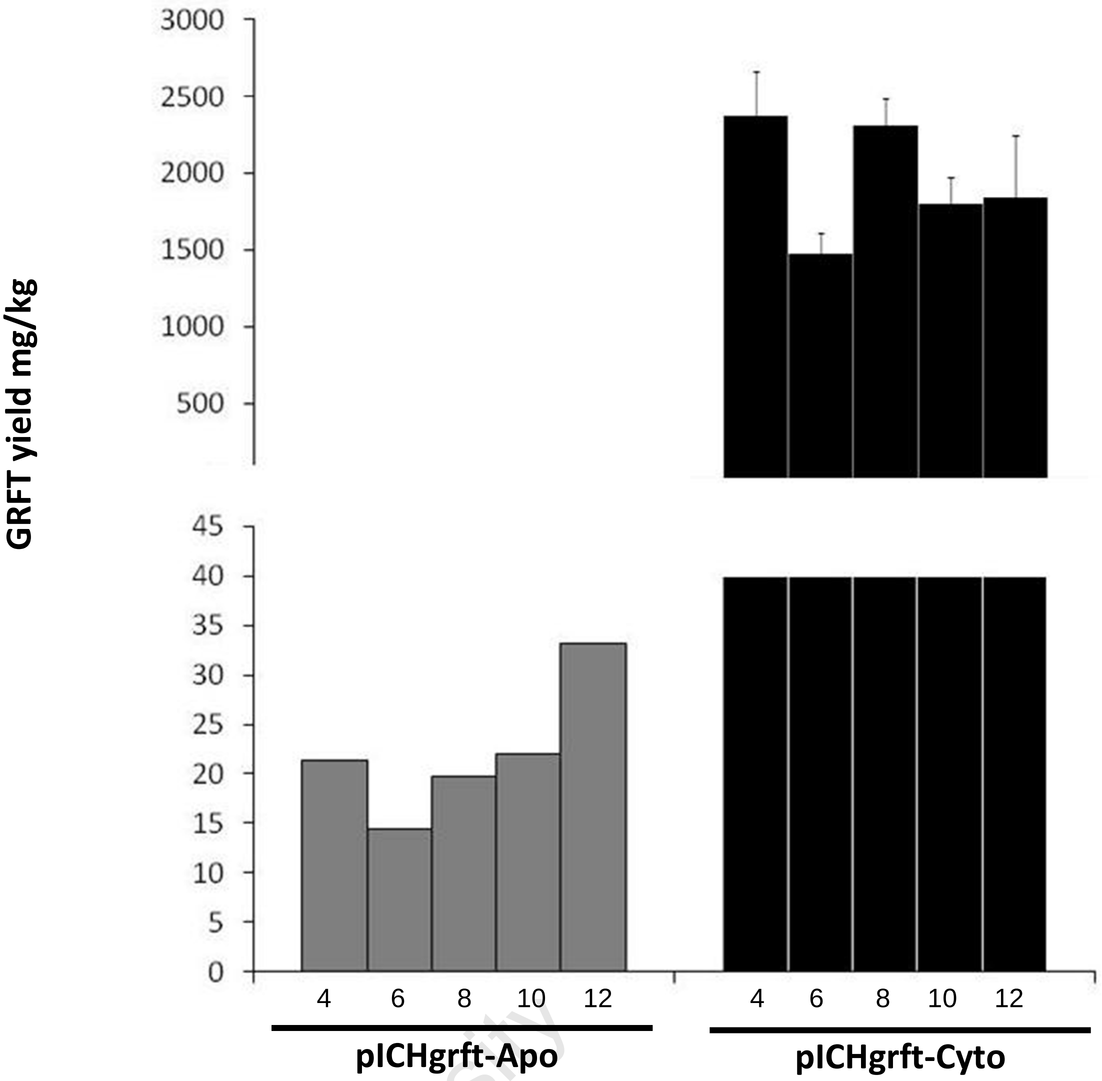

Fig. 3.6 GRFT expression with the magnICON system in the apoplast (pICHgrft-Apo) and cytosol (pICHgrft-Cyto) as measured by gp120 binding ELISA 4, 6, 8, 10 and 12 days post infiltration (dpi). Expression data is presented on two scales. Error bars indicate the difference in expression levels as calculated as standard error (SE) for three biological replicate experiments.

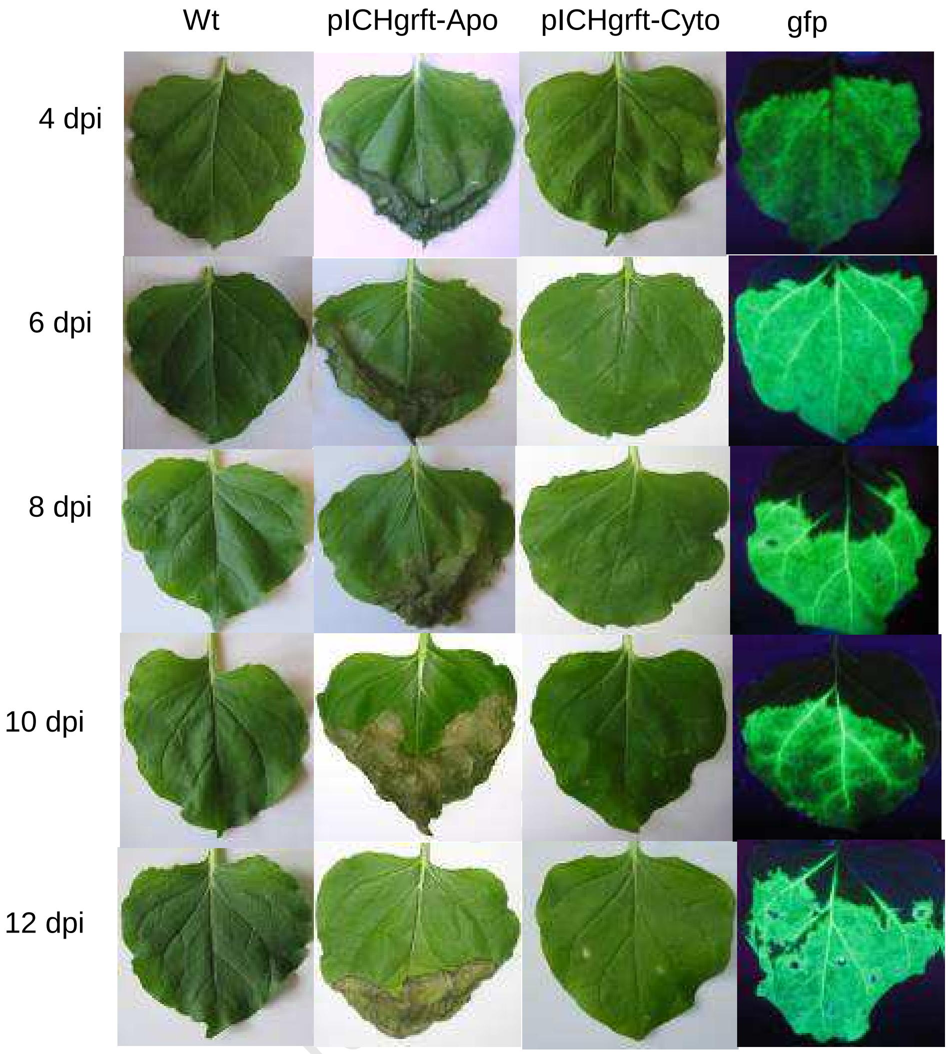

Fig. 3.7 The effect of subcellular location when griffithsin (GRFT) and GFP were expressed using magnICON provectors. The *gfp* provector was combined with the cytosol targeting provector, while the GRFT provector was combined with an apoplast (pICHgrft-Apo) and cytosol (pICHgrft-Cyto) targeting module. The GFP construct was visualised using UV illumination (what wavelength?)

3.3.4 Large scale expression of GRFT in N. benthamiana using pTRA and magnICON

The results detailed above were obtained using hand syringe-mediated infiltration experimentation. In industry syringe infiltration will not be practical, and a large scale vacuum infiltration process has to be applied. At the CSIR a vacuum system capable of infiltration of one whole plant was built and had to be assessed for infiltration efficacy and final product yield. Hence, the result of large scale infiltration on GRFT yield was assessed. *N. benthamiana* plants were vacuum infiltrated with the pTRA GRFT and GRFT magnICON vectors. Leaf material was harvested on days 4, 6 and 8 post infiltration and proteins were extracted from harvested material. For the pTRA vectors GRFT accumulation was significantly lower for all cellular compartments investigated (Fig. 3.8). In the cytosol approximately 50 mg/kg GRFT was obtained after large scale infiltration (Fig. 8A) vs. the 1 g/kg obtained with syringe infiltration. In the chloroplast GRFT amounts of 50 mg/kg (Fig. 3.8B) were obtained, which is 7-fold lower than that measured with hand infiltration. GRFT yields in both the apoplast and endoplasmic reticulum were below 10 mg/kg (Fig. 3.8C and 3.8D), more than a 10-fold reduction from syringe infiltrated levels.

With the large scale magnICON cytosol expression (Fig. 3.9B), GRFT levels were considerably lower than that observed with syringe infiltration. With large scale infiltration the maximum quantity of GRFT obtained in the cytosol was around 250mg/kg while hand infiltration resulted in over 1.5 g/kg. With apoplast expression, GRFT levels were similar as to those obtained with hand infiltration, reaching maximum levels of approximately 20 mg/kg for syringe infiltration and 25mg/kg for large scale infiltration (Fig.3.9A).

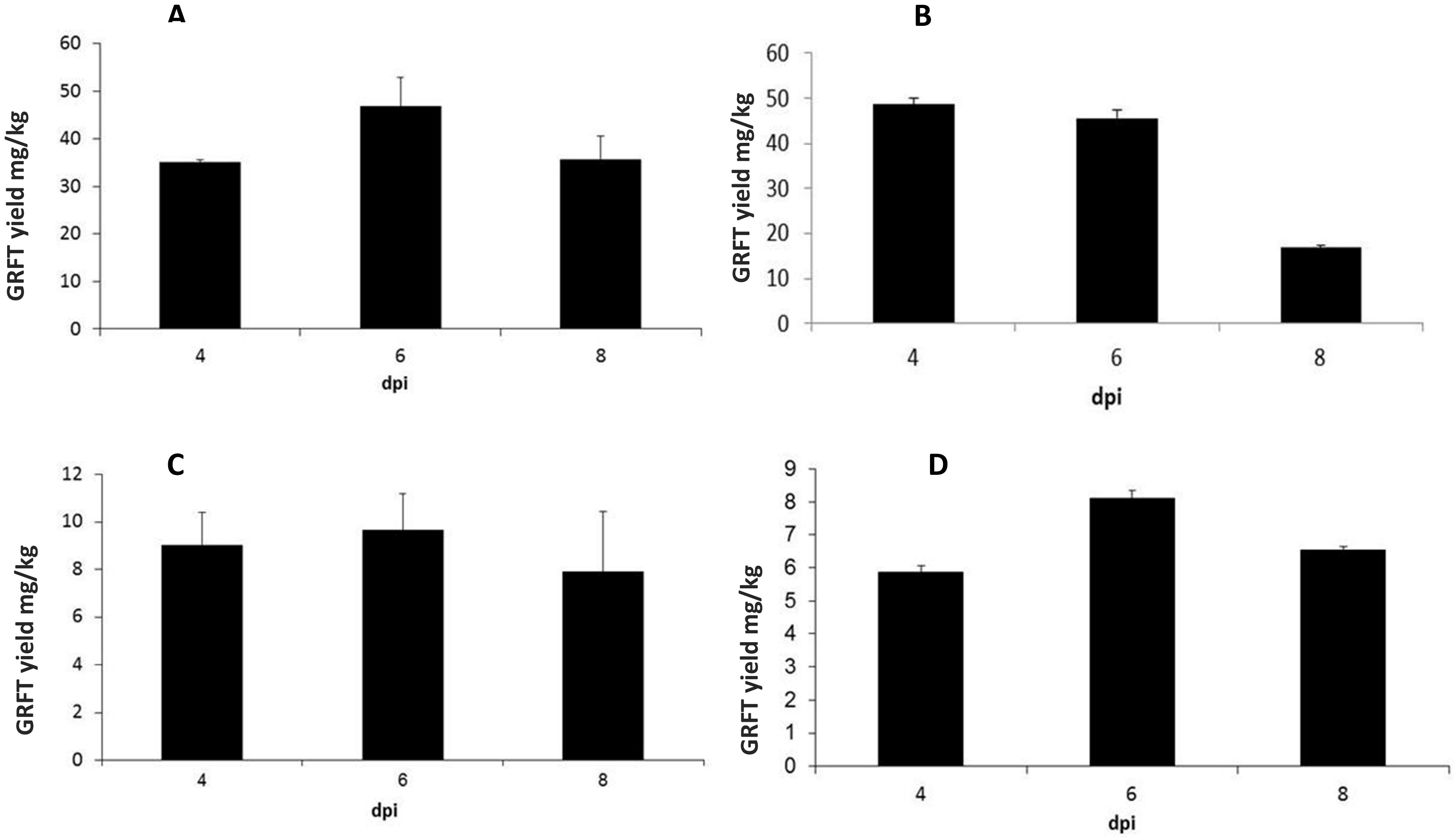

Fig. 3.8 Expression of GRFT with pTRA vectors following large scale infiltration of *N. benthamiana* leaves. Protein extractions were prepared from leaves harvested at days 4, 6, and 8 post infiltration (dpi). GRFT expression was measured for pTRA-Cgrft (A), pTRA-cTPgrft (B), pTRA-APOgrft (C) and pTRA-ERgrft (D) infiltrated with the NSs silencing inhibitor. Error bars indicate the difference in expression levels as calculated as standard error (SE) for two samples taken from the extract.

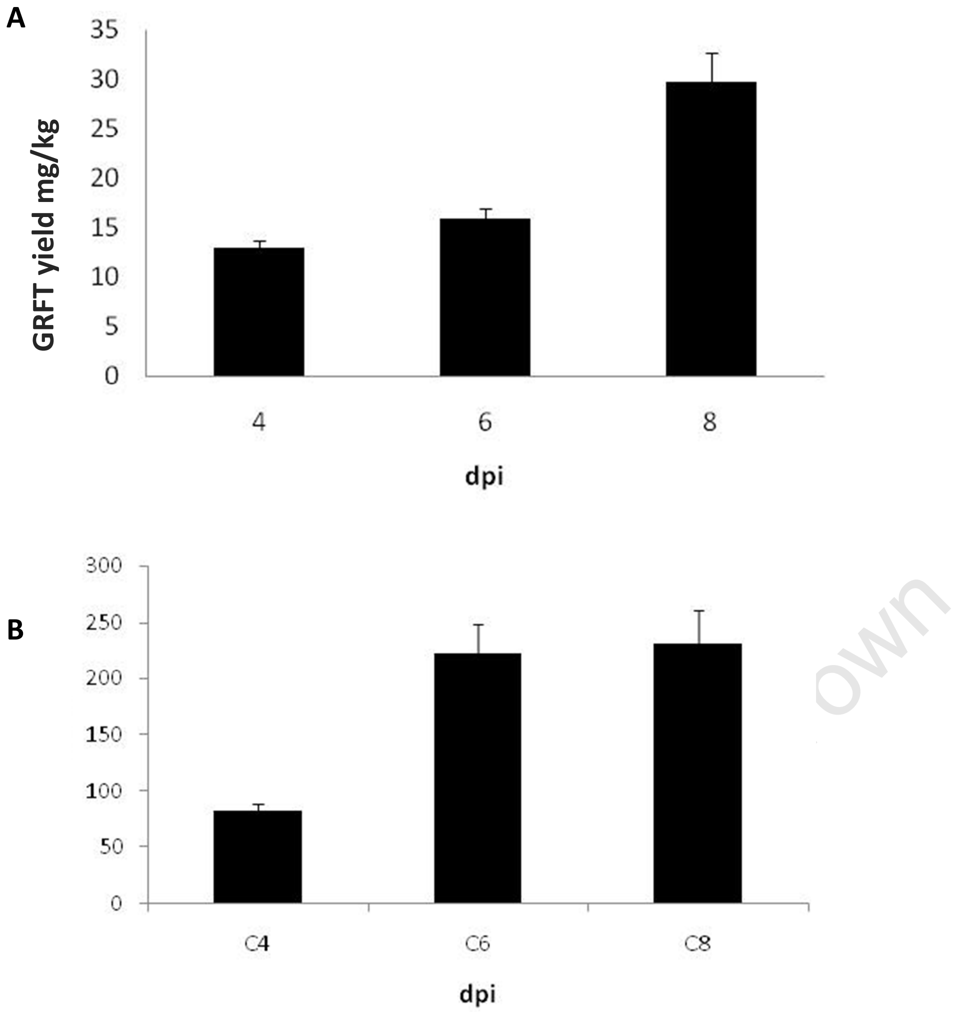

Fig. 3.9 Expression of GRFT (in mg/kg) with magnICON vectors were measured in large scale infiltrated *N. benthamiana* leaves. Expression for pICHgrft-Apo (A) and pICHgrft-Cyto (B) was measured 4, 6 and 8 days post infiltration (dpi) by gp120 binding ELISA. Error bars indicate the difference in expression levels as calculated as standard error (SE) for two samples taken from the extract.

3.3.5 Biochemical characterisation of magnICON produced GRFT by PAGE, immunoblot and MS analysis

PAGE and immunoblot analysis were used to biochemically characterise GRFT produced in the cytosol using the magnICON system. Proteins extracted from infiltrated leaves for cytosol expression were analysed under non-denaturing (Fig.3.10) and denaturing (Fig. 3.11) PAGE conditions and detected with GRFT specific antibodies in using both a colour (Fig. 3.10B) and chemiluminescence (Fig. 3.10C) substrate. Under non-denaturing PAGE conditions the *E. coli*-produced purified GRFT control had a size of approximately 75 kDa, no dimer band of 27 kDa was observed (Fig. 3.10). In the infiltrated samples several bands were detected that were absent in the uninfiltrated control. The sizes of these bands were approximately 40, 50, 60, 65 and 70 kDa. These also seem to react with the GRFT specific antibody. The bands detected with the antibody ranged from 40 kDa to bands larger than 70 kDa (Fig. 3.10 B and C). When the same samples were denatured, the *E. coli* GRFT appeared as a band of 15 kDa (Fig. 3.11). A small amount of dimer was also present at 27 kDa. Protein samples from GRFT infiltrated tobacco produced a band smaller than 15 kDa, which was absent from the wild type (Fig. 3.11). To obtain a better resolution of the smaller GRFT-specific band that is produced in *N. benthamiana*, plant protein extracts were resolved on a 12% acrylamide gel that was prepared with a 19:1 ratio of acrylamide:bis acrylamide (Fig. 3.12). The *E. coli* produced GRFT band was close to 15 kDa, while the plant produced GRFT bands again ran lower in the PAGE gel. Peptide bands 1, 2, 3 and 4 were excised and subjected to Peptide mass fingerprint sequencing (PMFS). No GRFT was detected in peptide bands 2 and 4, while in peptide bands 1 and 3 GRFT was strongly detected. In the latter however the His tag fragment at the N and C terminus was not detected (Fig. 3.13). To resolve if His tag cleavage occurred, a His and GRFT specific immunoblot was performed (Fig. 3.14). With both the GRFT and His-specific antibodies the *E .coli* and *N. benthamiana*-produced GRFT were detected.

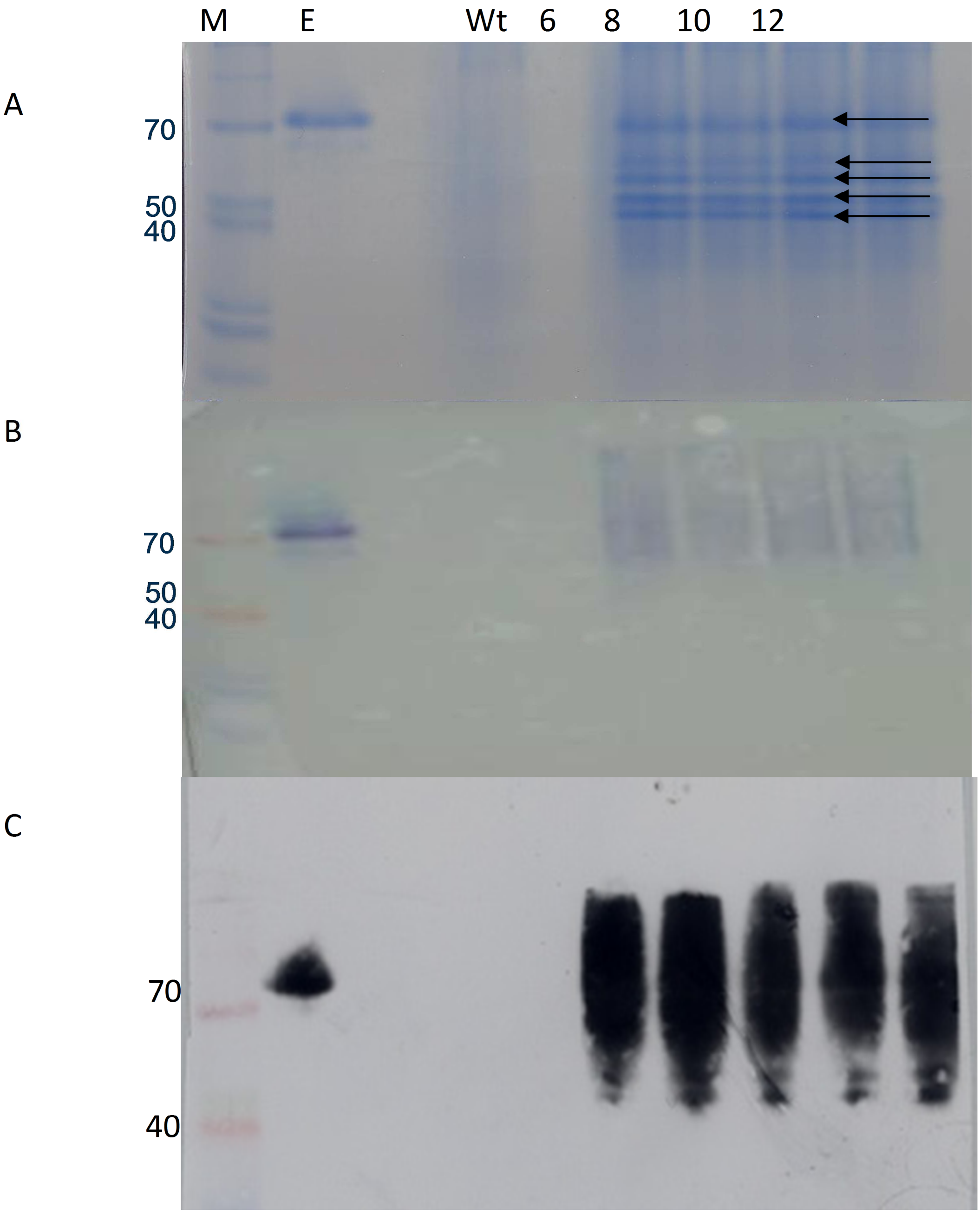

Fig. 3.10 Coomassie staining (A) and immune detection with colour (B) and chemiluminescence (C) of protein extracts of *N. benthamiana* GRFT magnICON plants separated under non-denaturing conditions. The lanes represents the following; protein marker (M); purified *E. coli* produced GRFT (E); uninfiltrated tobacco leaf extract (Wt); magnICON GRFT cytosol protein extracts from days 6, 8, 10 and 12 post infiltration. The arrow indicates the presence of GRFT.

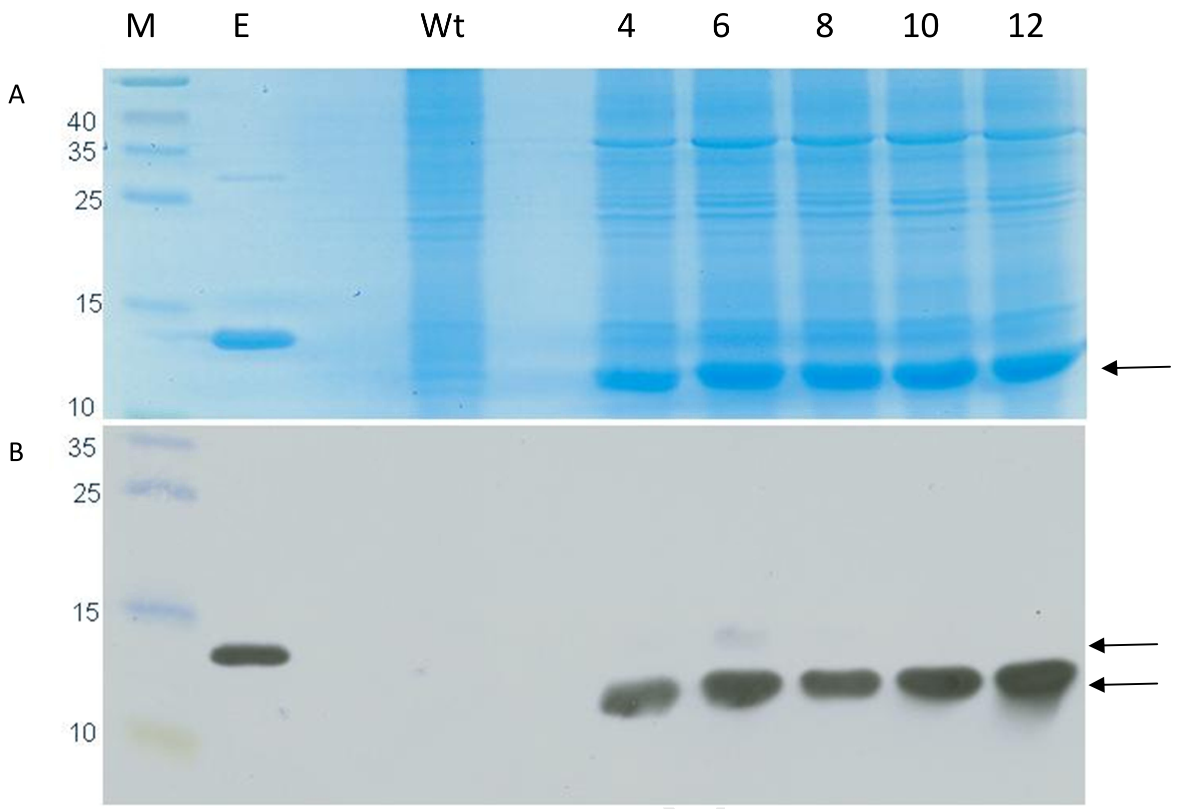

Fig. 3.11 Coomassie staining and immune detection of protein extracts of *N. benthamiana* GRFT magnICON plants separated under denaturing conditions. The lanes represents the following; protein marker (M); purified *E. coli* produced GRFT (E); uninfiltrated tobacco leaf extract (Wt); magnICON GRFT cytosol protein extracts from days 4,6,8,10 and 12 post infiltration. The arrow indicates the presence of GRFT.

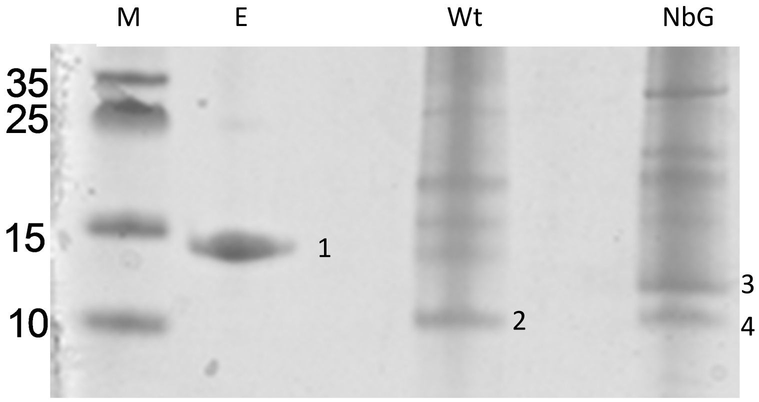

Fig. 3.12 Tris-tricine PAGE electrophoresis of *N. benthamiana* protein extract of cytosol produced GRFT. Lanes contain a molecular weight marker (M); purified *E. coli* produced GRFT as positive control (E), protein extract from unifiltrated wild type *N. benthamiana* (Wt) and cytosol produced GRFT (NbG).

a)

MGSSHHHHHHSSGLVPRGSLTHR**KFGGSGGSPFSGLSSIAVRSGSYLDAIIIDGVHHGGSGGNLSPTFTFGSGEY**
ISNMTIRSGDYIDNISFETNMGRRFGPYGGSGGSANTLSNVKVIQINGSAGDYLDSLDIYYEQY

b)

MGSLTHR**KFGGSGGSPFSGLSSIAVRSGSYLDAIIIDGVHHGGSGGNLSPTFTFGSGEYISNMTIRSGDYIDNIS**
FETNMGRRFGPYGGSGGSANTLSNVKVIQINGSAGDYLDSLDIYYEQYSSGLVPRGSHHHHHH

Fig. 3.13 Peptide Mass Fingerprint Sequencing (PMFS) results of the peptides that represent the *E. coli* produced GRFT band (a) and in the tobacco produced GRFT band (b). Sequences in green represents peptides that were detected with high confidence, sequences in red were peptides detected with low confidence while sequences in grey were peptides not detected.

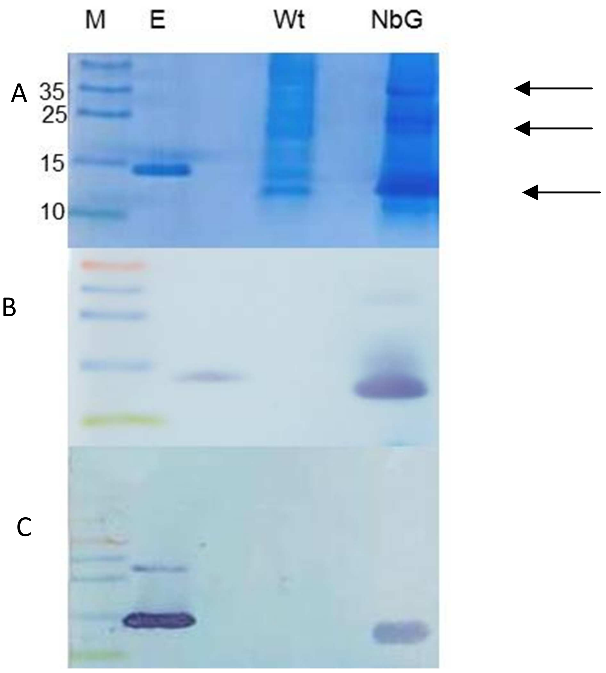

Fig. 3.14 Electrophoresis of GRFT cytosol extracts from *N. benthamiana* separated on a 12% Tris-tricine gel for Coomassie staining (A), GRFT specific immunoblotting (B) and His-specific immunoblotting (C).

3.3.6 Purification of GRFT from N. benthamiana infiltrated leaves

Total protein extract was applied to a Ni-IDA his affinity column. Fractions collected from the purification was collected and analysed *via* PAGE and immunoblot (Fig. 3.15 A and B). The crude extract had an intense band that corresponded to the GRFT monomer of 15 kDa. This band was also present in the flow through and the first wash fraction but was separate in the second wash fraction. The eluted fraction contained a band that corresponded to the GRFT monomer and dimer. From the immunoblot analysis no band was visible in the uninfiltrated control but bands corresponding to GRFT and its dimer were present in the purified eluted fraction.

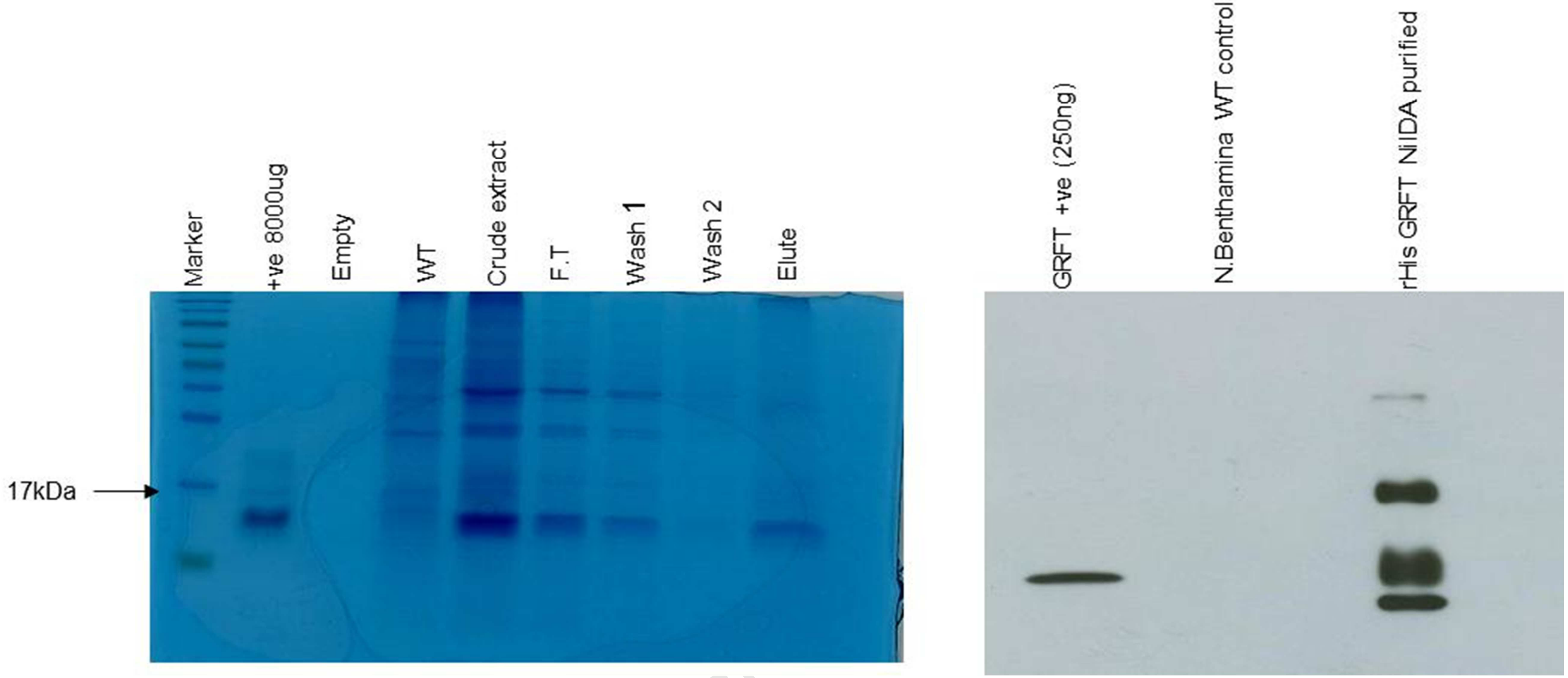

Fig. 3.15 SDS Page analysis of the recombinant plant produced His-GRFT extract of the protein purified by using a Protino® Ni-IDA purified, -10 column desalted and analysed by Coomassie blue staining on a 15% tris-glycine gel. The bands in the elute were positively identified as griffithsin via Mass Spec analysis. This was further reiterated by immunoblot analysis with anti-GRFT pAB.

3.3.7 Comparison of efficacy between N. benthamiana and E. coli produced GRFT

The efficacy of partially purified tobacco GRFT was tested in a TZM-bl pseudovirus assay against 9 HIV-1 subtype C viruses (Du151.2, COT6.15, COT9.6, CAP210.E8J, CAP45.G3J, CAP239.G3J, CAP270.cvl44, CAP261.cvl93 and CAP177.cvl96; K), including 3 from cervical vaginal lavages (CAP177.cvl96, CAP270.cvl44 and CAP261.cvl93cvl) of HIV positive women, and 2 HIV-1 subtype B viruses (QH0692.42 and PVO.4). GRFT inhibited both C and B HIV strains. Furthermore, the IC_{50} values of tobacco produced GRFT was comparable to that reported for *E. coli* produced GRFT (Alexandre *et al*. 2010) for the majority of the viruses tested (Table 3.3). Lower IC_{50}values were observed for 3 subtype C viruses (CAP210.E8J, CAP177.cvl96 and CAP261.cvl93cvl) and one subtype B virus (QH0692.42).

In a PBMC assay we tested *E. coli* GRFT and tobacco GRFT against one HIV-1 subtype C (CM1) and one subtype B (ADA) isolate. Table 3.4 shows that semi-purified tobacco GRFT had similar potency against the two tested viruses as the purified *E. coli* GRFT.

Further, we investigated the inhibition of tobacco-produced GRFT on HIV-1 transfer *via* DC-SIGN. Fig. 3.16 shows that GRFT in a crude tobacco extract could inhibit the transfer of DC-SIGN associated HIV-1 virus to TZM-bl cells as well as purified GRFT from *E. coli* (Alexandre *et al*., 2011).

Notably, both *E. coli* and *N. benthamiana* GRFT did not inhibit the control VSV-G virus indicating that no non-specific cell toxicity effect was associated with the observed IC_{50} values

Table 3.3 A comparison between crude *N. benthamiana* produced GRFT and purified *E. coli* produced GRFT as measured by a single-cycle neutralization assay (TZM-bl assay).

		IC$_{50}$ (nM)	
Envelope	***Subtype***	***E. coli GRFT***	***N. Bent.*** **GRFT**
Du151.2	C	1.5 ± 0.8	1.7 ± 0.5
COT6.15	C	0.4 ± 0.2	0.4 ± 0.10
COT9.6	C	1.2 ± 0.2	1.4 ± 0.01
CAP210.E8J	C	0.2 ± 0.0	0.05 ± 0.01
CAP45.G3J	C	0.2 ± 0.1	0.2 ± 0.04
CAP239.G3J	C	0.1 ± 0.0	0.09 ± 0.02
CAP177.cvl96	C	55.9 ± 5.0	30.8 ± 0.10
CAP270.cvl44	C	0.3 ± 0.1	0.2 ± 0.07
CAP261.cvl93	C	4.2 ± 0.4	1.9 ± 0.01
QH0692.42	B	0.2 ± 0.1	0.07 ± 0.02
PVO.4	B	0.1 ± 0.0	0.1 ± 0.04
VSV-G		No titer	No titer

Table 3.4 A comparison between crude *N. benthamiana* produced GRFT and purified *E. coli* produced GRFT as measured by Peripheral Blood Mononuclear Cell (PBMC)

		IC_{80}	
Virus	***Subtype***	***E. coli GRFT***	***N. Bent.* GRFT**
CM1	C	122.8 ± 5.1	73.8 ± 12.5
ADA	B	71.4 ± 10.2	51.7 ± 8.1

.

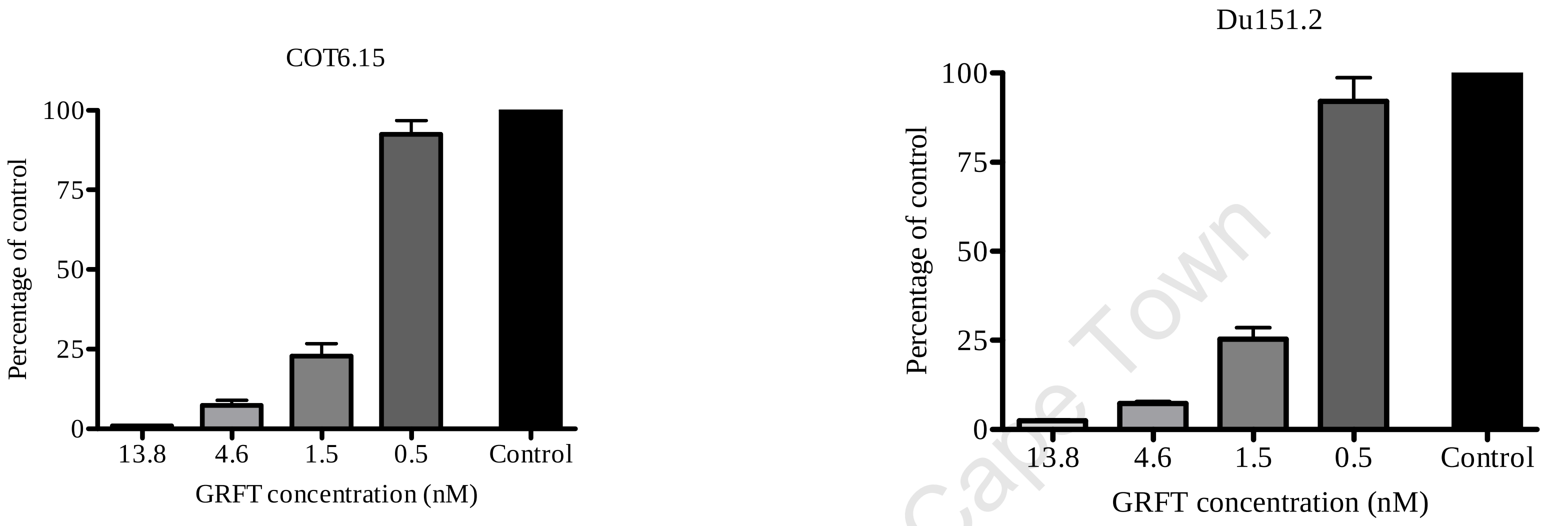

Fig. 3.16 Inhibition of DC-SIGN mediated HIV transfer by *N. benthamiana* produced GRFT. Crude *N. benthamiana* extract containing GRFT was tested for the ability to inhibit the transfer and subsequent infection by HIV stains COT6.15 and Du 151.2 as mediated by DC-SIGN.

3.3.8 Confocal microscopic evaluation of the subcellular location of GRFT

To confirm the subcellular location of GRFT in *N. benthamiana* cells, GRFT was fused to the green fluorescent protein (GFP) and expressed using different targeting sequences. The targeting was visualised using confocal laser scanning microscopy (Fig. 3.17). The GRFT-GFP fusion was expressed without any signal sequences as well as with apoplast, chloroplast and endoplasmic reticulum retention signals. GFP alone was included as a control for cytosol and apoplast expression. Confocal microscopy was conducted 6 days post infiltration and showed that GFP expression without any signal sequences resulted in the protein accumulating in the cytosol around the vacuoles (Fig. 3.17A). When GFP was expressed with the calreticulin apoplast targeting signal it was no longer located inside the cell, but seems to aggregate around the cells (Fig. 3.17B). Similar to GFP, the GRFT-GFP fusion expressed without targeting signal sequences also located inside the cell in the cytoplasm (Fig. 3.17C). In some cells the GRFT-GFP fusion was also present as a bright fluorescence in the nucleus. In contrast to GFP alone, the fusion formed aggregated masses in cytosplasm around the vacuoles. When the GRFT-GFP fusion was expressed with the apoplast targeting sequence, the protein was detected outside the cells (Fig. 3.17D). Expression of the fusion protein with the chloroplast targeted signal resulted in the accumulation of the protein in the chloroplasts (Fig. 3.17E). Targeting GRFT-GFP to the endoplasmic reticulum using a the LT-B signal peptide and SEKDEL retention sequence caused the fusion protein to accumulated throughout the cell cytoplasm as aggregated masses (Fig. 3.17F). No fluorescence was detected in the nucleus.

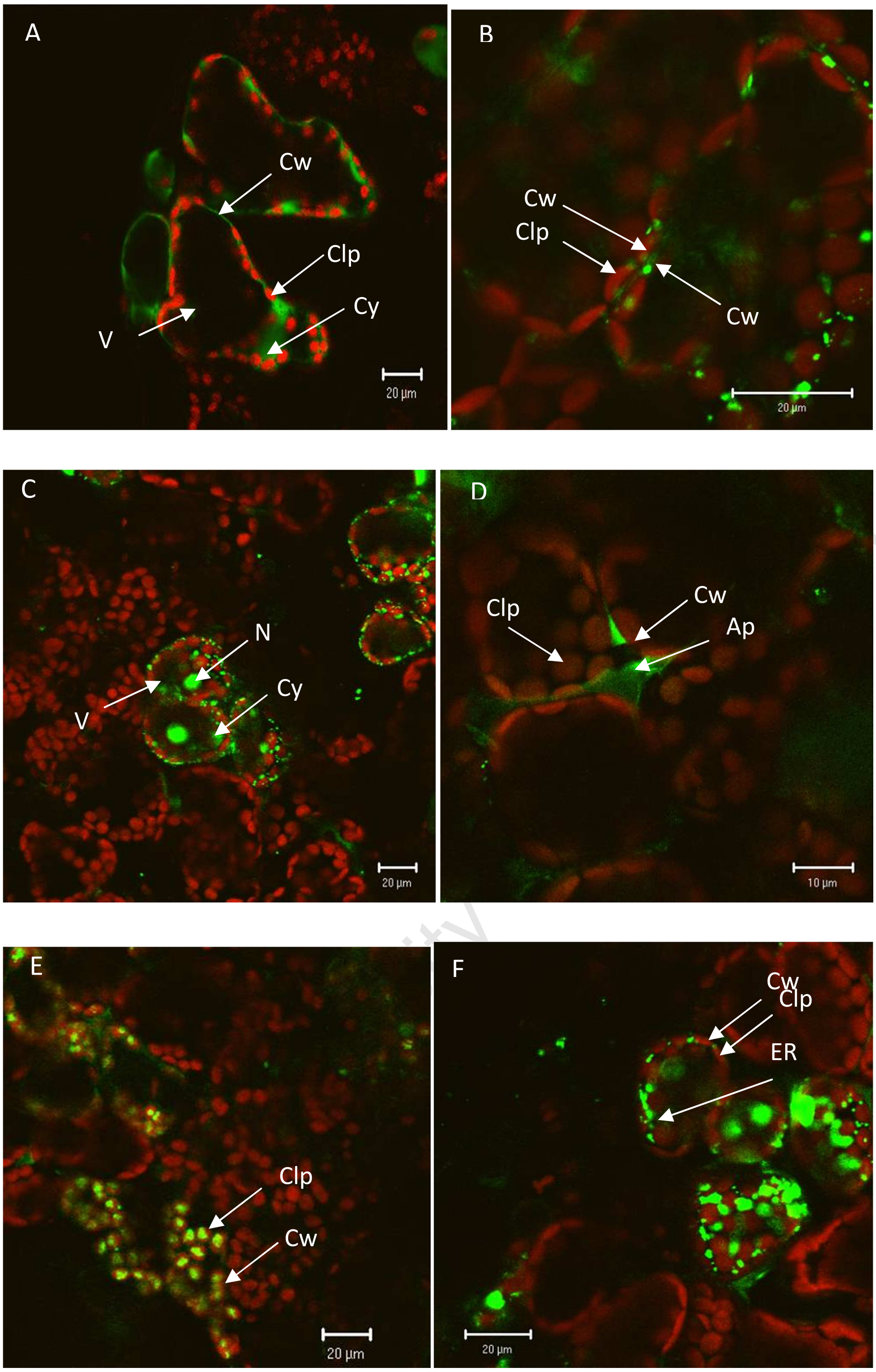

Fig. 3.17 CLSM (Confocal laser scanning microscopy) imaged transient infiltrated *N. benthamiana* cells displaying red autofluorescing chloroplast, GFP and GRFT-GFP targeted to different subcellular compartments. GFP located in the cytosol (A) and apoplast (B). GRFT-GFP fusion protein located in the cytosol/nucleus (C); apoplast (D); chloroplast (E)

and endoplasmic reticulum (F). Ap, apoplast; Clp, chloroplast; Cy, Cytosol; Cw, Cell wall; ER, endoplasmic reticulum; N, nucleus; and V, vacuole.

3.3.9 Transmission electron microscopic imaging of subcellular location of GRFT in N. benthamiana

Immunogold labelling together with transmission electron microscopy (TEM) imaging was used to determine where GRFT without GFP fused to it will localise inside cells (Fig. 3.18). Cytosol targeting resulted in expression of GRFT in the cytosol around cell organelles such as the nucleus and vacuoles (Fig. 3.18.1). No GRFT was detected in the nucleus in any of the cytosol targeted samples that were scanned. GRFT targeted to the chloroplast showed the presence of the lectin in the chloroplasts (Fig. 3.18.2). Apoplast targeted expression resulted in the accumulation of GRFT between cells (Fig. 3.18.3). GRFT targeted to the ER displayed immune-labeling in the cytoplasm where the ER is present (Fig. 3.18.4). The abundance of the lectin was much less than that observed in the cytosol (Fig. 3.18.1). Leaf material infiltrated with cytosol targeted GRFT-GFP were also subjected to gold labelling (Fig. 3.18.5). Labelled proteins were present in the cytoplasm and most definitely in the nucleus (Fig. 3.18.5). Both unlabelled and labelled (Fig. 3.18.6) uninfiltrated plants displayed minimal presence of gold particles.

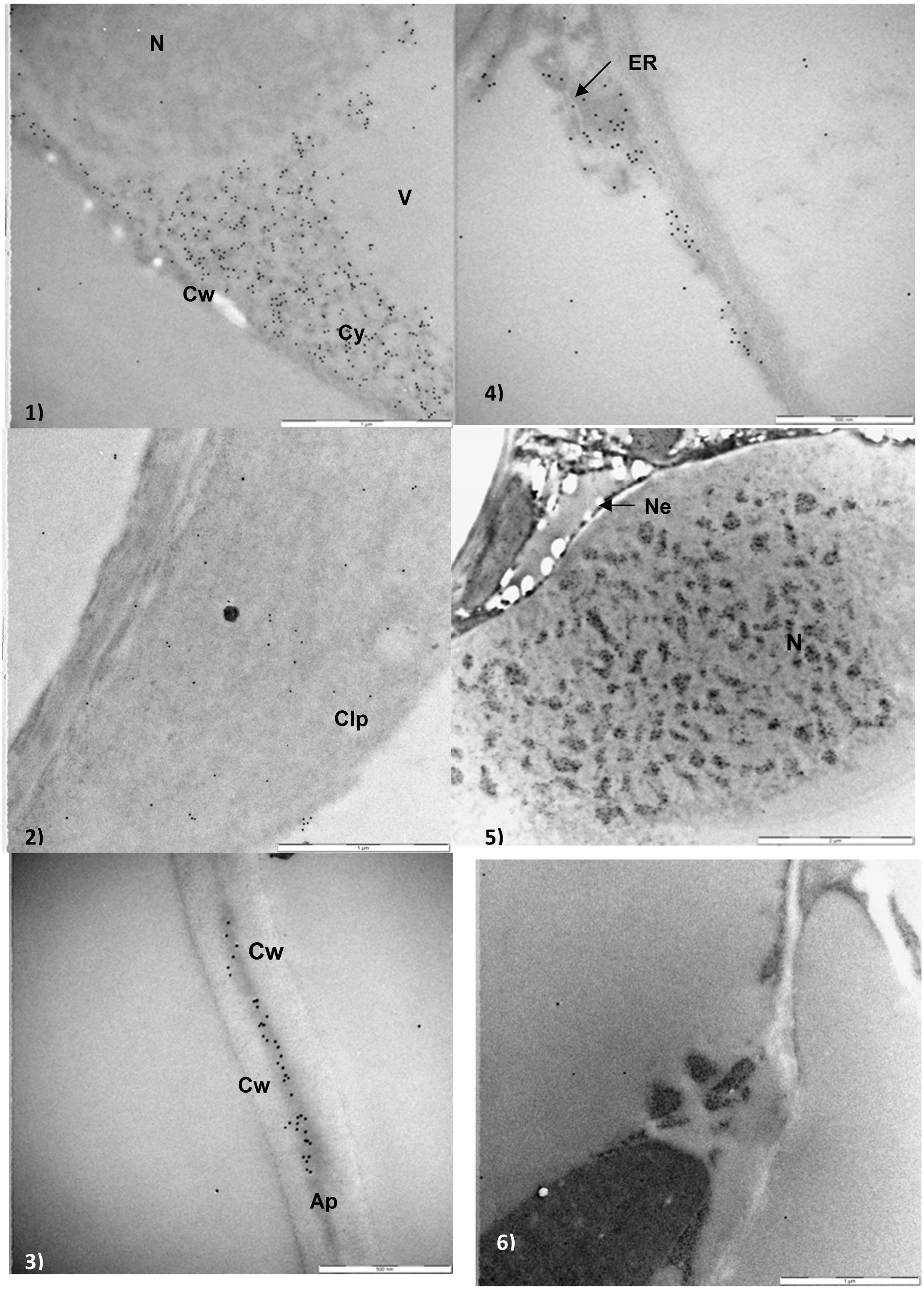

Fig. 3.18 Electron micrographs of sections of *N. benthamiana* infiltrated with magnICON and pTRA vectors for targeted expression of GRFT in the cytosol (1), chloroplast (2), apoplast (3) and endoplasmic reticulum (4). *N. benthamiana* was also infiltrated with a magnICON GRFT-GFP construct for expression in the cytosol (5). Uninfiltrated leaf

material was exposed to the labelling procedure as control (6). Ap, apoplast; Clp, chloroplast; Cy, Cytosol; Cw, Cell wall; ER, endoplasmic reticulum; N, nucleus; Ne, nuclear envelope and V, vacuole.

3.4 DISCUSSION

Several HIV-binding lectins have been isolated and are currently being investigated for microbicide application. Of these, GRFT is currently the most potent and promising. Plants are emerging as promising alternative expression platforms for pharmaceutical molecules. To obtain high protein expression in plants, several strategies can be applied such as subcellular targeted expression. Furthermore, new vector technologies have enabled plants to produce recombinant proteins to high levels. Although GRFT has been expressed with TMV vectors at high levels in *N. benthamiana*, a systematic analysis of the effect of subcellular targeting on expression and accumulation has not been reported previously. In this study I investigated two expression vector systems and different subcellular locations for optimal GRFT production in *N. benthamiana*. I expressed GRFT with enhanced binary pTRA vectors and the deconstructed magnICON viral vectors. Expression in the cytosol, chloroplast, apoplast and endoplasmic reticulum was evaluated. Additionally, the effect of silencing suppression on GRFT accumulation was assessed.

For both vector systems the optimal compartment for GRFT expression was the cytosol. The magnICON vector resulted in the highest GRFT yield reaching over 1.5 g/kg. This is slightly higher than levels previously reported for GRFT production in *N. benthamiana* using a tobacco mosaic viral (TMV) vector under control of a duplicated subgenomic promoter (O’Keefe *et al*., 2009). GRFT was produced in significantly lower amounts in all the other compartments.

Our results further support the notion that silencing suppression is needed to increase protein levels in transient expression systems (de Haan *et al*., 2010, Voinnet *et al*., 2003). In this study NSs enhanced accumulation of GRFT by approximately 3 fold in the cytosol, chloroplast and apoplast. De Haan *et al*. (2010) also reported an enhancing effect of silencing suppression on the production of cytosolic and secreted proteins. Interestingly, we observed no significant contribution of NSs on GRFT production in the endoplasmic reticulum. Theoretically, both the GRFT transcripts of the apoplast and ER follow the same cell sorting route to the ER. The maximum GRFT expression in both these compartments was very similar and peaked at 10 dpi. Given that naturally NSs is found in the cytosol (Kormelink *et al*., 1991), the effect of silencing suppression for GRFT ER expression should have followed a similar trend as that of the apoplast. It could be that in this study, the transit time for ER and apoplast transcripts differ in such a way that the effect of NSs silencing on ER targeted expression cannot act quickly enough to show an impact. In contrast to the cytosol, the pTRA vectors resulted in higher GRFT apoplast expression than for the magnICON system. It could be possible that with the higher expression levels associated with the magnICON system, the expression threshold is reached faster and thus GRFT only accumulates to a certain level, especially where cytotoxicity is observed.

No tissue damage was observed when GRFT was expressed in the cytosol and chloroplast; however, GRFT accumulated to significantly different amounts in these two compartments. GRFT accumulated to some extent in the chloroplast while it reached more than 2 g/kg in the cytosol. With the rbsc1-cTP target sequence, translation occurs in the cytosol whereafter the protein is transported to and folded in the chloroplast (Meyers *et al*., 2008). Since cytosol targeted GRFT is also translated in the cytosol it could be that the transcript level differs between cytosol and chloroplast targeted GRFT. Alternatively the unique fold of GRFT (Ziolkowska *et al*., 2006) may require eukaryotic advanced cellular machinery rather than what is provided in the prokaryotic derived chloroplasts. This leads to fewer stable GRFT dimers and more monomers that are less stable (Moulaei *et al*., 2010). An interesting observation of this study was that GRFT levels had a sudden drop after 8 days post infiltration in the presence of NSs and 10 days post infiltration in the absence of NSs. Since no visible leaf discolouring (i.e. chloroplast loss) was seen, it could only be surmised that the stability of GRFT in the chloroplast declined after a period of time.

Expression of GRFT in the apoplast and endoplasmic reticulum caused visible tissue damage. Leaf necrosis has previously been reported with use of the magnICON system for expression of the IgG human antibodies in the apoplast (Giritch *et al*, 2006) and human growth hormone (hGH) in the apoplast and cytosol (Gils *et al*., 2005). The necrotic effect has been attributed to high accumulation levels of protein in the cell; the IgG levels were relatively high despite the necrotic effect and for hGH the compartment that showed the highest necrosis also gave the highest expression levels (Gils *et al*., 2005). However, for GRFT this was not the case since the highest expression levels were observed in the cytosol without any tissue damage. It has been reported that GRFT binds mannose, N-acetylglucosamine and glucose (Mori *et al*., 2005; Ziolkowska *et al*., 2006 Ziolkowska *et al*., 2007). In a plant cell, N-glycosylation begins in the ER by the transfer of an oligosaccharide precursor, Glc3Man9GlcNAc2, to the nascent protein (Gomord *et al*., 2010). During the movement of the proteins through the secretory pathway, other modifications are carried out such as removal of mannose and glucose resides. Thus, if GRFT is transported to the ER via a signal peptide or apoplastic targeting signal, it may encounter sugars that it is able to bind. If these carbohydrates are crucial in the secretory pathway, it can cause cell death and subsequent tissue damage. When *Galanthusnivalus* agglutinin (GNA) were modified to locate to the ER in transgenic *Arabidopsis thaliana*, it was reported that plants displayed a dwarfed phenotype compared to those in which GNA located to the vacuole and that many plants died before setting seed (Fouquaert *et al*., 2007). The authors have also speculated that the observed cytotoxic effect of the lectin might be due to interference with synthesis and sorting of N-glycosylated proteins in the ER. I thus conclude that expression levels and effect on tissue is a combinatory effect from accumulation levels as well as native properties of the protein expressed.

Expression data of GRFT in *N. benthamiana* was gathered from plants that were syringe infiltrated with the constructs. In the commercial arena syringe infiltration will not be a practical way to introduce these constructs and large scale infiltration methods will be applied. At the CSIR we have developed a vacuum infiltration system to achieve large scale infiltration. GRFT expression using vacuum infiltration was thus subsequently investigated. Significantly lower GRFT amounts were obtained from large scale infiltrated plants. The lower observed levels could be attributed to two factors. First, with syringe infiltration the exact infiltrated zone was labelled and harvested. In large scale infiltration, whole plant material is harvested, which is not necessarily infiltrated to full capacity as demonstrated when assessing infiltration efficacy via GFP fluorescence (results not shown). Second, crushing of smaller masses of leaf material to a finer powder was easier than with larger amounts of leaf biomass. Thus, the resulting plant mass was possibly not fine enough for optimal contact with the extraction buffer. To address these issues, plant material can be processed using a disintegrator and screw press as reported by O'Keefe (*et al*. 2009). The cell culture densities used correlated to that used by Kentucky BioProcessing (KBP, personal communication); therefore, one could investigate the effect of different *Agrobacterium* OD_{600} used for infiltration on subsequent expression levels.

From SDS-PAGE analysis it appeared that GRFT that is expressed in the plant cytosol was smaller than purified *E. coli* produced GRFT used as a control in the gel electrophoresis. Previous plant produced GRFT was not electrophoresed alongside the *E. coli* produced GRFT (O'Keefe *et al.*, 2009), while *E. coli* produced GRFT was separated with native GRFT without a His tag (Giomarelli *et al.*, 2006), thus it is difficult to say why the plant-produced His tagged GRFT in this study migrated lower than *E. coli* produced GRFT, and if this is a common phenomenon. Peptide mass fingerprint sequencing (PMFS) of the tobacco produced GRFT showed that in both the *E. coli* and tobacco-produced GRFT the same GRFT peptide was detected with confidence. For both these GRFT bands the His tagged containing peptide was not detected with confidence, although an immunoblot with a His affinity antibody detected both. In the *E. coli* produced GRFT the His tag is present at the N-terminus, while in the tobacco produced GRFT it was present on the C-terminus. It could be possible that this affects the folding and thus migration through the PAGE gel. Under on-denaturing conditions, the protein extract from GRFT magnICON infiltrated samples contained clear high molecular weight proteins which were absent in the uninfiltrated sample. These also seem to be more immunoreactivce than the proteins in the negative control. Interestingly the purified *E.coli* produced GRFT was also detected as a high molecular weight protein that is more than double the size of the expected dimer. Furthermore, both the *E.coli* and tobacco produced GRFT were resuspended in the same buffer. Thus the higher molecular weight forms could be due to either a certain gel condition or potentially a strong aggregation of GRFT molecules in the sample. With the confocal microscopy, punctuate fluorescent aggregates were observed in the cytosol and ER. It is possible that GRFT aggregates with itself and/or with other cell proteins.

Subcellular localisation is regularly pursued to determine the optimal location to benefit yield and stability of recombinant produced proteins (Streatfield *et al*., 2007). However, targeted expression of recombinant proteins may result in the protein being differently located than anticipated (Chikwamba *et al*., 2003; Drakaki *et al*., 2006). This is sometimes dependent on the host of expression, plant organ or other inherent properties of the protein itself. Discrepancies in subcellular localisation could influence protein stability, activity and downstream purification which again influence downstream application and costs. For example, a protein that does not require glycosylation which is suddenly targeted to be glycosylated or *vice versa* can lose its function, or not be acceptable for use in the therapeutic market. Alternatively, the protein can find itself in a new cell location where it is broken down by proteases.

In plants two types of lectins are produced. Classical lectins, normally with signal peptides, are produced in abundance and are found in storage organelles such as vacuoles or in the extracellular compartments (Lannoo and Van Damme, 2010; Van Damme *et al*., 2004). These lectins were proposed to play a defence role in the plant. The second type of lectin, nucleocytoplasmic lectins, are generally produced in much lower quantities in response to a stress stimuli and are found in the cytosol or nucleus. It has been reported that recombinant expression of lectins from one biological source in another biological host can be differently targeted than originally observed: for example, Chao and Etzler (1994) reported that vacuolar lectins from the legume *Dolichos biflorus* were not associated with the vacuoles when expressed in the yeast *Saccharomyces cerevisiae*. Protein sorting to vacuoles involves many potential mechanisms and signal sequences (Vitale and Hinz, 2005; Vitale and Raikhel, 1999), all which can influence lectin trafficking in recombinant hosts. Nucleocytoplasmic lectins have also been found to be secreted. It would therefore be interesting to investigate the targeting of lectins when expressed in recombinant hosts, to determine if any differences in location occur than originally defined by the expression vector.

Accordingly, I fused GRFT to GFP in order to validate the subcellular location. Overall GFP and GRFT-GFP were found to be located as governed by the targeting signals in the expression vectors. Apart from the cytosol, the GRFT-GFP fusion was also detected in the nucleus, while no nuclear localisation for GFP alone was observed. This was also confirmed with immunogold labelling experiments. GFP is small enough to enter through the nuclear pores (Hanson and Köhler, 2001) and several sources report that cytosol-targeted GFP is detected in both the cytoplasm and the nucleus (Kim and Grierson, 2005; Li *et al*., 2006; Reisin *et al*.; 2003). With GFP fusion proteins, nuclear localisation indicates that the fusion partner is likely to be a nuclear-localised protein (Hanson and Köhler, 2001) and several studies show that nucleocytoplasmic type lectins fused to GFP are also detected in the cytoplasma and nucleus (Atalah *et al*., 2011; Fouquart *et al*., 2007; Lannoo *et al*., 2006). Thus, by expressing the GRFTGFP in the cytoplasm and observing the presence of the fusion in the cytoplasm and nucleus, GRFT might be classed as a nucleocytoplasmic lectin. Also, like GRFT, many plant nucleocytoplasmic lectins have an affinity for mannose or high mannose residues (Lannoo *et al*., 2010). Furthermore, nucleocytoplasmic lectins are normally produced in low quantities in their native hosts (Lannoo and van Damme, 2010). Although the native role of GRFT in its natural host is not known, it has been reported that lectins in algal species are present in low concentrations (Rogers and Hori, 1993).

It is imperative a plant-produced pharmaceutical protein should be as efficient if not better than its original form. Several studies have reported that *E. coli* and plant-produced GRFT have inhibited HIV strains from different clades (Alexandre *et al*., 2010; Emau *et al*., 2007; O'Keefe *et al*., 2009). MagnICON produced crude GRFT was tested for efficacy against several HIV strains and found in most cases to be as potent as *E. coli*-produced purified GRFT without any toxic affect to the cell lines used. Compared to purified *E. coli*-produced GRFT, *N. benthamiana*-derived GRFT was slightly less potent against 3 subtype C viruses (CAP210.E8J, CAP177.cvl96 and CAP261.cvl93cvl) and one subtype B virus (QH0692.42). The reason for this is not clear. All the viruses have different predicted glycosylation patterns (Alexandre *et al*., 2010) and if the tobacco extract contains some compound that acts synergistically with GRFT on these viruses, it should have had the same effect on pseudoviruses with a similar glycosylation pattern. This could be further investigated with affinity measurements of the magnICON produced GRFT against gp120. With HIV, DC-SIGN cells play an important role of disseminating HIV (Piquet and Steinman, 2007). The ability of plant-produced GRFT to prevent HIV infection *via* DC-SIGN was assessed and found to be similar to that of purified *E. coli*-produced GRFT.

In conclusion, this study shows that different vector systems and different subcellular targeting influenced the accumulation of GRFT in the plant cell after transient expression. However, the overall trend remained the same in that the cytosol is the optimal compartment for GRFT production, and that GRFT is toxic when it follows the secretory pathway. GRFT localised as was expected, and could be classed as a nucleocytoplasmic lectin. Finally, the lectin was produced to very high levels with the magnICON system as was as efficacious as the purified *E. coli*-produced lectin against HIV.

CHAPTER 4.

Concluding remarks and future prospects

Immense potential has been recognised in plants for the production of recombinant proteins, especially for medical applications. Numerous plant hosts have been deployed for this purpose with various levels of success and there has been continuous development of plant expression vectors to address shortcomings of or concerns about recombinant protein expression in plants. *Zea mays* (maize) and *Nicotiana benthamiana* (tobacco) were selected for this project due to their unique and common attributes for expressing recombinant proteins, as well as because they are among the best characterised plant species used for the purpose.

Several lectins have been identified, from various organisms. To date, 12 families of the proteins have been characterised (Van Damme *et al.*, 1998). Recombinant expression of lectins was initially pursued to improve plant resistance against insects, and very low levels of expression were adequate to achieve this (Peumans and van Damme, 1995). The discovery of lectins with anti-HIV activity, however, prompted the recombinant expression of these peptides in different expression platforms since very often these molecules were present in very low quantities in their native organism and/or the native organism would have been difficult to cultivate. One such lectin, griffithsin (GRFT) (Mori et al., 2005), which at this stage is the most potent anti-HIV lectin, was used in this study.

Maize was used to produce the first two commercialised products from plants; namely, avidin and β-glucuronidase (Hood *et al.*, 1997; Witcher *et al.*, 1998). Furthermore, several therapeutic proteins -including anti-HIV antibodies – have been produced in maize seed (Ramessar *et al*., 2008; Lotter-Stark *et al.*, 2012). Maize is the most cultivated cereal crop in the world (Naqvi *et al.*, 2011), meaning its growth and agronomic properties are very well understood. One can also tap into very well established molecular protocols when deploying this plant as an expression host for foreign proteins. If expression of GRFT in maize seed were to be commercially viable in this platform, it could mean that the seeds could provide a stable storage system for the lectin. The seeds can either be stored for long periods, or transported in a partially crushed form (to prohibit potential cross contamination with food sources), or as a maize meal to several areas all over the world for further purification. Extraction of GRFT could easily be done subsequently using existing maize seed processing technology. Accordingly, in this project I evaluated the expression of GRFT in maize seed endosperm using a gamma zein endosperm specific promoter, and different targeting signals for different cellular locations in the endosperm.

A debatable concern is the possibility that expression of foreign proteins in food crops will endanger food sources by potential cross contamination with seeds or pollen. There have been case studies where genetically modified maize did contaminate food crops (reviewed by Ramessar *et al.*, 2008). Furthermore expression of vaccines, vaccine subunits, antibodies in food crops in open fields may come in contact with native viruses leading to unwanted recombinations, or might reach individuals in which the expressed product might cause unwanted effects. Tobacco is a non-food crop that has been extensively used for the production of several human therapeutic proteins (Tremblay *et al.*, 2010). Apart from being a non-food crop, tobacco's popularity or potential as a biopharming platform has increased immensely with the development of transient expression methodology and refined transient expression vectors. The latter have enabled tobacco to express target proteins to very high levels in an extremely short period of time. Expression of GRFT in tobacco at very generous levels has already been done using a TMV based vector system. This study reports the expression of GRFT using the magnIcon (rTMV-based) and pTRA (agroinfiltration only) vector systems. Furthermore, a systematic investigation and comparison of the effect of subcellular targeting on expression levels, plant physiology and purification of GRFT was performed for the first time.

Expression of GRFT in the maize endosperm was 5 to 25 fold lower than that of other proteins reported to have been expressed in the endosperm (Chikwamba *et al.*, 2002b; Hood *et al.*, 1997; Ramessar *et al.*, 2008). Unlike other reports, there was not a significant difference in the expression levels of the lectin when targeted to different cellular locations in the endosperm. The low expression level made it difficult to obtain GRFT in a semi-pure form for efficacy testing. It has previously been reported that heterologous proteins might associate with other proteins and or molecules in the maize endosperm. Maize endosperm consists mostly of carbohydrate and insoluble protein (Ramessar *et al.*, 2008). The immunoblot showed numerous bands in the GRFT maize endosperm protein extracts, indicating a possible association with other endosperm proteins or components.

In tobacco, both the magnIcon and pTRA system gave exceptionally good expression levels when GRFT was expressed in the cytosol. When GRFT was secreted its expression levels were very low, and it had a toxic effect on the cell. Chloroplast accumulation levels were slightly higher with no toxicity, but expression patterns varied a lot. It could be that this difference was due to different transcript levels, differences in the cellular machinery between chloroplasts and cytosol or that the number of chloroplast differs or expression levels differ as these organelles are broken down at different rates in different leaves.

When comparing the two expression systems, it was obvious that they have some attributes in common such as post translational modification capability; large biomass; established stable or transient transformation methodology as well as some unique attributes such as the GRAS (Generally recognized as safe) status of maize and the storage capability in maize seed while tobacco has the advantage of not being a food crop and transient expression is more rapid than stable transformation of maize. For this study, the construction period of expression vectors for both maize and tobacco was more or less similar; however, the time period from introducing the genes to analysis of the protein extract from the plants differed significantly. This has been reported in literature as a huge advantage of transient technology over transgenic methodology (Kamarova *et al.*, 2010). Even though maize is considered as a better platform to purify proteins due to fewer contaminating components, a low expression yield will challenge even this attribute. Indeed in the case with maize expression of GRFT as well as potential cross reactivity of GRFT with other endosperm molecules, purification of the lectin was not achieved. However in tobacco the high expression of GRFT made it easy to purify GRFT on bench scale using a commercial kit. Efficacy testing of GRFT in maize showed that the seed extract contained a substance that was toxic to the cultured cells that were used in the tests. Therefore, whether or not maize-produced GRFT was efficacious against HIV remains inconclusive. On the other hand, GRFT in the crude tobacco extract was as efficacious as purified *E. coli*-produced GRFT without being toxic to the cells used in the test. Thus, from this study, I conclude that tobacco was the superior host for expression of GRFT.

A study conducted by O' Keefe *et al*. (2009) showed that 60g pure GRFT could be produced from 230 kg tobacco leaf material using transient vector technology. Given the potency of GRFT this could mean the production of several doses from a relatively small greenhouse facility. GRFT is currently undergoing several pre-clinical trials for its development as an anti- HIV microbicide (Ferir *et al.,* 2011; Kouokam *et al.,* 2011; O' Keefe *et al.,* 2009). Formulation plays a crucial role in the success of an anti-HIV microbicide. This will also apply to GRFT. Thus a continuation of this project could include obtaining a pure GRFT molecule from magnICON infiltrated samples and testing different formulations of GRFT. This will entail evaluating the stability, solubility and efficacy of GRFT in different intravaginal ring (IVR) materials by itself or in combination with other anti-retrovirals (ARVs). It will also be interesting to see the development of the first clinical trials with this notorious lectin. Furthermore it would be interesting to see if the fusion of GRFT and GFP had increased the expression levels in different cellular compartments. Elghabi *et al.* (2011) have reported that CV-N expression in tobacco chloroplasts was higher when fused to GFP than when it was expressed alone.

Several plant-produced molecules are in different stages of clinical development (recently reviewed by Nagels *et al*., 2012). Furthermore, the future of plant-made pharmaceuticals was recently strengthened by the FDA approval of carrot produced glucocerebrosidase. The carrot produced pharmaceutical proved to be as effective as the CHO produced version (Shaaltiel *et al.*, 2007). Given that several plant-produced pharmaceutical proteins has entered clinical trials and that their regulatory approval processes are smoothed out it probably will not be long before we see an established plant-made pharmaceutical industry.

References

Alexandre, K.B., Gray, E.S., Lambson, B.E., Moore, P.L., Choge, I.A., Mlisana, K., Karim, S.A., McMahon, J., O'Keefe, B., Chikwamba, R. and Morris, L. (2010) Mannose-rich glycosylation patterns on HIV-1 subtype C gp120 and sensitivity to the lectins, griffithsin, cyanovirin-N and scytovirin. Virology 402:187–96

Alexandre, K.B., Gray, E.S., Mufhandu, H., McMahon, J.B., Chakauya, E., O'Keefe, B.R., Chikwamba, R. and Morris, L. (2011) The lectins griffithsin, cyanovirin-N and scytovirin inhibit HIV-1 binding to the DC-SIGN receptor and transfer to CD4+ cells. Virology 423(2): 175-86

Alexandre, K.B., Gray, E.S., Pantophlet, R., Moore, P.L., McMahon, J.B., Chakauya, E., O'Keefe, B.R, Chikwamba, R., and Morris, L. (2013) Binding of the mannose-specific lectin, griffithsin, 1 to HIV-1 gp120 exposes the CD4-binding site. J. Virol. 85:9039-50

Armbruster, C., Stiegler, G.M., Vcelar, B.A., Jäger, W., Michael, N.L., Vetter, N., and Katinger, H.W.D. (2002) A phase I trial with two human monoclonal antibodies (hMAb 2F5, 2G12) against HIV-1. AIDS 16:227- 33

Armbruster, C., Stiegler, G.M., Vcelar, B.A., Jäger, W., Köller, U., Jilch, R, Ammann,C.G., Pruenster, M., Stoiber, H. and Katinger, W.D.K. (2004) Passive immunization with the anti-HIV-1 human monoclonal antibody (hMAb) 4E10 and the hMAb combination 4E10/2F5/2G12. J. Antimicrob. Chemother. 54:915-20

Atalah, B.A., Fouquaert, E., Vanderschaeghe, D., Proost, P., Balzarini, J., Smith, D.F., Rouge, P., Lasanajak, Y., Callewaert, N. and Van Damme, E.J.M. (2011) Expression analysis of the nucleocytoplasmiclectin “Orysata”from rice in Pichia pastoris. FEBS J. 278(12): 2064–79

Aviezer, D., Brill-Almon, E., Shaaltiel, Y., Hashmueli, S., Bartfeld, D., Mizrachi, S., Liberman, Y., Freeman, A., Zimran, A., and Galun, E. (2009) A plant-derived recombinant human glucocerebrosidase enzyme - a preclinical and phase I investigation. PLOS ONE 4:e4792

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

Baba, T.W., Liska, V., Hofmann-Lehmann, R., Vlasak, J., Xu, W., Ayehunie, S., Cavacini, L.A., Posner, M.R., Katinger, H., Stiegler, G., Bernacky, B.J., Rizvi, T.A. Schmidt, R., Hill, L.R., Keeling, M.E., Lu, Y., Wright, J.E., Chou, T-C., and Ruprecht, R.M. (2000) Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian–human immunodeficiency virus infection. Nat Med 6:200-6

Baily, M.R., Woodard, S.L., Callaway, E., Beifuss, K., Magallanes-Lundback, M., Lane, J.R., Horn, M.E., Mallubhotla, H., Delaney, D.D., Ward, M., Van Gastel, F., Howard, J.A. and Hood, E.E. (2004) Improved recovery of active recombinant laccase from maize seed. Appl. Microbiol. Biotechnol. 63:390-7

Baker, R.L., Brown, R.L., Chen, Z.Y., Cleveland, T.E. and Fakhoury, A.M. (2009) A maize lectin-like protein with antifungal activity against *Aspergillus flavus*. Journal of Food Protection 72(1):120–7

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

Balzarini, J. (2006b) Inhibition of HIV entry by carbohydrate-binding proteins. Antiviral Research 71:237–47

Balzarini, J., Van Herrewege, Y., Vermeire, K., Vanham, G., and Schols, D. (2007) 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. 71:3-11

Banerjee, K., Michael, E., Eggink, D., van Montfort, T., Lasnik, A.B., Palmer, K.E., Sanders, R.W., Moore, J.P., and Klasse, P.J. (2011) Occluding the mannose moieties on Human Immunodeficiency Virus Type 1 gp120 with griffithsin improves the antibody responses to both proteins in mice. AIDS Res. Hum. Retroviruses 28 (2):206-14

Banta, L.M. and Montenegro, M. Agrobacterium and plant biotechnology. In: Tzfira, T. and 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, E.J., Peumans, W.J., and Rouge, P. (1996) Structure-function relationship of monocot mannose-binding lectins. Plant Physiol. 112:1531-40

Barrientos, L.G., and Gronenborn, A.M. (2002) The domain-swapped dimer of cyanovirin-N contains two sets of oligosaccharide binding sites in solution. Biochem. Biophys. Res. Commun. 298:598–602

Barrientos, L.G., O'Keefe, B.R., Bray, M., Sanchez, A., Gronenborn, A.M., and Boyd, M.R. (2003) Cyanovirin-N binds to the viral surface glycoprotein, GP1, 2 and inhibits infectivity of ebola virus 1. Antiviral Res. 58:47–56

Barrientos, L.G., Lasala, F., Delgado, R., Sanchez, A., and Gronenborn, A.M. (2004) Flipping the switch from monomeric to dimeric CV-N has little effect on antiviral activity. Structure 12:1799–807

Barta, A., Sommergruber, K., Thompson, D., Hartmuth, K., Matzke, M.A., and Matzke, A.J.M. (1986) The expression of a nopaline synthase human growth hormone chimaeric gene in transformed tobacco and sunflower callus tissue. Plant Mol. Biol 6:347-57

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

Bendandi, M., Marillonnet, S., Kandzia, R., Thieme, F., Nickstadt, A., Herz, S., Fröde, R., Inogés, S., de Cerio, A. L.-D., Soria, E., Villanueva, H., Vancanneyt, G., McCormick, A., Tusé, D., Lenz, J., Butler-Ransohoff, J.-E., Klimyuk, V. and Gleba, Y. (2010) Rapid, high-yield production in plants of individualized idiotype vaccines for non-Hodgkin’s lymphoma. Ann.Oncol. 21: 2420-7

Bewley, C.A., and Otero-Quintero, S. (2001) 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.123:3892-902

Bewley, C.A., Cai, M., Ray, S., Ghirlando, R., Yamaguchi, M. and Muramoto, K. (2004) New carbohydrate specificity and HIV-1 fusion blocking activity of the cyanobacterial protein MVL: NMR, ITC and sedimentation equilibrium studies. J. Mol.Biol, 339(4): 901–14

Binley, J.M., Wrin, T., Korber, B., Zwick, M.B., Wang, M., Chappey, C., Stiegler, G., Kunert, R., Zolla-Pazner, S., Katinger, H., Petropoulos, C.J., and Burton, D.R. (2004) Comprehensive cross-clade neutralization analysis of a panel of anti-human immunodeficiency virus type 1 monoclonal antibodies. J. Virol. 78:13232-52

Bosch, D. and Schots, A. (2010) Plant glycans: friend or foe in vaccine development? Expert Rev. Vaccines 9(8), 835–42

Bokesch, H.R., O’Keefe, B.R., McKee, T.C., Pannell, L.K., Patterson, G.M.L., Gardella, R.S., Sowder, R.C. (II), Turpin, J., Watson, K., Buckheit, R.W.(Jr), and Boyd, M.R. (2003) A potent novel anti-HIV protein from the cultured cyanobacterium Scytonema Varium. Biochemistry 42: 2578-84

Bonifacino, J. S. (2011). Electrophoresis and immunoblotting. Current protocols in cell biology. 52:6.0.1–6.0.3

Bortesi, L., Rossato, M., Schuster, F., Raven, N., Stadlmann, J., Avesani, L., Bortesi, L., Rossato, M., Schuster, F., Raven, N., Stadlmann, J., Avesani, L., Falorni, A., Bazzoni, F., Bock, R., Schillberg, S., and Pezzotti, M. (2009) Viral and murine interleukin-10 are correctly processed and retain their biological activity when produced in tobacco. BMC Biotechnol. 9:22

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

Boyd, M.R., Gustafson, K.R., McMahon, J.B., Shoemaker, R.H., O'Keefe, B.R., Mori, T., Gulakowski, R.J., Wu, L., Rivera, M.I., Laurencot, C.M., Currens, M.J., Cardellina, J.H. (II), Buckheit, R.W.(Jr), Nara, P.L., Panell, L.K., Sowder, R.C. (II) and Henderson, L.E. (1997)

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. 41:1521-30

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-54

Buckheit, R.W. (Jr), Watson, K.M., Morrow, K.M., and Ham, A.S. (2010) Development of topical microbicides to prevent the sexual transmission of HIV. Antivir. Res.85:142–58

Bulgheresi, S., Schabussova, I., Chen, T., Mullin, N.P., Maizels, R.M., and Ott, J.A. (2006) A new C-type lectin similar to the human immunoreceptor DC-SIGN mediates symbiont acquisition by a marine nematode. Appl. Environ. Microbiol. 72:2950-6

Calarese, D.A., Scanlan, C.N., Zwick, M.B., Deechongkit, S., Mimura, Y., Kunert, R., Zhu, P., Wormald, M.R., Stanfield, R.L. Roux, K.H., Kelly, J.W., Rudd, P.M., Dwek, R.A., Katinger, H., Burton, D.R., and Wilson, I.A. (2003) Antibody domain exchange is an immunological solution to carbohydrate cluster recognition. Science 300:2065-71

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

Castilho, A., Strasser, R., Stadlmann, J., Grass, J., Jez, J., Gattinger, P., Kunert, R., Quender, H., Pabst, M., Leonard, R., Altmann, F. and Steinkellner, H. (2010). In planta protein sialylation through over-expression of the respective mammalian pathway. J Biol.

Chem. 285:15923-30

Castilho, A., Bohorova, N., Grass, J., Bohorov, O., Zeitlin, L., Whaley, K., Altmann, F., and Steinkellner, H. (2011) Rapid high yield production of different glycoforms of Ebola virus monoclonal antibody. PLOS ONE, 6(10):e26040

Chao, Q. and Etzler, M.E. (1994) Incorrect targeting of plant vacuolar lectins in yeast. J. Biol. Chem., 269:20866-71

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

Chiba, H., Inokoshi, J., Nakashima, H., Omura, S., and Tanaka, H. (2004) 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. 316:203–10

Chikwamba, R.K., Cunnick, J., Hathaway, D., McMurray, J., Mason, H., and Wang, K. (2002a) A functional antigen in a practical crop: LT-B producing maize protects mice against *Escherichia coli* heat labile enterotoxin (LT) and cholera toxin (CT). Transgenic Res. 11: 479–93

Chikwamba, R.K., McMurray, J., Shou, H., Frame, B., Pegg, S.E., Scott, P., Mason, H. and Wang, K. (2002b) Expression of a synthetic *E. coli* heat-labile enterotoxin B sub-unit (LT-B) in maize. Mol. Breeding 10: 253–65

Chikwamba, R.K., Scott, M.P., Mejía, L.B., Mason, H.S. and Wang, K. (2003) Localization of a bacterial protein in starch granules of transgenic maize kernels. Proc. Natl. Acad. Sci. USA, 100(19): 11127-32

Christou, P., and Kohli, A. (2005) Transformation method and transgenic plants produced thereby. US Patent No 6846970. 25 Jan, www.uspto.gov.

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

Coëffier, E., Clément, J., Cussac, V., Khodaei-Boorane, N., Jehanno, M., Rojas, M., Dridi, A., Mireille Latour, M., El Habib, R., Barre´-Sinoussi, F., Hofnung, M., and Leclerc, C. (2001) Antigenicity and immunogenicity of the HIV-1 gp41 epitope ELDKWA inserted into permissive sites of the MalE protein. Vaccine 19:684–93

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

Daniell, H., Streatfield, S.J. and Wycoff, K. (2001) Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends in Plant Sci. 6(5): 219-26

Davis, K.R. (2010) 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.

De Haan, P., Engel, A., de Vreis, W., van der Velden, J., van Holst, G-J. and Arnoldussen E. (2010) RNA silencing suppressors boost the production of recombinant proteins and viruses. Cells and culture, ESACT PROCEEDINGS 4: 9-12

Dellaporta, S.L., Wood, J., and Hicks, J.B. (1983) A plant DNA minipreparation:version II. Plant Mol. Biol. Rep. 1:19-21

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

De Rosny, E., Vassell, R., Jiang, S., Kunert, R., and Weiss, C.D. (2004) 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. 78:2627-31

Dey, B., Lerner, D.L., Lusso, P., Boyd, M.R., Elder, J.H., and Berger, E.A. (2000) 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. 74:4562-9

Doncel, G.F., and Clark, M.R. (2010) Preclinical evaluation of anti-HIV microbicide products: new models and biomarkers. Antiviral Res. 88:S10–S18

Doores, K.J., Bonomelli, C., Harvey, D.J., Vasiljevic, S., Dwek, R.A., Burton, D.R., Crispin, M., and Scanlanc, C.N. (2010) Envelope glycans of immunodeficiency virions are almost entirely oligomannose antigens. Proc. Natl. Acad. Sci.107:13800-5

Drakakaki, G., Marcel, S., Arcalis, E., Altmann, F., Gonzalez-Melendi, P., Fischer, R., Christou, P., and Stoger, E. (2006) The intracellular fate of a recombinant protein is tissue dependent. Plant physiol. 141(2):578–86

Eggink, D., Melchers, M., and Sanders, R.W. (2007) Antibodies to HIV-1: aiming at the right target. Trends Microbiol. 15:291–4

Elghabi, Z., Karcher, D., Zhou, F., Ruf., S., and Bock, R. (2011) Optimizing of the expression of the HIV fusion inhibitor cyanovirin-N from the tobacco plastid genome. Plant Biotechnol. J. 9:599-608.

Emau, P., Tian, B., O'Keefe, B.R., Mori, T., McMahon, J.B., Palmer, K. E., Jiang, Y., Bekele, G. and Tsai, C. C. (2007). Griffithsin, a potent HIV entry inhibitor, is an excellent candidate for anti-HIV microbicide. J. Med. Primatol. 36: 244–53

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

Férir, G., Palmer, K.E., and Schols, D. (2011) Synergystic activity profile of griffithsin in combination with Tenofovir, Maraviroc and Enfuvirtide against HIV-1 clade C. Virology, 417 (2): 253-8

Férir, G., Huskens, D., Palmer, K.E., Boudreaux, D.M., Swanson, M.D., Markovitz, D.M., Balzarini, J. and Schols, D. (2012) Combinations of griffithsin with other carbohydrate-binding agents demonstrates superior activity against HIV type 1, HIV type 2, and selected carbohydrate-binding agent-resistant HIV type 1 strains. AIDS Res. Hum. Retroviruses 28 (11): 1513-23.

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

Floss, D.M., Sack, M., Stadlmann, J., Rademacher, T., Scheller, J., Stöger, E., Fischer, R., and Conrad, U. (2008) Biochemical and functional characterization of anti-HIV antibody–ELP fusion proteins from transgenic plants. Plant Biotechnol. J.6:379–91

Francois, K.O., and Balzarini, J. (2010) Potential of carbohydrate-binding agents as therapeutics against enveloped viruses. Med. Res. Rev. 23:1-39

Franquelim, H.G., Chiantia, S., Veiga, A.S., Santos, N.C., Schwille, P., and Castanho, M.A. (2011) Anti-HIV-1 antibodies 2F5 and 4E10 interact differently with lipids to bind their epitopes. AIDS 25:419-28

Friend, D.R., and Kiser, P.F. (2013) Assessment of topical microbicides to prevent HIV-1 transmission:concepts, testing, lessons learned. Antiviral Research 99, 391-400

Fromme, R., Katiliene, Z., Giomarelli, B., Bogani, F., McMahon, J., Mori, T., Fromme, P., and Ghirlanda, G. (2007) A monovalent mutant of cyanovirin-N provides insight into the role of multiple interactions with gp120 for antiviral activity. Biochem. 46:9199-207

Fouquaert, E., Hanton, S.L., Brandizzi, F., Peumans, W.J., and Van Damme, E.J.M. (2007) Localization and topogenesis studies of cytoplasmic and vacuolar homologs of the Galanthus nivalus agglutinin. Plant cell Physiol. 48(7): 1010-21

Fouquaert, E., Smith, D.F., Peumans, W.J., Proost, P., Balzarini, J., Savvides, S.N., and Van Damme, E.J.M. (2009) Related lectins from snowdrop and maize differ in their carbohydrate-binding specificity. Biochem. and Biophys. Res. Comm. 380: 260-5

Fraley, R.T., Rogers, S.G., Horsch, R.B., Sanders, P.R., Flick, J.S., Adams, S.P., Bittner, M.L., Brand, L.A., Fink, C.L., Fry, J.S., Galluppi, G.R., Goldberg, S.B., Hoffmann, N.L., and Woo, S.C. (1983) Expression of bacterial genes in plant cells. PNAS (80):4803-7

Gallie, D.R., Tanguay, R.L., and Leathers, V. (1995) The tobacco etch viral 5' leader and poly (A) tail are functionally synergistic regulators of translation. Gene 165: 233 -8

Garg, A.B., Nuttall, J., and Romano, J. (2009) The future of HIV microbicides: challenges and opportunities. Antiviral Chem. Chemother. 19:143-50

Garg, S., Goldman, D., Krumme, M., Rohan, L.C., Smoot, S., and Friend, D.R. (2010) Advances in development, scale-up and manufacturing of microbicide gels, films, and tablets. Antiviral Res. S19-29

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

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

Giddings, G., Allison, G., Brooks, D., and Carter, A. (2001) Transgenic plants as protein factories. Curr. Opinion in Biotechnol. 12:450–4

Giomarelli, B., Provvedi, R., Meacci, F., Maggi, T., Medaglini, D., Pozzi, G., McMahon, J.B., Gardella, R., and Boyd, M.R. (2002) The microbicide cyanovirin-N expressed on the surface of commensal bacterium Streptococcus gordonii captures HIV-1. AIDS 16:1351-6

Giomarelli, B., Schumacher, K.M., Taylor, T.E., Sowder, R.C. (II), Hartley, J.L., McMahon, J.B. and Mori, T. (2006) Recombinant production of anti-HIV protein, griffithsin, by auto-induction in a fermentor culture. Protein Expr.Purif. 47(1): 194–202

Giritch, A., Marillonnet, S., Engler, C., Van Eldik, G., Botterman, J., Klimyuk, V., and Gleba, Y. (2006) Rapid high-yield expression of full-size IgG antibodies in plants co-infected with noncompeting viral vectors. PNAS 103:14701-6

Gils, M., Kandzia, R., Marillonnet, S., Klimyuk, V., and Gleba, Y. (2005). High-yield production of authentic human growth hormone using a plant virus-based expression system. Plant Biotechnol. J. 3:613–20

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

Golovkin, M., Spitsin, S., Andrianov, V., Smirnov, Y., Xiao, Y., Pogrebnyak, N., Markley, K., Brodzik, R., Gleba, Y., Isaacs, S.N., and Koprowski, H. (2007) Smallpox subunit vaccine produced in planta confers protection in mice. PNAS 104(16): 6864-9

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

Gomord, V., Fitchette, A., Menu-Bouaouiche, L., Saint-Jore-Dupas, C., Plasson, C., Michaud, D., and Faye, L. (2010) Plant-specific glycosylation patterns in the context of therapeutic protein production. Plant Biotechnol. J. 8(5): 564–87

Goldstein, I.J., and Hayes, C.E. (1978) The lectins: carbohydrate-binding proteins of plants and animals. Adv. Carbohydr. Chem. Biochem. 35:127-340

Gustafson, K.R., Sowder , R.C. (II), Henderson, L.E., Cardellina (II), J.H., McMahon, J.B., Rajamani, U., Panell, L.K., and Boyd, M.R. (1997). 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. 238: 223–8

Hanson, M.R., and Köhler, R.H. (2001) GFP imaging: methodology and application to investigate cellular compartementation in plants. J. of Exp. Bot. 54(356): 529-39

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

Hessell, A.J., Hangartner, L., Hunter, M., Havenith, C.E.G., Beurskens, F.J., Bakker, J.M., Lanigan, C.M.S., Landucci, G., Forthal, D.N., Parren, P.W.H.I., Marx, P.A., and Burton, D.R. (2007) Fc receptor but not complement binding is important in antibody protection against HIV. Nature 449:101-4

Hessell, A.J., Rakasz, E.G., Tehrani, D.M., Huber, M., Weisgrau, K.L., Landucci, G., Forthal, D.N., Koff, W.C., Poignard, Watkins, D.I., and Burton, D.R. (2010) 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. 84:1302-13

Hoorelbeke, B., Huskens, D., Ferir, G., Francois, K.O., Takahashi, A., Van Laethem, K., Schols, D., Tanaka, H., and Balzarini, J. (2010) Actinohivin, a broadly neutralizing prokaryotic lectin, inhibits HIV-1 infection by specifically targeting high-mannose-type glycans on the gp120 envelope. Antimicrob. Agents Chemother. 54:3287-301

Hoorelbeke, B., Xue, J., LiWang, P.J., and Balzarini, J. (2013) Role of the carbohydrate-binding sites of griffithsin in the prevention of DC-SIGN mediated capture and transmission of HIV-1. PLOS ONE, 8 (5) e64132

Hood, E.E., Witcher, D.R., Maddock, S., Meyer, T., Baszczynski, C., Bailey, M., Flynn, P., Register, J., Marshall, L., Bond, D., Kulisek, E., Kusnadi, A., Evangelista, R., Nikolov, Z., Wooge, C., Mehigh, R., Hernan, R., Kappel, W.K., Ritland, D., Li, C.P., and Howard, J.A. (1997) Commercial production of avidin from transgenic maize: characterization of transformant, production, processing, extraction and purification. Mol. Breeding 3: 291–306

Hood, E.E. (2004) Where, oh where has my protein gone? TRENDS in biotechnol. 22 (2):53-5

Hu, Q., Frank, I., Williams, V., Santos, J.J., Watts, P., Griffin, G.E., Moore, J.P., Pope, M and Shattock, R.J. (2004) Blockade of attachment and fusion receptors inhibits HIV-1 infection of human cervical tissue. J. Exp. Med. 199:1065-75

Huang, Z., LePore, K., Elkin, G., Thanavala, Y., and Mason, H.S. (2008) High-yield rapid production of hepatitis B surface antigen in plant leaf by a viral expression system. Plant Biotechnol. J. 6(2):202–9

Huang, Z., Phoolcharoen, W., Lai, H., Piensook, K., Cardineau, G., Zeitlin, L., Whaley, K.J. Arntzen, C.J., Mason, H.S., and Chen, Q. (2010) High-level rapid production of full-size monoclonal antibodies in plants by a single-vector DNA replicon system. Biotechnol Bioeng 106:9-17

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

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

James, C., and Krattiger, A.F. (1996) Global review of the field testing and commercialization of transgenic plants, 1986 to 1995: The First Decade of Crop Biotechnology. ISAAA Briefs No. 1. ISAAA: Ithaca, NY. pp. 31

Jankovic, M., Cuperlovic, M, and Hajdukovic, L. (1990) Salt-soluble lectins of corn grain. Plant Physiol. 93: 1659-62

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

Johnson, T.J., Clark, M.R., Albright, T.H., Nebeker,J.S., Anthony L. Tuitupou, A.L., Clark, J.T. Fabian, J. McCabe, R.T., Chandra, N., Doncel, G.F., Friend, D.R., and Kiser, P.F.(2012) A 90-Day Tenofovir Reservoir Intravaginal Ring for Mucosal HIV Prophylaxis. Antimicrob. Agents Chemother. 2012, 56(12):6272.

Karim, S.S.A., Richardson, B.A., Ramjee, G., Hoffman, I.F., Chirenje, Z.M., Taha, T., Kapina, M., Maslankowski, L., Coletti, A., Profy, A., Moench, T.R., Piwowar-Manning, E., Mâsse, B., Hillier, S.L., and Soto-Torres, L. (2011) Safety and effectiveness of BufferGel and 0.5% PRO2000 gel for the prevention of HIV infection in women. AIDS 25:957-66

Karim, Q.A., Karim,A.S.S., Frohlich, J.A., Grobler, A.C., Baxter, C., Mansoor, L.E., Kharsany, A.B.M., Sibeko, S., Mlisana, K.P., Omar, Z., Gengiah, T.N., Maarschalk, S., Arulappan, N.,Mlotshwa, M., Morris, L., Taylor, D., on behalf of the CAPRISA 004 Trial Group. (2010) Effectiveness and safety of Tenofovirgel, an antiretroviral microbicide, for the prevention of HIV infection in women. Science; 329(5996):1168-74

Karim, Q.A., Baxter, C. and Karim, S.A. (2013) Topical microbicides – what's new? Acquir Immune Defic Syndr **63,** Supplement 2.

Kelley, B.S., Chang, L.C., and Bewley, C.A. (2002) Engineering an obligate domain-swapped dimer of cyanovirin-N with enhanced anti-HIV activity. J. Am. Chem. Soc. 124:3210-1

Kim, S.H., and Grierson, D. (2005) Subcellular localisation and silencing of ripening-associated membraneprotein (TRAMP) in tomato Lycopersicone sculentum Mill. Plant Sci. 169: 1022-9

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

Ko, K,. Tekoah, Y., Rudd, P.M., Harvey, D.J., Dwek, R.A., Spitsin, S., Hanlon, C.A., Rupprecht, C., Dietzschold, B, Golovkin, M., and Koprowski, H. Function and glycosylation of plant-derived antiviral monoclonal antibody. PNAS100: 8013-8

Koprivova, A., Altman, F., Gorr, G., Kopriva, S., Reski, R., and Decker, E.L. (2003) N-glycosylation in the moss Physcomitrella patens is organized similarly to that in higher plants. Plant Biol. 5:582-91

Koshte, V.L., Van Dijk, W., Van Der Stelt, M.E., and Aalberse, R.C. (1990) Isolation and characterization of BanLec-I, a mannoside-binding lectin from Musa paradisiac (banana). Biochem. J. 272:721-6

Kouokam, J.C., Huskens, D., Schols, D., Johannemann, A., Riedell, S.K., Walter, W., Walker, M.J., Matoba, N., O'Keefe, B.R., and Palmer, K.E. (2011) Investigation of griffithsin's interaction with human cells confirms its outstanding safety and efficacy profile as a microbicide candidate. PLOS ONE, 6, e22635

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

Kusnadi, A.R., Nikolov, Z.L., and Howard, .JA. (1997) Production of recombinant proteins in transgenic plants: practical considerations. Biotechnol. and Bioeng. 56(5):473-84

Kormelink, R., Kitajima, E.W., De Haan, P., Zuidema, D., Peters, D., and Goldbach, R. (1991) The non-structural protein (NSs) encoded by the ambisense S RNA segment of tomato spotted wilt virus is associated with fibrous structures in infected plant cells. Virology 181(2): 459 -68

Kouokam, J. C., Huskens, D., Schols, D., Johannemann, A., Riedell S.K., Walter, W., Walker, M.J., Matoba, N., O'Keefe B.R., and Palmer, K.E. (2011) Investigation of griffithsin's interaction with human cells confirms its outstanding safety and efficacy profile as a microbicide candidate. PLOS ONE, 6, e22635

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4". Nature, 227(5259): 680–5

Lannoo, N., Peumans, W.J., Pamel, E.V., Alvarez, R., Xiong, T.C., Hause, G., Mazars, C., and Van Damme, E.J.M. (2006) Localization and in vitro binding studies suggest that the cytoplasmic/nuclear tobacco lectin can interact in situ with high-mannose and complex N-glycans. FEBS letters 580(27):6329–37

Lannoo, N., and Van Damme, E.J.M. (2010) Nucleocytoplasmic plant lectins. Biochim. Biophys. Acta. 1800:190–201

Lau, O.S., and Sun, S.S.M. (2009) Plant seeds as biorectors for recombinant protein production. Biotechnol. Adv. Epub, May 18

Lenz, O., Dittmar, M.T., Wagner, A., Ferko, B., Varauer-Uhl, K., Stiegler, G., and Weissenhorn, W. (2005) Trimeric membrane-anchored gp41 inhibits HIV membrane fusion. J. Biol. Chem. 280:4095-101

Li, H.Y., Ramalingam, S., and Chye, M.L. (2006) Accumulation of recombinant SARS-CoV spike protein in plant cytosol and chloroplasts indicate potential for development of plant-derived oral vaccines. Exp. Biol. Med., 231(8):1346–52

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

Li, Y., O'Dell, S., Walker, L.M., Wu, X., Guenaga, J., Feng, Y., Schmidt,S.D., McKee, K., Louder, M.K., Ledgerwood, J.E., Graham, B.S., Haynes, B.F., Burton, D.R. Wyatt, R.T., and Mascola, J.R. Mechanism of neutralization by the broadly neutralizing HIV-1 monoclonal antibody VRC01. J. Virol. 85: 8954-67

Liu, X., Lagenaur, L.A., Simpson, D.A., Essenmacher, K.P., Frazier-Parker, C.L., Liu, Y., Tsai, D., Rao, S.S., Hamer, D.H., Parks, T.P., Lee, P.P., and Xu, Q. (2006) Engineered vaginal lactobacillus strain for mucosal delivery of the human immunodeficiency virus inhibitor cyanovirin-N. Antimicrob. Agents Chemother.50:3250-9

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

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

Loos, A., Van Droogenbroeck, B., Hillmer, S., Grass, J., Kunert, R., Cao, J., David G. Robinson, D.G., Depicker, A., and Steinkellner, H. (2010) Production of monoclonal antibodies with a controlled N-glycosylation pattern in seeds of Arabidopsis thaliana. Plant Biotechnol. J. 9:179–92

Lotter-Stark, H.C.T., Rybicki, E.P., and Chikwamba, R.K. (2012) Plant made anti-HIV microbicides-A field of opportunity. Epub 2012 Jun 28

Luo, X.M., Lei, M.Y., Feidi, R.A., West (Jr), A.P., Balazs, A.B., Bjorkman, P.J., Yang, L., and Baltimore, D. (2010) Dimeric 2G12 as a potent protection against HIV-1. PLOS Pathog 6:1661–71

Ma, J.K., Drake, P.M.W., and Christou, P. (2003) The production of recombinant pharmaceutical proteins in plants. Nature 4:794-805

Maclean, J., Koekemoer, M., Olivier, A.J., Stewart, D., Hitzeroth, I.I.,Rademacher, T., Fischer, R., Williamson, A-L., and Rybicki, E.P. (2007) 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. Gen. Virol. 88:1460–9

Marillonnet, S., Giritch, A., Gils, M., Kandzia, R., Klimyuk, V., and Gleba, Y. (2004) In planta engineering of viral RNA replicons: efficient assembly by recombination of DNA modules delivered by Agrobacterium. PNAS 101:6852-7

Marillonnet, S., Giritch, A., Gils, M., Kandzia, R., Klimyuk, V, and Gleba, Y. (2004) In planta engineering of viral RNA replicons: efficient assembly by recombination of DNA modules delivered by Agrobacterium. PNAS 101(18): 6852-7

Marks, M.D., Lindell, J.S., and Larkins, B.A. (1985) Quantitative Analysis of the Accumulation of Zein mRNA during Maize Endosperm Development. The Journal of Biol. Chem. 260 (30): 16445-50

Mascola, J.R., Lewis, M.G., Stiegler, G., Harris, D., VanCott, T.C., Hayes, D., Louder, M.K., Brown, C.R., Sapan, C.V., Frankel, S.S., Lu,Y., Robb, M.L., Katinger, H., and Birx, D.L. (1999) Protection of macaques against pathogenic simian/human immunodeficiency virus 89.6 PD by passive transfer of neutralizing antibodies. J. Virol. 73: 4009-18

Mascola, J.R., Stiegler, G., VanCott, T.C., Katinger, H., Carpenter, C.B., Hanson, C.E., Beary, H., Hayes, D., Frankel, S.S., Birx, D.L., and Lewis, M.G. (2000) Protection of macaques against vaginal transmission of a pathogenic HIV- 1/SIV chimeric virus by passive infusion of neutralizing antibodies. Nat. Med. 6:207-10

Mason, H.S., Haq, T.A., Clemets, J.D., and Arntzen, C.J. (1998) Edible vaccine protects mice against Escherichia coli heat-labile enterotoxin(LT): potatoes expressing a synthetic LT-B gene. Vaccine 16(13): 1336-43

Matoba, N., Husk, A.S., Barnett, B.W., Pickel, M.M., Arntzen, C.J., Montefiori, D.C., Takahashi, A., Tanno, K., Omura, S., Cao, H., Mooney, J.P., Hanson, C.V., and Tanaka, H. (2010) HIV-1 neutralization profile and plant-based recombinant expression of Actinohivin, an env glycan-specific lectin devoid of T-Cell mitogenic activity. PLOS ONE, 5(6), e11143

Mayer, K.H., Peipert, J., Fleming, T., Fullem, A., Moench, T., Cu-Uvin, S., Bentley, M., Chesney, M., and Rosenberg, Z. (2001) Safety and tolerability of BufferGel, a novel vaginal microbicide, in women in the United States. Clin. Infect. Dis. HIV/AIDS 32:476–82

McFadden, K., Cocklin, S., Gopi, H., Baxter, S., Ajith, S., Mahmood ,N., Shattock, R., and Chaiken, I. (2007) A recombinant allosteric lectin antagonist of HIV-1 envelope gp120 interactions. Proteins: Struct. Funct. Bioinf. 67:617–29

McFeeters, H., Gilbert, M.J., Wood, A.M., Haggenmaker, C.B., Jones, J., Kutsch, O., and McFeeters, R.L. (2013) Scytovirin engineering improves carbohydrate affinity and HIV-1 entry inhibition. Biochem Physiol, S2

McGaughey, G.B., Citron, M., Danzeisen, R.C., Freidinger, R.M., Garsky, V.M., Hurni, W.M., Joyce, J.G., Liang, X., Miller, M., Shiver, J., and Bogusky, M.J. (2003) HIV-1 vaccine development: constrained peptide immunogens show improved binding to the anti-HIV-1 gp41 MAb. Biochem. 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.115-33

Mett,V., Farrance, C.E., Green, B.J., and Yusibov, V. (2008) Plants as biofactories. Biologicals 36:354–8

Meuleman, P., Albecka, A., Belouzard, S., Vercauteren, K., Verhoye, L., Wychowski, C., Leroux-Roels,G., Palmer, K.E., and Dubuisson, J. (2011) Griffithsin has antiviral activity against Hepatitis C virus. Antimicrob. Agents Chemother. 55 (11): 5159-67.

Meyers, A., Chakauya, E., Shephard, E., Tanzer, F.L., Maclean, J., Lynch, A., Williamson, A., and Rybicki, E.P. (2008) Expression of HIV-1 antigens in plants as potential subunit vaccines. BMC Biotechnol. 8:53

Minces, L.R., and McGowan, I. (2010) Advances in the development of microbicides for the prevention of HIV Infection. Curr. Infect. Dis. Rep. 12:56-62

Montefiori, D.C. (2004) Evaluating neutralizing antibodies againts HIV, SIV and SHIV in luciferase reporter gene assays. In: Coligan, J.E., Kruisbeek, A.M., Margulies, D.H., Shevach, E.M., Strober, W., Coico, R. (Eds.), Current Protocols in Immunology. John Wiley & Sons, pp. 12.11.1–12.11.15

Mori, T., Shoemaker, R.H., McMahon, J.B., Gulakowski, R.J., Gustafson, K.R., and Boyd, M.R. (1997) Construction and enhanced cytotoxicity of a cyanovirin-N-pseudomonas exotoxin conjugate against human immunodeficiency virus-infected cells. Biochem. Biophys. Res. Commun. 239:884–8

Mori, T., and Boyd, M.R. (2001) 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. 45:664 -72

Mori, T., O'Keefe, B.R., Raymond, C., Sowder, R.C. (II), Bringans, S., Gardella, R., Berg, S., Cochran, P., Turpin, J.A., Buckheit, R.W. (jr), McMahonn, J.B., and Boyd , M. R. (2005) Isolation and characterization of griffithsin, a novel HIV-inactivating protein, from the red alga Griffithsia sp. J. Biol. Chem. 280(10): 9345–53

Morris, G., Chindrove, S., Woodhall, S., Wiggins, R., Vcelar, B., and Lacey, C. (2010) 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 (Pittsburgh, PA), LB1, p. 229

Morris, G.C., and Lacey, C.J.N. (2010) Microbicides and HIV prevention: lessons from the past, looking to the future. Curr. Opin. Infect. Dis. 23:57-63

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

Moulaei, T., Shenoy, S. R., Giomarelli, B., Thomas, C., McMahon, J.B., Dauter, Z.,O'Keefe, B. R., and Wlodaver, A. (2010) Monomerization of viral entry inhibitor griffithsin elucidates the relationship between multivalent binding to carbohydrates and anti-HIV activity. Structure 18:1104-15

Muster, T., Steindl, F., Purtscher, M., Trkola, A., Klima, A., Himmler, G., Ruker, F., and Katinger, H. (1993) A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. J. Virol. 67:6642-7

Nabatov, A.A., de Jong, M.A., de Witte, L., Bulgheresi, S., and Geijtenbeek, T.B. (2008) C-type lectin Mermaid inhibits dendritic cell mediated HIV-1 transmission to CD4+ T cells. Virology 378:323–8

O'Keefe, B.R. (2001) Biologically active proteins from natural product extracts. J. Nat. Prod. 64:1373-81

O'Keefe, B., Vojdani, F., Buffa, V., Shattock, R.J., Montefiori, D.C., Bakke, J., Mirsalis J., d'Andrea A-L., Hume S.D., Bratcher, B., Saucedo, C. J., McMahon, J.B., Pogue, G.P., and

Palmer, K.E. (2009) Scaleable manufacture of HIV-1 entry inhibitor griffithsin and validation of its safety and efficacy as a topical microbicide component. PNAS 106 (15):6099–104

O'Keefe, B.R., Giomarelli, B., Barnard, D.L., Shenoy, S.R., Chan, P.K.S., McMahon, J.B., Palmer, K.E., Barnett, B.W., Meyerholz, D.K., Wohlford-Lenane, C.L., and McCray, P.B. (Jr). (2010) Broad-spectrum in vitro activity and in vivo efficacy of the antiviral protein griffithsin against emerging viruses of the family Coronaviridae. J. Virol. 84:2511-21

O'Kennedy, M.M., Burger, J.T., and Botha, F.C. (2004) Pearl millet transformation system using the positive selectable marker gene phosphomannose isomerase. Plant Cell Rep. 22:684–90.

Parren, P.W., Marx, P.A., Hessell, A.J., Luckay, A., Harouse, J., Cheng-Mayer, C., Moore, J.P., and Burton, D.R.. (2001) Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro. J. Virol. 75:8340-7

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

Paul, M., and Ma, J.K-C. (2011) Plant-made pharmaceuticals: Leading products and production platforms. Biotechnol. Appl. Biochem. 58:58-67

Piguet, V., and Steinman, R. M. (2007) The interaction of HIV with dendritic cells: outcomes and pathways. Trends Immunol. 28(11): 503 -10

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

Peumans, W.J., and Van Damme, E.J. (1995) Lectins as plant defense proteins. Plant Physiol. 109:347-52

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

Pogue, G.P., Lindbo, J.A., Garger, S.J., and Fitzmaurice, W.P. (2002) Making an ally from an enemy: plant virology and the new agriculture. Annu. Rev. Phytopathol. 40:45–74

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

Rademacher, T., Sack, M., Arcalis, E., Stadlmann, J., Balzer, S., Altmann, F. Quendler, H., Stiegler, G., Kunert, R., Fischer, R., and Stoger, E. (2008) Recombinant antibody 2G12 produced in maize endosperm efficiently neutralizes HIV-1 and contains predominantly single-GlcNAc N-glycans. Plant Biotechnol. J. 6:189–201

Ramessar, K., Rademacher, R., Sack, M., Stadlmann, J., Platis, D., Stiegler, G., Labrou, N., Altmann, F., Ma, J., Stoger, E., Capell, T., and Christou, P. (2008) Cost-effective production of a vaginal protein microbicide to prevent HIV transmission. PNAS 105 (10): 3727–32

Ramessar, K., Sabalza, M., Capell, T., and Christou, P. (2008) Maize plants: An ideal production platform for effective and safe molecular pharming. Plant Science 174: 409–19

Ransohoff, T.C., Mittendorff, R.E. (II), and Levine, H.L. Forecasting industrywide biopharmaceutical manufacturing capacity requirements. Advances in Large-Scale Biopharmaceutical Manufacturing and Scale-Up Production, 2nd Edition , ASM Press [American Society for Microbiology] and BioPlan Associates, Inc., November 2007

Regnard, G.L., Halley-Stott, R.P., Tanzer, F.L., Hitzeroth, I.I., and Rybicki, E.P. (2010) High level protein expression in plants through the use of a novel autonomously replicating geminivirus shuttle vector. Plant Biotechnol. J. 8:38–46

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

Reisen, D., Leborgne-Castel, N.,Özalp, C., Chaumont, F., and Marty, F. (2003) Expression of a cauliflower tonoplast aquaporin tagged with GFP in tobacco suspension cells correlates with an increase in cell size. Plant Mol. Biol. 52, 387–400

Rhee, Y., and Staswick, P.E. (1992) Nucleotide sequence of a soybean vegetative storage protein vspB gene1. Plant Physiol. 98: 794-5

Rogers, D.J., and Hori, K. (1993) Marine algal lectins: new developments. Hydrobiologia 260/261, 589-93

Rodriguez, M., Ramirez, N.I., Ayala, M., Freyre, F., Perez, L., Triguero, A., Cristina Mateo, C., Selman-Housein, G., Gavilondo, J.V., and Pujol, M. (2004) Transient expression in tobacco leaves of an aglycosylated recombinant antibody against the epidermal growth factor receptor. Biotechnol. Bioeng. 89:188-94

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

Sack, M., Paetz, A., Kunert, R., Bomble, M., Hesse, F., Stiegler, G., Fischer, R., Katinger, H, Stoeger, E., and Rademacher, T. (2007) Functional analysis of the broadly neutralizing human anti-HIV-1 antibody 2F5 produced in transgenic BY-2 suspension cultures. FASEB J 21:1655-64

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

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

Sainsbury, F., Sack, M., Stadlmann, J., Quendler. H., Fischer, R., and Lomonossoff, G.P. (2010) Rapid transient production in plants by replicating and non-replicating vectors yields high quality functional anti-HIV antibody. PLOS ONE, 5:1-9

Sato, Y., Okuyama, S., and Hori, K. (2007) Primary structure and carbohydrate binding specificity of a potent anti-HIV lectin isolated from the filamentous cyanobacterium Oscillatoria agardhii. J. of Biol. Chem., 282(15): 11021-9

Scanlan, C.N., Pantophlet, R., Wormald, M.R., Saphire, E.O., Stanfield, R., Wilson, I.A., Katinger, H. Raymond A. Dwek, R.A., Rudd, P.M., and Burton, D.R. (2002). 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. 76:7306–21

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

Scherer, E.M., Leaman, D.P., Zwick, M.B., McMichael, A.J., and Burton, D.R. (2010) Aromatic residues at the edge of the antibody combining site facilitate viral glycoprotein recognition through membrane interactions. PNAS 107:1529-34

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

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

Sexton, A., Drake, P.M., Mahmood, N., Harman, S.J., Shattock, R.J. and Ma, J.K. (2005) Transgenic plant production of cyanovirin-N, an HIV Microbicide. FASEB J. 20(2): 356-8

Sexton, A., Harman, S., Shattock, R.J., and Ma, J.K. (2009) Design, expression, and characterization of a multivalent, combination HIV microbicide. FASEB J. 23:3590 -600

Shaaltiel, Y., Bartfeld, D., Hashmueli, S., Baum, G., Brill-Almon, E., Galili, G., Galili, G., Dym, O., Boldin-Adamsky, S.A., Silman, I., Sussman, J.L., Futerman, A.H., and Aviezer, D.

(2007) Production of glucocerebrosidase with terminal mannose glycans for enzyme replacement therapy of Gaucher’s disease using a plant cell system. Plant Biotechnol. J. 5: 579–90

Shenoy, S.R., O'Keefe, B.R., Bolmstedt, A.J., Cartner, L.K., and Boyd, M.R. Selective interactions of the human immunodeficiency virus-inactivating protein cyanovirin-N with high-mannose oligosaccharides on gp120 and other glycoproteins. J. Pharmacol. Exp. Ther. 297:704-10

Shevchenko, A., Tomas, H., Havlis, J., Olsen, J.V., and Mann, M. (2006) In-gel digestion for mass spectrometriccharacterization of proteins and proteomes. Nat. Protoc. 1(6): 2856-60

Simek, M.D., Rida, W., Priddy, F.H., Pung, P., Carrow, E., Laufer, D.S., Lehrman, J.K., Boaz, M., Tarragona-Fiol, T., Miiro, G., Birungi, J., Pozniak, A., McPhee, D.A., Manigart, O., Karita, E., Inwoley, A., Jaoko, W., Dehovitz, J., Bekker, L.G., Pitisuttithum, P., Paris, R., Walker, L.M., Poignard, P., Wrin, T., Fast, P.E., Burton, D.R., and Koff, W.C. (2009) 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. 83:7337-48

Smee, D.F., Bailey, K.W., Wong, M.H., O'Keefe, B.R., Gustafson, K.R., Mishin, V.P., and Gubareva, L.V. (2008) Treatment of influenza A (H1N1) virus infections in mice and ferrets with cyanovirin-N. Antiviral Res. 80:266–71

Southern, E. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503–17

Sreevidya, V.S., Hernandez-Oane, R.J., So, R.B., Sullia, S.B., Stacey, G., Ladha, J.K., and Redddy, P.M. (2005) Expression of the legume symbiotic lectin genes psl and gs52 promotes rhizobial colonization of roots in rice. Plant Science 169:726–36

Stöger, E., Vaquero, C., Torres, E., Sack, M., Nicholson, L., Drossard, J., Williams, S., Keen, D., Perrin, Y., Chrisrou, P., and Fischer, R. (2000) Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. Plant Mol. Biol. 42:583–90

Streatfield, S.J., Lane, J.R., Brooks, C.A., Barker, D.K., Poage, M.L., Mayor, J.M., Lamphear, B.J., Drees, C.F., Jilka, J.M., Hood, E.E., and Howard, J.A. (2003) Corn as a production system for human and animal vaccines. Vaccine 21: 812–15

Streatfield, S.J. (2007) Approaches to achieve high-level heterologous protein production in plants. Plant Biotechnol. J. 5:2–15

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

Strasser, R., Castilho, A., Stadlmann, J., Kunert, R., Quendler, H., Gattinger, P., Jez, J., Rademacher, T., Altmann, F., Mach, L., and Steinkellner, H. (2009) Improved virus neutralization by plant-produced anti-HIV antibodies with a homogeneous 1,4-galactosylated N-glycan profile. J. Biol. Chem. 284:20479-85

Takeda, A., Sugiyama,K., Nagano, H., Mori,M., Kaido, M., Kazuyuki, K., Tsuda, S., and Okuno, T. (2002) Identification of a novel RNA silencing suppressor, NSs protein of tomato spotted wilt virus. FEBS Lett., 532:75-9

Tanaka, H., Chiba, H., Inokoshi, J., Kuno, A., Sugai, T., Takahashi, A., Ito, Y., Tsunoda, M., Suzuki, K., Akio Takenaka, A., Sekiguchia, T., Umeyama, H., Jun Hirabayashi, J., and Omurae, S. (2009) Mechanism by which the lectin actinohivin blocks HIV infection of target cells. PNAS 106:15633-8

Tavladoraki, P., Benvenuto, E., Trinca, S., De Martinis, D., Cattaneo, A., and Galeffi, P. (1993) Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack. Nature 366:469–72

Triguero, A., Cabrera, G., Cremata, J.A., Yuen, C., Wheeler, J., and Ramírez, N.I. (2005) 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. 3:449–57

Trkola, A., Purtscher, M., Muster, T., Ballaun, C., Buchacher, A., Sullivan, N., Srinivasav, K., Sodroski, J., Moore, J.P., and Katinger, H.(1996) Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. J. Virology 70:1100-8

Trkola, A., Kuster, H., Rusert, P., Joos, B., Fischer, M., Leemann, C., Manrique, A., Huber, M., Rehr, M., Oxenius, A., Weber, R., Stiegler, G., Vcelar, B., Katinger, H., Aceto, L., and Günthard, H.F. (2005) Delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies. Nat. Med. 11:615-22

Tsai, C.C., Emau, P., Jiang, Y., Tian, B., Morton, W.R., Gustafson, K.R., and Boyd, M.R. (2003) Cyanovirin-N gel as a topical microbicide prevents rectal transmission of SHIV89. 6P in macaques. AIDS Res. Hum. Retroviruses 19(7):535–41

Tsai, C.C., Emau, P., Jiang, Y., Agy, M.B., Shattock, R.J., Schmidt, A., Morton, W.R., Gustafson, K.R., and Boyd, M.R. (2004) Cyanovirin-N inhibits AIDS virus infections in vaginal transmission models. AIDS Res. Hum. Retroviruses 20:11-18

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

Twyman, R.M., Schillberg, S., and Fischer, R. (2005) Transgenic plants in the biopharmaceutical market. Expert Opin.Emerg. Drugs 10(1):185–218

Twyman, R.M., Schillberg, S., and 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, 2012 p 145-59

UNAIDS. Report on the global HIV-1/AIDS epidemic. In: UNAIDS, 2010

UNAIDS. Report on the global HIV-1/AIDS epidemic. In: UNAIDS, 2011

Van Damme, E.J.M., Allen, A.K., and Peumans, W.J. (1987) Isolation and characterization of a lectin with exclusive specificity towards mannose from snowdrop (Galanthus nivalis) bulbs. *FEBS* Lett. 215:140-4

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

Van Damme, L., Ramjee, G., Alary, M., Vuylsteke, B., Chandeying, V., Rees, H., Pachara Sirivongrangson, P., Mukenge-Tshibaka, L., Ettiègne-Traoré, V., Uaheowitchai, C., Karim, S.S.A., Mâsse, B., Perriëns,J., Laga, M. (2002) Effectiveness of COL-1492, a nonoxynol-9 vaginal gel, on HIV-1 transmission in female sex workers: a randomised controlled trial. The Lancet 360:971–7

Van Damme, E.J., Barre, A., Rougé, P., and Peumans, W.J. (2004) Cytoplasmic/nuclear plant lectins: a new story. Trends in Plant Sci., 9(10), 484-9

Van Der Veen, M.J., van Ree, R., Aalberse, R.C., Akkerdaas, J., Koppelman, S.J., Jansen, H.M., and van der Zee, J.S. (1997) Poor biologic activity of cross-reactive IgE directed to carbohydrate determinants of glycoproteins. J. Allergy Clin. Immunol. 100:327-34

Van Montfort, T., Nabatov , A.A., Geijtenbeek, T.B.H., Pollakis, G., and Paxton, W.A. (2011) Efficient capture of antibody neutralized HIV-1 by cells expressing DC-SIGN and transfer to CD4+ T lymphocytes. J. Immun. 178:3177-85

Veazy, R.S., Shattock, R.J., Pope, M., Kirijan, J.C., Jones, J., Hu, Q, Ketas, T., Marx, P.A., Klasse, P.J., Burton, D.R., and Moore, J.P. (2003) Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120. Nat. Med. 9:343-6

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

Vitale, A., and Raikhel, N.V. (1999) What do proteins need to reach different vacuoles. Trends in Plant Sci. 4(4):1360-85

Vitale, A., and Hinz, G. (2005) Sorting of proteins to storage vacuoles: how many mechanisms? Trends in Plant Sci. 10(7):1360-85

Voinnet, O., Rivas, S., Mestre, P., and Baulcombe, D. (2003) An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J., 33(5): 949–56

Walker, L.M., Phogat, S.K., Chan-Hui, P., Wagner, D., Phung, P., Goss, J.L., Wrin, T., Simek, M.D., Fling, S., Mitcham, J.L., Lehrman, J.K., Priddy, F.H., Olsen, O.A., Frey, S.M., Hammond, P.W.; Protocol G Principal Investigators, Kaminsky, S., Zamb, T., Moyle, M., Koff, W.C., Poignard, P., and Burton, D.R. (2009) Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. Science 326:285-8

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

West, A.P., Galimidi, R.P., Foglesong, C.P., Gnanapragasam, P.N.P., Huey-Tubman, K.E., Klein, J.S., Maria D. Suzuki, M.D., Tiangco, N.E., Vielmetter, J, and Bjorkman, P.J. (2009) Design and expression of a dimeric form of human immunodeficiency virus type 1 antibody 2G12 with increased neutralization potency. J. Virol. 83:98-104

Wu, X., Yang, Z., Li, Y., Hogerkorp, C., Schief, W.R., Seaman, M.S., Zhou, T., Schmidt, S.D., Wu, L., Xu, L., Longo, N.S., McKee, K., O'Dell, S., Louder, M.K., Wycuff, D.L., Feng, Y., Nason, M., Doria-Rose, N., Connors, M., Kwong, P.D., Roederer, M., Wyatt, R.T., Nabel, G.J., and Mascola, J.R. (2010) Rational design of envelope identifies broadly neutralizing human monoclonal antibodies to HIV-1. Science 329:856–61

Xiong, C., O’Keefe, B.R., Byrd, R.A., and McMahon, J.B. (2006) Potent anti-HIV activity of scytovirin domain 1 peptide. Peptides; 27(7):1668–75

Xiong, C., O’Keefe, B.R., Botos, I., Wlodawer, A., and McMahon, J.B. (2006a) Overexpression and purification of scytovirin, a potent, novel anti-HIV protein from the cultured cyanobacterium Scytonema varium. Protein Expr Purif 46: 233–39

Xiong, C. O’Keefe, B.R., Byrd, A., and McMahon J.B. (2006b) Potent anti-HIV activity of scytovirin domain 1 peptide. Peptides 27: 1668-75.

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

Xu, J., Xymeng, G., and Dolan, M.C. (2011) Towards high-yield production of pharmaceutical proteins with plant cell suspension cultures. Biotechnol. Adv. 29:278–99

Xu, H., Song, L., Kim, M., Holmes, M.A., Kraft, Z., Sellhorn, G. Reinherz, E.L., Stamatatos, L., and Roland K. Strong, R.K. (2010) Interactions between lipids and human anti-HIV antibody 4E10 can be reduced without ablating neutralizing activity. J. Virol. 84:1076-88

Yang, J., Barr, L.A., Fahnestock, S.R., and Liu, Z-B. (2005) High yield recombinant silk-like protein production in transgenic plants through protein targeting. Transgenic Res. 14(3):313-24

Yang, J., Barr, L.A., Fahnestock, S.R., and Liu, Z-B. High yield recombinant silk-like protein production in transgenic plants through protein targeting. (2005) Transgenic Res. 14:313-24

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

Zappe, H., Snell, M.E., and Bossard, M.J. (2008) PEGylation of cyanovirin-N, an entry inhibitor of HIV. Adv. Drug Del. Rev.60:79-87

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

Zhou, J. Y., and Montefiori, D.C. (1997). Antibody-mediated neutralization of primaryisolates of human immunodeficiency virus type 1 in peripheral blood mononuclearcells is not affected by the initial activation state of the cells. J. Virol. 71(3): 2512–7

Zhou T, Xu L, Dey B, Hessell AJ, Van Ryk D, Xiang SH, Yang, X., Zhang, M.Y., Zwick, M.B., Arthos, J., Burton, D.R., Dimitrov, D.S., Sodroski, J., Wyatt, R., Nabel, G.J., and Kwong, P.D. (2007) Structural definition of a conserved neutralization epitope on HIV-1 gp120. Nature 445:732–7

Ziółkowska, N.E., and Wlodawer A. (2006) Structural studies of algal lectins with anti-HIV activity. Acta Biochim. Pol. 53:617–26

Ziółkowska, N., O'Keefe, B., Mori, T., Zhu, C., Giomarelli, B., Vojdani, F., Palmer, K., McMahon, J., and Wlodawer, A. (2006) Domain-swapped structure of the potent antiviral protein griffithsin and its mode of carbohydrate binding structure. Structure 14(7):1127-35

Ziolkowska, N.E., Shenoy, S.R., O'Keefe, B.R., and Wlodawer, A. (2007) Crystallographic studies of the complexes of antiviral protein griffithsin with glucose and N-acetylglucosamine. Protein Sci. 16:1485-9

Zwick, M.B., Labrijn, A.F,, Wang, M., Spenlehauer, C., Saphire, E.O., Binley, J.M., Moore, J.P., Stiegler, G., Katinger, H., Burton, D.R., and Parren, P.W. (2001) Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. J Virol.75:10892-905

Appendix A

Nutrient	**N6E** Callus initiation 7-10 days	**N6OSM** Osmoticum medium for Bombardment	**N6S 12/6** Selection medium 2 weeks	**N6S 13/4** Selection medium 4 weeks	**Reg I 26/8** 2-3 weeks	**Reg II 13/4** Until ready for hardening-off
MS salts (Sigma)					4.3 g/l	4.3 g/l
MS vitamin stocks (Sigma)					1 ml/l	1 ml/l
N6 salts (Sigma)	4 g/l	4 g/l	4 g/l	4 g/l		
N6 vitamin stock	+	+	+	+		
Sucrose	30g/l	30 g/l	6 g/l	4 g/l	8 g/l	4 g/l
pH	5.8	5.8	5.8	5.8	5.8	
Gelrite	2.5 g/l	2.5 g/l	2.5 g/l	2.5 g/l	3 g/l	3 g/l
$AgNO_3$ (silver nitrate)	4.2 mg/l	4.2 mg/l	0.85 mg/l	0.85 mg/l		
2,4-D (1mg/l stock)	2 mg/l	2 mg/l	2 mg/l	2 mg/l		
L-Proline (stock = 4g/10 ml)	25 mM (7.25 ml/l)	6 mM (1.73 ml/l)				
Casein hydrolysate	100 mg/l	100 mg/l				
Myo-inositol	100 mg/l	100 mg/l	100 mg/l	100 mg/l	100 mg/l	100 mg/l
D-Mannitol		36.4 g/l				
D-Sorbitol		36.4 g/l				
Mannose			12 g/l	13 g/l	26 g/l	13 g/l

Micronutrients (use 5ml/l media)	**N6** **g/500ml**	**MS** **g/l**
H_3BO_3	0.16	1.24
$MnSO_4.4H_2O$	0.33	4.46
$ZnSO_4 .7H_2O$	0.15	1.72
KI	0.08	0.166
$Na_2MoO_4.2H_2O$	0.025	0.05
$CuSO_4.5H_2O$	0.0025	0.005
$CoCl_2.6H_2O$	0.005	0.005

Macronutrients (use 50ml/l media)	**N6** **g/l**	**MS** **g/l**
KNO_3	56.60	38
$CaCl_2$ x $2H_2O$	3.32	8.8
$MgSO_4$ x $7H_2O$	3.7	7.4
KH_2PO_4	8.0	3.4
$(NH4)_2SO_4$	9.26	
NH_4NO_3		33

Vitamin composition (use5ml/l media)		
	N6 **(mg/200ml)**	**MS** **(mg/200ml)**
Thiamine-HCl	40	4
Pyridoxine-HCl	20	20
Nicotinic Acid	20	20
Glycine	80	80
Myo-inisitol		4000

Fe-source (use5ml/l media)	**N6** **(g/l)**	**MS** **(g/l)**
$Na_2EDTA.2H_2O$	5.56	5.56
$FeSO_4.7H_2O$	7.46	7.46